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Antibacterial Activity of the Silver Nanoparticles Synthesized From Extracts of Brassica Compestris

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ABSTRACT: Microbes are the common cause of infection and mortality in the world. Antibiotics are used as potent and cost effective tool to treat antimicrobial diseases.Over or Misuse of antibiotics led to antibiotic resistance. The same antibiotic remains no more effective against killing that same pathogen or microbe or bacteria causing rise in mortality and cost of treatment as well.

In bacteria, both Gram positive and Gram negative show faster rate of antibiotic resistance. The discovery and development of new sources and therapies against antibiotic resistant microbes are in progress.

Most commonly, the medicines are not properly prescribed by physician due to inadequate knowledge, improperly available treatment guidelines by pharmaceutical companies, patient load and his or her socio-economic status. Another leading cause of transmission of antibiotic resistant microbes is nosocomial infections. Intrinsic type is developed due to structural and functional modification in microbes or bacteria. The acquired type is developed by horizontal gene transfer from resistant to non-resistant strain. Antibiotic resistance is developed by modification at level of protein synthesis, DNA, extracellular matrix, and by forming thicker biofilms etc.

Nanotechnology is greatest emerging field of research. Bio-nanotechnology is the combination of biotechnology with nanotechnology; opening new research avenues in the field of medicine, diagnostics and treatment, bioengineering etc. Nanoparticles operating in biological systems run through simplistic and eco-friendly technique. Role of silver nanoparticles is under research and exploration nowadays. Silver itself is highly toxic for microorganisms while less toxic to mammal cells due to their different cellular composition and complexity.

I. INTRODUCTION

Microbes are among topmost cause of infection and mortality in the world. Antibiotics are used as potent and cost effective tool to treat antimicrobial diseases (Wang et al., 2017). Over a period of time, over dose or misuse of antibiotics led to antibiotic resistance particularly in developing world. The same antibiotic remains no more effective against killing that same pathogen or microbe causing rise in mortality and cost of treatment as well (Wikaningtyas et al., 2016).

In bacteria, both Gram positive and Gram negative show faster rate of antibiotic resistance, i.e, methicillin resistant Staphylococci and vancomycin resistant Enterococci species are found a decade ago (Kurmarasamy et al., 2010). The discovery and development of new sources and therapies against antibiotic resistant microbes are in

progress (Frieri et al., 2017). According to a study conducted in Pakistan by WHO, 95% participants were found positive to multidrug resistant. The contributing factors are lack of knowledge, un-prescribed medication practice and doubts of physician towards health complication of patient (Sarwar et al., 2018).

Moreover, improper knowledge of physician, improperly available treatment guidelines by pharmaceutical companies, patient load and his/her socio-economic status promote antibiotic resistance in microbes particularly in bacteria (Faizullah et al., 2017). Another leading cause of transmission of antibiotic resistant microbes is nosocomial infections or hospital acquired infections (HAIs). Invasive mechanical devices cause ventilator associated pneumonia along with surgical site infections, bacteremia and septicemia and urinary tract infections (Ali et al., 2017). Acinetobacter baumannii has arisen prime cause of HAIs; it has increased frequency of being Multi drug resistant (MDR) as well as extensively drug resistant (XDR) (Shazly et al., 2015).

There are two types of drug resistances: Intrinsic and Acquired. Intrinsic is developed due to structural and functional modification in microbes or bacteria. The acquired one is result of horizontal gene transfer from resistant to non-resistant strain. (Li and Webster, 2018). Antibiotic resistance is developed by modification at level of protein synthesis, DNA, extracellular matrix, extracellular proteins modification or by forming thicker biofilms etc. (Fair and Tor, 2014)

This alarming situation has tempted scientists to explore new antimicrobial sources mainly the new medicinal plants more effective than conventional herbal medicines. Many plants have shown polyphenols mediated antioxidant activity as well as antimicrobial properties. Even waste and by-products of agro-industry have been observed containing significant amount of polyphenols (Farah et al., 2015). Another approach is to involve nanotechnology in biological systems.

Nanotechnology is the use of particles having size range of 1-100nm (10-9m), that makes 40,000 times thinner than human hair or equal to size of typical virus. Nanotechnology is greatest emerging field of research and revolutionizing areas of electronics, optoelectronics and information storage etc. (Jena et al., 2013). Nanoparticles (NPs) operating in biological systems run through simplistic and eco-friendly techniques (Kaegi et al., 2010). Theragnostics or smart drugs involve whole systems related to prevention, diagnosis, and treatment of diseases. Nano-engineered devices are used for molecular targeting and precise drug delivery to the infected cells.Silver itself is highly toxic for microorganisms while less toxic to mammal cells due to their different cellular composition and complexity. Silver ions form complexes with less stability which offer their effect for very short period of time, this problem has been overcome by using silver nanoparticles which are stable, inert and show longer effect (Sahayaraj and Rajesh, 2011).

Ethnobotany is the relationship between humans and plants. Besides food, shelter, clothing and ornamental use, plants are also providing healthcare to us. People-Plant relationship is growing in various dimensions like pharmacology, ecology, medicine and public health. The powerful compounds are extracted from plants since ancient times to make folk medicines. In currently used medicines, 50% are of natural origin (Kirbag and Zengin 2009).

Silver (Ag) is well-known for showing antibacterial activity. For over decades, Ag colloidal solution has been used to curtail infections, even in modern day ointments and creams Ag is added to prevent bacterial infections on burns and wounds. Comparative analysis of NPs, silver nitrate and silver chloride has revealed that NPs show significantly higher antibacterial potential than free silver ion (Choi et al., 2008).

II. MECHANISM OF ANTIMICROBIAL ACTION OF NANOPARTICLES

Studies have revealed that NPs are performing bactericidal action on antibiotic resistance bacteria. Silver ions interact with peptidoglycan of bacteria cell wall (Yamaka et al., 2005) causing lysis of cell (Monores et al., 2005), they also interact with bacterial plasma membrane (Jung et al., 2008), bacterial DNA as well as bacterial proteins (Shrivastava et al., 2007). By binding with DNA it controls the expression of enzymes required for cellular respiration of bacteria as well as affects functionality of cell membrane receptors (Ellis et al., 2007).

Nano-silver (NS) has intrinsic bactericidal property independent of Ag+ elution. One more study revealed that NS produces reactive oxygen species (ROS) to kill living cells whether bacterial cell. This property is potentially harmful for human cells also (Park et al., 2009). Additionally, researches also revealed that AgNPs targets the thiol group of enzymes to obstruct bacterial cell division, they form low molecular weight regions in the core of bacteria and change cellular signaling via dephosphorylating the key peptide substrates on their tyrosine residues by

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attacking respiratory activity of cell (Rajkuberan et al., 2015).

III. PHYTONANOTHERAPY- A GLANCE AT HISTORY

The escalating level of antibiotic resistance in infectious bacteria has triggered the exploration of potent, simple, easily available and cost effective alternatives such as modified herbal medicinal products (HMP) which are based on pharmacologically important biochemical constituents (Tumbas et al., 2012).

In 2014, evaluation of synergistic effect of antibiotics and silver nanoparticles (AgNPs), synthesized from water extract of flowers against E. coli, S. aureus, B. cereus. Results indicate that they possess antibacterial activity (Pedelia et al., 2014).

Soon after this, in another research, methanol extract of banana peel was used to prepare silver nanoparticles and its synergistic effect with levofloxacin was observed, which showed better results against pathogenic strains. Their minimum inhibitory concentration (MIC) value was 10.2µg/ml (Ibrahim, 2015).

In another study, aqueous extract from the fruit extract of Thevetia peruviana was used to synthesize AgNPs to evaluate their antibacterial activity. AgNPs act as a reservoir of Ag+ ions, which led to degradation of cell membrane by incorporating into the bacterial cell protein. It was confirmed by the 20mm zone of inhibition for E. coli (Chakraborty et al., 2016).

Furthermore, aqueous extracts of healthy leaves of Trichodesma indicium has been used to prepare the AgNPs to evaluate their anti-larval activity against Mythimna separate. Results indicated that AgNPs possess larvicidal activity at different concentrations. Mortalities of 46.67% and 83.33% were caused at different concentrations of 200 and 300 ppm, respectively (Buhroo et al., 2017).

IV. AIMS OF STUDY

- The purpose of this study is to reduce threats of multi-drug resistant bacteria (MDR) by treating them with natural antimicrobials found in flowers of Brassica compestris locally grown plants.
- To my knowledge, this is the first study done in South Punjab of Pakistan. This includes making aqueous and methanolic extracts of flower, then finding out antibacterial activity of biosynthesized silver-nanoparticles against MDR bacteria, and then evaluating the MIC of selected plants against bacterial isolates.
- The plants selected for this study is Brassica compestris. It contains plentiful of phenolic antioxidants that are 1-4% of entire chlorogenic acids. Brassicacompestris belongs to family Brassiceae which produces edible flowers. Its oil is being used for cooking purposes and for topical uses. This plant has been found to have antibacterial, antimicrobial properties as well as show anti-viral action against Herpes simplex virus type-1, measles virus and few RNA viruses due to presence of active compound called brassino-steroid produced by this plant (Dalal et al., 2014).

V. MATERIALS& METHODS

i. Sample Collection

Sample collection was done from different parts of Multan and its floral parts were separated for making its aqueous and methanolic extracts. B. compestris flowers collected from The women university Multan.

ii. Collection of Sewage Water

Sewage water was collected from Nishter Hospital, Multan; Family hospital, Multan was collected in air tight bottles. This was done in order to gather antibiotic resistant bacteria growing under constant exposure of antibiotic use in health care centers.

iii. Culture Media preparation

Different Types of media are required for this study. These were:

- Nutrient agar
- McConkey agar
- Eosin and Methylene Blue (EMB) agar
- Besides them, Mannitol Salt Agar (MSA)
- MR-VP broth, Simon Citrate agar
- Triple sugar iron (TSI) media
- N-agar supplemented with starch
- Gelatin medium,
- Tryptophan supplemented SIM agar with kovac's reagent
- Mueller-Hinton agar (MHA)

Additionally, stains and solutions for gram staining and acid indicator dyes were also used Methanol and Distilled water were also used for extraction

iv. Antibiotics

Antibiotics used include Methicillin, Ceftazidime, Imipenem, Vancomycin, Imidazole, and Augmentin with different potencies like 20-to 30µg.

VI. RESULTS

I. identification and Isolation of Bacteria

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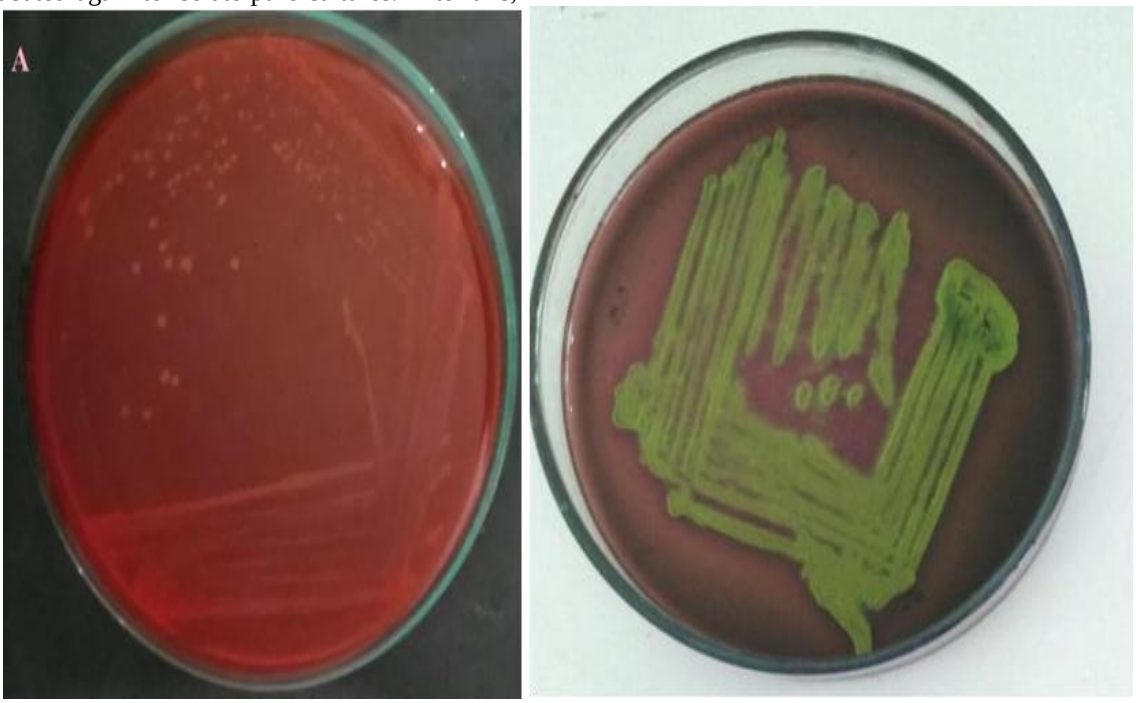
Sewage waste water dilutions were made and separate plate method was applied to culture waste water bacteria on nutrient agar for 24 hours at 37°C in order to identify bacterial colonies and their external morphology. These grown colonies were transferred strain wise to fresh plates and incubated again to isolate pure cultures. After this, the isolated colonies were grown on Macconkey agar for identifying lactose fermenters bacteria. Pink color colonies appeared on plate was lactose fermenters and was suspected to be E. coli as shown below:



II. Biochemical Analysis

Biochemical analysis of bacterial colonies was done to confirm their identity on the basis of their biochemical properties.

III. Mannitol Salt agar test

One of the tests is mannitol salt agar test, which was positive for salt tolerant Staphylococci.

IV. Other Important tests

The other tests performed were methyl red-voges proskauer (MR-VP) test, triple sugar iron test, citrate utilization test, starch hydrolysis, gelatin hydrolysis, and indole production test. The results confirmed our strains till genera. Bacterial identification based on all the above mentioned analyses is given in Table below:

Organism	Gram stain	Indole Production	MR reaction	VP reaction	Citrate Utilization	Starch Hydrolysis	Gelatin Hydrolysis	TSI
E. coli	Rod (-)	+	+	-	-	-	-	AG +
S. aureus	Cocci (+)	_	+	+	-	-	_	A
Pseudomonas	Rod(-)	-	+	-	+	-	+ Rapid	-
Klebsiella	Rod (-)	-	-	-	+	-	-	AG
Salmonella	Rod (-)	+	-		+	-	-	AG
Shigella	Rod (-)	+	-		-	-	-	+, -
Proteus	Rod (-)	+	-		+	-	+	A +, -

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V.**Preparation of Extract of Plant**

To prepare plant extract (methanol), petals were washed and cleaned thoroughly with distilled water and then oven dried at 45 °C for 3-5 days. After drying these were grind into fine powder, while for the preparation of aqueous extract fresh petals were used.

VI.**Reflux of water**

For aqueous extraction, 20g of fresh petals were added to 60ml of distilled water and constantly boiled on slow heat for 1 hour with constant stirring. Then it was filtered through filter paper. Then the filtrate was collected in a sterilized, dried conical flask. It was cooled at room temperature, and then was stored at 4°C for future use.

VII.**Extract of Methanol**

For methanolic extraction, 20g of oven-dried powder of each separately was added to 60ml of methanol and was mixed with continuous shaking in shaking incubator, at 15000 rpm, for 72 hours. Then it was evaporated, to obtain concentrated solution. It was stored in refrigerator at 4 °C for future use. When plant extract was prepared the phytochemicals dissolve in the solvent, hence color changed from yellow to blackish color for B. compestris.

VII. NANOTECHNOLOGY

Nanoparticles can be synthesized from bottom-up and top-bottom technique. In bottom-up technique elemental constituents are used to make nanostructures. This technique covers physical, chemical and biological methods. Top-down approach involves lithographic and non-lithographic technique. This is commonly used to manufacture micro and nano-system components.

I. Preparation of Silver nanoparticles

To prepare silver nanoparticles, the silver nitrate solution (5mM), was made for each extract, which was colorless solution. Then stock solutions of plant extract were taken and added to silver nitrate solution separately. Color changed from original was an indication for the successful synthesis of nanoparticles.

Change in color was observed after 5 minutes and 10 minutes. The color changed after 10 minutes was darker than earlier. These solutions were centrifuged at 12000 rpm for 15 minutes and supernatant was discarded to obtain purified particles. Centrifugation was repeated for 2-3 times and every time pellet was collected (Padalia et al., 2014). After this experiment, characterization of nanoparticles was made in following ways.

II. Visual analysis

The change in color is the characteristic for the formation of nanoparticles. Color change from colorless to dark color is due to surface plasmon resonance. . As time increased resonance increased as well, hence the color darkens as the time passes. Dark green color appeared for B. compestris.

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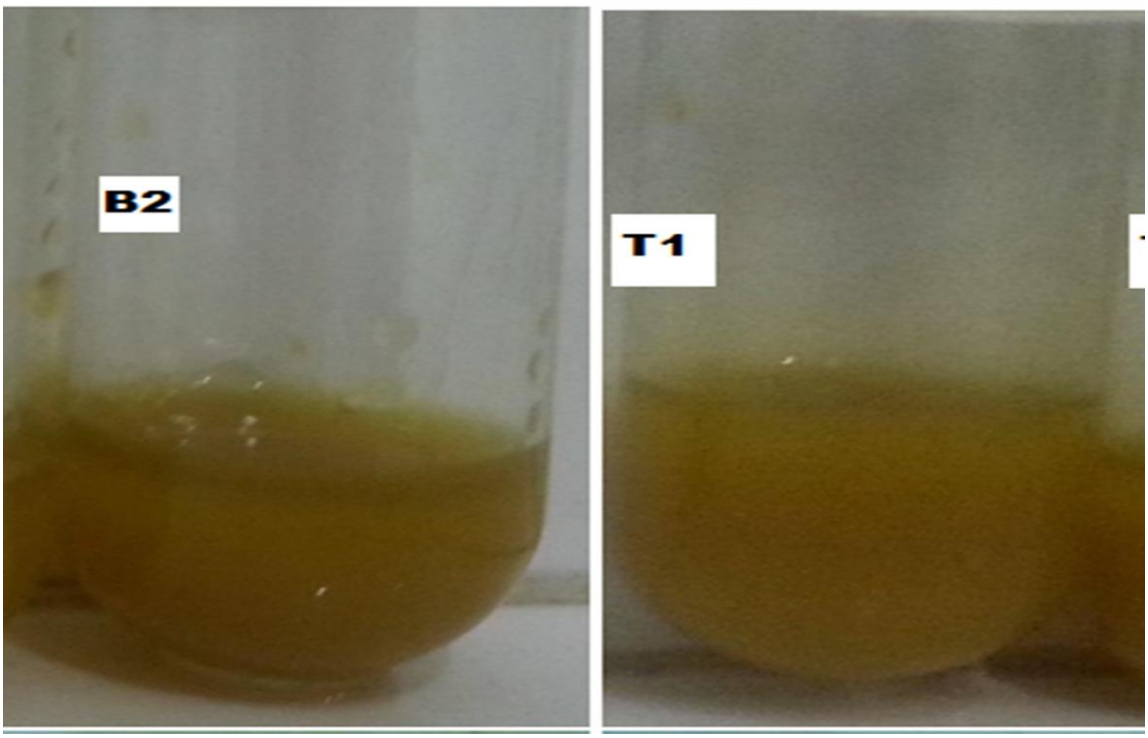
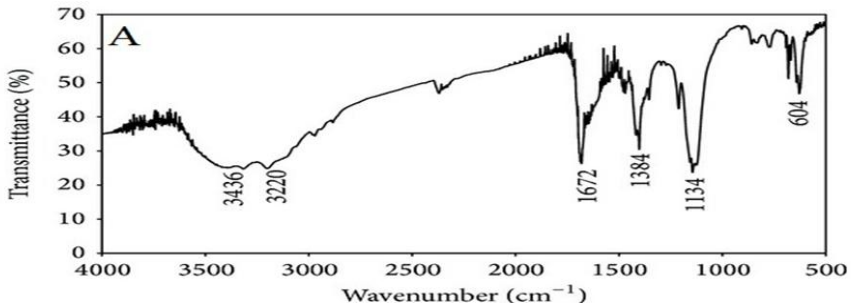


Fig. B.compestris after 5 min, B2) B.campestris after 10 min, T1)

III. Fourier transform infrared spectroscopy (FTIR) analysis

FTIR was done to evaluate the characteristic phytochemicals in each flower extract work as a reducing as well as capping agent. Sample preparation for FTIR was done by evaporating the solution of AgNPs and collecting pellet after centrifugation to get pure concentrated solution. The FTIR spectrum was recorded in the range of 500-4000 cm-1. The presence of vital groups was confirmed by FTIR in transmittance mode, over the range of 500-4000 wavenumber cm-1. FTIR bands were observed in the regions of 4000-500 were 3436, 3220 for B. compestris. The broadened absorbance of bands at 3436 and 3220 was observed.



FTIR of B.campestris. These peaks exhibited the presence of phytochemicals present in plant extracts, which act as capping plus reducing agent.

IV. UV-Visible spectroscopy

The reduction from Ag+ to Ag° was observed by measuring the UV-Vis spectrum within the range of 400– 800nm wave length at a resolution of 10nm in the UV-Vis spectrophotometer. For this purpose, concentrated solution of AgNPs was taken in glass cuvette and pure methanol was used as control. Wavelength scan was used to obtain the graph. It was done to confirm the presence of silver nanoparticles. Color change happened due to excitation of surface Plasmon vibrations. This was the characteristic feature of silver which gave maximum absorbance in the range of 425 nm. Absorbance for the flower was in between 1.0 and 1.5

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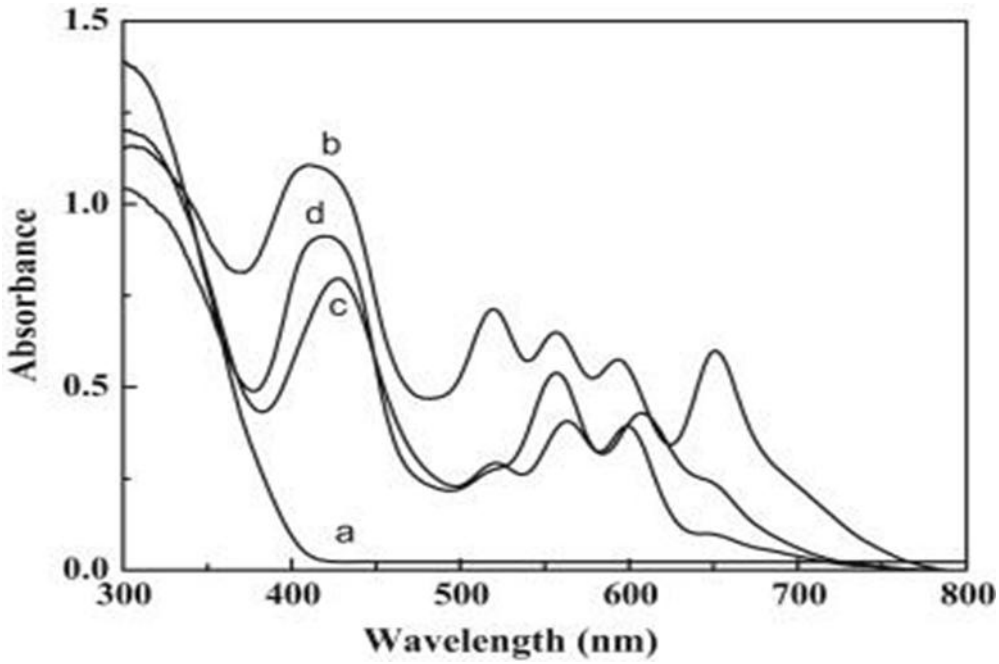


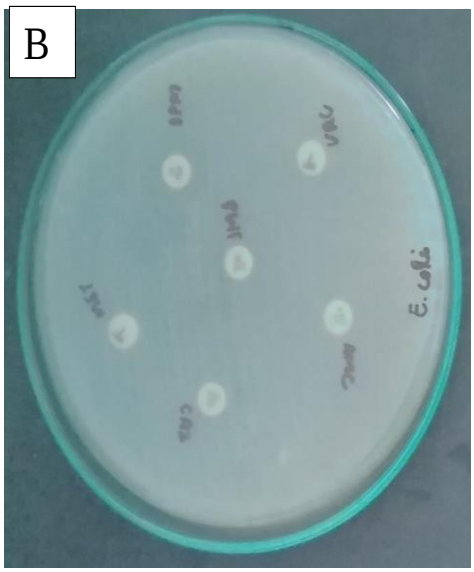
Fig b) peak of B. compestris

i. Preparation of Inoculum

Active cultures for tests were prepared by transferring loopful cultures to L-agar plate and incubated for 24 hours at 37 °C. After incubation fresh cultures were obtained, for further experiment.

ii. Antibiotic Activity Analysis

To evaluate the antibiotic activity against isolated strains, disc plate method was used. The antibiotic discs were positioned on Mueller-Hinton agar which has been inoculated with the test organism (swabbed petri plates). During incubation, these antibiotic diffuse outward from disc creating concentration gradient. After 18-24 hours, the zone of inhibition formed on agar was measured and then reference tables were used for determination that whether the bacteria are sensitive (S), resistant (R) or intermediate (I), to the antimicrobial drugs. Among all of these antibiotics used S. aureus was found to be resistant to methicillin. Most of these strains were resistant to the Vancomycin, Augmentin,Ceftazidime and Imidazole as well.



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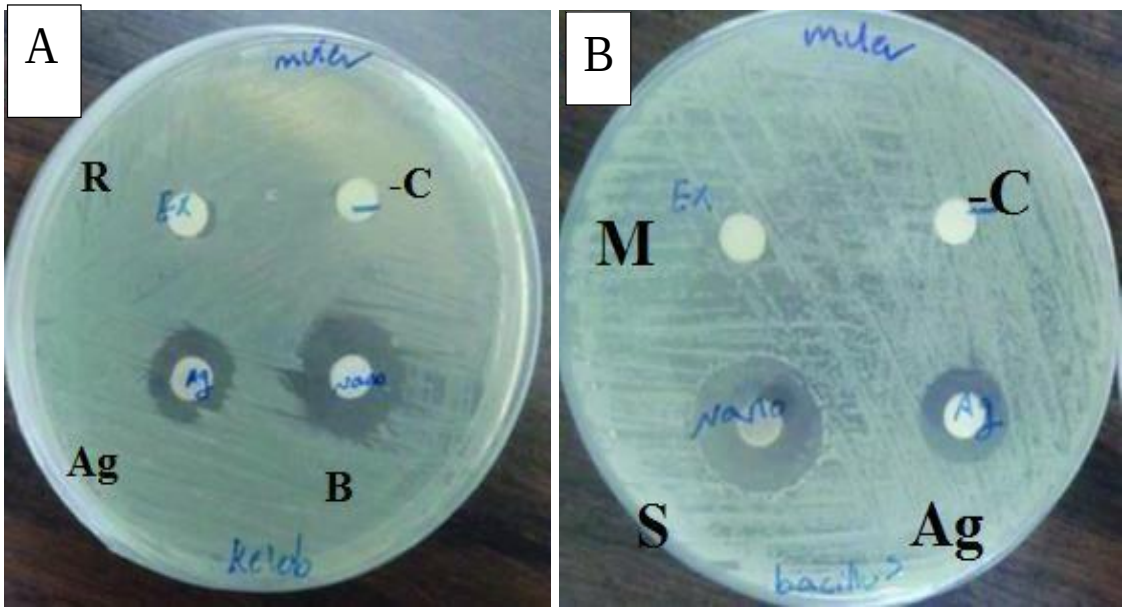
VIII. SILER NANOPARTICLE ACTIVITY

Antibacterial activity of biosynthesized nanoparticles was analyzed by agar disc diffusion method and well diffusion method.

i. Paper disc diffusion analysis

Testing microorganisms were swabbed on surface of the prepared Mueller-Hinton Agar plates.
Filter paper discs were prepared by soaking disks in solution of AgNPs for 30 minutes
Plates were dried and placed on agar plate having microorganisms inoculated over it.
These plates were further incubated at 37 °C for the duration of 24 hours.
After 24 hours these were observed



ii.Well diffusion method

Testing microorganisms were swabbed on the surface of solidified MHA plates and the inoculum could dry for 5 minutes. With the help of glass Pasteur pipette 6mm holes were made on the seeded agar plates. Aliquot of 20µl from solution of each AgNPs was added and plates could remain on bench for 1 hour for appropriate diffusion. Then plates were incubated at 37 °C up to 24 hours. After incubation resulting zone of inhibition was measured in millimeters.

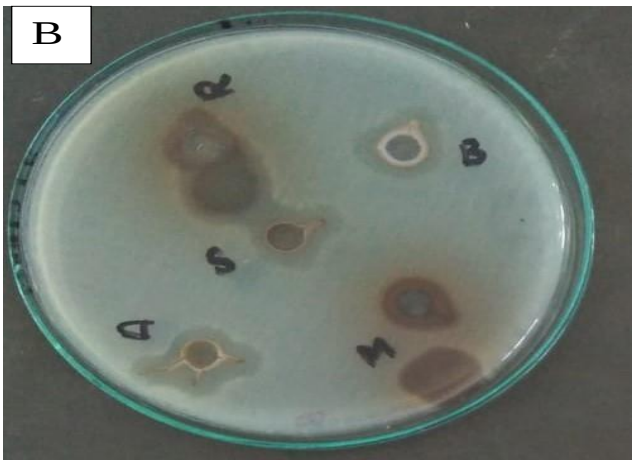


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Fig.clearly depicted that AgNPs exhibited effect against both gram positive along with gram negative bacteria. It can be vividly seen that nanoparticles based on bioactive compound extracted from flowers of B. compestrishad very strong effect against selected antibiotic resistant strains.

iii.Determination of Minimum Inhibitory Concentration (MIC)

MIC for all the synthesized nanoparticles was determined using flasks containing nutrient broth media.Supplemented with different concentrations of silver nanoparticles (50, 25, 12.5, 6.25 and 3.12µl/ml) were inoculated with 100µl of microbial culture broths. The flasks were then incubated in the shaking incubator for 24 hours at 37 °C on 150 rpm. Silver nanoparticles-free broth was used as controls. B. compestris showed even best results at 10µl/ml concentration.

IX. DISCUSSION

In the present study, different strains isolated from hospital`s sewage water were characterized by different biochemical tests. As mannitol salt agar identifies a salt tolerant bacterium which hydrolyses mannitol into acid which can be seen with the use of dye indicator. This test identified Staphylococci. The MR-VP test, with the help of indicator dyes, glucose fermenting and acetoin producing bacteria were identified. Completely positive result indicated presence of Klebsilla while negative result identified presence of Pseudomonas. Simon citrate agar culture identified those bacteria which utilize citrate as energy source while TSI test identified those which ferment sucrose and unable to ferment lactose. Negative results showed presence of Pseudomonas while Salmonella and Proteus gave positive results. Starch hydrolysis and gelatin hydrolysis identified amylase and gelatinase exoenzyme producing bacteria respectively. Indole production test picked identity of tryptophan hydrolyzing bacteria.

Later, optimization of different parameters involved in this study experiments was done. The parameters with significant effect on the formation and working of plant extracts and silver nanoparticles were boiling time, pH, and concentrations of used extracts and solutions (Zayed et al., 2012) Mechanical agitation method (magnetic stir or ultra sonication) also affect the results significantly (Lu et al., 2016). Concentrated plant extracts, (Ibrahim, 2015), High concentration of AgNO3 required for nanoparticle synthesis (Vimala, et al., 2015), alkaline pH of solutions (Roopan, et al., 2013), increase in reaction time (Aragao, et al., 2016) produced larger number of smaller nanaoparticles having dark colored solution due to increased surface Plasmon vibration. Therefore, optimum conditions for the synthesis of silver nanoparticle by flower extract of B. compestris was maintained.

After nanofabrication at optimum conditions, nanoparticles characterization was done by FTIR and UV-Vis spectrophotometer. FTIR spectrum is carried out to identify the functional groups responsible for the reduction of silver ions to silver nanoparticles. It was recorded in the range of 500-4000 cm-1. FTIR spectrum for B. compestris showed peaks at 3436, 3220.These peaks correspond to the different functional groups like flavonoids, saponins, terpenoids etc. These bioactive compounds are responsible for the formation of silver nanoparticles by the reduction of Ag+ ions to Ag^{o} (Fig. 5).

Besides this, UV-Vis spectrophotometry, Plasmon resonance vibrations were oobserved for all the nanoparticles (NP). Fig. showed that there are narrow peaks for Brassica compestris. Lastly, the analysis of antimicrobial activity of AgNPs showed that and B. compestris showed better antibacterial activity. But these AgNPs showed better results against gram negative bacteria than gram positive bacteria due to thicker layer of peptidoglycan in the cell wall of gram positive bacteria leading to the decreased penetration ability of silver nanoparticles, while peptidoglycan layer is thin in gram negative bacteria (Shrivastava et al., 2007).

Antimicrobial activity was determined by MIC by using agar dilution and broth dilution approaches. MIC can be defined as the minimum concentration of present antimicrobial agent responsible for inhibiting the visible growth of microorganisms under defined conditions (Ibrahim, 2015) Silver nanoparticles showed more bactericidal effect than silver salt. The inhibition zones were 15-20mm and 8-10mm respectively. The higher bactericidal activity of these silver nanoparticles was due to larger surface area, responsible for better contact with the microorganisms.

X. FUTURE PROSPECTS

Nanotechnology continues to develop and hoard more attention; its application in treating ESBLs is cheaper. Thus, biogenetically combined silver nanoparticles will bring about a substantial result for the field of bionanomedicine. As nanoparticles can change the pharmacokinetics of drug, therefore, they are the good source of drug

delivery. These should be used to deliver those drugs, which causes high resistance to present antimicrobials or are have failed to treat disease.

CONCLUSION

Plants based compounds have pharmacological importance. Their secondary metabolites act as anti- oxidant, anti-blood clotting agents and in food additives (Anjukarishna et al., 2015). Various parts of plants are non-toxic, safe, eco-friendly and economic for the synthesis of these potent nanoparticles. Use of flowers for the nanoparticles synthesis has an additional advantage of being environmental friendly. In the present study, nanoparticles were made from B.campestris. Their phytochemical constituents enhance their value for medicinal purpose.

In the present study, we concluded that prevalence of infections caused by ESBL-MDR is increasing day by day and antibiotics are failed to treat. So, the alternate method to control the prevalence rate of MDR is of great interest, because antibiotics are expensive too. From our studies it can be concluded that biosynthesis of nanoparticle would be promising technique to overcome increasing resistance to current therapies for bacteria. In our study we have seen that nanoparticles of Brassica compestris showed better results against all the tested organisms.

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