Biogenic Silver Nanoparticles as an Antibiofouling (Bactericidal), Cytotoxic And Anticancereous Agents

By

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Department of Microbiology

Faculty of Biological Sciences

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A thesis submitted in partial fulfillment of the requirements for the Degree of

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IN

MICROBIOLOGY

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Author's Declaration

I **Mr. Aftab Rafiq** hereby state that my Ph.D. thesis titled **"Biogenic Silver Nanoparticles as an Antibiofouling (Bactericidal), Cytotoxic and Anticancereous Agent"** is my own work and has not been submitted previously by me for taking any degree from Quaid-i-Azam University, Islamabad, Pakistan.

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Date: 30-11-2021

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This is to certify that the research work presented in this thesis, entitled titled **"Biogenic Silver Nanoparticles as an Antibiofouling (Bactericidal), Cytotoxic and Anticancereous Agent"** was conducted by **Mr. Aftab Rafiq** under the supervision of **Prof. Dr. Naeem Ali** and co-supervision of **Prof. Dr. Zafar Mahmood Khalid (IIU, Islamabad).** No part of this thesis has been submitted anywhere else for any other degree. This thesis is submitted to the Department of Microbiology, Quaid-i-Azam University, Islamabad in partial fulfillment of the requirements for the degree of Doctor of Philosophy in field of **Microbiology**.

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DEDICATED

TO

My Great Father, Loving Mother,

Sisters & Helpful Brothers

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List of Acronyms

Ag^{+}	Silver ion
$AgNO_3$	Silver Nitrate
AgNPs	Silver nanoparticles
ATCC	American Type Culture Collection
ATP	adenosine triphosphate
Au^{3+}	Gold ion
BA	Blood agar
BHI	Brain Heart Infusion
BSA	Bovine serum Albuimn
C.albican	*Candida albican*
CLSI	Clinical & Laboratory Standards Institute (CLSI)
CPS	counts per second
CRA	Congo Red Agar
E.coli	*Escherasia coli*
Eco	Ecology
EDTA	Ethylenediaminetetraacetic acid
EFTEM	Energy-Filtering Transmission Electron Microscoy
ELIZA	Enzyme Linked Immuno-Sorbent Assay
EPS	Extra polymeric Substances
Etc	Etcetera
FCC	Face Cubic Centered
FIC	Fractional Inhibitory Concentration

FTIR	Frustrated Total Internal Reflection
FWHM	full-width at half maximum
k.pneumonaie	*Klebsiella pneumonaie*
LB	Luria Bertani
LPS	Lipopolysacchride
MALDI-TOF MS	Matrix-assisted Laser Desorption Ionization-Time-of-Flight MS
MDR	Multiple drug resistance
MHA	Muller Hinton Agar
MIC	Minimum Inhibitory Concentrations
MPA	Microtittre plate Assay
MRSA	Methicillin Resistant *Staphlococcus aureus*
MRSE	Methicillin resistant *Staphylococcus epidermidis*
NaCl	Sodium Chloride
NADH	Nicotinamide adeninine di- hydide
NADPH	Nicotinamide adeninine di-phosphate hydide
NaOH	Sodium Hydroxide
NM	Nanao-Materials
NPs	Nanoparticles
OD	Optical Density
P. aeruginosa	*Pseuomonas aeruginosa*
PBS	Phosphate Buffer Saline
PEG	Poly (ethylene glycol)
PET	Positron Emission Tomography

PLGA	Poly (lactic-co-glycolic acid
PLH	Poly (l-histidine)
PMS	Phenazine metho-sulfate
PVP	polyvinyl pyrolidone
RBCs	Red Blood Cells
ROS	Reactive Oxygen Species
S.aureus	*Staphylococcus aureus*
SDA	Sabroud dextrose agar
SDS	Sodium Dodecyl Sulphate
SEM	Scanning Electron Microscopy
TBE	Tris/Borate/EDTA
TEM	Transmission electrons Microscopy
TSC	Tri-Sodium Citrate
UV-Vis	UV-visible spectral analysis
WHO	World Health Organization
XRD	X-Rays Diffraction
XTT	2, 3-bis [2-Methoxy-4-nitro-5-Sulfophenyl]-2H-tetrazolium-5-carboxanilite inner salt

List of Publications

Rafiq, A., Zahid, K., Qadir, A., Khan, M. N., Khalid, Z. M., & Ali, N. (2020) Inhibition of microbial growth by silver nanoparticles synthesized from Fraxinus xanthoxyloides leaf extract. *Journal of Applied Microbiology*

Rafiq, A., Saimoon, K., Khalid, Z. M., & Ali, N. (2021) Antiseptic and healing activity of Chitosan hydro film coated with silver nanoparticles synthesized from Bischofia javanica leaf extract (Under review)

Rafiq, A., Saimoon, K., Khan, M. N., Khalid, Z. M., & Ali, N. (2021) Anti-bacterial and Anti metabolic activity of silver nanoparticles synthesized from Bischofia javanica leaf extract (In submission)

Rafiq, A., Z. M., & Ali, N. (2021) Evaluation of anticancer and cytotoxic ability of Biogenic silver nanoparticles (In submission)

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Aftab Rafiq

Abstract

Nanotechnology is an emerging field of research with numerous roles in science and technology. It is used specifically for designing new agents and development of nanoparticles (NPs) with unique characteristics and broad-spectrum antibacterial activities. Nano-materials have diversified improved chemical composition and properties exhibiting a wide range of applications like agriculture, food processing and healthcare techniques. Nanotechnology is the fabrication of materials and devices under a 100 nm length scale. It is interrelated with the principle of chemistry, physics and engineering, which involves the processing, manufacturing and their applications at the nanometer scale. Currently, nanoparticles are gaining interest to address antibiotic resistance-related concerns as they can be used as a better alternative antimicrobial agent. Conventional antibiotics have been failed to treat infectious diseases due to the emergence of multidrug resistance (MDR) in some common pathogens. The current study aimed to formulate new antimicrobials from greener sources. In the midst of these efforts, nanotechnology is a newly emerged field, in which the synthesis of new nanoparticles through novel and efficient means is on the rise. The current work has been carried out to assess the potential of bacterial and plant species in the biosynthesis of silver nanoparticles. Cell-free culture supernatant of Bacteria i-e *P. aeruginosa* and leaf extracts of plants including *Fraxinus xanthoxyloides* (FX) and *Bischofia j avanica* (BJ) were used for biosynthesis of silver nanoparticles. This method is an economical and simple one-step approach to synthesize AgNPs. Characterization of Biogenic Silver nanoparticles (B-AgNPs) has been done by UV–Visible spectroscopy, X-ray diffraction (XRD), transmission electronic microscope (TEM) and Fourier transform Infrared spectroscopy (FT–IR).The formation of silver nanoparticles by *P. ae ruginosa* (PA-AgNPs) and FX–AgNPs has confirmed through UV–Visible spectroscopy (at 430 nm and For BJ-AgNPs at 422nm) by change of colour owing to surface Plasmon resonance. Based on the XRD pattern, the crystalline property of B-AgNPs has established. Functional groups existing in *F. xanthoxyloides*, *B. javanica* leaf extract and Cell-free supernatant of P. aeruginosa is confirmed by FT-IR spectrum. TEM authenticated morphology of the

AgNPs. Comparatively Silver nanoparticles synthesized from *F. xanthoxyloides* were better in terms of nm as compared to Silver nanoparticles synthesized from *B. javanica* which have an average size of 10±2.5 nm while Silver nanoparticles synthesized from *P. aeruginosa* (PA-AgNPs) have an average size of 20±9nm. The newly synthesized nanoparticles were evaluated for their antimicrobial potential. Minimum inhibitory concentration was determined against *Escherichia co li,* Methicillin-resistant *Staphylococcus aur eus* (MRSA) strains, *Pseudomonas ae ruginosa, Candida al bicans an d K . pneumoniae* by microtiter plate assay and well diffusion method. The lowest inhibition (69%) observed against *MRSA* was at a concentration of 50 ppm FX-AgNPs and maximum inhibition (81%) observed was against *P .aeruginosa*. For PA-AgNPs highest susceptibility was observed in the case of *K. pnemoniae* which exhibited a zone of inhibition 15mm against 300 and 400 ppm concentration. For BJ-AgNPs, the minimum zone of inhibition (6mm), was recorded for 100ppm of *C. albicans* for the highest concentration of *K. pneumoniae* (300ppm) 13mm zone of inhibition was recorded. Gram-negative bacteria were more susceptible toward AgNPs as compared to Gram-positive bacteria. Synergistic behaviour of AgNPs + Ciprofloxacin and AgNPs + Ampicillin were also observed. The biosynthesized AgNPs triggered an 86.6% drop in the biofilm formation of *P. aeruginosa* as compared to the control, while chemically synthesized nanoparticles (C-AgNPs) showed a 70.87 drop. Metabolic activity of *P. aeruginosa* was investigated by XTT assay, Cells exposed to nanoparticles produced fewer amounts of reductase enzyme and displayed compromised metabolic activity by 62%. The hemolysis test measures the lysis of the red blood cells when exposed to an environmental agent. As the results indicate lysis was dose-dependent but still far less as compared to control. The PsF membranes were fabricated with different concentration of silver and utilized for the anti-biofouling study. Membranes were characterized by the FTIR, and SEM. At the concentration of 1.66 weights % highest reduction is observed against all the MDR strains. Membrane effectiveness for the long run was observed under the two conditions i.e. shaking and static under the time period of 7, 14 and 21 days. Overall results showed that the silver nanoparticles impregnated Psf membrane did not allow dense growth of biofilm as compared to Plain Psf membrane. The biogenic silver nanoparticles exhibit

exceptional anticancer activity against the HeLa cell line and showed a reduction in cellular proliferation at 25 µM, further increase in concentration proved cytotoxic. In another study, we have developed hydrogels their antimicrobial and wound healing potential was evaluated through in-vivo studies. Silver nanoparticles coated hydrogel showed effective bactericidal activity against gram negative and gram positive bacteria, In rat models it could be clearly observed that chitosan has enhanced the wound healing and eliminated the risk of skin contraction or scar formation after healing, which could be clearly seen in control or sham group. Furthermore silver nanoparticles loaded hydrogels completely eliminated the bacterial infection in infected group and reduces the risk of sepsis leading to death which is the major issue in septic wounds. This study imparts a useful insight into the development of a new antimicrobial agent from a novel source

Introduction

1. Introduction

Nanotechnology is an emerging field of research, with numerous roles in science and technology, used specifically for designing new agents with desired characteristics and broad spectrum antibacterial activities (Sánchez-López et al. 2020). It is interrelated with principle of chemistry, physics and engineering, which involves the processing , manufacturing and their applications at nanometre scale. Nano-materials have diversified and improved chemical composition and properties exhibiting wide range of applications like agriculture, food processing and healthcare techniques (Sirelkhatim et al. 2015). Currently, nanoparticles are gaining interest to address the antibiotic resistance related concerns as they can be used as better alternative antimicrobial agent (Beyth et al. 2015).

1.1. Characteristics of Nanoparticles

Nanotechnology is basically synthesis of materials at nano-scale (1-100nm) and their characterization followed by application (Mansoori and Soelaiman 2005). When materials are scaled down to atomic level, their surface area is increased as compared with large materials (Huh and Kwon 2011). Unlike their bulk materials, nanoparticles possess significantly different size-related characteristics (Buzea et al. 2007). Due to smaller size, nanoparticles have bigger surface area than their counterparts. This discrete property is also a reason behind their probable applications in bio-nanotechnology, biosensors and Nano-medicine (Ashe 2011).

1.2. Synthesis of nanoparticles

There are several methods for preparation of nanomaterials that include physico-chemical methods and hydrothermal methods. Physico-chemical method include sole gel synthesis (Dercz et al. 2008), aqueous precipitation, thermal decomposition (Callister and Rethwisch 2011) and hydrothermal methods (Suciu et al. 2006) which requires very high temperatures. In comparison, a biological synthesis of nanoparticles is low cost procedures, more beneficent and easily attainable under mild conditions. Synthesising nanoparticles by chemical and physical method can cause adverse effects on environment and consume more energy. (Parveen et al. 2016; Sathishkumar et al. 2016; Khan and Lee 2020). The continuous demand for the reliable, biocompatible and ecofriendly processes to synthesize NPs have attracted more researchers to exploit biological systems.

Biogenic method for synthesizing nanoparticles provides more ecofriendly, biocompatible and less cytotoxic way (Logeswari et al. 2013; Verma and Mehata 2016; Khan et al. 2020) and also are more economical and can produce nanoparticles in bulk (Mittal et al. 2013)

A search for an ecologically viable synthesis procedure has led to a few biomimetic approaches. It is well known that the biological systems (especially microbes and plants) can uptake number of metals or metal-containing compounds and convert them into less toxic forms through bioremediation (Saratale et al. 2018). Based upon this strategy, up till now, various microbes from bacteria to fungi have been investigated to fabricate inorganic nanomaterial's both intra as well as extracellular ways and thus can act as potential ecofriendly bio Nano factories. Most metal ions

have proven toxic to bacteria, so a defensive mechanism formed by bacteria to combat such toxicity is the reduction of ions or the creation of water insoluble complexes (Hassan 2018). Enzymatic reactions by microorganisms are responsible for the degradation of harmful reducing products that are formed during the synthesis of nanoparticles (Bloch et al. 2021).

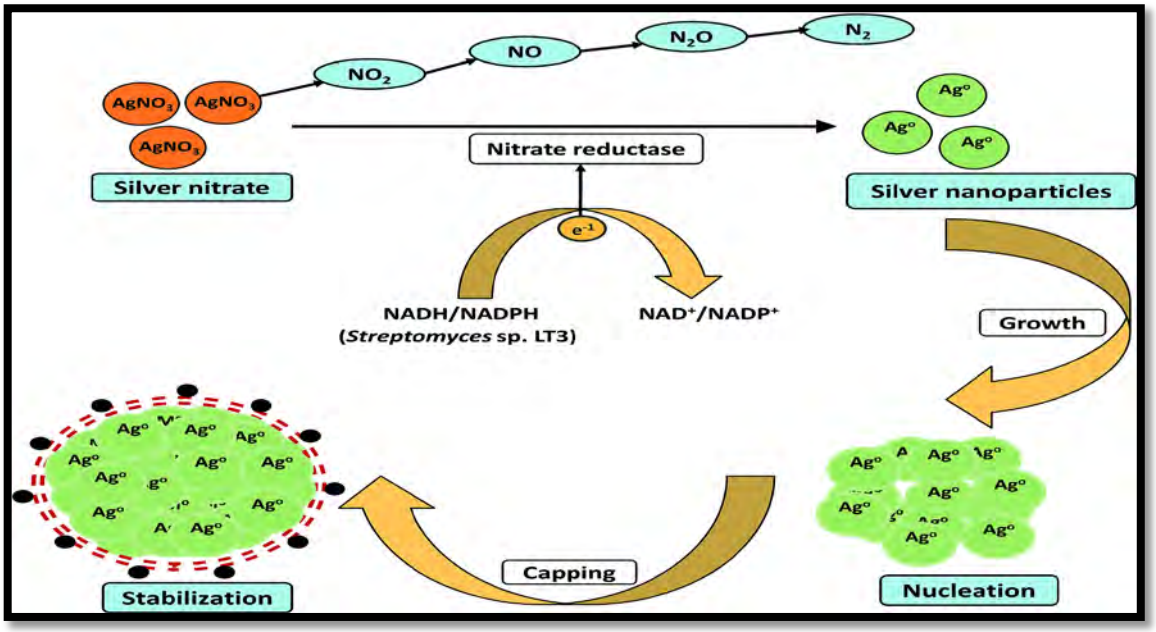


Figure1.1Proposed schematic bio reduction mechanism of the Nano scale particles and stabilization by nitrate reductase enzyme (Gahlawat & Choudhury, 2019)

Similar study was reported in Synthesis of silver (Ag) Nanoparticles from *Pseudomonas* (Kumar and Mamidyala 2011; Shivaji et al. 2011; Thamilselvi and Radha 2013; Baker et al. 2015).

It was already established that lot of active biomolecules are secreted by microorganisms which have played promising roles in reducing and capping NPs during biosynthesis (Singh et al. 2020) During present study the successful fabrication of AgNPs from cell free culture supernatant of *Pseudomonas aeruginosa*

was reported.

Despite of all the ecological benefits, process of synthesis and conversion of metal into nanoparticles by microbes is slow as it can take several days to synthesize nanomaterial.in comparison to that green synthesis by plant is time saving quick procedure as conversion of metal in nanoparticles requires only few hours.

Many plants such as *Luffa ac utangula* (Taruna et al. 2016), *Bauhiniatomentosa* (Ramar et al. 2018), *Theobroma cacao* (Thatikayala et al. 2019), *Aloe vera* (Ahmadi et al. 2018), and *Bridelia retusa* (Vinayagam et al. 2018) have shown potential to reduce silver nitrate and form AgNPs.

Current study also reports first ever synthesis of silver nanoparticles through the reduction of aqueous Ag with *Fraxinus xanthoxyloides* and *bischofia j avanoca* leaf extract. *Fraxinus* plant has been conventionally utilized for the treatment of malarial and pneumonia infections. A list of chemical components has been extracted from *Fraxinus* plant comprising of secoiridoids, phenyl ethanoids, lignans, flavonoids, and coumarins. Certain biologically active ingredients were obtained from *Fraxinus* plant i.e. Catechin Fraxetin, Esculetin, Syringin, Oleoside 11methyl ester, Calceolarioside B, Tannic acid and Quercetin Rutin (Sarfraz et al. 2017).It is also stated that methanol extract of *F. xanthoxyloides* leaves possess anti-inflammatory, analgesic (Younis et al. 2016b), anti-leishmanial (Younis et al. 2016c) and hepatoprotective capabilities (Younis et al. 2016a). *Bischofia javanica* is an edible plant natural inhabitant of South Asian countries specially India and Pakistan. The seeds, bark, leaves and roots of *Bischofia* plant has been conventionally utilized as a source of food treatment option for sores, tooth ache and eye infections (Panda et al. 2018). A list of chemical

components has been extracted from *Bischofia* comprising of secoiridoids, phenyl ethanoids, lignans, flavonoids, and coumarins. (Indra et al. 2013) These plants were never used before for production of silver nanoparticles.

1.3. **Antimicrobial potential of nanoparticles**

Recent developments in nanotechnology has encouraged the potential applications of NPs against infectious diseases by exploring their antibacterial mechanisms (Huh and Kwon 2011). For instance, nanoparticles tend to modify the bacterial metabolic activity starting from contact with bacterial surface and ultimately end with cell death (Chatzimitakos and Stalikas 2016). This feature of NPs has prompted their use to cure infectious diseases. Nanoparticles (NPs) interact with bacterial surface through different electrostatic interactions (Li et al. 2015a), such as hydrophobic interactions (Luan and Zhou 2016), receptor ligands (Gao et al. 2014) and van der Waals forces (Armentano et al. 2014). NPs cross bacterial membrane and manipulate the metabolic pathways of cell by altering the structural physiology of cell. Afterward, nanoparticles trigger cell death by binding themselves to ribosomes, lysozymes, DNA and enzymes intern inhibit enzymes, causes electrolyte imbalance, deactivation of protein and altered permeability of membranes (Shrivastava et al. 2007; Yang et al. 2009; Xu et al. 2016)

In cytotoxic studies, reactive oxygen species are considered to have an active role in both in vitro and in vivo studies(Nathan and Cunningham-Bussel 2013). It causes serious oxidative stress and damages macromolecules, induces lipid peroxidation, and nucleic acid damage. Such as ZnO NPs generate H_2O_2 and OH in contrast to calcium oxide and magnesium oxide NPs which can produce only O instead of H_2O_2. Similarly, CuO NPs can generate all above mentioned types of ROS. It has been revealed that

AgNPs inhibit biofilm by hindering the colonization of bacteria (Thomas et al. 2015).other nanoparticles have also shown biofilm inhibitory capabilities (Yu et al. 2016), Ag-based NPs (Markowska et al. 2013), Mg-based NPs (Lellouche et al. 2012b), NO NPs (Hetrick et al. 2009; Slomberg et al. 2013), ZnO NPs (Hajipour et al. 2012), CuO NPs (Miao et al. 2016), Fe_3O_4 NPs (Chifiriuc et al. 2012), and YF NPs (Lellouche et al. 2012a).

1.4. Cytotoxicity of nanoparticles

Nanoparticles could have cytotoxicity's at certain cellular, molecular and tissue level. As nanoparticles circulate through the body they come in contact with various biological moieties such as cellular organelles, cytoplasm, extracellular matrix and blood. Consequently, the interaction of nanoparticles with biological components at submicron level may influence the biological functions of cell. It has been suggested that certain nanoparticles are prone to elicit a toxic response based upon their morphological features (Johnston et al. 2010) and size (Pan et al. 2007). Moreover, metal nanoparticles have also manifested cytotoxic activity in previous studies. For instance, it has been reported that silver and iron oxide nanoparticles have harmful impact both in vivo and in vitro, primarily due to the production of ROS. Moreover, dose dependency is also related with cytotoxicity (Hansen and Baun 2012). However, characteristics of nanoparticles which decide the inherent hazards of NPs are still unknown.

Current study reported successful biogenic fabrication of silver nanoparticles by using bacterial cell free cultures supernatant and plant leaf extract, after characterization, synthesized nanomaterials were evaluated for their antimicrobial and

cytotoxic potential. Plants used in this study were never been reported before for silver nanoparticles synthesis.

1.5. Aims and Objectives

The study was designed to synthesize silver nanoparticles by biogenic approach under controlled laboratory conditions. Later, antimicrobial potential of synthesized silver nanoparticles was assessed (with and without antibiotics) and also as antibiofouling agent. Furthermore, nanomaterials were also evaluated for potential anti-cancer and cytotoxic properties and their role as anti-infective and wound healing agent

The research objectives are summarized below

- Biogenic fabrication of silver nanoparticles by
    1. Cell free culture supernatant of *Pseudomonas aeruginosa.*
    2. From leaf extracts of *Bischofia javanica* and *Fraxinus xanthoxyloides.*
- Characterization of nanoparticles by UV-Vis spectrometry, X-rays Diffraction (XRD). Fourier-transform infrared spectroscopy (FTIR) and Transmission electron microscopy (TEM) to confirm size, crystalline nature, stability and morphology
- Evaluation of minimum inhibitory concentration (MIC) of nanoparticles against selected bacterial and fungal strains by microtiter plate assay and well diffusion method.
- Estimation of synergistic antimicrobial effects of nanoparticles in combination of antibiotics by checkerboard micro titration method
- Assessment of anti-biofilm activity of nanoparticles against *P. aeruginosa* by microtiter plate assay (MPA).

- Determination of metabolic and extra polymeric substance (EPS) activity of nanoparticles treated *P. aeruginosa* through microtiter plate assay (MPA)
- Investigation of role of AgNPs as antibiofouling agent in water purification filter membrane
- Determination of cytotoxic and anticancer activity of AgNPs on HeLa cell line by flow cytometry.
- Evaluation of Silver nanoparticles loaded Chitosan hydrogel as anti-infective and wound healing agent in Rat model

Review of Literature

2.1. Nanoparticles-An Introduction

Nanotechnology is manipulation of materials at Nano scale of about 1-100 nm (Hasan 2015). It has become promising field of science which is capable of providing various novel applications in almost all aspects of life. Nanoparticles are the basic components of nanotechnology. Reducing the size particles to Nano-scale can modify their chemical biological and physical properties compared with their precursors. These properties are due to their improved mechanical strength, enhanced reactivity and large surface area to volume (Balbus et al. 2007). Because of these intrinsic properties, nanoparticles are at the foremost edge of rapidly emerging field of nanotechnology. A nanoparticle had various dimensions depending upon the parameters such as length, breadth and height (Cho et al. 2013). For example zero dimensional where at single point all the parameters are fixed e.g. Nano dots, one dimensional with one parameter such as nanowire and nanotubes, two dimensional having two parameters such as breadth and length e.g. graphene and three dimensional owning all three parameters for example gold nanoparticles. The nanoparticles can be flat, spherical, conical, cylindrical, tubular, spiral, core and hollow characterized by their size, shape and structure. Moreover, they are made up of three layers (a) The core, which is the central part of nanoparticle; (b) The surface layer, containing metal ions, small molecules, polymers and surfactants; (c) The shell layer (Shin et al. 2016)

2.2. Classification of Nanoparticles

The complex characteristics of nanoparticles like morphology, physical and chemical structure, agglomeration, shapes can be used to classify nanoparticles. In terms of dimension, particles can be categorized as Dimensionality relates to its form or morphology, which can be classified as one-dimensional, two-dimensional, three-dimensional (Ealia and Saravanakumar 2017) Nanoparticles can be obtained from natural or anthropogenic sources like designed or undesirable/incidental NPs. It is possible to categorize into two types (a) Natural and (b) Engineered nanoparticles (Van Broekhuizen et al. 2012) Also, the process of biodegradation and bio mineralization, occurring naturally in the universe can synthesize nanoparticles (De Castro and Mitchell 2002) Naturally occurring process including forest fires, strokes of the marine wave, erosion, volcanic eruption physical and chemical rock weathering, friction also results in nanoparticle formation. Human activities like automotive exhaust, burning of fossil fuel, fumes from welding operation, and industrial effluents can result in unintentional production of Nanoparticles.

Nowadays, mostly metals and carbon based particles are designed for benefit of mankind. Although standard techniques can chemically synthesize NPs, biosynthesis avoids pollution in the atmosphere. It is possible to control the shape and size of NPs and to produce a required form of NP by regulating the medium's temperature and concentration. It is possible to classify engineered NPs into primarily carbon-based NPs, inorganic NPs and organic NPs. Ecofriendly approach using plants extracts results in formation of nanoparticles with fine size and shape. The enormous variety of

engineered NPs resulting from their multiple characteristics such as chemical nature, shape, dispersion state, dispersion medium and surface changes contribute to making this a significant active science field. The NPs are generally intended with surface changes that are tailored in accordance to particular applications. The structure, dimension, and morphology of engineered NPs allow their use in a multitude of fields such as the science of electronic, biomedical, pharmaceutical, cosmetic, power, environmental, catalysis, and equipment. Because nanotechnology is an innovative and science development sector with exponential manufacturing, more data is required about the environmental effects of these nanomaterials (NMs). Therefore, a study on NPs as contaminants is a new environment health field (Remédios et al. 2012)

2.3. SILVER NANOPARTICLES (AgNPs)

Silver is a lustrous smooth white water insoluble element, but salts of silver like nitrate and chloride are water soluble. Metallic silver in a finely distributed form shows distinctive characteristics similar to noble metals like chemical stability, outstanding electrical conductivity, and catalytic activity along with other more precise properties such as bacteriostatic and bactericidal. Nano-sized metal particles display properties that are different from both ion and bulk material imparting higher catalytic activity due to active facet morphologies (Yacamán et al. 2001). The ease of synthesis makes AgNPs more unique. Their applications in technological world, material science, biomedical field make them an excellent research candidate and scientists around the world are conducting more research on AgNPs due to their extensive applications in different areas (Safaepour et al. 2009) In the medical and environmental field,

nanomaterials especially Nano silver are regularly used as an antimicrobial and anticancer agent.

2.4. PRODUCTION OF SILVER NANOPARTICLES

NPs can be manufactured using different techniques, including biological, physical, chemical and other electrochemical, and sonolytic techniques (Zakir et al.). AgNPs can be synthesized through both top-down approaches (physical) and bottom-up approaches (chemical processes and green pathways) (Prabhu and Poulose 2012) It is possible to divide these techniques of synthesis into intra and extracellular. It would be more economical to synthesize extracellular NPs using plant leaf extracts rather than whole crops due to easier downstream processing(Annamalai et al. 2011).

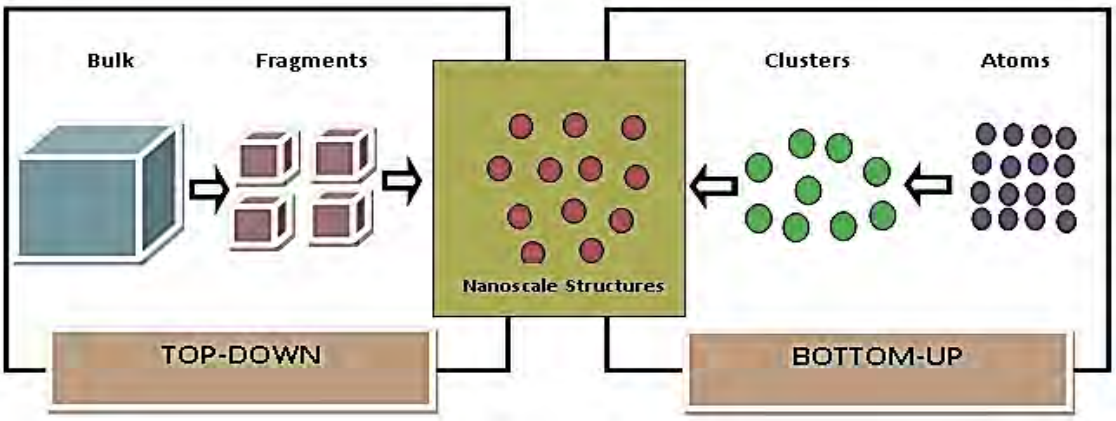


Figure2. 1Approaches for silver nanoparticle synthesis

2.4.1. NEED AND SIGNIFICANCE OF AgNPs BIOSYNTHESIS

There are different methods of preparation of nanoparticles that include physico-chemical methods and hydrothermal methods. Physico-chemical method include sole gel synthesis (Dercz et al. 2008), aqueous precipitation m thermal decomposition (Callister and Rethwisch 2011) and hydrothermal methods (Suciu et al. 2006) requires very high temperatures, on the contrary biological synthesis skips most of costly procedures with low cost, more beneficent and easily attainable mild conditions ,proceeds the synthesis beneficially at low cost in mild condition Production and assemblage of nanoparticles via chemical or physical means have substantial adverse effect on the environment as major of their impurities are hard to decontaminate and often consume more energy (Parveen et al. 2016; Sathishkumar et al. 2016; Khan and Lee 2020) Biological techniques for the synthesis of NPs were then tried and demonstrated to be a boost to the nanotechnology sector. Biological systems including bacteria, fungi, actinomycetes, algae, crops, etc. have been examined for their NP production ability. Produced NPs have a longer shelf life and stability as natural capping occurs. The continuous demand for the development of clean, reliable, biocompatible and benign processes to synthesize NPs have attracted more and more researchers to exploit biological systems. Nanoparticle created by biogenic method is the most reasonable option because of their eco-friendliness, biocompatibility, and reduced toxicity (Logeswari et al. 2013; Verma and Mehata 2016; Khan et al. 2020). The biological synthetic method is also economical, ecologically-sound, and easily can be produced in bulk amount (Mittal et al. 2013).

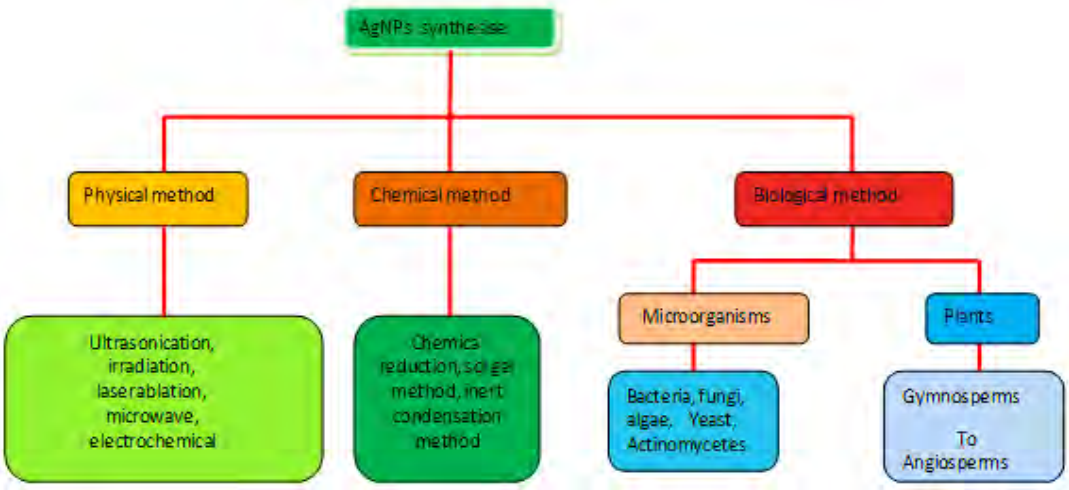


Figure2. 2. Schematic representation of different methods of AgNPs synthesis

2.5. Biosynthesis Of Nanoparticles

Despite their effectiveness, synthesis of nanoparticles by physical and chemical methods have certain drawbacks, including the increased production costs, release of unfriendly by-products, duration of synthesis, higher temperature requirement (Nagajyothi and Lee 2011). Global warming and climate change have contributed to a worldwide understanding of eliminating toxic and hazardous waste products, thereby aggressively raising development in the fields of science and industry through the green synthesis path. (Kathiraven et al. 2015) the name indicates aid in the synthesis of very complex reactions within a fraction of m inutes, nanoparticles biosynthesis has now drawn attention to the need for environment ally benign material science technologies in synthesis grievance (Tarannum and Gautam 2019) The biogenic method for the synthesis of silver nanoparticles can prove to best alternative and clean approach. It includes plant extract (leaf, bark and root) cell

free culture supernatant of microorganisms and this route has proven to be nontoxic, ecofriendly, and economical (Mittal et al. 2013) Enzymes from living organism can catalyze chemical reactions and act as strong reducing agent.Because of their presentprospect, the biological system has acquired commercial significance. (Bloch et al. 2021)

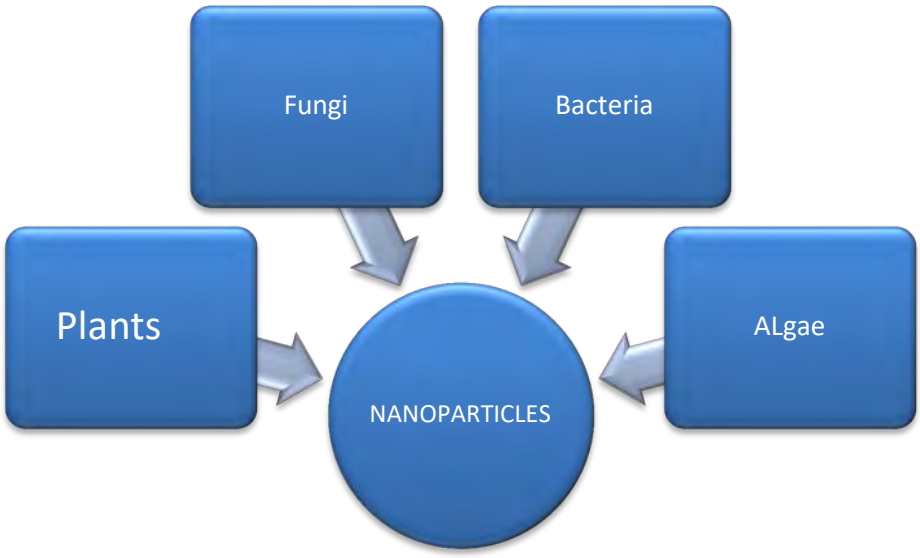


Figure2. 3.Different routes for biosynthesis of nanoparticles

2.5.1. Silver nanoparticles-Synthesizing Fungi

In contrast to bacteria, greater quantities of nanoparticles can yield from fungi as amount of proteins they can secret is much more in turn means more fabrication of nanoparticles (Rana, 2008). (Govindappa et al. 2020) the reduction of silver ion to silver is thought to be facilitated by extracellular enzymes anthraquinones. it is believed that for formation of nanoparticle, the NADPH dependent nitrate reductase and a shuttle quinine extracellular process are involved (Birla et al. 2013) However the precise mechanism of silver nanoparticle fabrication by fungi is not entirely understood

it is supposed that the above stated mechanism is responsible for the method. A foremost disadvantage of microbes used for silver nanoparticles synthesis is duration as compared to plant extracts.

2.5.2. Silver nanoparticles-Synthesizing Algae

Several algal species revealed extracellular synthesis of AgNPs. Extract of *Chlorella vulgaris* unicellular green algae is used at room temperature to synthesize NPs (Rajkumar et al. 2021) Also studied for synthesizing AgNPs using red marine seaweed,(de Aragao et al. 2019) and Sargassum polycystum (Palanisamy et al. 2017) extract from algae are reported to have Carboxyl groups in aspartic and/or glutamine clusters and hydroxyl groups in protein tyrosine clusters are responsible for the reduction of metal ions (Płaza et al. 2014)

2.5.3. AgNPs Synthesis From Bacteria

Production nanoparticles with the help of microbes such as fungi, green algae, bacterium, and viruses have been reported. These microorganisms are famously said to be as bio-nano factories Utilization of bio-nano factories to produce nanoparticles is highly recommended because from this approach nanoparticles formed are sustainable, eco-friendly, and inexpensive. Enzymatic reactions by these bio-nano factories are responsible for the degradation of harmful reducing products that are formed during the synthesis of nanoparticles. The fabrication of silver with the help *of Pseudomonas* is also reported in previous researches (Kumar and Mamidyala 2011; Shivaji et al. 2011; Thamilselvi and Radha 2013; Baker et al. 2015). Many microorganisms can be used to synthesize metal nanoparticles. For example gold ions were reduced to gold nanoparticles with size range of 5–25 nm by incubating gold salt inside the *Bacillus*

subtilis cells (Alsamhary 2020) Another bacterial strain Lactobacillus, commonly found in buttermilk was used to synthesize silver nanoparticles (Rajoka et al. 2020) Synthesis of metallic nanoparticles of silver using *Klebseilla pneumonia, Escherichia coli* and *Enterobacter cloacae* as cultural supernatants has been reported. Most metal ions have a toxic effect on bacteria, so a defensive mechanism formed by bacteria to combat such toxicity is the reduction of ions or the creation of water insoluble complexes(Hassan 2018) It is usually thought that the bio reduction process are performed by the enzymes but few studies have revealed inconsistent outcomes. The presence of alpha NADP eliminates the downstream processing step consequently through the reduction, nitrate is converted into nitrite and the electron is transferred to the silver ion and reduced to silver. (Tan et al. 2021)

2.5.4. AgNPs Synthesis From Plants

In the genesis of NPs, plants are regarded to be green nanofactories. The synthesis of plant-based AgNPs has been under the focus of most researchers as well as biologists, chemists and technicians. Plant extracts are readily accessible, secure and non-toxic, contain of metabolites that can reduce silver ions in less time than microbes. The growing interest in cost, time, waste minimization and the implementation of sustainable processes to develop environmentally friendly and easy techniques for AgNPs manufacturing lead to the implementation of photobiological approaches (Acay and BARAN 2019) Nanoparticle created from plants extracts is the most reasonable option because of their eco-friendliness, biocompatibility, and reduced toxicity (Logeswari et al. 2013; Verma and Mehata 2016; Khan et al. 2020). The biological synthetic method is also economical, ecologically-sound, and easily can be produced in

bulk amount (Mittal et al. 2013). Many plants such as *Luffa acutangula* (Taruna et al. 2016),*Bauhiniatomentosa* (Ramar et al. 2018), *Theobroma cacao* (Thatikayala et al. 2019), *Aloe vera* (Ahmadi et al. 2018), and *Bridelia retusa* (Vinayagam et al. 2018) have shown potential to reduce silver nitrate and form AgNPs. In search of broad activities, efficiency, cost reduction and eco friendliness, researchers are continuously investigating new plants for the production of novel nanoparticles.

Initially, whole plants were used in metal NPs ' green synthesis. Use of isolated pure Phyto-compounds such as cellulose(Li et al. 2015b)and glucose has been reported (Tang et al. 2013) (Baker et al., 2013) investigated the role of *Brassica juncea* germinating seeds in the successful green synthesis of Ag-Au-Cu alloy. A cost-effective and environmentally friendly method for AgNPs green synthesis from $AgNO_3$ solution using papaya fruit extract as both a reduction and a capping agent was created and the synthesized NPs discovered to be extremely toxic to pathogenic organisms (Balavijayalakshmi and Ramalakshmi 2017).

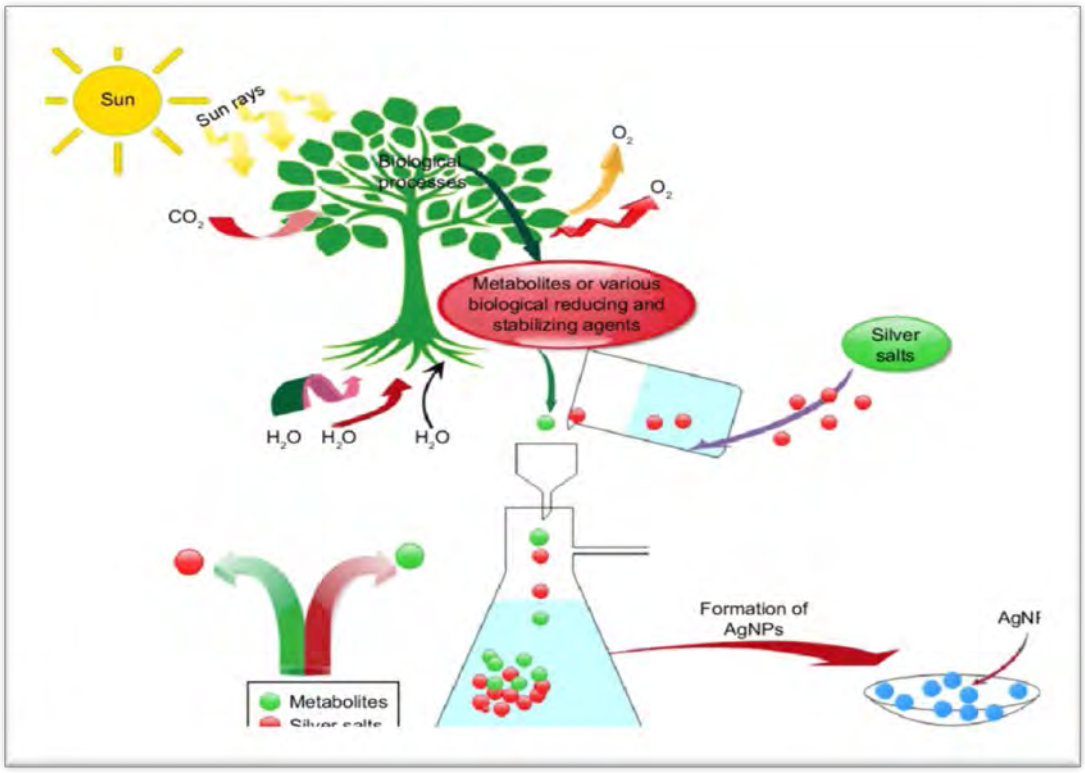


Figure2.4.Graphical representation of silver nanoparticles synthesis by plants(Khan et al. 2018)

Green synthesis includes the biomass of plants for reduction of silver ions to AgNPs. AgNPs production was intended to happen with functional groups and silver salts ionic interactions. Solvent medium, reduction, capping and stabilization agents are the main parameters of AgNPs formation (Khan et al. 2020) The aqueous extract is regarded as more environmentally friendly than organic extracts and discovered to be more suitable in AgNPs ' green synthesis. Almost all plants (herbs, shrubs, or trees) containing latex, flavonoids, phenols, alcohols, and proteins are known to be able to generate AgNPs from silver salts. The nature of plant extract affects the type of NPs that are synthesized with the plant extract source being the most important factor influencing synthesized NP morphology (Pirtarighat et al. 2019)

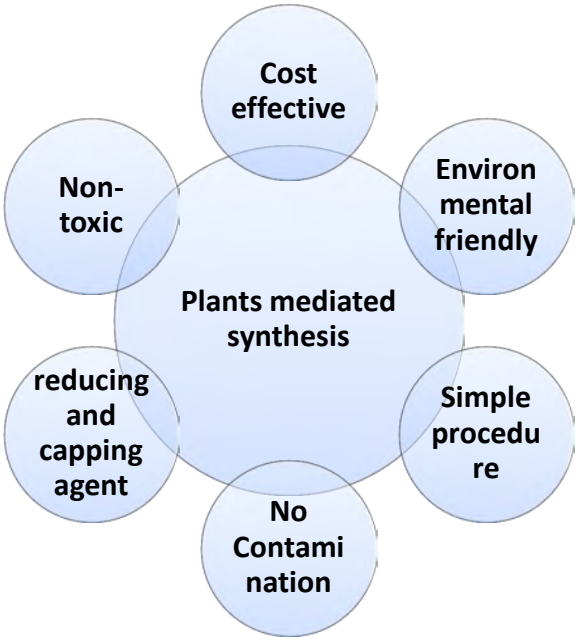


Figure2.5. Advantages of Plant mediated synthesis of AgNPs by plant extracts

2.5.4.1. The Role of Plant Biomolecules in AgNPs ' Green Synthesis

Plants produce certain metabolites that can act as reducing and stabilizing agents including proteins, amino acid and simple and complex sugars like glucose and starch (Ahn et al. 2008) (Makarov et al. 2014) studied the effectiveness of sugars as reducing agents in the synthesis of metal NPs even, green and black tea extracts contain both a reductant and a surfactant, Some studies have reported use a variety of tea extracts like *C. sinensis* (Deepika et al. 2013). Turmeric is commonly used as a cosmetic and spice. It is now a global famous medicinal plant, curcumin, the primary element of turmeric acts as an anti-oxidant, anti-bacterial, anti-fungal, anti-parasitic, anti-inflammatory, anti-mutagenic, anti-carcinogenic and detox characteristics medicine (Akamine et al. 2007)Ginger is renowned for its medicinal values such as ginger for the treatment of skin diseases, colorectal cancer, arthritis, heart disease and antibacterial characteristics.

Volatile oil (zingiberene, zingiberol, D-camphor), shogaols, diarylheptanoids, gingerols, paradol, zerumbone, 1-dehydro-(10) gingerdione, terpenoids, and ginger flavonoids are the main active ingredients of ginger(Kipyegon 2020). The use of raspberry extract as a source of ellagic acid, as it has powerful antioxidant impacts comparable to many other polyphenols, provides the option of direct silver ion reduction. The existence of other organic compounds from the polyphenols, tannins and flavonols group makes it possible to stabilize developing suspensions without additional variables inhibiting the development of metallic silver agglomerates(Viskelis et al. 2012) Being the biggest polyphenol in C.aromaticus, rosmarinic acid was more likely to be responsible for most of the antioxidant activity observed, with the possibility of reducing silver ions (Ag+) to AgNPs (Ag0) reported (Selvan et al. 2018).

A wealthy source of flavonoids and phenolic acid derivatives is Dioscoreabulbifera (*D. bulbifera*) tuber. Flavonoids play a key role in the NP synthesis reduction process. Consequently, in the phytochemical analysis, Dioscoreabulbifera tuber extract, a wealthy source of flavonoids and phenolic acid derivatives, highly promotes Ag+ to Ag^0 bio reduction. Pulicariaglutinosa plant extract includes numerous phenolic compounds, known to play a crucial role in silver ion decrease. Curry leaf has recently been discovered to be a powerful antioxidant owing to elevated carbazole levels, a water-soluble heterocyclic compound, plausibly responsible for metal ion decrease and stability.(Kora and Arunachalam 2012) verified that the oxidation in Astragalusgummifer aqueous extract of the hydroxyl and carbonyl groups involved in the decrease of silver ions. There are several variables such as pigments, enzymes, alkaloids, polyphenol found in plant extract are involve in synthesis of silver

nanoparticles. These molecules reduce the silver ion to neutral silver and other molecules surround the aggregated silver atoms to form stable nanoparticles (Ganaie et al. 2014) The wealthy source of metabolites with negatively loaded functional groups may be accountable for reducing metal ions and stabilizing synthesized NPs efficiently under natural circumstances(Banerjee and Nath 2015)

2.6. Nanoparticles-Biomedical Aspect

With the emergence of nanotechnology, nanoparticles have been gaining much interest in biomedical applications with the concern of significant improvement in diagnosis and treatment of diseases.

The distinctive properties such as their potential to absorb and carry other compounds, the surface to mass ratio and their quantum properties make them significantly attractive in biomedical applications. Moreover, they have relatively huge surface area to bind and carry other compounds such as proteins, drugs and probes. Due to their small size, nanoparticles can circulate throughout the body and bind with the specific cells as well. This feature has allowed the development of enhanced imaging of organs and diseased tissues in body and brings a revolution in diagnostics. Magnetic nanoparticles are not only used as an alternative to radioactive technetium to detect spread of cancer cells in body along with lymph nodes but also has the ability to kill tumors via hyperthermia. In addition, nanoparticles have the ability to increase the fluorescent imaging along with the enhanced images in ultrasound and positron emission tomography (PET).Their miscellaneous applications in medicine includes neutraceutical, both in vitro and in vivo diagnostic, drug delivery and production of

better biocompatible materials (Duncan 2003; de Jong et al. 2005; Ferrari 2005) Recently, with advancement in drug delivery, they are considered as promising treatment of neurological disorders including Alzheimer disease, Parkinson disease and multiple sclerosis. Nanoparticles and Nano fibers have their applications in tissue repair therapies as they have significant role in design and manufacture of innovative framework for bone and tissue repair. Only biocompatible nanostructures such as calcium hydroxide can be used for therapeutic purpose. Also, nanoparticles play their role in the progression of health-related products such as a sunscreen; Optisol was designed with titanium dioxide nanoparticles along with manganese to block the ultraviolet rays on skin. Although, nanoparticles are likely to have improved diagnostic and treatment approaches in medicine but they also have significant impact on human health that needs to be considered regarding their biomedical applications in future. For example, very few studies were conducted about the effect of nanoparticles after they get entered in our body or whether they have any harmful effects on our body. The concerns related to the effectiveness and safety of nanoparticles can be resolved by clinical trials. Nanoparticles required for medical applications should be manufactured under sterile conditions.

2.6.1. Nanoparticles as an Antimicrobial Agent

Nanoparticles are highly auspicious particles and gaining interest to address the concerns related to the antibiotic resistance as they can be used as better alternative antimicrobial agent. Antimicrobial Nano-materials have diversified and improved chemical composition and properties. This is the reason behind their several modes of

actions. Besides, sensitivity of bacteria to nanoparticles is directly linked with the many bacterial factors such as their physiological state, genetics, metabolic pathways and many other components (Baek and An 2011b; Nath et al. 2013) Moreover, environmental factors including temperature, pH and aeration have significant impact on their antimicrobial activity. The physiochemical properties of particles including size, shape and concentration are also associated with the lethality of nanoparticles to bacteria (Gatoo et al. 2014). Metal nanoparticle has been revealed the most potent antimicrobial agent against conventional pathogens. Of them, silver, zinc oxide and titanium dioxide has gained much attention as therapeutic purpose in health care and industries (Loomba and Scarabelli 2013). The antimicrobial activity of silver nanoparticles was investigated by several studies against *Escherichia co li* and *Staphylococcus aureus*. It has been suggested that silver ions prompt similar morphological changes in both Gram-positive and Gram-negative organisms. The cytoplasmic membrane get detached from cell wall and deoxyribonucleic acid molecule containing region appears in the center of cell (Kim et al. 2011) The antimicrobial activity of silver nanoparticles has been studied against multi drug resistant pathogens. Among them, Methicillin resistant *Staphylococcus aur eus* (MRSA) showed greater activity than Methicillin resistant *Staphylococcus e pidermidis* (MRSE) and *Streptococcus py ogenes* (Thomas et al. 2014). *Klebsiella pne umonia* and *Salmonella typhi* showed little activity against Ag nanoparticles (Dobrucka and Długaszewska 2016). They also possess significant activity against *Salmonella e nteritidis, L isteria monocytogenes,* and *E. coli* O157:H7. In addition, there are several other studies which confirmed the catalytic activity against food borne pathogens such as *Salmonella*

typhimurium and *Staphylococcus aureus* (Tayel et al. 2011; Espitia et al. 2013). Another study on ZnO nanoparticles has suggested the disintegration and enhanced membrane permeability against *E. coli.*

2.6.1.1. Interaction of NPs with Biomolecules

There are many variations between a microorganism and a human cell, and these are at the root of several toxicity symptoms. In living organism, Proteins are synthesized in ribosomes with N-formyl methionine as the initiator codon in bacteria and methionine in human cells. There are also variations in transcription and mRNA translation. The cell architecture of living cells, on the other hand, has similarities. Protein production is identical in all aspects of life; however, proteins , nucleic acids and membrane structure of bacteria and human differ structurally (Sohaebuddin et al. 2010; Hajipour et al. 2012)

In general, because of their small size and high surface-to-mass ratio, inorganic NPs can interact with biomolecules. Due to their small scale, NPs can translocate via membrane structures in microorganisms. Inorganic NPs with a diameter of less than 100 nm have been proposed to enter cells, and inorganic NPs with a diameter of less than 40 nm have been proposed to reach the nucleus membrane and associate with DNA in humans. In addition, NP can be taken up by microorganisms in a variety of ways: phagocytosis, macro pinocytosis, or endocytosis are all terms for the same thing (Sohaebuddin et al. 2010)

2.6.1.2. Interaction with Protein

Proteins are polymer macromolecules (polypeptides) made up of one or more amino acids, each of which has a unique conformation and a different net surface charge depending on the pH. Proteins are structural and functional elements of living cells (Saptarshi et al. 2013) Proteins are involved in the movement and locomotion of cells and microorganisms, as well as catalyzed reactions for biomolecule synthesis and DNA replication processes. Proteins also make up the cell structure and the DNA containing extracellular matrix. Protein adsorption on inorganic NPs involves a number of interaction forces, including hydrogen bonds. In general, not only NP properties, but also protein interactions and the chemical environment, affect the growth of NP-protein systems (Worrall et al. 2006; Bardhan et al. 2009; Chatterjee et al. 2010)

According to reports, NPs influence protein interactions, cellular signaling, and DNA transcription by causing structural differences in adsorbed proteins, all of which are critical for enzyme and biomolecule synthesis. Furthermore, the NP surface can induce instability in the adsorbed protein molecule, rendering it vulnerable to chemical denaturation and conformational changes in the active site, which can result in enzyme activity loss. As a result, the intermediate, tertiary, and quaternary configurations of proteins would be altered by the NP surface, altering their biological role. Furthermore, morphological properties of NPs have been related to protein modifications; for example, curved surfaces favor conformational changes caused by greater flexibility and surface area over planar surfaces. However, experimental findings show that the existence of NPs plays a critical role. (Lundqvist et al. 2004; Worrall et al. 2006;

Gheshlaghi et al. 2008; Kopac et al. 2008; Bardhan et al. 2009; Chatterjee et al. 2010; Turci et al. 2010; Saptarshi et al. 2013) .

Although studies have shown that AuNPs cause conformational changes in the structure of bovine serum albumin, no significant conformational changes have been observed with carbon C60 fullerene NPs. Protein adsorption on TiO2NPs caused conformational changes and decreased tubulin polymerization, while protein adsorption on ZnONPs caused only minor conformational changes. ZnONPs also serve as a chaotropic agent. ZnONPs also act as a chaotropic agent, disrupting the hydrogen bonding network in the periplasmic region of *V. cholera* proteins.(Lundqvist et al. 2004; Kopac et al. 2008; Turci et al. 2010)

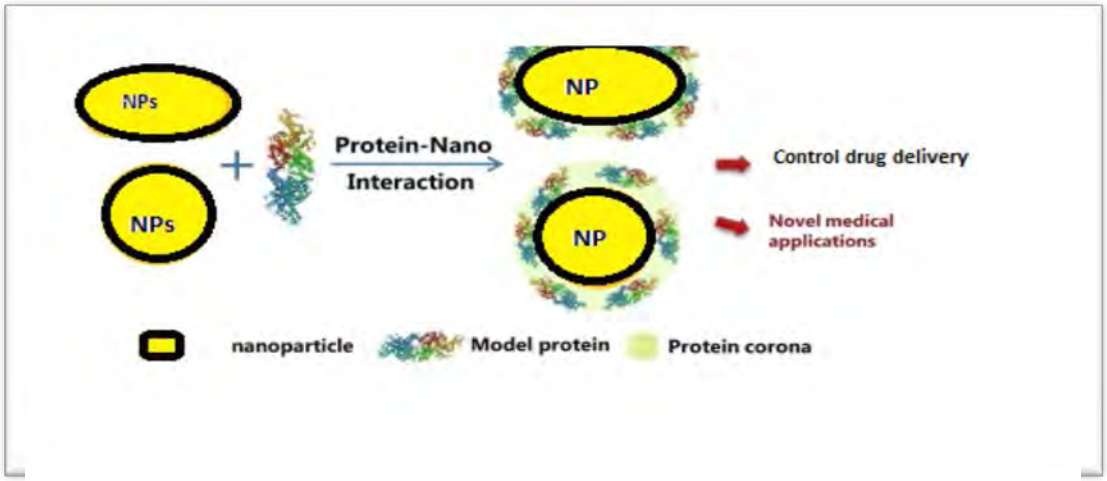


Figure2.6. Interaction of protein with nanoparticles

Nanoparticle absorption by bacterial cell is affected by physical properties like functional group, protein absorption, pH and temperature. The uptake kinetics of the same NPs varies based on the cell type, as has been discovered. Agglomeration of colloidal NPs is often caused by NP size, concentration, and surface charge, with NP size, concentration, and surface charge promoting agglomeration. NP agglomerates may have a different detectable effect on biological properties than dispersed NPs. (Oberdörster et al. 2004; Lacerda et al. 2009; Li et al. 2010b; Sohaebuddin et al. 2010; Cho et al. 2011)

2.6.1.3.Interaction With Lipid

Bacterial membranes are composed of lipid bilayer structures made up of amphiphilic lipids, which are glycerol phospholipids with two fatty acids, a phosphate group and a variable head group. While both Gram-positive and Gram-negative bacteria use plasma membranes as their main biochemical organelles, the outer membranes serve as barriers. Many NPs have been shown to interact with lipid bilayers, causing structural changes and modulating substance permeability (Roiter et

al. 2008; Karlsson et al. 2009; Lin et al. 2010; Hajipour et al. 2012; Santhosh et al. 2012; Wang et al. 2012) According to recent research, NPs may have a close interaction with cell membranes, either through adsorption on the membrane surface or by interacting with membrane proteins, compromising the membrane's integrity. When NPs come into contact with membranes, they can cause physical disturbances such as holes and thinned areas. The NPs may also be partially or completely included in the lipid bilayer, depending on their size, electrostatic charge, and hydrophilic properties. Three different types of NP entrapment have been identified (partially entrapment of NPs in the bilayer, NP stretches between hydrophobic regions, and complete entrapment of NP in the center of the bilayer as a result of hydrophobic interactions (Wang et al. 2012).

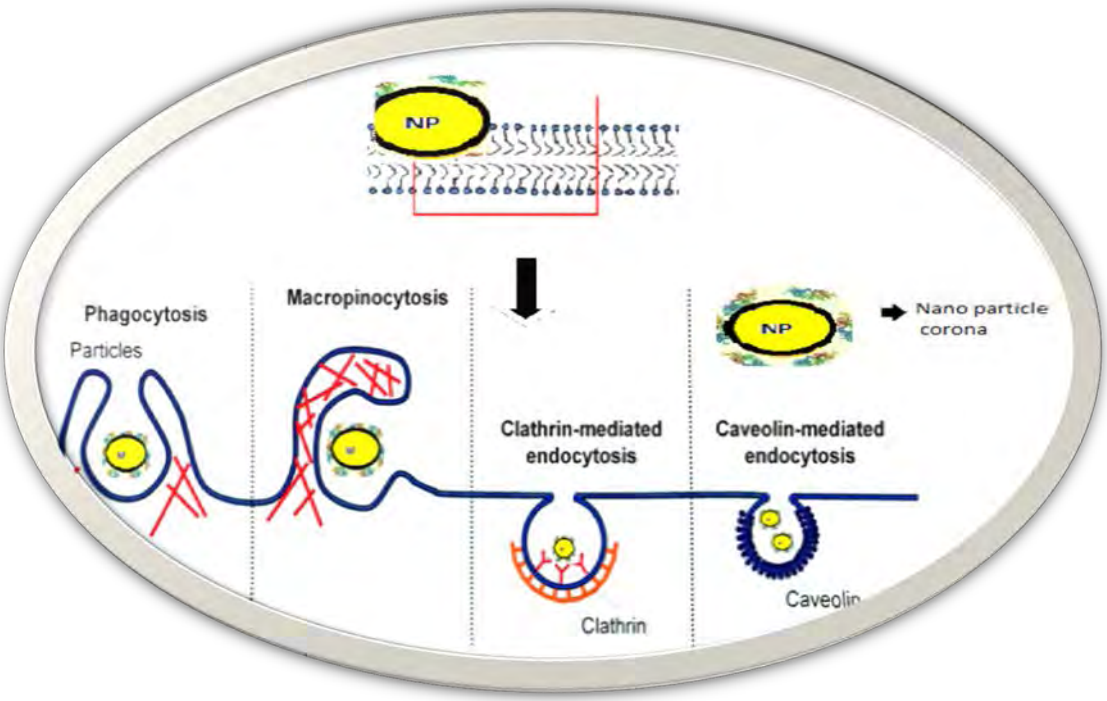


Figure2. 7. Possible mechanisms for uptake of silver nanoparticles by cell membrane

2.6.1.4.Interaction With Nucleic Acid

The same interaction forces that control protein-NP interactions also govern the interaction of NPs with nucleic acids (Forces such as Van der Waal's and steric forces, electrostatic forces, solvation/hydration forces, and so on) As a result, numerous studies have focused on the development of DNA sensors, with semiconductors as the primary NPs (quantum dot). Quantum dot Nano crystal-induced DNA damage, on the other hand, appears to be related to Cd release after CdNP photo degradation. CdSeNPs have been found to have a negative impact on the growth of Gram-positive bacteria. (Railsback et al. 2012)

For nanoparticles to interact with DNA, nanomaterials should be placed inside the nucleus; NPs can interfere with proteins and cause indirectly genetic damage. TiO2NPs

and SiO_2 NPs have been shown to generate intra nuclear protein aggregates that affect the replication, transcription, and cell proliferation processes(Li et al. 2008b; An and Jin 2012; Glibitskiy et al. 2012; Railsback et al. 2012; Hu et al. 2014) .Functional NPs with -OH, -NH_2, or -COOH groups will covalently bind (specifically bind) DNA to the surface. -NH2 oligonucleotides, for example, can be used to functionalize AgNPs and AuNPs. Furthermore, non-specific binding between DNA and NPs can be achieved using simple adsorption by non-covalent interactions. Functional NPs with -OH, -NH_2, or -COOH groups will covalently bind (specifically bind) DNA to the surface. NH_2 oligonucleotides, for example, can be used to functionalize AgNPs and AuNPs. Moreover, nanoparticles can be bound with DNA nonspecifically by non-covalent interaction like simple adsorption (Shang et al. 2007; Basu et al. 2008)

Two relevant pathways correlated with the genotoxic effects induced by NPs are oxidative stress processes (i.e., enhanced ROS causes cellular redox imbalance) and a reduction in antioxidants. ROSs are highly reactive molecules that negatively react with DNA, proteins, and lipids, disrupting homeostasis. Single- and double-stranded DNA breaks, base changes, and DNA cross-links have all been identified as ways for ROSs to cause DNA damage(Shang et al. 2007; Basu et al. 2008; Li et al. 2008b; An and Jin 2012; Glibitskiy et al. 2012; Railsback et al. 2012; Hu et al. 2014) . Indeed, transition metal-based NPs have the ability to transform cellular oxygen metabolic products such as H_2O_2 and superoxide anions to hydroxyl radicals (OH), which are one of the most common DNA-damaging species(Basu et al. 2008).

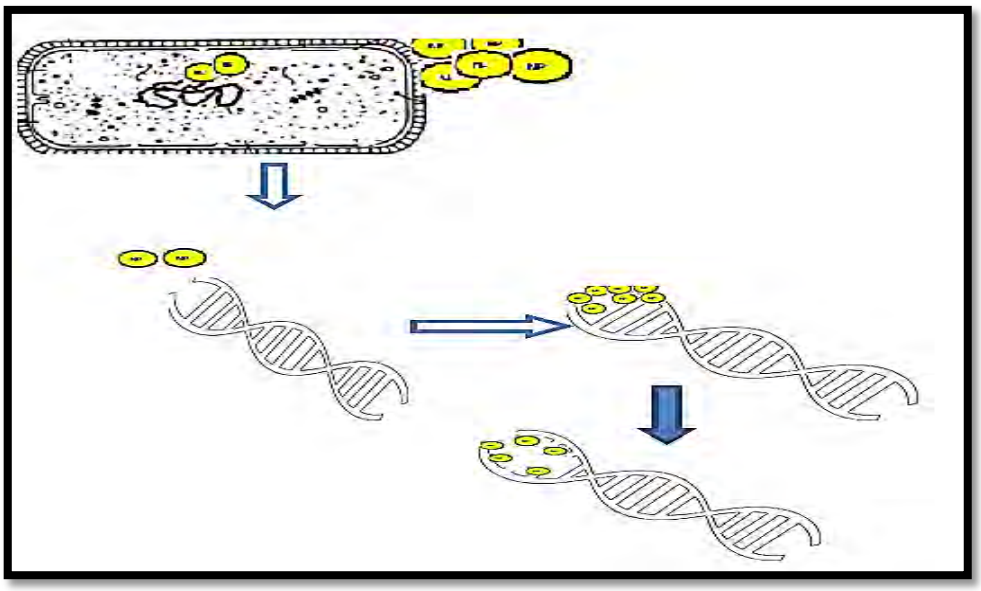


Figure2.8. Illustration of DNA-NP interaction and unwinding of DNA

2.6.2. Factors Associated With The Nanoparticles Antimicrobial Activity

In order to explain the behavior of inorganic NPs, two types of factors are widely used. Factors linked to the NP as well as factors linked to the microorganism. While these are essential aspects of the biomolecules-nanoparticles relationship, other medium-related aspects should be considered as well. However, there is a scarcity of knowledge on this, and several antimicrobial activity studies are conducted in vitro. CuNPs' antimicrobial activity, for example, is influenced by a variety of physical and chemical factors, including pH, oxygen, NP, and bacteria concentrations. Increased temperature, high aeration and, as a result, a high oxygen concentration, and acid pH have all been shown to reduce NP agglomeration and improve their toxicity.(Karlsson et al. 2009; Nowacs 2009; Hajipour et al. 2012; Chen et al. 2014; Dizaj et al. 2014)

2.6.2.1. Factors Associated To Microorganism

Several factors associated with microbial cells, such as cell wall, growth rate, and biofilm formation, have been identified in several studies to influence NPs' microbial activity (Karlsson et al. 2009; Hajipour et al. 2012; Dizaj et al. 2014).

2.6.2.2. Bacterial Cell Wall

The cell wall of bacterial gives microorganism's strength, rigidity and protecting them from osmotic and mechanical damage. Gram-positive and gram-negative bacteria cell walls are distinguished by their composition, elements, and functions (Scott and Barnett 2006; Fabrega et al. 2009; Baek and An 2011a; Guzman et al. 2012) Gram-positive bacteria hold the Gram reagent's Their cell wall is made up of several layers of peptidoglycan or murein, and they retain purple crystal violet dye. Furthermore, the cell wall is 15-80 nanometers thick and made up of multiple peptidoglycan layers. Gram-negative bacteria, on the other hand, have a more complex cell wall that does not hold the Gram reagent's crystal violet dye. A thin peptidoglycan coating and an external membrane make up the cell wall of this type of bacteria. Lipopolysaccharides are a form of polysaccharide found in gram-negative bacteria's outer membrane. These biopolymers boost the surface negative charge density of cell membranes, which is crucial for the bacteria's structural integrity and viability. Furthermore, as compared to gram-positive bacteria, the cell wall is relatively small (10 nm)(Lu et al. 2009). The effect of NPs on microorganisms is clearly influenced by the shape and chemical composition of the cell wall. It is expected that various interactions between NPs and the cell wall surface will arise as a result of the chemical composition differences. Differences in AgNPs susceptibility between *E. coli* and *S. aureus*, for example, are

explained by aspects of cell wall composition. However, it has been determined that the resistance of bacteria to inorganic NPs is not entirely dependent on cell wall structural features. (Jiang et al. 2011) Other variables, according to studies, may affect bacteria's vulnerability or resistance to NPs. Gram-negative bacteria, such as *E. c oli*, are particularly susceptible to CuONPs, whereas gram-positive bacteria, such as *S. aureu*s and *B. s ubtilis*, are less susceptible. *S. aur eus* and *B. s ubtilis,* on the other hand, are more vulnerable to NiONPs and ZnONPs than *E. c oli*. .(Scott and Barnett 2006; Fabrega et al. 2009; Baek and An 2011a; Guzman et al. 2012)

2.6.2.3. Growth Rate

The rate of bacterial growth is also influenced by microorganism's biological characteristics and tolerance to inorganic NPs. This can be looked at from two angles: (a) at the cellular or organism level (regulatory mechanisms related with increases in volume and differentiation) and (b) at the population level. Microscopic techniques are often used to detect morphology and relative size changes during a microbial growth analysis. Antibiotics and inorganic NPs are more vulnerable to fast-growing bacteria like *X. m altophilia,* and *P. anaerobius* than slow-growing bacteria like *Mycobacterium tuberculosis* (Karlsson et al. 2009) It has been proposed that the slow-growing bacteria's resistance property is linked to genetic factors that regulate the expression of stress response genes (Brown et al. 1988; Lu et al. 2009)

2.6.2.4. Biofilm Formation

To live in drastic and changing ecological situations, microbes have mechanisms of sticking to surfaces and aggregating in groups, known as biofilms. Hence, biofilm is a bacterial community that is shaped as a consequence of bond to a compact exterior

and by release of a EPS that covers the bacterial inhabitants (bacteriological EPS is formed by proteins, DNA, and polysaccharides). It has been evidenced that genomic and physiological features are responsible for biofilm associated specific functions. It has been supposed that, NP- biofilm interaction would be controlled by the physiochemical characteristics of both the NPs and biofilms (usually, bacteria contain a negative charged biofilm, though, this is not an authoritative situation; *e.g*., *S. epidermidis* has a polycationic biofilm) (Mah and O'Toole 2001; Stewart 2002). It has been stated that silver nanoparticles could inhibit the accumulation of new bacterial cells onto an existing biofilm and decrease the expansion of the biofilm. But, the effectuality of NP dispersion into the biofilms may be halted by the physiochemical features of the microbial cell wall (such as, due to its hydrophobic nature). The hydrophobic nature of the biofilm is due to a combined effect of exopolymeric and intracellular compounds (like the most known peptidoglycan and protein) (Mah and O'Toole 2001; Stewart 2002; Landini et al. 2010; Applerot et al. 2012).

2.6.3. Factors Associated To Nanoparticles

Chemical and physical structure, concentration, size and shape of the NPs are significant constraints influencing the antimicrobial characteristics.

2.6.3.1. Chemical Composition

Physical appearance and chemical structure of the NPs are the vital features associated with the antimicrobial activities. To highlight this aspect, researches have compared the bactericidal activity of various Nanoparticles. Such as, Adams *et al.* (2006) compared

antibacterial activity of the TiO_2, SiO_2 and ZnO using microbial cells of *B. subtilis* and *E. coli.* It was observed that the bactericidal activity associated with these three compounds gradually reduced from ZnO to TiO_2 to SiO_2 (Karlsson et al. 2009; Shah and Belozerova 2009; Somasundaran et al. 2010; Zhang et al. 2011; Applerot et al. 2012). Jones *et al.* (2008) evaluated the antimicrobial activity of MgONPs, TiO_2NPs, Al_2O_3NPs, CuONPs, CeO_2NPs, and ZnONPs against *S. aureus*; where they recorded comparatively high antibacterial effect to compounds Al_2O_3 and ZnO (Dizaj et al. 2014). Various authors have also compared the antibacterial activity associated with different nanoparticles (Karlsson et al. 2009; Hajipour et al. 2012; Dizaj et al. 2014). Similarly, NPs of same nature can be prepared with various chemical structures and arrangements. Hence, these can be obtained via reduction reactions with numerous reductive chemicals (*e.g*., NaH_4B and sodium citrate) and stabilizing agent (*e.g*., , polyvinylpirolidone, polyvinyl alcohol or other polyols); also, these can be simply functionalized via thiol groups or other functionalizing agents (Khan et al. 2012; Mukherjee et al. 2012). The chemical configuration of NPs can also be changed if mixed with NPs of different types. This type of configuration has been adopted to enhance the physiochemical and biocidal effects of the NPs. (Karlsson et al. 2009; Hajipour et al. 2012; Dizaj et al. 2014).

2.6.3.2. Concentration, Size, and Shape of nanoparticles

Till now, it has been known that the size and chemical configuration of NPs are the vital properties of NPs which define their *iv-vivo* diffusion, toxicant level, durability and specificity (Fabrega et al. 2009; Somasundaran et al. 2010) NPs, due to Nano-

scaled size when compared to other compounds have relatively high intracellular diffusion abilities (Oberdörster et al. 2004; Lacerda et al. 2009; Li et al. 2010b; Cho et al. 2011) In connection to biocidal characteristics, it has been widely known that it is dependent on different parameters like their concentration (increased concentration have a greater antimicrobial effect), size (reduced size promotes high antimicrobial activity) and shape (real evidences are not conclusive) (Sohaebuddin et al. 2010).

Dosage-based antimicrobial effect has been explored for AgNPs (0.2–33 μmol/L) and Fe_2O_3NPs (0.1–2 mg/L) (Sohaebuddin et al. 2010). Size-based antimicrobial activity also has been widely investigated for AgNPs. Recently, Liu *et al*. studied antimicrobial activity of different sized AgNPs (i.e. 4, 5-7, and 10-40nm) and found that antibacterial activity of these particles reliant on the concentration of the particles. Size-based activity was recorded only at the concentration of 0.1 mg/L. Moreover, it was also seen that reduce sized particles (4nm) were the most active, that showed activity at lower concentration (0.01 mg/L) as well as at high concentrations (1-10 mg/L). Nanoparticles of various sizes had comparable activity, separately (Gojova et al. 2007; Skebo et al. 2007; Sohaebuddin et al. 2010) Studies on the shape-dependent effect of NPs are rare because of experimental effort to acquire specific shape. Significance of shape was reported for rod and tube shaped TiO2 particles that were synthesized via assimilation trails on plasma proteins. Shape-based antibacterial experiments were conducted by Pal et al. (2007) using AgNPs against pathogenic *E .coli*. Three main shapes spherical, rod and triangular were used for the experiments, It was observed that high-atom-density, large surface area and different geometrical arrangements of silver presented comparatively excellent activity (Gojova et al. 2007;

Skebo et al. 2007; Sohaebuddin et al. 2010).

2.6.4. Effect of environment on antimicrobial activity of nanoparticles

Many studies have been conducted to highlight the role of environmental situations on the spectrum of antimicrobial activities associated with different nanoparticles. It has been noted that environmental temperature has a significant effect on antimicrobial activity because it has a direct effect on the rate of reactive oxygen species formation. When ZnO NPs were exposed to temperature, electrons were taken by their active sites that later on, interacted with free oxygen and formed reactive oxygen species, thus improved the antibacterial effect. Similarly, low pH speeds up the suspension frequency of ZnO NPs, that marks better antimicrobial properties (Saliani et al. 2015). The pH was particularly observed to be linked within a range of 3.5±0.2 to 5.8±0.1 folds rise in NP attachment to the microbial membranes. Furthermore, a decrease in the efficiency of poly (lactic-co-glycolic acid) (PLGA)-poly (l-histidine) (PLH)-poly (ethylene glycol) (PEG)-encapsulated vancomycin has been observed when were exposed to an acidic pH. The outcomes proposed that certain protonation of the imidazole groups of PLH in low pH firmly controlled NP surface charge inter conversion. The acidic pH turned the surfaces positive, that excellently interacted with the negatively charged surfaces of bacterial cells, persuaded robust multivalent electrostatic regulation (Radovic-Moreno et al. 2012). Another research predicted an oxidative cessation protocol for AgNPs via the reaction of Ag+ with dissolved oxygen and protons. Deviation in water chemistry could trigger AgNPs, enhancing the antimicrobial activity of the Ag NPs because of releasing Ag ions. The research confirmed that the solubility of NPs was improved in acetic acid solution when

compared to neutral water (Peretyazhko et al. 2014).

2.7. Mechanisms of action of NPs

A growing use of nanoparticles in the field of medicine has resulted an increased amount of studies discovering possible antimicrobial mechanisms of NPs (Huh and Kwon 2011). It has been studied that metal nanoparticles disrupt the metabolic activity of bacteria (Armentano et al. 2014). That could be much beneficial when used against pathogenic bacteria. The NPs have been also proved to the enter the bacterial biofilm interferes with genes responsible for biofilm formation and thus results in the inhibition of biofilm formation (Zhao and Ashraf 2015). NPs interact with bacterial cells through different mechanisms that comprise electrostatic grip (Li et al. 2015a), van der Waals forces (Armentano et al. 2014), receptor–ligand interaction (Gao et al. 2014) and hydrophobic interactions (Luan et al. 2016). The nanoparticles are known to cross the microbial membranes and interact with the metabolic pathway, affecting the integrity and functionalities of the membranes. Once the bacterial shape and protecting ability is compromised, nanoparticles can easily affect the vital components of bacterial cell like DNA, ribosomes, lysosomes, enzymes, leading to generation of oxidative stress with the cell, heterogeneous alterations, enhanced membrane permeability, electrolyte imbalance, enzyme destruction, protein inactivation, and hampered gene expression (Shrivastava et al. 2007; Li et al. 2010b; Xu et al. 2016) The subsequent mood of actions has been frequently explored: oxidative stress (Gurunathan et al. 2012), metal ion release, (Zakharova et al. 2015) and non-oxidative mechanisms.(Leung et al. 2014)

2.7.1. Oxidative stress

The reactive oxygen radical's made oxidative stress is a vital antibacterial effect

associated with NPs. The ROS is a general term describing the positive redox potential of many molecules and their proactive mediates, and various kinds of NPs promote different types of ROS by reducing oxygen molecules. The four most known types of ROS are the superoxide radical (O^{-}), hydroxyl radical (·OH), hydrogen peroxide (H_2O_2), and singlet oxygen (O_2), that express diverse level of dynamics and activities, Such as the oxide-nanoparticles of calcium and magnesium can produce O^{-}, while zinc can produce H_2O_2 and OH but not O^{-}. Meanwhile, copper can generate all the four aforementioned types of reactive oxygen. From different studies it has been concluded that O^{-} and H_2O_2 generate a smaller amount of stress reactions that can be nullified by endogenic antioxidants, such as superoxidases and catalases, while ·OH and O_2 can enough lethal to kill the attacked microbe. The main causes of ROS production are reorganization, defect sites, and oxygen valances in the crystal (Malka et al. 2013). In hemostatic situations, generation and neutralization of the ROS are kept balance in microbial cells. While an increased generation of ROS, without neutralization results in oxidation off the bacterial cell, that ultimately leads to oxidative stress, and causes oxidative destruction of bacterial essential components (An and Jin 2012; Peng et al. 2013).

Oxidative stress has been proved as a major factor in altering the porosity of the cell membrane, which results in cell membrane disruption. An study proved that Al_2O_3 NPs cross cell membrane and it's interaction the with the cell membrane ultimately triggers loss of membrane chemical configuration (Ansari et al. 2015). Correspondently, nano-silver ions are utilized as catalysts for activation of the oxygen in air or water, resulting

in the generation of hydroxyl radicals and reactive oxygen ions, that stop the propagation of microbes or kill them (Shrivastava et al. 2007; Yang et al. 2009). Many studies have shown the ability of ROS to interact with bacterial DNA and cell membranes (Pramanik et al. 2012). Furthermore, the ROS are known promoters of an increased gene expression, which is a significant mechanism in bacterial cell apoptosis (Wu et al. 2011). Moreover periplasmic enzyme activity is inhibited by ROS which affects the morphology of cell and physiological processes. (Padmavathy and Vijayaraghavan 2011)

2.7.2. Non-oxidative mechanisms

To evaluate the antibacterial mechanisms, many researches have used advanced techniques like transmission electron microscopy (TEM), liquid chromatography, mass spectrometry; proteomics tools, electron spin resonance, flat cultivation and Fourier transform infrared (FTIR) analysis. Under UV, natural light and in dark conditions, three types of magnesium nanoparticles have shown good antibacterial efficacy on *E. coli* and were completely different from membrane lipid peroxidation caused by oxidative stress, established on the following points: 1) magnesium oxide nanoparticles are not detected in the cell as surface pores are clearly obvious and the bacterial cell membrane is broken,. Furthermore, no ion ns are visible in energy-dispersive X-ray spectroscopy spectra did not report extreme Mg ions in excess. 2) In the cell wall, Phosphatidyl ethanolamine and Lipopolysaccharide are not expressively altered by MgO NP treatment, which specifies that the lipid peroxidation was not caused by MgO nanoparticles. Furthermore, there was negligible change in the quantity of ROS-associated protein. (Leung et al. 2014)

2.7.3. Dissolved metal ions

Metal ions like (Ag^{+}, Cu^{2+}, Cd^{2+}, Zn^{2+} and Pb^{2+}) (Chudobova et al. 2015) are gradually released by metal oxide and are absorbed through the cell membrane. These ions directly interact with the functional groups of nucleic acids as well as proteins such as amino, mercapto (–SH), and carboxyl (–COOH) groups. This interaction damages enzyme activity and altering cell structure and eventually deterring the microorganism. As inside lipid vesicles metal oxide has small impact on PH during antibacterial activity in suspension form, so dissolved metal ions are not the foremost antimicrobial mechanism.(Yu et al. 2014) likewise, a study revealed that super paramagnetic iron oxide interacts with microbial cells by entering the cell membrane and affecting the of trans membrane electron transfer. (Hussein-Al-Ali et al. 2014)

2.7.4. Variations in genetic expression by Nanoparticles

Metabolic processes are vital to maintaining cell growth. Cell membrane may be subject to damage due to disturbances in metabolic processes, resulting in oxidative stress that eventually leads to cell death. Targeted changes can be made in metabolic processes of the microbe to control their pathogenicity. Several mechanisms have been suggested involving nanoparticles to interfere microbial metabolism, including Metal ion dissolution mechanism and Reactive Oxygen Mechanism. Magnesium oxide NPs, were reported to alter various metabolic proteins expression; like down regulation of a critically important metabolic proteins as well as up regulation of riboflavin metabolic protein and a weak thiamine ester-binding protein resulting in destruction of metabolism of cell (Padmavathy and Vijayaraghavan 2011; Yu et al. 2014). Copper Oxide NPs can suppress the nitrogen metabolism by deregulating the expression of

related proteins leading to the inhibition of nitrate reductase activity (Su et al. 2015). TiO_2 affects the adhesion of bacteria during the biofilm formation]. NPs can also affect the metabolite levels of bacterial communities (Peng et al. 2013; Roguska et al. 2015). NPs can also affect the metabolite levels of bacterial communities (Peng et al. 2013). Regulation of bacterial metabolism is essential to biofilm formation, like, metabolism of D-alanine is important in establishing *S. mutans* biofilm (Qiu et al. 2016).

2.8. Biofouling of Membranes

Regardless of the extraordinary advantages of membrane applications, numerous researchers concluded that the extreme decline in water flux is because of biofouling can leads toward the membrane replacement which is very expensive (Bos et al. 1999; Al-Ahmad et al. 2000; Escobar and Randall 2000; Gorey et al. 2008).These are some generalized mechanisms through which the fouling would be occurring on the membranes:

1. Clogging of the membrane pores leads towards the membrane blockage.
2. By the substances superimposition moderate sought clogging is occur
3. Particulate matter is entrapped in the membrane pores which in turns reduce the membrane pore size; this is known as standard blocking.
4. Larger particulate matter is dumped on the surface of the membrane which in turns blocks the membrane this is known as cake layer formation. When a newly functionalized membrane is encountering permeate that possess the particulate matter and having bacterial colonization in it, all these things leads towards the development of biofouling.

Cake layer formation is occurred due to the accumulation of natural organic matter (NOM). This cake layer formation works as nutritional reservoir for the microorganisms and eases their proliferation and adhesion. Extracellular polymeric substance (EPS) is secreted by microorganism which provides the favorable conditions for the biofilm formation. When the width of biofilm surges to an interference threshold, the surface of the membrane changes and appears to be encapsulated. Many case studies suggested that the primary stage of biofouling, adhesion of microorganisms on the membrane surface, occurs comparatively in an immediate manner i.e. within two hours, nevertheless, the succeeding stages such as microbial growth, can occurs in weeks or months (Al-Ahmed et al., 2000). Majority of the reported studies (Bos et al. 1999; Al-Ahmad et al. 2000; Escobar et al. 2005; Ivnitsky et al. 2005) have documented the key phases of biofilm development including conveyance to the solid-liquid edge, initial impeachment, attachment, propagation, and biofilm establishment

There are primarily two types of fouling mechanisms are there i.e. organic and inorganic fouling procedures. Inorganic fouling mechanisms comprise of the fouling of colloidal and particle sought of fouling while the organic ones involve the biological fouling. Biofouling of membrane is comparatively more intricate mechanism with respect to other sought of fouling. If the microorganisms are said to be completely removed from the feed solution even, then they ensure their survival because they have enough in-built mechanisms to adapt their surroundings. They have considerably good propagation rates (Flemming et al. 1997; Ivnitsky et al. 2005) When the infiltrate is passing from the surface of the membrane at that time tangential forces are applied on its surface which helps the microorganisms for their settlement and transportation on the membrane

surface. In this way biofilm formation is occur on the membrane surface which in turn reduces the flux rate of the permeate (Sablani et al. 2001)).

In the biofilm formation the first step is the preliminary adherence of the microorganisms to the membrane surface. There are several aspects that could distress bacterial adherence to the membrane surface but among the entire key factor or the aspect is the surface properties which are possess by the membrane itself. These surface properties in turns affect the swiftness and capability of adherence possess by the bacterial cells (Pasmore et al. 2001).

Biofouling is started by irrevocable attachment of few or several bacteria on to surface of the membrane, progression and proliferation of the cells occur at the expenditure of nutrients present in the feed stream (Kang et al. 2006a). Organic fouling is accredited to NOM which comprises of proteins, fulvic, humic acids and polysaccharides accompanying with microorganisms movement (Tu et al. 2005). On surface of the membrane humic acid is accumulated particularly its water repellent portion. This hydrophobic portion is responsible for the formation of biofilm that act as solid liquid interface. Deposition of humic substances and precipitation of calcite present on the its surface are the main causes that subjected to a considerable increment in pH of the respective feed stream solution (Goosen et al. 2005). Combination of inorganic and organic fouling is occurred during the biofilm manufacturing that can't be distinguished easily. This combination is responsible for pores fouling. Flux reduction is mainly due to the calcite substances precipitation such as of calcium humate. Pore magnitudes of UF membranes ranges from 1 to 100 nm. Though, majority of the humic particulate matter have representative lengths that extend from 1 to 10 nm only. Humic substances are

adhere at the internal site areas of membranes pores with respect to their capability; thus, they initiate to bound on its surface and causes the extensive pores clogging. This could be clarified by the detail that the retaining of NOMs is because of the joint consequence of sieving and adsorption on its surface. A gel layer irretrievable structure is formed by the particulate matter which is attached and accumulated on the membrane surface (Chang and Benjamin 2003). Hence, the Pore sizes and their respective allocation on its surface are the most influential aspects of biofouling because of the deposition of NOM. The preliminary association of particulate matter ,humic substances, calcite, organic and inorganic molecules, and bacteria with the membrane be subjected to its surface features, such as : water repellent and loving characteristic, molecular weight cut-off (MWCO), charge, chemical composition and coarseness, microbial interactions, it's also involves environmental factors, for example pH, temperature, ionic strength, nutrient availability(Childress and Elimelech 1996; Fonseca et al. 2007).

Biofouling could have numerous destructive effects on membranes which are presented below:

• Membrane flux decline because of the biofilm formation which in turns decreases its permeability.

• To produce the treated product under the same rate there is an immense need of enhanced differential and feed pressure.

• Acidic by-products strenuous on the membrane surface are chiefly responsible for membrane biodegradation.

• Enhanced energy consumption because of increased pressure requires restricting the biofilm confrontation and the flux reduction (Ridgway and Flemming 1988; Flemming 1992; Kramer and Tracey 1995; El Aleem et al. 1998; Murphy et al. 2001).

2.8.1. Nanoparticles as antibiofouling agents

Numerous types of nanoparticles, formed by diverse procedures, are useful as raw constituents in diverse areas. Nanoparticle integration in the construction and manufacturing of substances has been an vast area of research in recent decades for instance mechanical (Okada and Usuki 1995), thermal (Gilman 1999) and magnetic (Godovski 1995). Therefore, one of the up to date technologies includes the integration of nanoparticles into the polymeric membranes so that it will increase the performances of the membranes for instance cross flow through the membrane, its capability , and fussiness. For example , polyvinylidene fluoride membranes unified with silica nanoparticles can tolerate the elevated temperature, relatively higher selection criteria and greater diffusion rates (Yu et al. 2009b), chitosan/zinc possess greater antibacterial characteristics (Li et al. 2010a) polysulfone membranes integrated with silica nanoparticles displayed boosted gas permeation performance; polyethersulfone/aluminum oxide membranes (Ahn et al. 2008) unveiled lesser flux reduction, increased porosity (Maximous et al. 2009);and polybenzimidazole/silica nanoparticles membranes presented enhanced permeation and selection in gas segregation (Sadeghi et al. 2009).

Some previous research studies suggested that the polymers integrated into the membranes provided the stability (Hu et al. 1997) . Integration of nanoparticles into the synthesized polymeric membranes has some disadvantages. Among one of the

restraining aspects is the dispersal of the nanoparticles in the polymers. The dispersal control, which is the first procedure for the formation of materials integrating nanoparticles, is very problematic for nanoparticles that have diameters smaller than 100 nm. Though researchers comprehend the surface interaction models, but the aspects that would subsidize to increase the agglomerations remains uncertain. Due to this nanoparticle's dispersion becomes difficult during the course of membrane synthesis. Yu et al. recommended that the enhancement in density of nanoparticles could enhance nanoparticles accumulation (Yu et al. 2009a). Benjamin et al. proposed the accumulation of the nanoparticles is also inducted by the two environmental factors such as ionic strength and pH of the feed stream(Gilbert et al. 2009) Most of the times these recommended methods are utilized for the integration of nanoparticles into the membrane such as casting procedures in which polymers are mixed with the nanoparticles in an appropriate ratio(Bae and Tak 2005; Kang et al. 2008; Maximous et al. 2009; Soroko and Livingston 2009; Yu et al. 2009b).

Absorption of the glass plate into a water bath at room temperature condition is vital for membranes polymers organization with the help of phase inversion method (Ren et al. 2004; Blanco et al. 2006; Cao et al. 2006; Yang et al. 2006; Zheng et al. 2006; Li et al. 2008c; Gao et al. 2009; Li et al. 2009) Polymers have been utilized in the manufacturing of nano- particles because of their of specified functional groups which are present on the back bone of the polymeric substance chain (Khayet et al. 2005), Fe_3O_4 (Wu et al. 2004), ZrO_2 (Bottino et al. 2002) , TiO_2 (Kim et al. 2003; Sun et al. 2004; Bae and Tak 2005; Losito et al. 2005), CdS (Xu et al. 2002; Trigo et al. 2004)

All the above-mentioned nanoparticles could be impregnated during the membrane synthesis and the unified effect of the polymers with nanoparticles in turns produces a synthesized polymeric membrane impregnated with certain nanoparticle possess the specific properties (Z.-K. Xu, Xiao, Wang, & Springer, 2002)

2.8.1.1.-Silver Nanoparticles Impregnated Membranes:

Biofouling of polymeric membranes comprises of EPS and proteins that is secreted by the microorganisms. It is also responsible for the flux decline i.e. it affects the permeability of the membrane which in turns lead to the enhanced cost and energy usage for the production of the membrane (McDonogh et al. 1994). Pretreatment procedures and certain conventional methods such as cleaning and back washing are utilized for the mitigation of biofouling. Even the pretreatment can serve as an adequate manner of biofouling prohibition, numerous polymeric membranes don't possess the ability to tolerate the chemical cleaners corrosiveness (Zodrow et al. 2009). Integration of antimicrobial agents unified with the nano-particles into membranes suggests a state-of-the-art solution for the biofouling mitigation (Savage and Diallo 2005; De Prijck et al. 2007; Li et al. 2008a). Silver nanoparticles possess a substantial antimicrobial property due to this reason they are integrated into the polymeric membranes during their synthesis. Interaction of the silver nanoparticles with the Sulphur and phosphorous group which is mainly the part of thiol group that belongs to cysteine and certain other compounds(Davies and Etris 1997). Silver nano particles are responsible for the destruction of bacterial proteins, interference in the ETC and dimerization of DNA by forming the disulfide bond or S–Ag (Trevors 1987; Russell and Hugo 1994; Feng et al. 2000). In spite of that, silver nanoparticles also facilitate the selective permeation that means it will only allow the passage of certain components. This distinctive feature allows the polymeric membranes integrated with silver Nano particles to be used in advance application that are subjected for the transportation of certain products. Silver ions and silver nanoparticles (AgNPs) have been utilized for an extensive range of water

treatment procedures that includes the filtration of water by these membranes. Silver nanoparticles have been impregnated into the cellulose acetate (Chou et al. 2005), polyamide (Damm et al. 2007), polyimide (Deng et al. 2008) and poly (2-ethyl-2-oxazoline) (Kang et al. 2006b) polymeric membranes. Though, the impregnation of silver nanoparticles into the polymeric membranes and the utilization of these membranes for the mitigation of biofouling in a long run has not been discussed and established yet. Only a small portion of research is conducted in order to determine the antibacterial assessment of silver nanoparticles impregnated in the polymeric membrane, for the contaminated water filtration. Thus, detail study is conducted on the depiction and usage of polysulfone membranes permeated with silver-based nanoparticles reported by Zodrow et al. could deliver us an improved understanding of the concepts (Zodrow et al. 2009). The amalgamated ultrafiltration membrane was manufactured by utilizing the wet phase-inversion procedure as reported by Mulder. Silver nanoparticles ranges from 1–70 nm was integrated into the polysulfone membrane by dissolving nanoparticles in the dope solution, polysulfone resin is dissolves already dissolved in the casting solution. 10% of polyvinyl pyrrolidone (PVP) were employed for the creation of pores (Mulder 2012).

According to reports polysulfone membranes infused with 0.9 wt.% silver Nano particle keeps the same permeability rates and membrane surface charges as hold by the control polysulfone membranes (Zodrow et al. 2009). The composite membranes were proved to be more hydrophilic comparatively to the membranes which serve as a control, 10% of decline is observed in the contact angles. The asymmetric structure of silver-nanoparticles permeated membranes was deceptive. Integration of silver nanoparticles is

responsible for the effective decline in colony forming unit of *Escherichia coli* which is present on the membrane surface. Furthermore, several properties were improved by the integration of the silver nanoparticles beside this there are some weaknesses which are left behind to be addressed. Leaching of the silver was determined by the help of the transmission electron microscopy (TEM) and performing inductively coupled plasma (ICP) spectrophotometer. A single trace of silver was not found in filtrate with inductively coupled plasma spectrophotometer even after the filtration of 0.31 L/cm2 water. A total of 10 % of silver was leached by the membrane which was determined by the TEM. Silver leaching was determined by the TEM analysis in the form of silver. Also, it was concluded that the silver loss was generally from the surface. Membrane characteristics such as its antibacterial properties are affected by the leaching of the silver from the membrane and these properties are eventually decreases and lost due to this leaching. silver nanoparticles leaching also leads towards the water poisoning due to its cytotoxicity effects (Zodrow et al. 2009).

Silver nanoparticles are impregnated into the membranes they possess the ability to abridge the segregation of olefin. Olefin segregation is predictably attained by fractional distillation, which is a very energy consuming and expensive procedure. Sung pet et al., defined that oxidized pyrrole , it is a polymer which is electrically conductive in nature .It was found to be proficient of altering the permanent electronic environment which is existing between the silver and the counter ion. They also allow the silver ions to do the complex formation with ethylene when water is absent. As well as allowing silver (I) ions to form a complex with ethylene in the absence of water. Nafion membranes are

made by the polymerization of pyrrole. It is formed by utilizing a solution comprising of two ingredients such as pyrrole and hydrogen peroxide (Sungpet et al. 1997).

At present, there are several silver compounds which are pertinent to the manufacturing of polymeric composite membranes, for instance silver tetrafluoroborate (AgBF4), silver triflate (AgCF3SO3), silver nitrate (AgNO3), and many more (Kim et al. 2005). What sought of silver compounds are utilized in the manufacturing of the membrane is reliant on alterable complexity of silver ions with the chemical additives i.e. olefin to be transported, which unswervingly hinge on the connections of the silver with its pledge anion and with polymer (Kim et al. 2002). Research has been conducted to regulate the capability of silver bromide nanoparticles (Koh et al. 2009).

Polymeric synthesized membrane fabrication was categorized mainly into three basic steps: first the formation of PVC-g-P4VP, it is a grafting copolymer, fabrication of N-PVC-g-P4VP, and it is succeeded by the formation of N-PVC-g-P4VP/AgBr. Hence the precipitation occurs in the chains of polymer, the synthesizing Ag Br nano composites are alleviated and prohibited from accumulating by the capping mechanisms of pyridine groups (Sambhy et al. 2006). Even if there are several existing advantages of integrating silver nano composites into polymeric substances, researchers are not satisfy yet because of the safety concerns. Still they are not recommended for the commercial use. Enhanced utilization of silver nano composites demands risk assessment in terms of its environmental safety use and its use in medical appliance i.e. health related products.(Dreher 2004) Silver nanoparticles are extremely reactive in nature, which is the major feature that is possessed by any sought of nanoparticle.

Utilization of polymeric substances impregnated with the silver Nano composites in potable drinking water filtration plants and units, it may lead towards the leaching of silver ions into the water. Leaching of the Nano composites into the water is occur mainly due to two reasons first, the physical damage and second, the inappropriate integration of nanoparticles into the polymeric membrane. When the body is exposed to the silver for the longer duration of the time, than the discoloration of the skin is occurred which is characterized by blue gray discoloration of the skin. This condition is known as argyria beside this when our body is exposed to the lower concentration of the silver than the silver also get accumulated in the skin and the other body parts(Hyun et al. 2008). *In vitro* investigation designated that the silver nano composites are responsible for the DNA mutilation and cellular death in embryonic stem cells of mouse and its fibroblasts (Ahamed et al. 2008).

2.9. Futuristic approaches

The application of nanoparticles in the field of medicine is still in initiative phase but is gaining much interest to researchers because of their nanostructures and distinctive characteristics. They can be used for multiple purposes such as to deliver biomolecules and drugs which prompt the inhibition or stimulation of biological processes. In these processes, a delivery system is needed because of short half-life of biomolecules, fast degradation and lethal at high dosage. There should be a technology which can actively stimulate the right process at right time. Nanoparticles have the capability to trigger the tissue formation process and overcome the harmful inflammation process. Development of multifunctional nanoparticles having applications in specific protein targeting, temporal control of cargo and drug delivery will bring an innovation in field

of medicine as well as in tissue engineering and cancer treatment. Secondly, nanoparticles can be easily being cooperated into other materials and technologies. In comparison with the micro scale material Nanomaterials have improved biological activities, regenerative outcomes and cell survival. To further enhance their activity in future work there is need of better understanding related to the material, chemistry and topographical properties. Moreover, stem cell therapy is a promising therapy to combat chronic diseases. Still there is need to develop a technology which can regulate the fate of transplanted stem cell, bio distribution and mechanism of action of stem cell therapies. Nanoparticles have potential to modulate and track the stem cell behavior. The significance of this system is that it is a combination of both theranostic capacity and drug delivery capabilities to regulate the stem cell approaches. In addition, nanoparticles also provide a platform for potential imaging; however, in-vivo applicability and long-term toxicity need to be considered. Likewise, the capacity to execute functional and high-resolution imaging complex organism remains barrier. However, the knowledge of manipulation of nanoparticles, and their functionalization for cancer therapy, gives reassurance that the above tasks can be fulfilled.

EXPERMENTAL

EXPERIMENTAL 1

3. Biogenic synthesis of Silver Nanoparticles (AgNPs) from *P. aeruginosa*

3.1. Introduction

The continuous demand for the development of clean, reliable, biocompatible and benign processes to synthesize NPs have attracted more and more researchers to exploit biological systems. A search for an ecologically viable synthesis procedure has led to a few biomimetic approaches. It is well known that the biological systems (especially microbes and plants) can uptake and remediate a number of metals or metal-containing compounds and convert them into less toxic forms. Based upon this strategy, up till now, various microbes from bacteria to fungi have been investigated to fabricate inorganic nanomaterial's both intra as well as extracellularly and thus can act as potential bio Nano factories. It was already established that the lot of active biomolecules are secreted by fungi which played promising roles in reducing and capping NPs during biosynthesis.

In this study, extracellular synthesis of AgNPs was carried out by using the cell free culture filtrates of previously isolated bacteria *Pseudomonas aeruginosa.*

The conversion of silver ions (Ag^{+}) to elemental silver (Ag^{o}) was investigated by visual observations and UV-Vis spectrophotometry. AgNPs size was measured by using techniques of X-Ray Diffraction (XRD) and Transmission Electron Microscopy (TEM).

3.1.1. MATERIALS AND METHODS

Silver nitrate of analytical grade, Potato dextrose agar and Luria Bertani (LB) broth and agar was bought from Sigma Aldrich. Germany. *P. aeruginosa* ATCC 27853 was obtained from the laboratory of Microbiology, Quaid-I-Azam University Islamabad

3.1.1.1. Silver Nanoparticle synthesis

Extracellular synthesis of silver nanoparticle were carried out by following (Yang et al. 2020) with modification. Synthesis was performed by inoculating loop full of 24hour fresh culture of *Pseudomonas aeruginosa* into 100mL of TSB broth. Erlenmeyer flask was incubated for the next 48 hours by placing them in shaking incubator at 28 ºC and 120 rpm. Centrifugation was performed at 10,000 rpm for 10m in order to obtain the cell filtrates and after that decantation was performed. The supernatant was added by the final concentration of 1 mM $AgNO_3$ it was followed by the incubation of supernatant for the next 24 hours, in darkroom conditions.

3.1.1.2.Characterization

For the characterization of Nanoparticles; following techniques were used.

3.1.1.2.1. Visual Observations

Through visual observations, change in color of the reaction mixture was monitored. Images were taken at the beginning and at the end of the experiments.

3.1.1.2.2. UV-Vis Spectrophotometry of AgNPs

Samples taken at different time's interval during experiments were scanned in a range of 200 – 800 nm λ by using UV-Vis spectrophotometer in order to detect the presence and concentration of AgNPs

3.1.1.2.3. XRD analysis of AgNPs

XRD is a rapid analytical technique principally used for phase identification of a crystalline material. XRD was carried out to measure the mean size of silver colloidal suspension synthesized by exposing cell free (culture) filtrates .Particle size was studied by X- Ray powder diffraction (PANalytical X'pert PRO XRD, Netherlands), available in the Department of Chemistry, Quaid-i-Azam University Islamabad.

3.1.1.2.4. TEM of AgNPs

The Transmission electron microscope (FEI Tecnai G2 Spirit Twin TEM instrument USA) was used to observe the shape and size of AgNPs. In this technique, bright field image mode was used to analyze the samples. Pure ethanol based dilute suspensions of AgNPs were prepared by ultra-sonication. Diffraction patterns of samples were recorded by using diffraction focus knob to focus the diffraction rings and spots. Standard developing procedure was used to process exposed photographic plates, and scanned (flatbed high resolution scanner) to get the final positives of the images were obtained.

3.1.2. RESULTS

3.1.2.1.Silver nanoparticle synthesis

3.1.2.2.Visual Observation and UV-Visible Spectroscopy

A color change was observed when the supernatant of the *Pseudomonas aeruginosa* is incubated with the salt of silver nitrate $AgNO_3$.Tryptone Soy Broth changes its coloration from darkish yellow to darkish brown. Change in color clearly specified that silver nanocomposites were manufactured by the reduction of the silver ion, a strong broad peak was observed at 430 nm with the help of UV spectrophotometer.

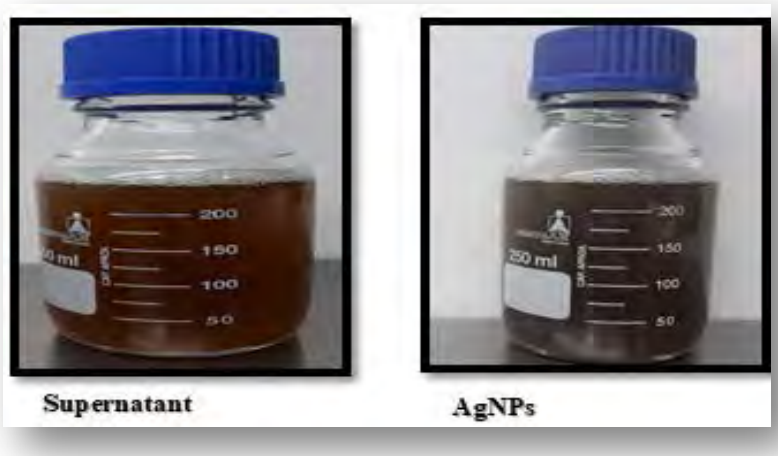


Figure3. 1. Biogenic synthesis of silver nanoparticles by *P. aeruginosa*

Change in the color shade indicated development of silver nanoparticles. It was due to surface Plasmon resonance (SPR) of the silver nanoparticles. The net yield of nanoparticle obtained was 60.7%

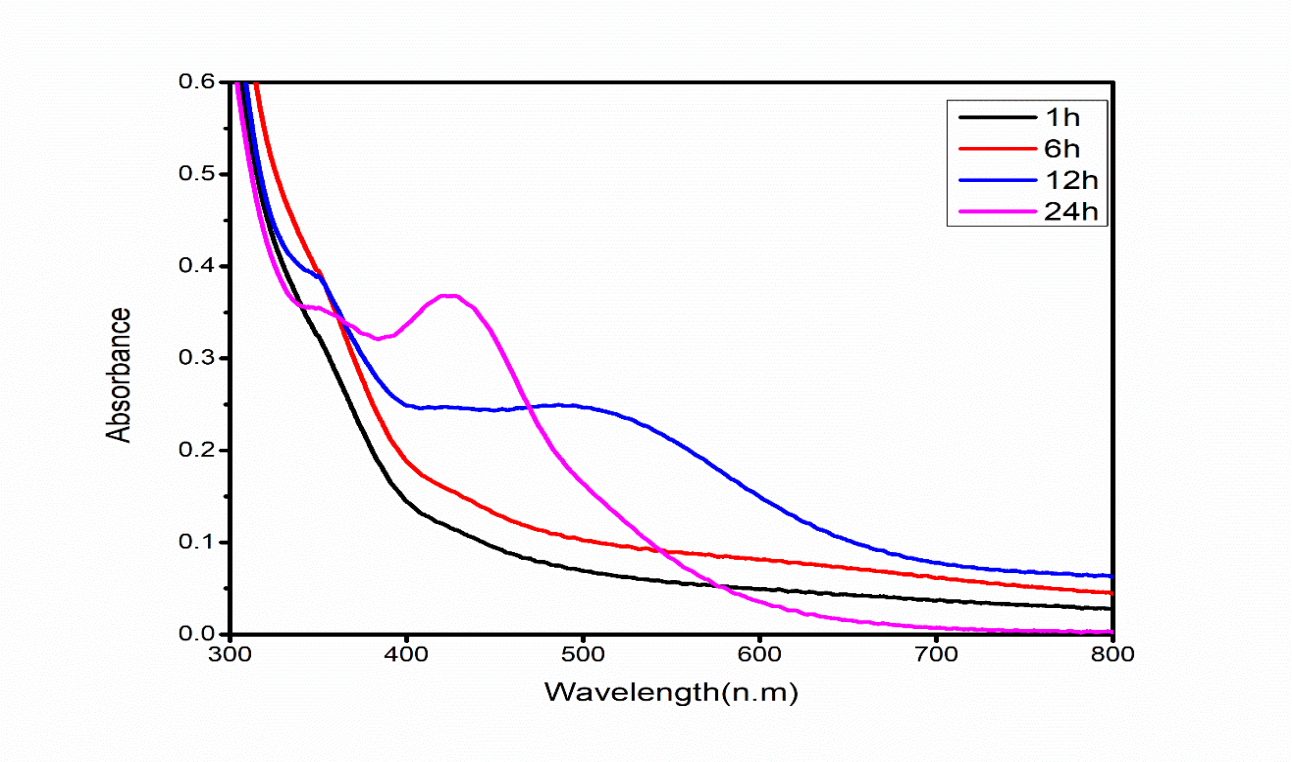


Figure3. 2. UV-visible absorption spectra of silver nanoparticles synthesized from P. aeruginosa at different time intervals; (a) 1 hour (b)6 hour (c) 12 hours (c) 24 hours

3.1.2.3.Fourier Transforms Infrared Spectroscopy(FT-IR)

FT-IR was performed. (Figure 3.3**)** shows the FT-IR spectra of cell free culture supernatant of *P. ae ruginosa* and AgNPs with many peaks. The spectra show the number of peaks that were positioned at 3273, 2981, 2135, 1635, 1379 and 1074 cm^{-1}.

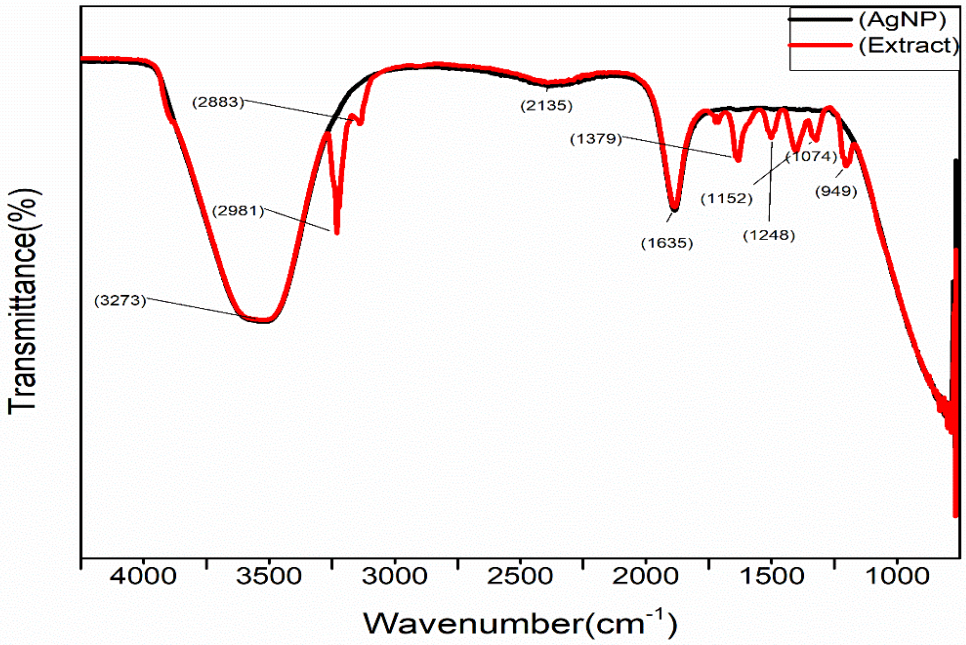


Figure3. 3. Fourier-transform infrared spectra of *Pseudomonas aeruginosa* cell free culture supernatant and PA-AgNPs

3.1.2.4.X-Ray Powder Diffraction (XRD)

The XRD analysis confirms the crystal-like nature of PA-AgNPs, that shows characteristic peaks at 2ɵ values of diffraction peaks at 32.16, 46.16, 6.41 and 76.71 corresponding to XRD planes of (111), (200), (220), and (311).

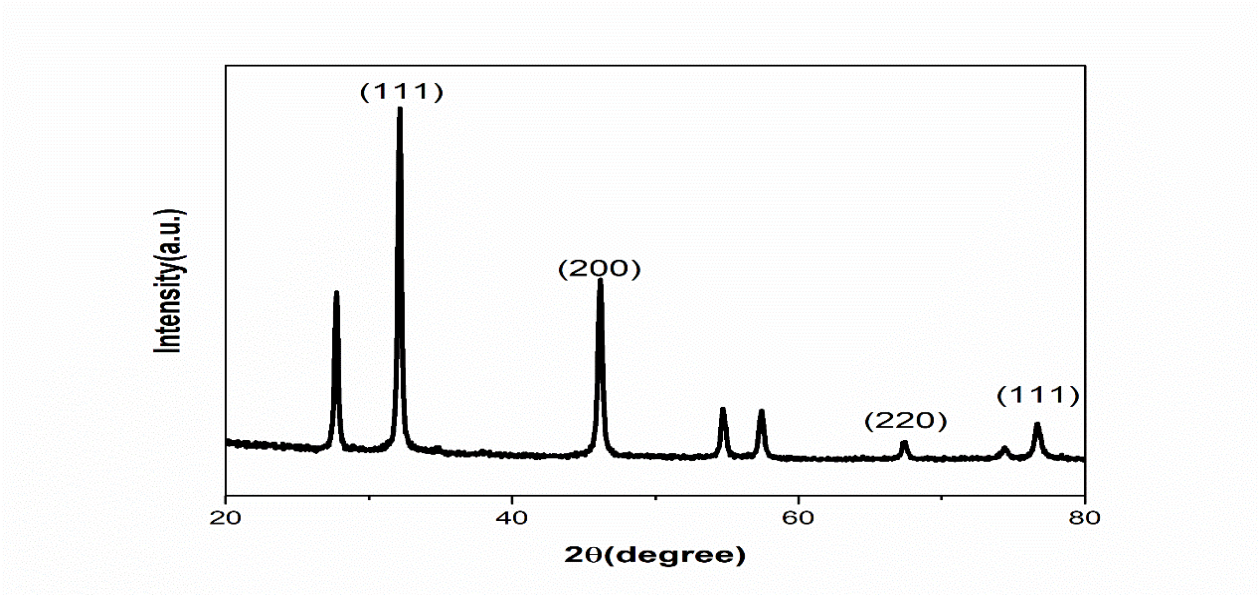


Figure3. 4. X-Ray Diffraction Pattern of AgNPs synthesized from *P. aeruginosa*. XRD peaks observed from 20° to 80° confirmed successful formation of the crystalline AgNPs

3.1.2.5.Transmission Electron Microscopy (TEM)

TEM micrographs revealed that spherical shaped silver nanoparticles were formed while very few particles have irregular shape and predominantly spread. Particles size ranges from 5 nm to 50nm (Figure 3.5)

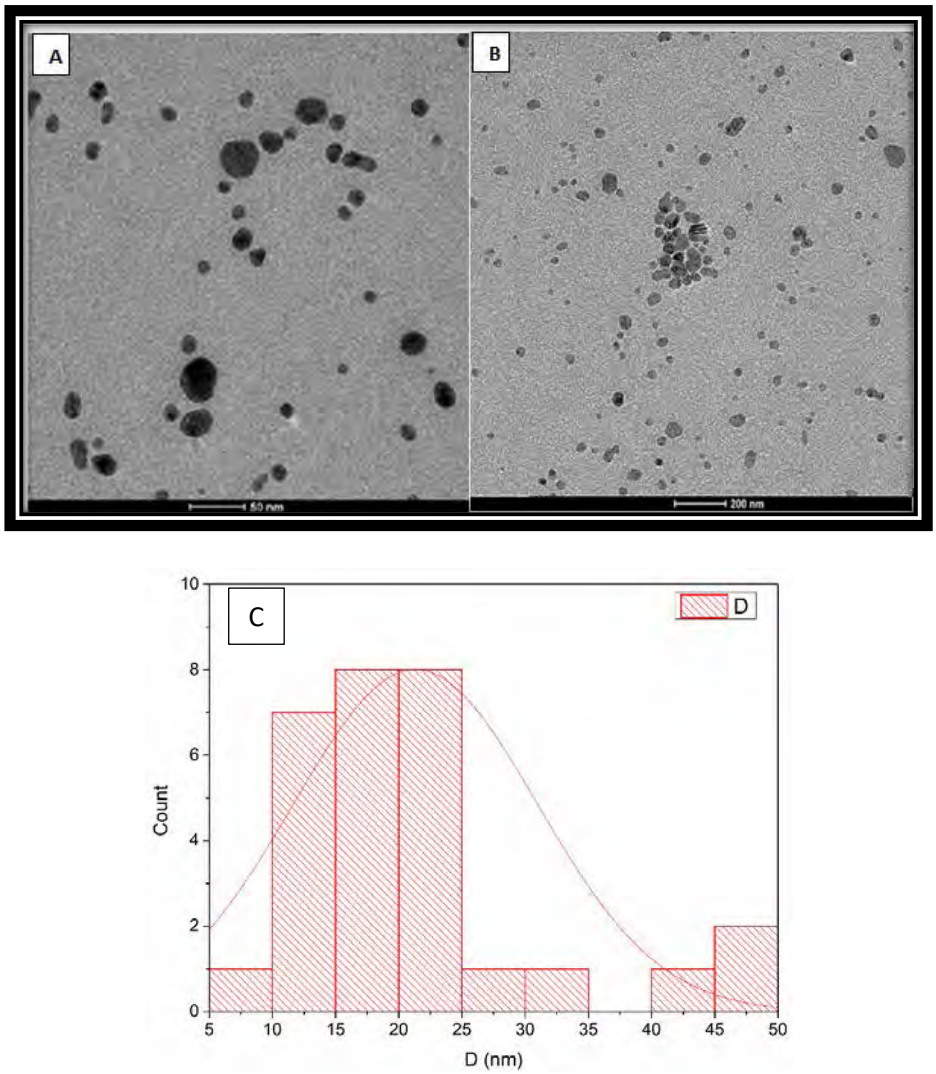


Figure3. 5TEM image of AgNPs synthesized by *P. aeruginosa* shows spherical shape particles at scale bar of (a) 50 nm, (b) 200nm and (c) shows size distribution of AgNPs at 50nm

EXPERIMENTAL II

3.2. Synthesis of Silver Nanoparticles (AgNPs) from Plants

3.2.1. Introduction.

Production and assemblage of nanoparticles via chemical or physical means have substantial adverse effect on the environment as major of their impurities are hard to decontaminate and often consume more energy (Parveen et al. 2016; Sathishkumar et al. 2016; Khan and Lee 2020). In contrast, nanoparticle created from plants extracts is the most reasonable option because of their eco-friendliness, biocompatibility, and reduced toxicity (Logeswari et al. 2013; Verma and Mehata 2016; Khan et al. 2020). The biological synthetic method is also economical, ecologically-sound, and easily can be produced in bulk amount (Mittal et al. 2013).

Many plants such as *Luffa acutangula* (Taruna et al. 2016), *Bauhiniatomentosa*(Ramar et al. 2018), *Theobroma c acao* (Thatikayala et al. 2019), *Aloe ver a* (Ahmadi et al. 2018), and *Bridelia r etusa* (Vinayagam et al. 2018) have shown potential to reduce silver nitrate and form AgNPs. In search of broad activities, efficiency, cost reduction and eco friendliness, researchers are continuously investigating new plants for the production of novel nanoparticles.

In connection with this effort, this study report the synthesis of silver nanoparticles through the reduction of aqueous Ag+ with *Fraxinus xanthoxyloides* and *Bischofia javanica* leaf extract

Fraxinus xanthoxyloides naturally present in Hindu Kush and Himalayan mountains of Pakistan. In northern parts of Pakistan, bark, leaves and roots of *Fraxinus* plant has been conventionally utilized for the treatment of malarial and pneumonia infections. A

list of chemical components has been extracted from *Fraxinus* plant comprising of secoiridoids, phenyl ethanoids, lignans, flavonoids, and coumarins. Certain biologically active ingredients were obtained from *Fraxinus* plant i.e. Catechin Fraxetin, Esculetin, Syringin, Oleoside 11methyl ester, Calceolarioside B, Tannic acid and Quercetin Rutin (Sarfraz et al. 2017).It is also stated that methanol extract of *F. xanthoxyloides* leaves possess anti-inflammatory, analgesic (Younis et al. 2016b), anti-leishmanial (Younis et al. 2016c)and hepatoprotective capabilities (Younis et al. 2016a).

Bischofia j avanica is an edible plant natural inhabitant of South Asian countries specially India and Pakistan. The seeds, bark, leaves and roots of *Bischofia* plant has been conventionally utilized as a source of food treatment option for sores, tooth ache and eye infections (Panda et al. 2018). A list of chemical components has been extracted from *Bischofia* comprising of secoiridoids, phenyl ethanoids, lignans, flavonoids, and coumarins. (Indra et al. 2013)

3.2.2. Synthesis of Silver Nanoparticles (AgNPs) by *Fraxinus xanthoxyloides* Leaf Extract

3.2.2.1.Materials And Methods

Leaves were plucked from the *Fraxinus xanthoxyloides* plants present in the Bio Lawn of Quaid-I-Azam University, Islamabad and were brought to the laboratory. Silver nitrate of analytical grade, Potato dextrose agar and Luria Bertani (LB) broth and agar was bought from Sigma Aldrich. Germany.

3.2.2.2.Preparation of Plant Leaf Extract

The obtained *Fraxinus x anthoxyloides* leaves were 25g in weight, which were subjected to washing thrice by distilled water, after that they were exposed to Millipore water so that all the dirt and other pollutants could be removed from these. Shredded leaves were added into 100ml of Millipore water in 200ml Erlenmeyer flask, followed by boiling for 15 min. Whatman (No.1) filter paper was utilized for filtration of the plant extract(Farooqui et al. 2010; Nagati et al. 2012; Rafiq et al. 2020).

3.2.2.3.Synthesis of AgNPs from Extract of *F. xanthoxyloides* Leaves

To synthesize AgNPs, a solution of 0.001M $AgNO_3$was prepared in 100ml of Millipore water. The fabrication of AgNPs was confirmed through turning light brown color to dark brown when 10ml of the leaf extract (*F. xanthoxyloides*) was transferred to 90ml of 1mM silver nitrate solution. The silver ion reduction was determined by measuring UV–Vis spectra at different intervals ranged from 200-800nm (Perugu et al. 2016; Rafiq et al. 2020).

3.2.2.4.Characterization of AgNPs

The effective biosynthesis of silver nanoparticles was then validated through the use of different instruments. The optical density of FX-AgNPs was measured by UV-Visible Cary 7000 spectrophotometer (Agilent technologies United States). The crystallinity of processed samples was checked through Rigaku Ultima IV X-ray diffractometer working at 45kV voltages. Morphological structure of silver nanoparticles was determined through Scanning electron microscope (SEM, JSM-JEOL USA). Transmission electron microscope (FEI Tecnai G2 Spirit Twin TEM instrument USA) was used to assure the size of particle. Polydespersity index (PDI) was calculated by the equation (1)

$$PDI = \left(\frac{\sigma}{\chi}\right)^2 \quad (1)$$

Equation 1, σ and χ represent the standard deviation and mean size diameter, correspondingly. Fourier transform infrared spectrum was obtained by FT-IR instrument (Vector 22, Bruker Germany) (Rafiq et al. 2020)

3.2.3. RESULTS

3.2.3.1. Visual Observation and UV-Visible Spectroscopy of FX-AgNPs

Gradual change in the color from light brown to dusky blackish brown was experienced when Plant extract was mixed with the salt of silver nitrate (**Figure 3.6)**.

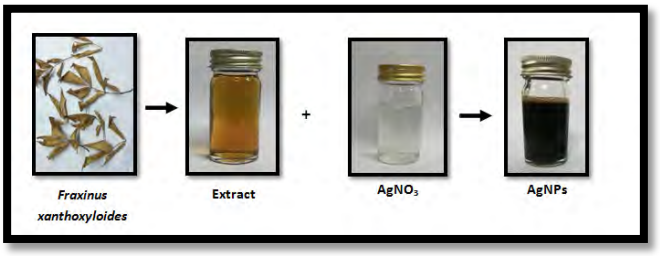


Figure3. 6 Graphical representation of FX-AgNPs biosynthesis

Change in the color shade indicated development of silver nanoparticles. It was due to surface Plasmon resonance (SPR) incitement of the silver nanoparticles. The optical properties of biosynthesized AgNPs were examined in the wavelength range of 250 to 800 nm UV-Vis spectrophotometry. Sharp absorption peak could be seen slightly above 430nm (Figure 3.7). The net yield of nanoparticle obtained was 53.64%.

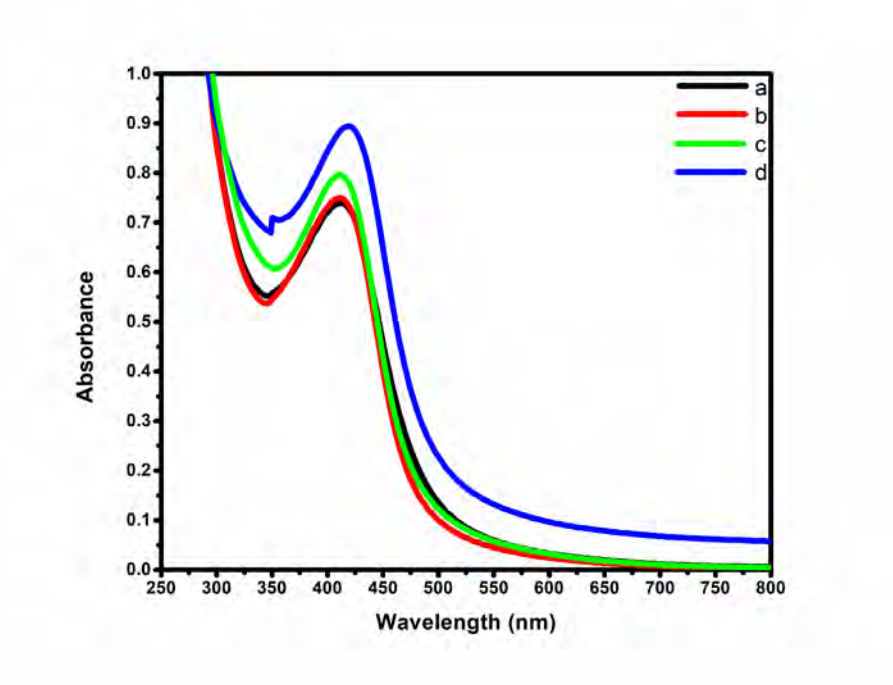


Figure3. 7 UV-visible absorption spectra of silver nanoparticles synthesized from *Fraxinus xanthoxyloides* leaf extract at different time intervals; (a) 30 min (b)1 hour (c) 2 hours (c) 4 hours

3.2.3.2. XRD Analysis

The crystallinity of synthesized silver nanoparticles from FX leaf extract was checked by XRD. The XRD pattern showed diffraction peaks at 77.51°, 64.94°, 38.15°, and 44.27° can be indexed to the planes (311), (220), (111) and (200) respectively (Figure 3.8).

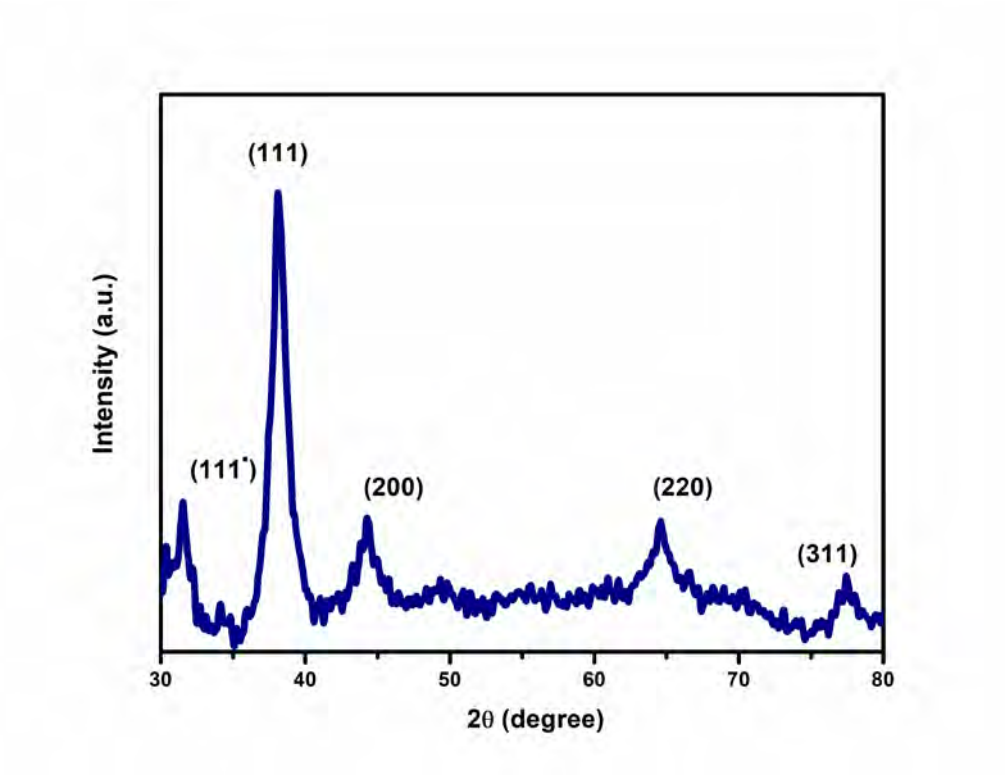
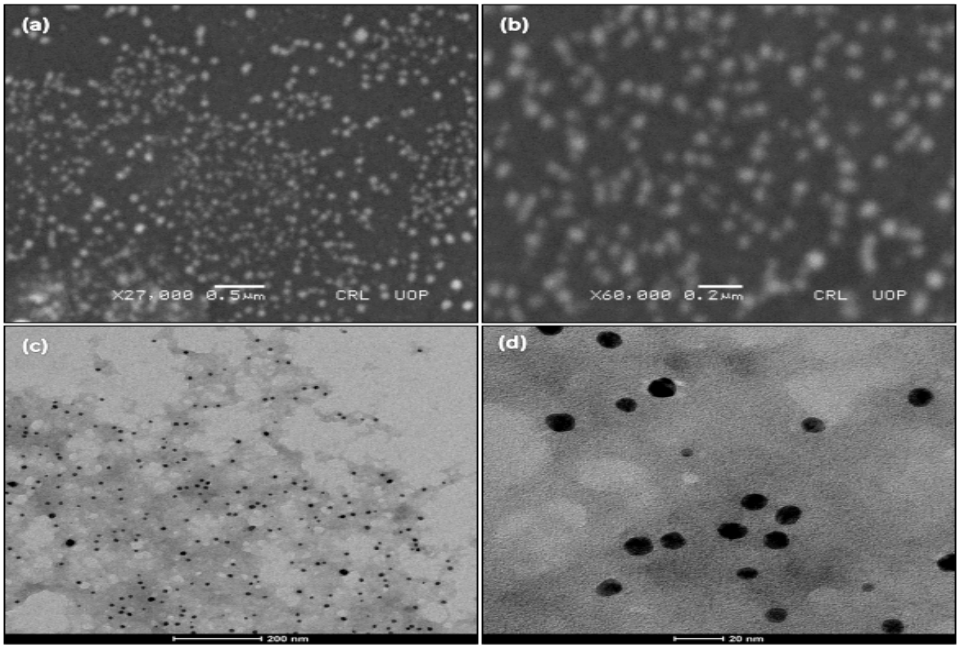


Figure3. 8 X-ray Diffraction Pattern of AgNPs synthesized from *Fraxinus xanthoxyloides* leaf extract. XRD peaks observed from 30° to 80° confirmed successful formation of the crystalline AgNPs

3.2.3.3. Scanning and Transmission Electron Microscopy

To explore the morphology and microstructure of newly prepared particles, scanning and transmission electron microscopy was done to analyze the shape and dispersion of the Ag nanoparticles. Figure (3.9A and B) portrays spherical morphology with dispersed nature of the AgNPs. The particle size of the AgNPs was calculated by ImageJ software with a typical diameter of 72nm.TEM micrographs revealed that the most of the AgNPs possess regular ball-shape, while some of them have irregular shape and predominantly spread (Figure 3.9C**)**. It confirms the spherical morphology of Ag nanoparticles and is in accordance with SEM images.



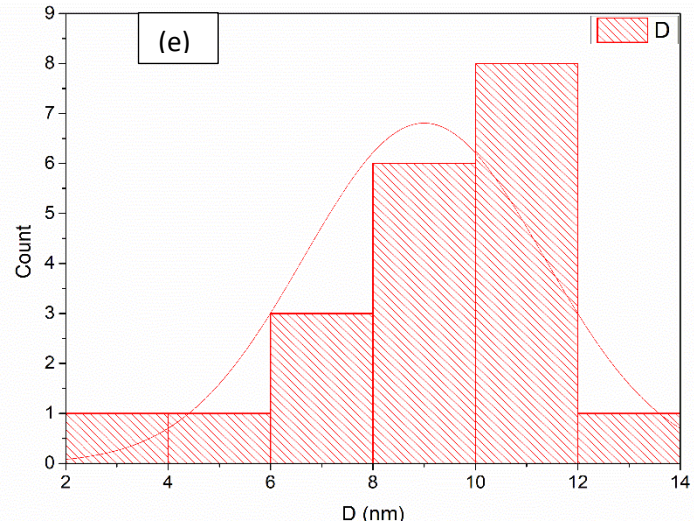


Figure3. 9(a)-(e) (A) SEM image of FX-AgNPs; scale bar is 0.5µm, (B) SEM image of FX-AgNPs illustrates morphology of nanoparticles; scale bar is 0.2µm,(C) TEM image of FX-AgNPs; scale bar is 200 nm, and (d) TEM image shows spherical shape of FX-AgNPs; scale bar is 20nm while (e) shows size distribution at 20 nm

3.2.3.4. FT-IR Analysis

In addition, Figure 3.10 shows the FT-IR spectrum of prepared nanoparticles, and FX-leaf extract, which revealed the functional group responsible for stabilizing and reducing silver nanoparticles. FT-IR spectra of AgNPs prepared from *Fraxinus xanthoxyloides* leaf extract showed different absorption peaks at 3207, 2907,1600, 1312, 1038 and 764 cm^{-1}. FX-leaf extract showed peaks at 3211, 2927, 1575, 1443and 1034 cm^{-1} respectively.

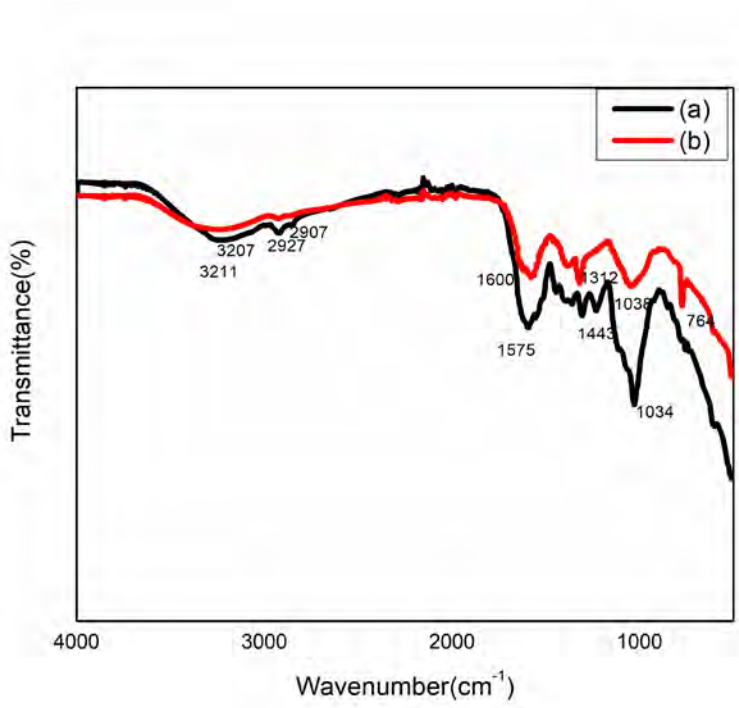


Figure3.10Fourier-transform infrared spectra of (a) *Fraxinus xanthoxyloides* leaf extract (b) FX-AgNPs

3.3. Synthesis of Silver Nanoparticles (AgNPs) from *Bischofia javanica* Leaf Extract

3.3.1.MATERIALS AND METHODS

Leaves were plucked from the *Bischofia j avanica* plant present in the Bio Lawn of Quaid-I-Azam University, Islamabad and were brought to the laboratory. Silver nitrate of analytical grade, Potato dextrose agar and Luria Bertani (LB) broth and agar was bought from Sigma Aldrich. Germany.

3.3.1.1. Preparation of Plant Leaf Extract

The obtained *Bischofia javanica* leaves were 25g in weight, which were subjected to washing thrice by distilled water, after that they were exposed to Millipore water so that all the dirt and other pollutants could be removed from these. Shredded leaves were added into 100ml of Millipore water in 200ml Erlenmeyer flask, followed by boiling for 15 min. Whatman (No.1) filter paper was utilized for filtration of the plant extract(Farooqui et al. 2010; Nagati et al. 2012; Rafiq et al. 2020).

3.3.1.2.Synthesis of AgNPs from Extract of *Bischofia javanica* Leaves

To synthesize AgNPs, a solution of 0.001M $AgNO_3$was prepared in 100ml of Millipore water. The fabrication of AgNPs was confirmed through turning light brown color to dark brown when 10ml of the leaf extract (*Bischofia j avanica*) was transferred to 90ml of 1mM silver nitrate solution. The silver ion reduction was determined by measuring UV–Vis spectra at different intervals ranged from 200-800nm (Perugu et al. 2016).

3.3.2. Characterization of AgNPs

The effective biosynthesis of silver nanoparticles was then validated through the use of different instruments. The optical density of AgNPs was measured by UV-Visible Cary 7000 spectrophotometer (Agilent technologies United States). The crystallinity of processed samples was checked through Rigaku Ultima IV X-ray diffractometer working at 45kV voltages. Transmission electron microscope (FEI Tecnai G2 Spirit Twin TEM instrument USA) was used to assure the size of particle. Fourier transform infrared spectrum was obtained by FT-IR instrument (Vector 22, Bruker Germany).

3.3.3. Results

3.3.3.1. Visual Observations and UV-Vis Spectrophotometry of AgNPs

Through visual observations, change in color of the reaction mixture was monitored. Images were taken at the beginning and at the end of the experiments.

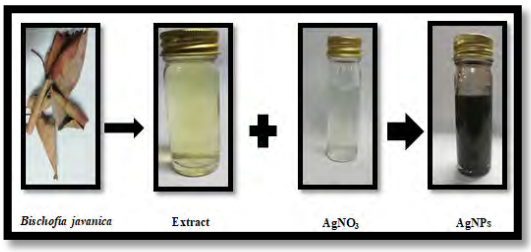


Figure3.11Graphical representation of BJ-AgNPs biosynthesis

The optical properties of biosynthesized AgNPs were examined in the wavelength range of 250 to 800 nm UV-Vis spectrophotometry. Absorption peak could be seen around 420nm (Figure 3.12). The net yield of nanoparticle obtained was 55.5%

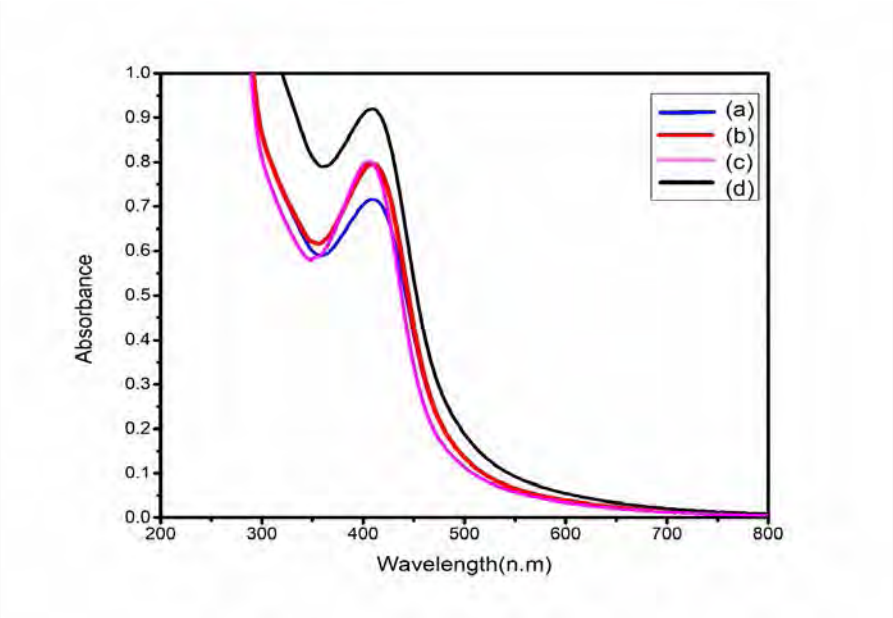


Figure3.12UV-visible absorption spectra of silver nanoparticles synthesized from *Bischofia javanica* leaf extract at different time intervals; (a) 30 min (b)1 hour (c) 2 hours (d) 4 hours

3.3.3.2. XRD

The crystalline structure of silver nanoparticles synthesized from B.J leaf extract was confirmed by XRD. The XRD pattern showed diffraction peaks at 77.08°, 64.02°, 43.96° and 37.76°, can be indexed to the planes (311), (220), (200) and (111) respectively (Figure 3.13).

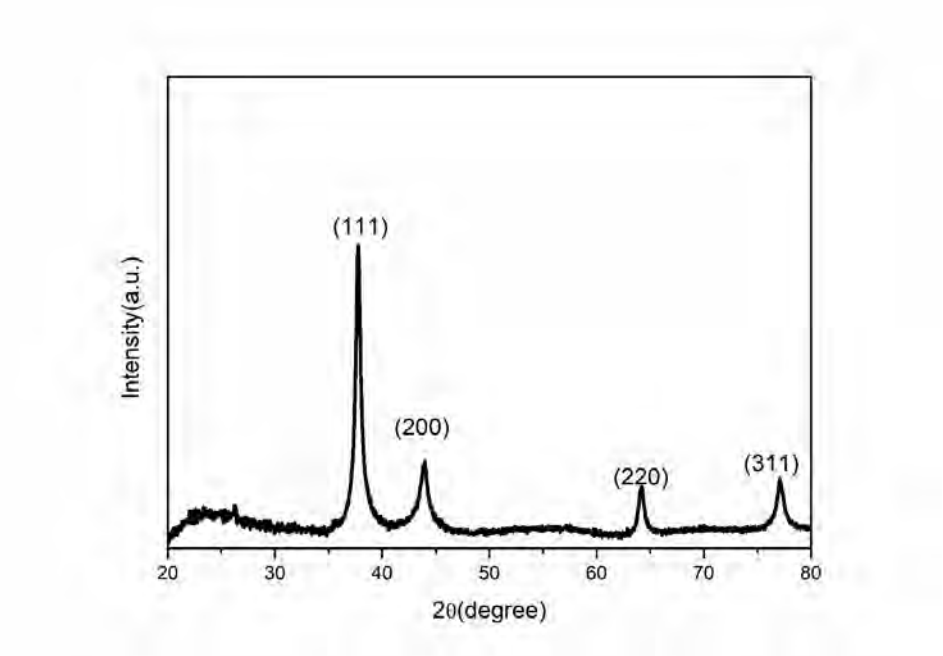


Figure3.13X-ray Diffraction Pattern of AgNPs synthesized from *Bischofia* javanica leaf extract. XRD peaks observed from 20° to 80° confirmed successful formation of the crystalline AgNPs

3.3.3.3. FT-IR

(Figure 3.14) shows the FT-IR spectrum of nanoparticles and B.J-leaf extract, which revealed the functional group responsible for stabilizing and reducing silver nanoparticles. FT-IR spectra of AgNPs and leaf extract of *Bischofia javanica* displayed absorption peaks at3296, 2943,2883, 1209, 1340 and 988 cm^{-1} respectively.

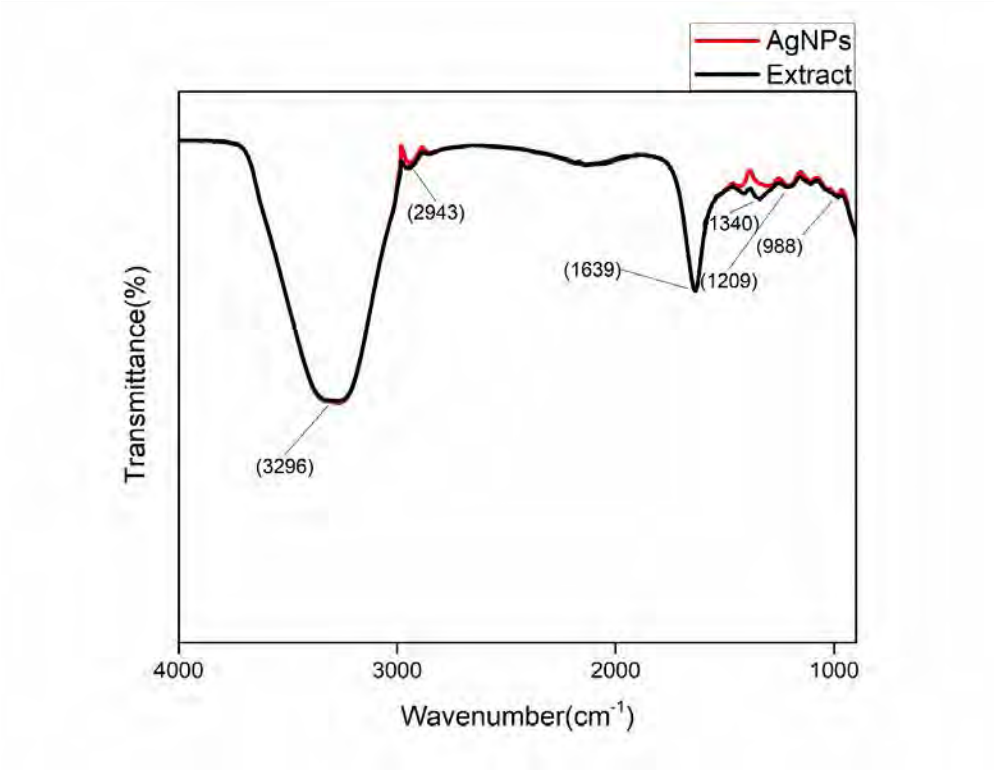
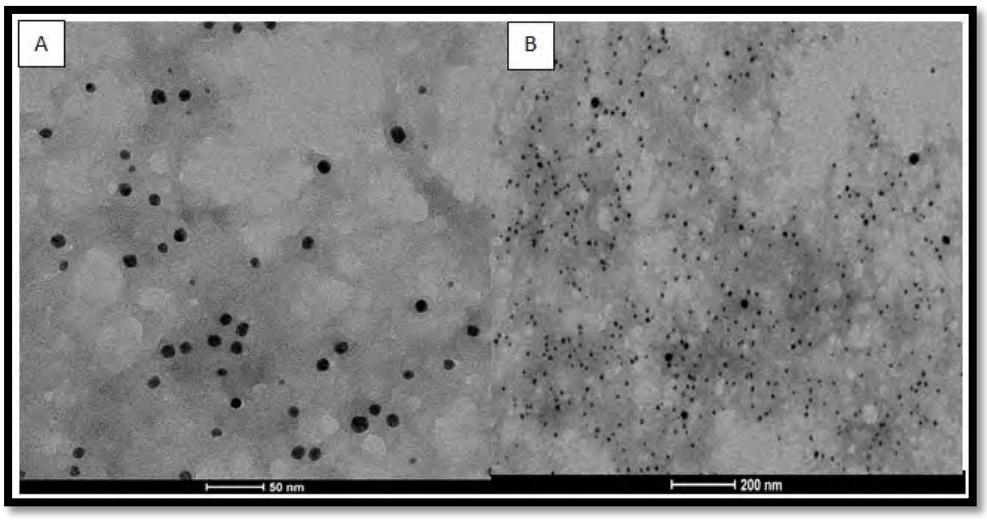


Figure3.14Fourier-transform infrared spectra of (a) *Bischofia javanica* leaf extract (b) BJ-AgNPs

3.3.3.4. TEM

Morphology and microstructure of newly prepared silver nanoparticles were explored by transmission electron microscopy. It was done to analyze the shape and dispersion of the Ag nanoparticles. Figure (3.15A and B) shows spherical and well dispersed particle. The particle size of the AgNPs was calculated by ImageJ software with a typical diameter range of TEM micrographs revealed that the most of the AgNPs possess regular ball-shape, and very few are irregular in shape. Particles size ranges from 4 nm to 16 nm



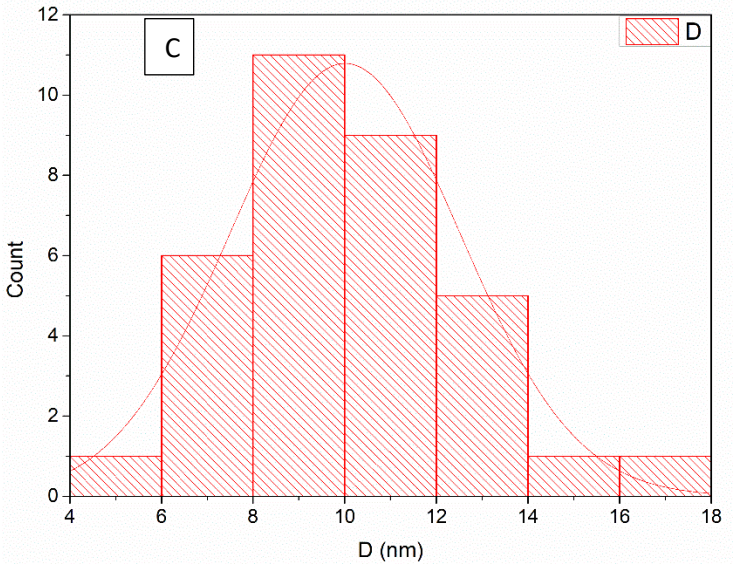


Figure3.15(A) TEM image of BJ-AgNPs; scale bar is 200 nm, (B) TEM image shows spherical shape of FBJ-AgNPs; scale bar is 20nm and (c) shows size distribution at 50 nm

EXPERIMENTAL III

3.4.Applications of Nanoparticles

3.4.1.Introduction

Nanoparticles are highly auspicious particles and gaining interest to address the concerns related to the antibiotic resistance as they can be used as better alternative antimicrobial agent (Beyth et al. 2015). Nano-materials have diversified and improved chemical composition and properties. Therefore, they have wide range applications from agriculture and food processing to healthcare techniques (Sirelkhatim et al. 2015). Nanotechnology is basically of synthesis and characterization followed by the assessment and application of materials at nano-scale (1-100nm) (Mansoori and Soelaiman 2005). When materials are scaled down at atomic level, their surface area is increased as compared with large materials (Huh and Kwon 2011). Nanoparticles are manipulated or controlled particles at nano-scale and the key element of nanotechnology. Unlike their bulk materials, nanoparticles possess significantly different size-related characteristics (Buzea et al. 2007). Along with their small size, nanoparticles have larger surface area than their precursor. This discrete property is also a reason behind their probable applications in bio-nanotechnology, biosensors and Nano-medicine (Ashe 2011). Recent developments in nanotechnology has encouraged the potential applications of NPs against infectious diseases by exploring their antibacterial mechanisms (Huh and Kwon 2011). For instance, nanoparticles have tendency to alter the metabolic activity of bacteria starting from contact with bacterial surface and ultimately end with cell death (Chatzimitakos and Stalikas 2016). This feature of NPs has prompted their use to cure infectious

diseases. Based upon the aforesaid implication, biologically synthesized AgNPs were investigated as antibacterial and antifungal agents separately and in synergism with antibiotics. In addition, in-vitro cytotoxic and antibiofouling activities of B AgNPs were evaluated.

3.4.2. Assessment of different biological activities of Biogenic AgNPs

3.4.2.1. Materials

Sabouraud dextrose broth, Potato dextrose agar and Commercial silver nanoparticles (C AgNPs) (100 nm, 99.5 %) nAg metal were purchased from Sigma-Aldrich Chemicals Co. (St. Louis, MO, USA), whilst Potato dextrose broth, Mueller Hinton Agar and Nutrient Agar were obtained from BD® Difco (Franklin lakes, NJ, USA).

3.4.2.2.Methods

Assays for the determination of biological activities

In order to assess the biological activities of AgNPs, following assays were performed:

Minimum inhibitory concentration

Antibiofilm assay

Cell viability assay

EPS estimation

Hemolysis assay

Antibiofouling assay

Cytotoxicity

3.4.2.3. Antimicrobial Activity of Biogenic silver nanoparticles

3.4.2.3.1.Minimum Inhibitory Concentration of Biogenic silver nanoparticles (P.A-AgNPs) by Well diffusion method

Well diffusion method (S. Harikumar, 2016) is extensively utilized for antibiotic susceptibility testing. *Escherichia coli, Staph Aureus, Pseudomonas aeruginosa Klebseilla pneumoniae* were used to test the activity of prepared nanoparticles. Plates were poured with 50ml of Muller Hinton Agar which is used specifically for antibiotic susceptibility. Plates were then allowed to solidify. Bacterial suspension was prepared in 1ml normal saline. . Optical density (O.D) of different bacterial isolates was adjusted at 625nm and concentration of bacterial suspension was kept at 10^6 CFU respectively (Balouiri et al. 2016) After that bacterial lawn was made with the help of sterile cotton swab. Wells are produced with sterile tip. The 30 µl of inoculum and nanoparticles with four different concentrations like 100ppm, 200ppm, 300ppm and 400ppm were added in each well. Plates were allowed to incubate for 24hours. Next day zone of inhibition was measured for all the samples tested.

3.4.2.3.2.Minimum Inhibitory Concentration of Biogenic silver nanoparticles (FX-AgNPs) by Microtiter plate assay

All tested strains were cultured overnight, bacterial strains at LB agar and candida on PDA. Fresh cultures of tested microorganisms were used i.e. *P. aeruginosa, S. aureus*, MRSA and *C. albicans*. Broth culture of the microorganisms was centrifuged for 10min at 10,000 g and 4°C for obtaining the pallet. The pallet was washed with phosphate buffer saline (PBS). Supernatant from all the falcons was discarded and pellets were suspended in the fresh media i.e. Luria Bertani (LB) broth. Optical density (O.D) of

different bacterial isolates was adjusted at 625nm and concentration of bacterial and fungal suspension was kept at 10^8 and 10^6 CFU respectively (Balouiri et al. 2016) The 100 µl of inoculum and nanoparticles with four different concentrations like 50ppm, 100ppm, 150ppm and 200ppm were added in each well of a 96 well microtiter plate .The microtiter plate was covered and incubated for 24h at 37°C. Next day, O.D was taken at 492nm for *C. al bicans (Scorneaux e t al . 201 7)* and 600nm for bacterial strains by Thermo Multiskan EX Microplate Photometer (Thermo Fisher Scientific) (Rafiq et al. 2020).

3.4.2.3.3.Minimum Inhibitory Concentration of Biogenic silver nanoparticles (BJ-AgNPs) be well diffusion method

All tested strains were cultured overnight. *P. aeruginosa*, *S. aureus, K. pneumoniae* and *E. coli* were four bacteria and one fungi candida albicans used to check the zone of inhibition with nanoparticles. Muller Hinton Agar (MHA) was poured in each petri plates and wait for half hour to solidify the media. 1mL of normal saline was added in an eppendorf using micropipette. The bacterial colony was taken through a sterile wire loop and infused into normal saline to form a suspended material. The inoculum turbidity was controlled via comparing it by 0.5 McFarland standards. Lawn of bacterial isolate was bacterial lawn was made over MHA petri plate utilizing sterile swab. Wells were rigged on the petri plate utilizing aseptic cork borer. Different nanoparticle concentrations 100ppm, 200ppm and 300ppm were made in sterile deionized water. Afterwards, 20 µl of each nanoparticle's solutions were dropped in each prepared well on inoculated plate. Test was executed in replica to confirm the inhibitory zone. Prepared plates were then inverted and stored in an

incubator for 18-24 hours at 37°C. The inhibitory zone was measured via reading scale.

3.4.2.4. Determination of synergistic effect of nanoparticles(P.A-AgNPs) with antibiotic

Two-dimensional micro dilution assay was performed to investigate the antimicrobial activity of silver nanoparticles synthesized from *Pseudomonas aeruginosa*(B-AgNPs) and chemically synthesized silver nanoparticles (C- AgNPs) in combination with antibiotics(Hwang et al. 2012) Minimum Inhibitory Concentration (MIC) of nanoparticles particle was determined, and the Fractional Inhibitory Concentration (FIC) of combination of nanoparticles with antibiotics was subsequently determined in 96-well microtiter plate with the help of checkerboard micro titration method. Nutrient broth was prepared as a growth medium and inoculated with overnight culture of bacteria. The bacterial suspension was adjusted to 1×10^8 cells by comparing it with McFarland or by taking the OD at 600 nm and100 µl of suspension were added in all the wells. The solutions of B-AgNPs and C-AgNPs (100 ppm) with antibiotics (20 µg/ml) were prepared according to the Clinical & Laboratory Standards Institute (CLSI) guidelines and 20 µl of each solution of nanoparticles and antibiotics were added in combination and alone as well. The plates were incubated for 18-24 hours at 37°C and results were analyzed by quantifying optical density (OD) at 600 nm. The combined antibiotic effect of agents A and B (where A is AgNPs, and B is one of two antibiotic agents) was calculated as follows:

The FIC index: =MIC (A in combination with B)/MIC (A alone) + MIC (B in combination with A)/ MIC (B alone)

The FIC index (FICI), calculated as the sum of each FIC, was interpreted as follows: FICI<0.5, Synergy; 0.5 ≤ FICI <1, Partial synergy; FICI5 = 1, Additive; 2 ≤ FICI < 4, Indifferent; 4 < FICI, Antagonism (Odds, 2003).

3.4.2.5. Antibiofilm properties of nanoparticles

3.4.2.5.1.Screening of biofilm producing bacteria

3.4.2.5.1.1. Congo red assay

The frequently used qualitative method for the detection of biofilm forming bacteria is Congo red assay (Bellifa *et al.,* 2016) Congo Red Agar (CRA) was prepared by adding brain heart infusion (BHI) 37 g/L, sucrose 36g/L, Congo red dye 0.8 g/L and agar 10.5 g/L. Autoclaved Congo red stain was added separately in autoclaved BHI agar at 55^0C temperature. The media was poured into petri plates when reached at touchable range. 5 strains were streaked on CRA plates followed by incubation at 37^0C for overnight. Results were recorded after 24 hours interval.

3.4.2.5.1.2.Quantification of biofilms by Microtiter plate assay

The most commonly used method for the quantification of biofilm former bacteria is microtiter plate assay (MPA). The assay comprises of measurement of optical density of stained bacteria by using 96 well plates and comparing it with cut off value afterwards. **(**Saxena *et al.,* 2014**)** Biofilms were quantified by taking optical density at

595nm with ELISA auto reader and compared with cutoff value. Cut off (ODc) value was determined by using formula:

ODc = Average OD of negative control+ 3x Standard deviation of negative control**.**

Table3. 1 Classification of *Pseudomonas biofilms* by the MTP method

Mean OD value	**Adherence**	**Biofilm formers**
>0.320**. 1**	Strong	Strong
0.120-0.320	Moderate	Moderate
<0.120	Non	Non/weak

3.4.2.5.2. *P. aeruginosa* biofilm production and treatment with nanoparticles

For the development of *Pseudomonas ae ruginosa*, bacterial cell suspension was prepared in LB broth supplemented with 2% glucose and 100 µL of suspension was added in microtiter plates. The plates were incubated at 37^0C for 24 hours. The wells were replaced with fresh media after 10-15 hours to observe the activity of nanoparticles at intermediate stage. After 24 hours, plates were washed with PBS to wash out the planktonic cells from plate twice. 100 µl of nanoparticle solution was added in it and incubated for 24 hours at 37^0C. Wells without nanoparticles and inoculum were designated as negative control and wells without nanoparticles but with bacterial suspension were considered as positive control.

3.4.2.5.2.1.Anti-biofilm Assay

For testing the Antibiofilm activity of biogenic AgNPs synthesized from *Fraxinus xanthoxyloides* leaf extract, the microtiterplate assay was performed following (Masum

et al. 2019; Rafiq et al. 2020) with some modifications. Cell suspension of *P. aeruginosa* was cultured in fresh LB broth in a shaker incubator. Optical density (O.D) was adjusted at 625nm and concentration of suspension was kept at 10^8 CFU (Balouiri et al. 2016) Each well of microtiter plate was inoculated with 100 µL of inoculums. The plates were then incubated for 24 hrs. at 37°C. Wells were replaced with fresh media after 10-15 hours of incubation of planktonic cells. After 24 hrs, plates were rinsed twice with PBS in order to eliminate the planktonic cells from plate. 100µl of 50ppm nanoparticle suspension was pipetted in it and incubated for 24 hrs at 37°C. Each well was then emptied from the liquid media and rinsed softly with sterilized double distilled H_2O. Additionally, to stain the biofilm in plate, a 100µl of crystal violet (0.1%, w/v) dye was poured to well at ambient temperature and incubated for 45min. The unattached dye was removed from the plate using double distilled H_2O.Wells without nanoparticles and Inoculum were designated as negative control and wells without nanoparticles but with bacterial suspension were considered as positive control. The OD was accessed at 570 nm wavelength using a microtiter plate reader.

$$\%\text{age inhibition} = \frac{\text{OD in control- OD in treatment}}{\text{OD in control}} \times 100$$

3.4.2.6. Cell viability assay

Cell viability assay is the evaluation of redox status of cell which comprises of reduction of tetrazolium dyes e.g. 2, 3-bis [2-Methoxy-4-nitro-5-Sulfophenyl]-2H-tetrazolium-5-carboxanilite inner salt (XTT). Oxidoreductase enzyme in bacteria reduces the colorless salt of XTT into bright orange substance in the presences of electron transporter Phenazine metho-sulfate (PMS). The effect of nanoparticles on

metabolic activity of bacterial cells was investigated through quantification of viable cells. The procedure contains following steps;

i. *Pseudomonas aeruginosa* was inoculated in 3-5 ml of LB broth supplemented with 2% of glucose and incubated at 37^0C for 24 hours.

ii. The cultures were centrifuged at 10,000 rpm for 10 minutes and pallet was harvested by decanting supernatant.

iii. Pallets were washed with 5 ml of PBS and centrifuged again at 10,000 rpm for 10 minutes.

iv. Bacterial suspension was made by addition of 5ml of fresh LB broth and adjusted to 1×10^8 cells by comparing the turbidity with 0.5 McFarland standards.

v. 100 µl of cell suspension was added in 96 well microtiter well plates along with negative control containing LB broth and incubated for 24 hours at 37^0C.

vi. After incubation, media was decanted from wells and washed twice with PBS

vii. 100 µl of fresh LB broth with 2% of glucose was added in all the wells of microtiter plate and subjected with treatment of 100 µl of B-AgNPs and C-AgNPs suspensions along with positive control without nanoparticles and incubated for 24 hours at 37^0C.

viii. Media was removed and wells were washed twice with PBS.

ix. 90 µL of XTT was added in each well followed by addition of 10 µL of PMS. Plate was incubated in dark at room temperature for 1 h.

x. Absorbance was measured at 492 nm by using multi-scan go microtiter plate reader.

xi. Cell viability was assessed by comparing absorbance values with control.

3.4.2.7.EPS estimation

Loop full of overnight culture was taken and added into test tubes having 5 ml of LB broth supplemented with 2% of glucose. Cultures were subjected to treatment with 200 µl of B-AgNPs and C-AgNPs suspensions along with cultures without nanoparticles served as control. The cultures were incubated at 37^0C for 24 hours. Next day, the cultures were centrifuged at 10,000 rpm for 10 minutes to obtain the supernatant. 1ml of supernatant was collected in fresh vial and absolute chilled ethanol was added by two volumes to precipitate out the EPS and incubated at 4^0C for overnight. The suspension was centrifuged at 10,000 rpm for 10 minutes to harvest EPS pallet by decanting supernatant and dried it at room temperature. The dry weight was measured by weighing balance.

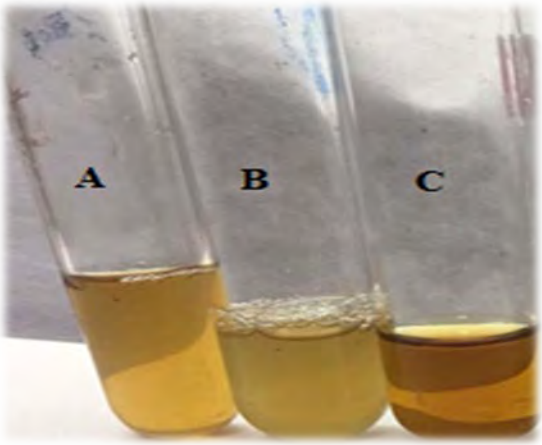


Figure3. 16EPS estimation A) P. aeruginosa treatment with B-AgNPs B) *P. aeruginosa* treatment with C-AgNPs, C) *P. aeruginosa* with no treatment

3.4.2.8.Hemolysis Assay

The cytotoxicity of nanoparticles on human cells can be investigated by performing hemolytic essays. Hemolytic assay was carried out for the purpose of investigation of hemolytic activity of Biogenic AgNPs with human erythrocytes. 10 ml of human blood sample was taken in a sterile tube containing Ethylenediaminetetraacetic acid (EDTA). After collection of blood sample, it was centrifuged for 15 min at 3000rpm to obtain Red Blood Cells (RBCs). RBCs were separated from other cells by discarding supernatant. The pallet of RCs was then washed and diluted phosphate buffer saline (PBS). Different concentrations of nanoparticles ranging from 10 to 100ppm ppm were added in RBCs. A positive control was prepared by mixing 1.5ml of Tween 80 with RBCs. The test samples and control were then incubated for 1h at 37°C followed by centrifugation for 15 min at 3000 rpm. The supernatant was recovered for spectroscopic analysis at 540 nm.

The percentage of hemolysis was calculated from the formula:

$$OD_{540}\,(\text{sample}) - OD_{540}\,(0\%\ \text{Lysis})/OD_{540}\,(100\%\ \text{Lysis}) - OD_{540}\,(0\%\ \text{Lysis}) \times 100\%$$

3.4.2.9. Statistical Analysis

All the experiments were performed in triplicates. IBM SPSS 25 was used for analysis of results. The concentrations of nanoparticles for each microorganism were taken as independent variable while the percentage of inhibition was taken as dependent

variable and one-way ANNOVA was applied. One sample t test was performed to know the level of biofilm reduction statistically.

3.4.3. Results

3.4.3.1. Antimicrobial Properties of B-AgNPs

3.4.3.1.1.Minimum Inhibitory Concentration of PA-AgNPs against gram positive and gram negative bacteria by well diffusion method

MIC of B-AgNPs was assessed by well diffusion method against Gram positive and Gram negative bacteria. It was perceived from the results that as the concentration of PA-AgNPs increases the diameter of zone of inhibition also increased. The maximum inhibition was observed against *Klebseilla. pnemoniae* presenting inhibition zone of 10mm at 100ppm and 200ppm concentration and 15mm at concentration 300ppm and 400ppm (Fig 4.12). Minimum inhibition was seen in case of *Staph aureus* which exhibited inhibition zone of 4mm at 100ppm concentration and 9mm at 200 ppm concentration (Fig 4).Zone of inhibition was enlisted against all four species tested. Significant inhibition was shown at 300 ppm as p value was less than 0.005. Detail results of MIC of are shown in Figure 3.17

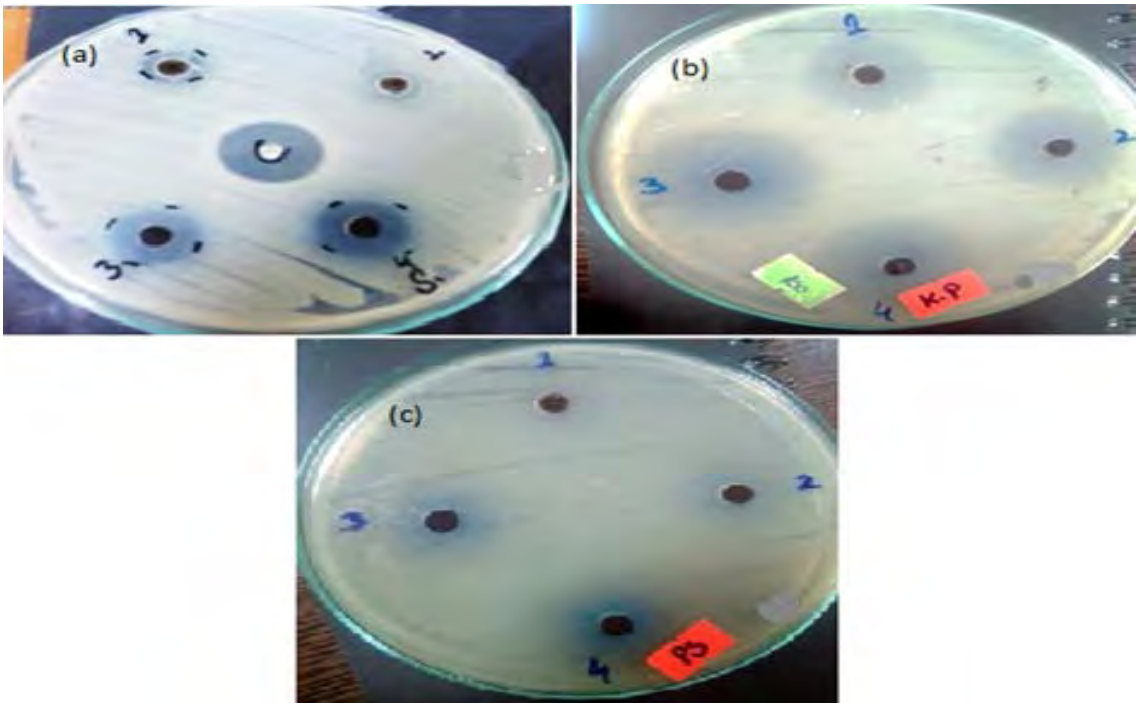


Figure3 17MIC of PA-NPs by well diffusion method zone of inhibition against (a) *s. aureus* (b) *K. Pneumoniae* and (c) *P. aeruginosa*

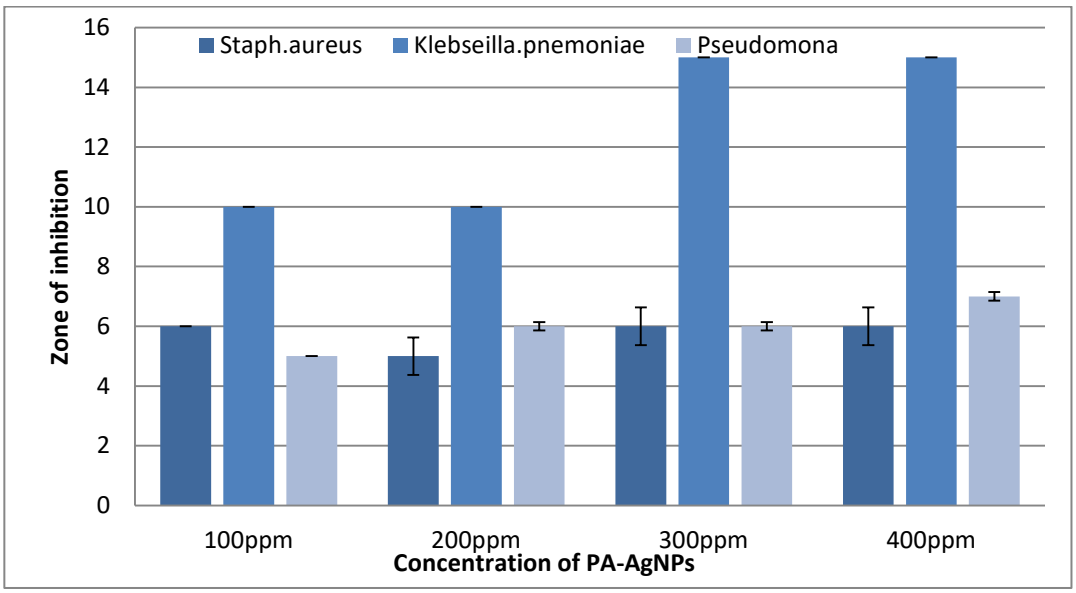


Figure3. 18Antimicrobial Activity of B-AgNPs synthesized from *P. aeruginosa* against MDR strains at different concentration ranges from 100 ppm to 400 ppm

3.4.3.1.2. Minimum Inhibitory Concentration by microtiter plate assay

Four concentrations of Biogenic-AgNPs(synthesized from F.X leaf extract) i.e. 50ppm, 100ppm, 150ppm and 200ppm were used against tested microorganisms. AgNPs displayed noticeable activity at each concentration. The maximum microbial reduction

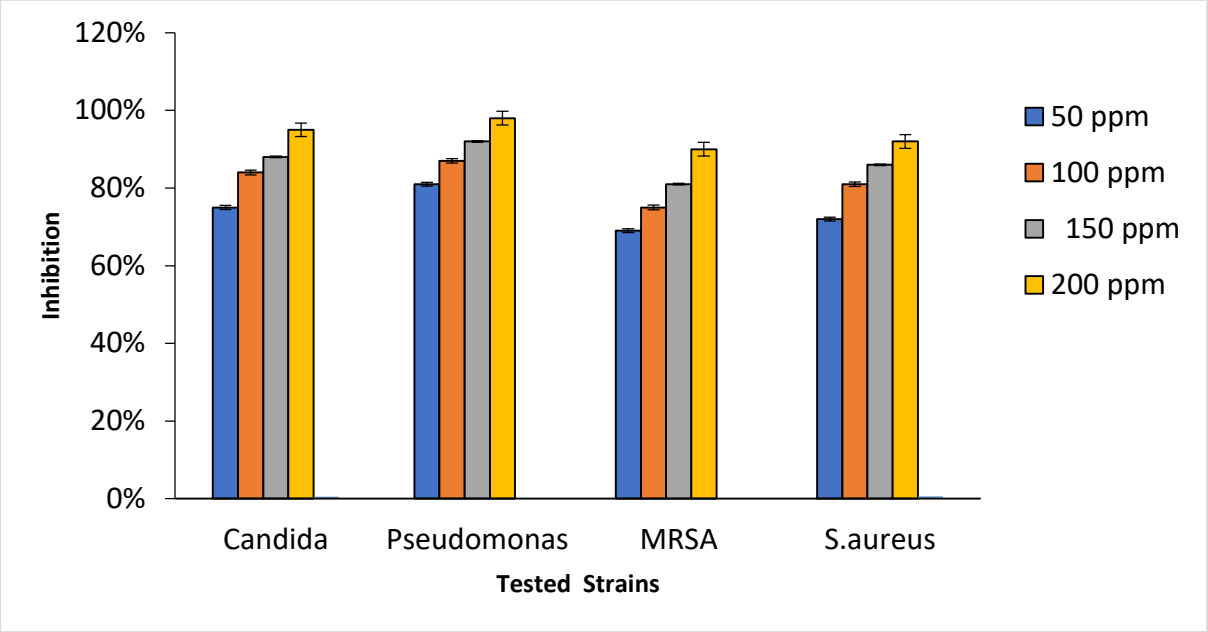


Figure3.19Antimicrobial Activity of FX-AgNPs against MDR strains at different concentration ranges from 50 ppm to 200 ppm

was observed at 200ppm and lowest reduction was observed against MRSA at a concentration of 50ppm i.e.69%. It has been observed that increase in the concentration of nanoparticle increase inhibition of test microorganism significantly. The p-value for 50ppm was recorded 0.155, for 100ppm and 150 ppm $P<0.05$ and for 200ppm $P<0.01$(Figure3.19).

3.4.3.1.3. Antimicrobial susceptibility of BJ-AgNPs against fungi, gram +ve and gram –ve microorganisms

This antimicrobial activity was checked by well diffusion method. AgNPs showed MIC against *S. aureus*, *E. coli, K. pneumoniae* and *C. albicans* at 100ppm concentration. However, AgNPs also indicated high susceptibility at 200pppm and 300ppm against *S. aureus*, *E.coli, K. pneumoniae* and *C .albicans.* (Table 3.2)

Table3.2Antimicrobial Activity of BJ- AgNPs against isolated microorganisms

microorganisms	Activity of AgNps in different concentrations		
	100ppm	200ppm	300ppm
Klebsiela pneumoniae	10mm	11mm	13mm
Staph.Aureus	7mm	9mm	9mm
E.coli	7mm	8mm	10mm
Candida albican	6mm	8mm	10mm
Pseudomonas aeruginosa	7mm	9mm	12mm

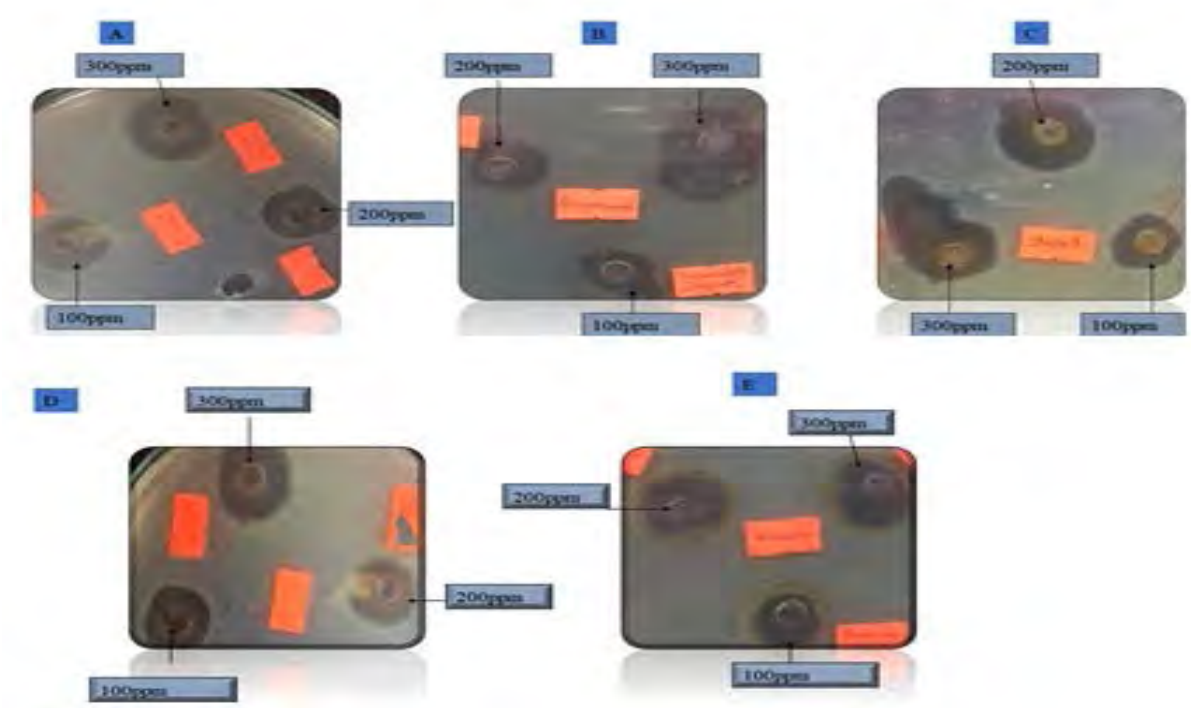


Figure3. 20 Antibacterial Activity of BJ-AgNPs against isolated microorganisms: a) E. coli b)*P. aeruginosa* c*) S .aureus* d) *C albicans* e) *K. pneumoniae*

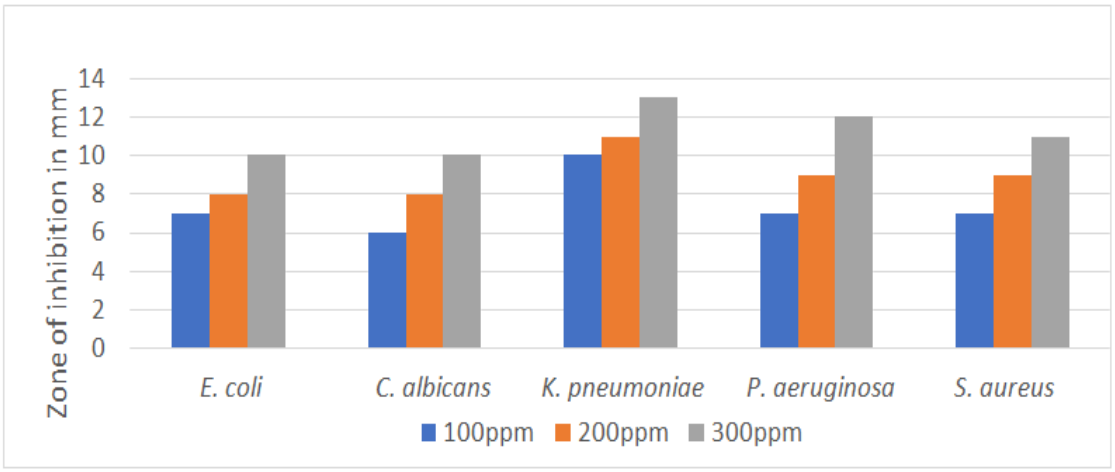


Figure3. 21Bar chart representing the MIC of each pathogen as analyzed by well diffusion method

3.4.3.2.Determination of synergistic effect of nanoparticles with antibiotic

The Minimum inhibitory concentration of *P. aeruginosa* based silver nanoparticle (B-AgNPs) in combination with antibiotics was evaluated by broth micro dilution chequer board method in accordance with CLSI standards. The results were compared with commercially available silver oxide nanoparticles. The results of combination assay are represented against *S. aureus, E. coli* and MRSA in Table 3.3, 3.4 and 3.5, respectively. Most of the combinations exhibited effectiveness against respected pathogen. Synergistic behavior of AgNPs + Ciprofloxacin was observed whereas the synergistic effect of AgNPs + Ampicillin, Other particles showed only partial synergistic or additive effect. Moreover, synergistic interaction of Nano + Ciprofloxacin was not found in any particle, whearas AgNPs + exhibited synergistic effect against *E. coli*

S, Synergistic; PS, Partial Synergistic; A, Additive; I, Indifferent

Table3.3Activities of combinations of Nanoparticles + Antibiotics against *S. aureus*

	FICI (µg/ml)	
Nanoparticles	**Nano + Ciprofloxacin**	**Nano +Ampicillin**
B-AgNPs	0.35 (S)	0.08 (S)
C :AgNPs	2.63 (I)	0.29 (S)

Table3.4Activities of combinations of Nanoparticles + Antibiotics *against E. coli*

	FICI (µg/ml)	
Nanoparticles	**Nano + Ciprofloxacin**	**Nano +Ampicillin**
B-AgNPs	1.04 (A)	0.38 (S)
C :AgNPs	1.89 (I)	0.77 (PS)

Table3.5Activities of combinations of Nanoparticles + Antibiotics against MRSA

	FICI (µg/ml)	
Nanoparticles	**Nano + Ciprofloxacin**	**Nano +Ampicillin**
B-AgNPs	1.00 (A)	0.35 (S)
C-AgNPs	0.92 (PS)	0.50 (PS)

3.4.3.3. Biofilm studies

3.4.3.3.1. Qualitative Screening of Biofilm Formers by Congo Red Assay

All the five strains were tested for their biofilm formation potential by the Congo red plate assay. CRA assay plates were incubated for 24-h, 48-h and 72-hours results were in the following Figure 3.22. *Pseudomonas* strain showed the highest potential of biofilm formation while *S. aureus* shows the potential for biofilm formation beside rest of the strains i.e. MRSA and *Candida al bicans* showed the medium ability for biofilm formation.

Figure3. 22 Qualitative Screening of Biofilm Formers

3.4.3.3.2.**Inhibition of Pseudomonas biofilms by nanoparticles**

The effect of nanoparticles on biofilm of pseudomonas was evaluated by using 50 ppm concentration through broth micro dilution method. Biofilm of *P. ae roginusa* was quantified after incubation at 30°C without agitation. Inhibition was observed against 50 ppm of FXAgNPs and C-AgNPs in comparison with control(Figure 3.23).

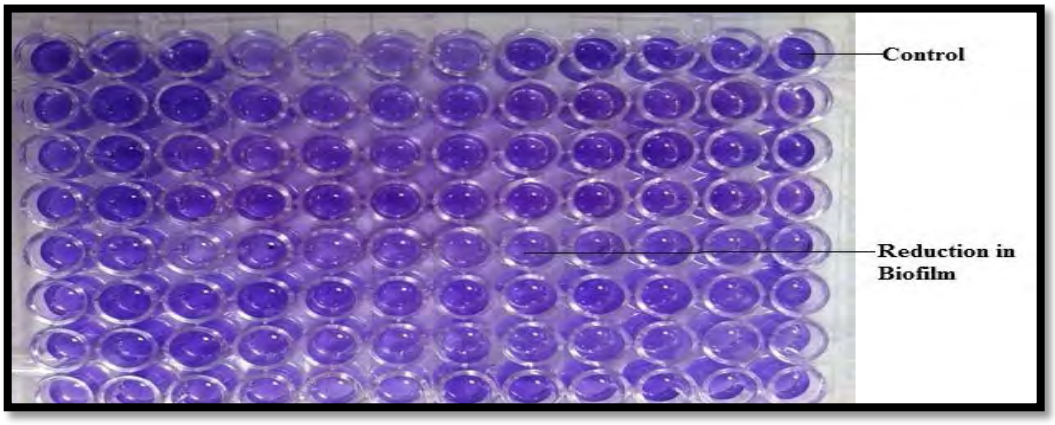


Figure3.23Effect of FX-AgNPs and C-AgNPs on biofilm formation of *P. aeruginosa,* light purple stained well indicate reduction in biofilm in comparison to dark purple stained control wells

The OD_{570} value of *P. aerogenousa* was 1.03 without AgNPs and with FX-AgNPs had an OD value 0.137. while with C-AgNPs 0.3. Hence, the green synthesized FX-AgNPs triggered 86.8% drop in the biofilm as compared to the control (Figure 3.24**)**. The t-test showed a p value of 0.041 for reduction of biofilm formation.

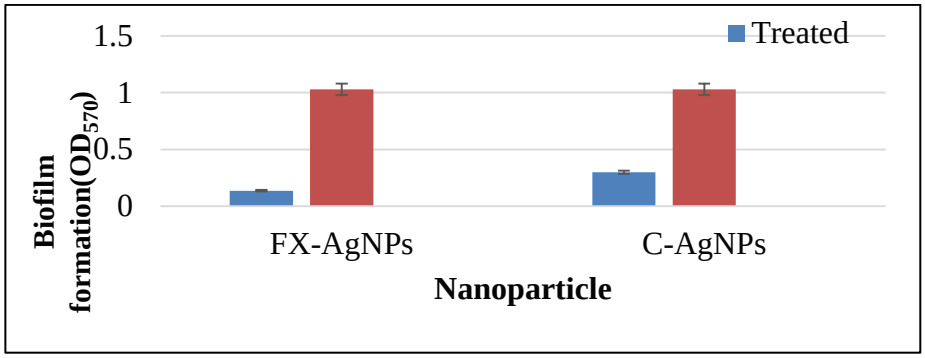


Figure3.24Biofilm formation of *P. aeruginosa*, test sample with FX-AgNPs and control without FX-AgNPs. Biofilm reduction of 86% was observed in test sample as compared to control

3.4.3.4.Reduction in metabolic activity

Along with biofilm formation ability of bacteria, metabolic activity was assessed as well by using XTT metabolic assay. In this assay, substrate is converted into colored product as a result of metabolic activity of mitochondrial enzymes. Reduction in metabolic activity was displayed by nanoparticles. BJ-AgNPs showed optimal reduction i.e. 62% whereas in C-AgNPs displayed 50%

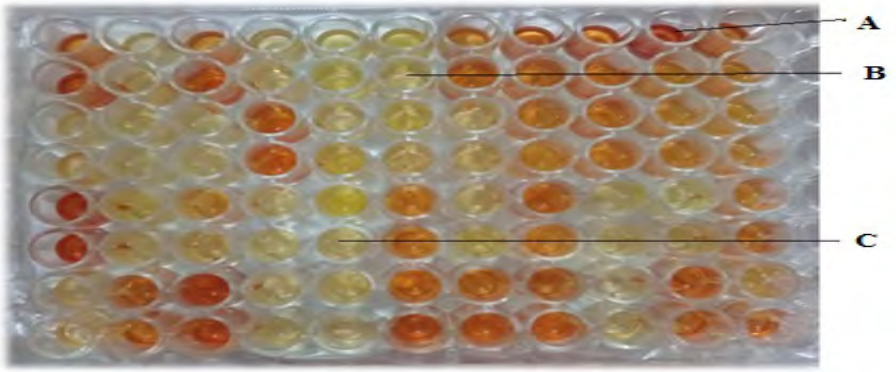


Figure3.25 Shows a representative result for reduction in metabolic activity performed for *Pseudomonas aeruginosa*. A) Control B) change in color from orange to yellow is clear indication of reduction in metabolic activity C) Pale yellow color represent significant reduction in metabolic activity

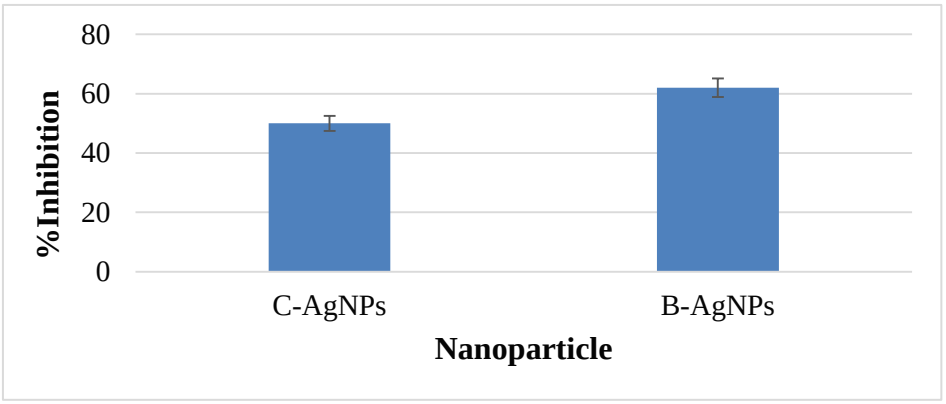


Figure3.26 Graphical illustration of reduction in metabolic activity by BJ-AgNPs and C-AgNPs against *P. aeruginosa*

3.4.3.5. Reduction in extracellular polymeric substances (EPS)

The influence of nanoparticles on extracellular polymeric substance was quantified by determining the dry weight of EPS obtained after the centrifugation of precipitated solution. A comparison was made between the EPS pallet of positive control with the pallet of treated samples. A decrease in extra polymeric substance was observed in case of both nanoparticles as compared to the control. However, B-AgNPs showed greater reduction in dry weight of EPS of *Pseudomonas aeruginosa*.

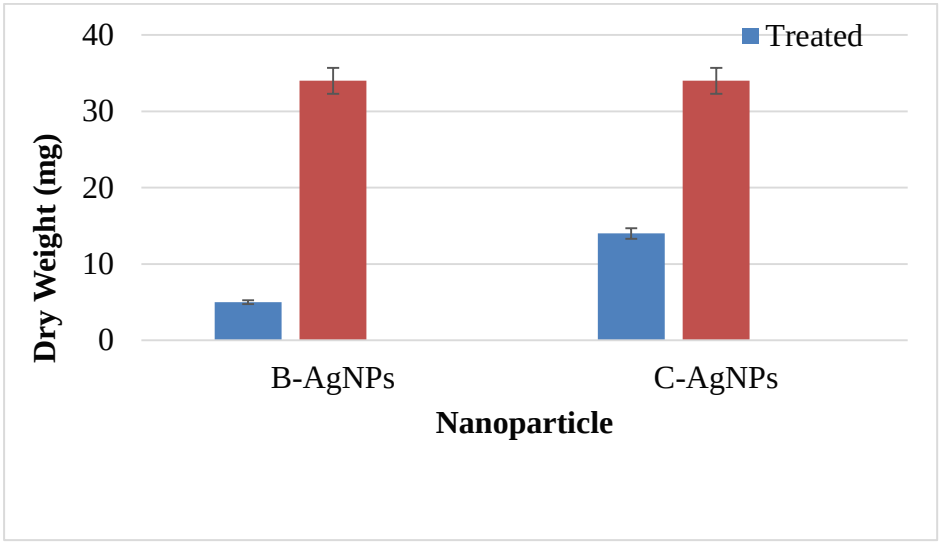


Figure3. 27Graphical illustration of reduction in EPS by B-AgNPs and C-AgNPs *against P. aeruginosa* in comparison with control

3.4.3.6.Cytotoxic effect on Human red blood cells (RBCs)

The hemolysis assay was employed to monitor the extent of cytotoxicity of nanoparticles. Human red blood cells (RBCs) were treated with nanoparticles and incubated for one hour at 37°C. A positive control of RBCs exposed to tween 80 was also set along with test samples. After incubation, the collected supernatant of test samples was compared with positive control. It was observed cytotoxicity increased in dose dependent manner.

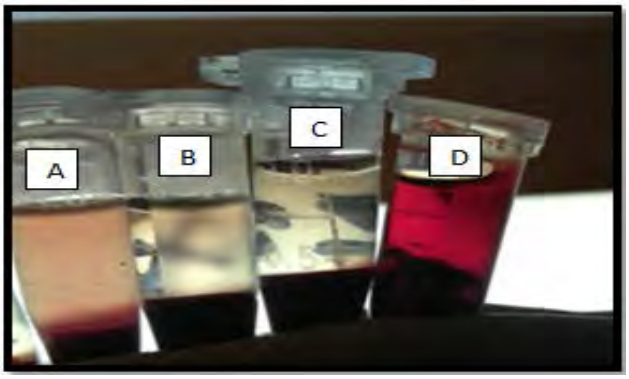


Figure3.28 Hemolysis assay at A) 100ppm B) 50ppm C) 10ppm D) Control. The clear supernatant is indication of zero hemolytic activity.

Table3. 6Hemolysis of RBCs by nanoparticles

Treatment	Percentage hemolysis (10 ppm)	Percentage hemolysis (50 ppm)	Percentage hemolysis (100 ppm)
RBCs + Tween 80	100 ± 0.0	100 ± 0.0	100 ± 0.0
RBCs + AgNPs	1.3 ± 0.03	4.09 ± 0.49	7.03 ± 0.89

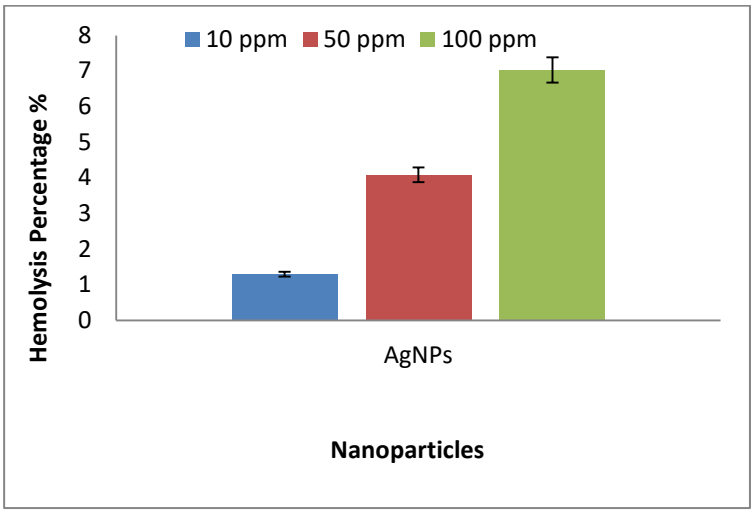


Figure3.29Graphical illustration of Hemolytic activity by B-AgNPs

EXPERIMENTAL IV

3.5. Anti-biofouling capability of Biogenic AgNPs in polysulfone membranes

3.5.1. Materials

Majority of the chemicals and media compounds were purchased from Sigma-Aldrich Chemicals Co. (St. Louis, MO, USA), BDH Laboratory Chemical Division (Poole Dorset, England), Oxoid Laboratory Chemicals (Hampshire, U.K), Difco Laboratory (Detroit Michigan, USA) and E.Merck (Darmstadt, Germany)

3.5.2. Membrane Fabrication

Polyvinylchloride (PVP) 10 % was firstly dissolved in N-Methyl-2-Pyrrolidone (NMP) 75 with constant rousing for half an hour at 50ºC. Polysulfone beads 15% and silver nanoparticles (BJ-AgNPs) 0.22g were slowly added in the solution with the constant stirring at 150 º C for six hours on a hot plate in 250 ml glass beaker. The dope solution allowed cooling at room temperature. The dope solution was cast with the help of an aluminum knife on a solid glass support. Immediately after casting the dope solution the glass solid support was transferred to the water container. Membranes were then allowed to be dehydrated at room temperature. Membranes were then preserved by transferring them to 2 % formaldehyde solution. Kept them at room temperature (Zodrow et al. 2009).

3.5.3. Membrane characterization

Scanning electron microscope (SEM) was used to establish membrane thickness, porous substructure and other morphological features. The specimen subjected to examination was cut into small sized pieces and desiccated before placing on the

copper stub in order to reduce the noise generated during microscopic analysis. The transverse-section of membrane was acquired by "liquid nitrogen freeze fracturing" then samples were subjected to "gold sputtering" (Bio-rad Polaron Division) before examination on a Scanning Electron Microscope (Jeol JSM 5910). Magnifications in the range of 500-7000x and 10-20 kV voltages were applied for the study. Fourier Transform Infra-Red (FTIR) Spectrometry was performed for detection of the structural and functional changes in the framework of membranes with and without nanoparticle after the impregnation of nanoparticles.

3.5.4. Anti-bacterial Assessment Assay by Membrane filtration

Firstly, in L.B broth 3ml from the stationary phase cultures of *S. a ureus*, MRSA, *Pseudomonas aeruginosa* and *Candida albicans* were serially diluted from the stock of 10^9 C.FU/ml. O.D.600 was adjusted at 2.5. Ag-PSF membrane and Psf membrane and vacuum filtration assembly were sterilized by autoclaving at 121^0C for 10 min it is followed by the filtration of required dilution from both membranes. On L.B agar plated membranes were overlay and incubated for overnight at room temperature. At the very next after 24 hours colony forming units (C.F.U) were counted (Zodrow et al. 2009).

3.5.4.1.Biofouling Assay

The considerable ability of microorganisms to form the biofilm was determined by utilizing the procedure described by (De Prijck et al. 2007). *Pseudomonas aeruginosa* was used in this experiment as it was known as the strong biofilm former microorganisms (Jayasekara et al. 1999) *Pseudomonas* strain was used and diluted to 10^{-7} at O.D.600 and membrane coupons were submerged in this diluted of 5ml of culture for

7,14 and 21 days. Both the planktonic and sessile cells were counted as this is the characteristic of the biofilm.

3.5.4.1.1. Planktonic cells

For the enumeration of the planktonic cells membranes coupons submerged in the medium was placed in shaking incubator. In order to evaluate the planktonic cells, 10^7 dilutions were plated on to the L.B agar plate. At the very next day, CFU were notified.

3.5.4.1.2.Sessile Cells

In order to determine the microbial cellular growth on both the membrane surface first, these membrane coupons were detached from the medium and washed with the deionized water followed by placing these membrane coupons in the fresh media i.e. 2 ml. Then these membranes were subjected to a vortex. This whole procedure of biofilm disturbance was repeated twice. Aliquots were obtained straight from the supernatant and placed and spread on the L.B agar plate. The colony-forming units were enumerated after overnight incubation. This experiment was slightly modified according to our respective study.

3.6. Results

3.6.1. Membrane Fabrication

Two different types of membranes were obtained by phase inversion method:

1. Polysulfone membrane with nanoparticles

2. Polysulfone Membrane without nanoparticles

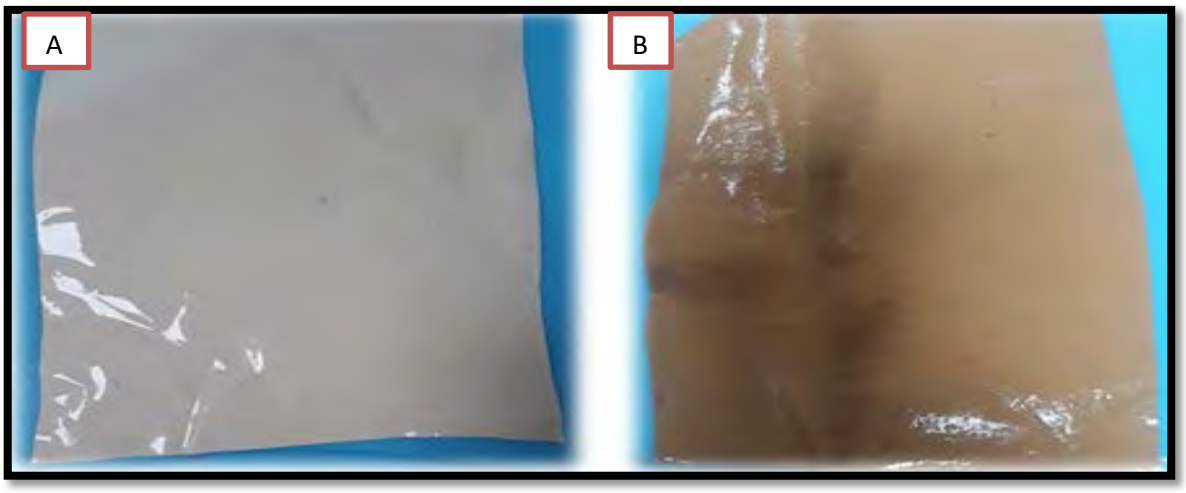


Figure3. 30(a): Polysulfone membrane without nanoparticles (b): Polysulfone membrane with nanoparticles

3.6.2. Characterization of Psf membrane

3.6.2.1.Scanning electron microscopy

Plain and AgNPs impregnated Psf membranes were found to be 110 µm thick. SEM figures indicated that most of the AgNPs were congregated into membrane voids. Membrane's cross section image provided an overview of its porous arrangement. In addition to this, it also indicated that the integration of AgNPs did not impede the development of porous structure. The pore size as was determined in the Psf membrane was around 0.5 to 5 µm. The pores observed were generally spherical in shape

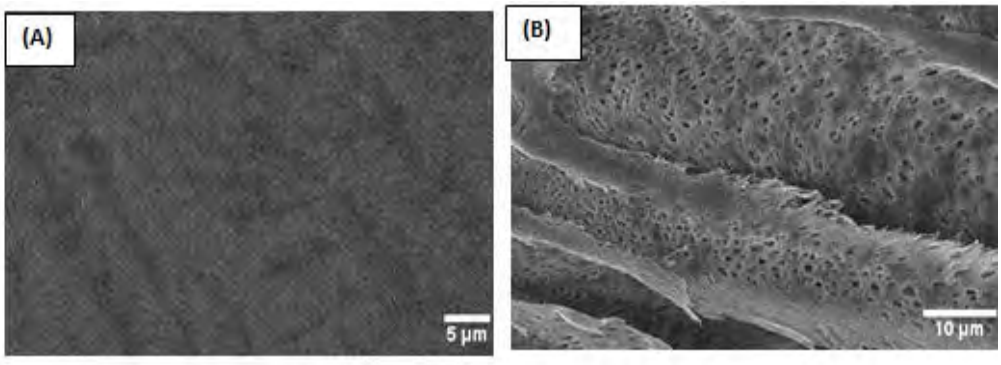


Figure3.31SEM images of plain Psf membrane surface views at different resolutions

3.6.2.2. FTIR of Psf membranes

In comparison to the standard spectrograph (available from Sigma-Aldrich), slight changes in some of the peaks were observed in the FTIR spectrum of synthesized Psf membrane. These changes occurred due to the addition of some other constituents during the membrane preparation. Major peaks corresponding to different groups were observed in the FTIR spectrum of synthesized Psf membrane e.g. Peak corresponding to ester (benzoate) group was observed at 1241cm-1, while at 1150 cm-1 and 1488 cm-1, peaks for sulfone and CH3 group were observed. These groups are chief components of Psf membranes and are also in accord with Sigma-Aldrich standard spectrographs (Figure 3.33 and 3.34).

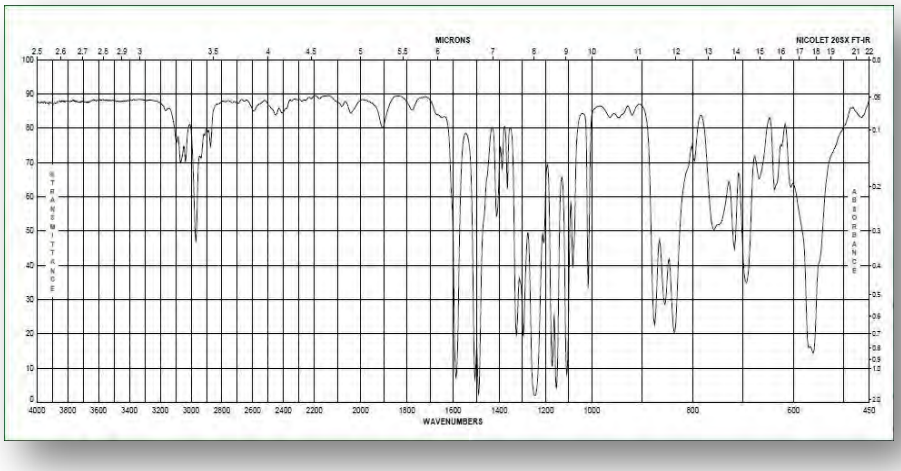


Figure3.32FTIR transmission spectrum of polysulfone (Sigma Aldrich)

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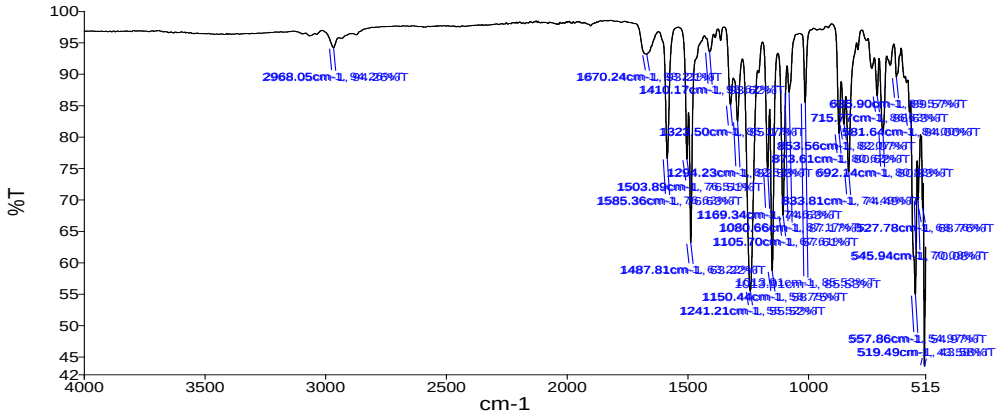

Figure3.33FTIR transmission spectrum of fabricated membrane

3.6.3. Anti-bacterial Assessment Assay by Membrane filtration

Adjusted inoculums of all the test organisms were passed through control (membrane without nanoparticles) and test membranes (impregnated with the B-AgNPs). It was detected that with the increase of silver nanoparticles concentration in polysulfone membrane there was a decrease in the bacterial count passing through these membranes.

At 0.22 % concertration of AgNps, highest number of bacterial colonies were observed and lowest bacterial counts were observed at 1.6 wt% by silver. This trend was observed in all four tested organisms, therefore, it was established that membrane impregnated with silver nanoparticles possesses considerable antimicrobial activity against these tested microorganisms i.e. *P aeruginosa, S aureus, E.coli*, and *Candida albicans.*

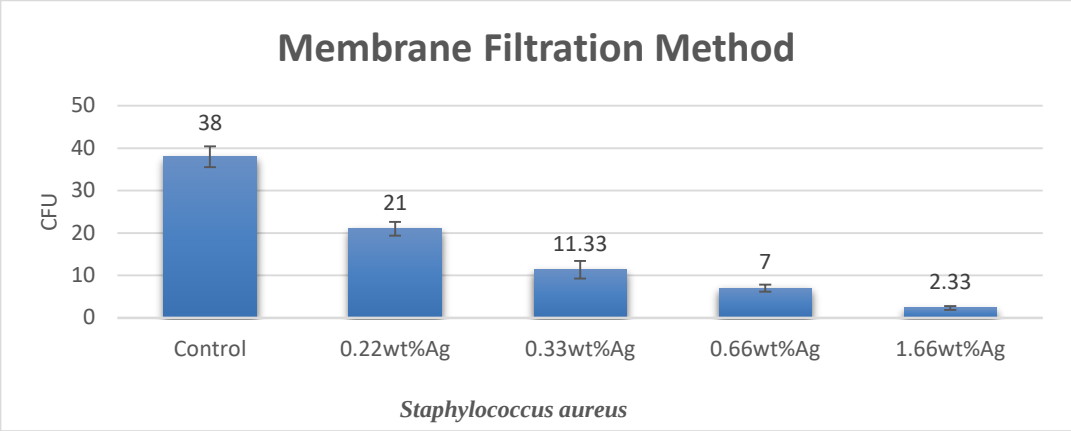

Figure3. 34 Filtration of *S aureus* culture through the Psf membranes impregnated with different concentration of B-AgNPs

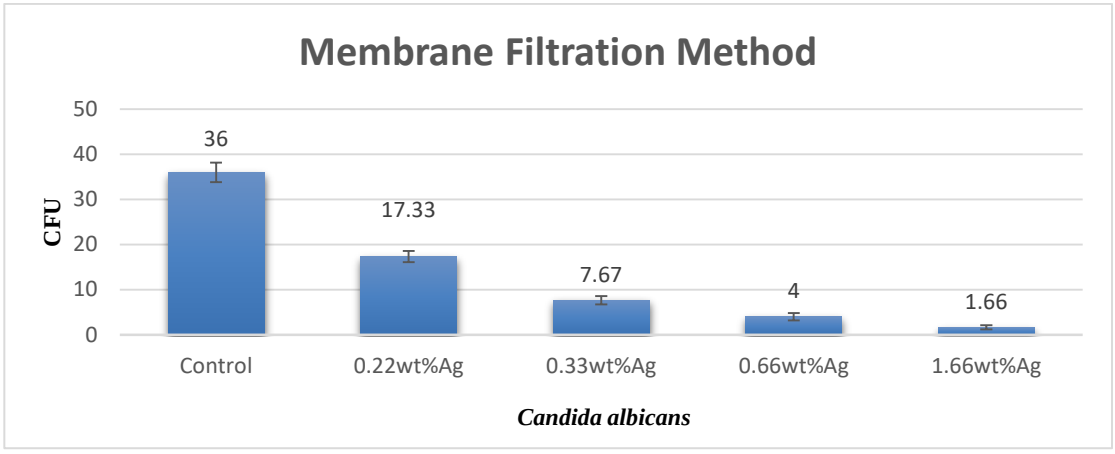

Figure3.35 Filtration of *C. albicans* culture through the Psf membranes impregnated with different concentration of B-AgNPs

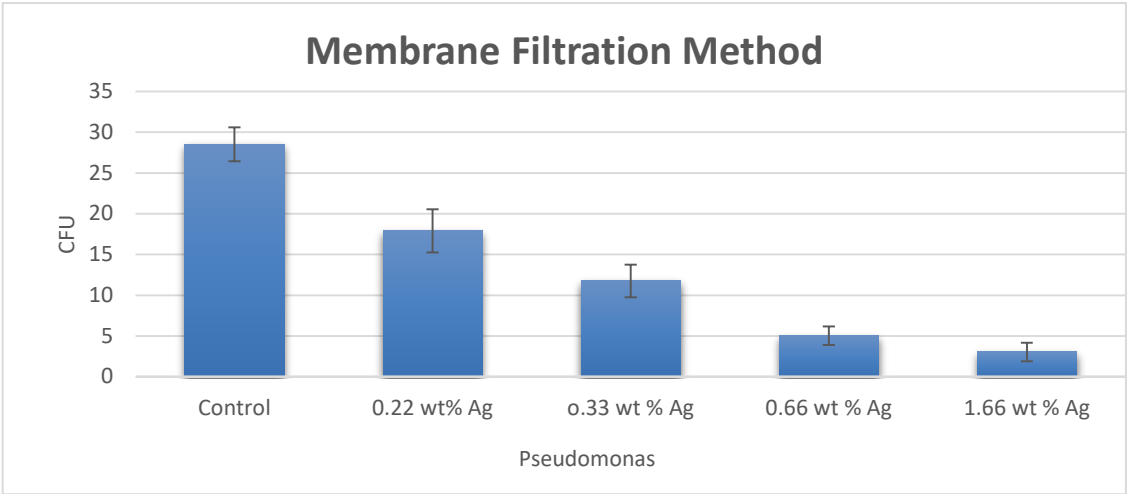

Figure3.36Filtration of *P. aeruginosa* culture through the Psf membranes impregnated with different concentration of B-AgNPs

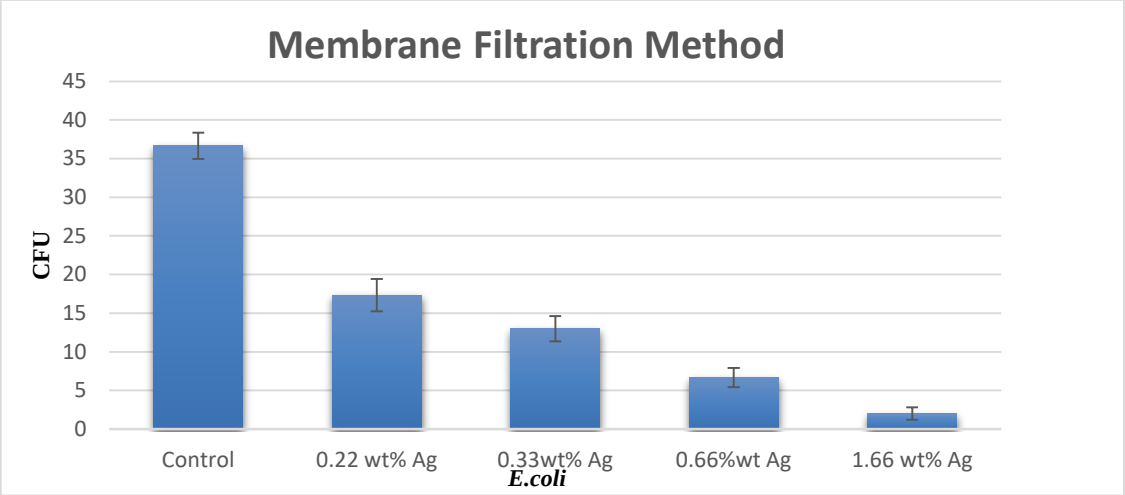

Figure3.37Filtration of E. coli culture through the Psf membranes impregnated with different concentration of AgNPs

3.6.4. Bacterial count on Psf membranes

Psf membranes with and without of AgNPs were processed separately and the CFU on an area of 1 $cm^2$of the membrane was determined. A different cfu count was observed between the samples from Psf membrane and Ag-Psf membrane. Results are described below in Table 3.7

Table3.7Biofilm formations on the Psf membranes under the shaking and static conditions

Incubation Time			
Membranes	**Seven Days**	**Fourteen Days**	**Twenty-one Days**
Psf-Static conditions(cfu)	5.3± 1.5	14.3 ±3.21	29.3±4.16
B-AgNPs-Psf Shaking conditions	3±0.7	12± 3	18.3± 3.5
B-AgNPs-Psf Static conditions	1.6± 0.5	11.6 ±3.05	14.6 ±3.5

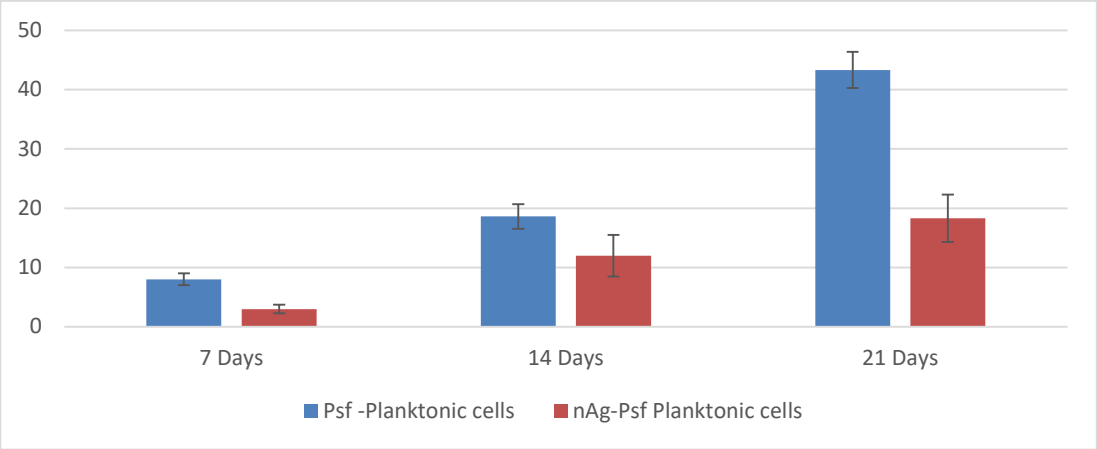

Figure3.38Biofilm formations on the Psf membranes under the shaking conditions

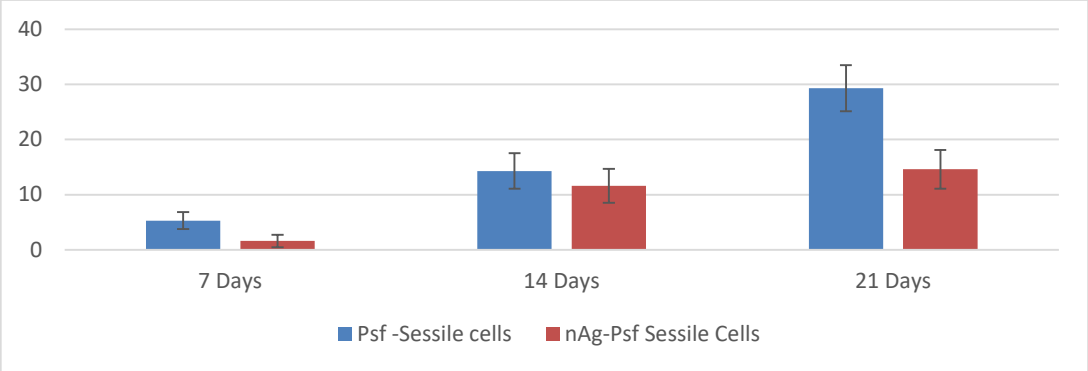

Figure3.39Biofilm formations on the Psf membranes under the shaking conditions

3.6.4.1.SEM of plain Psf membranes during biofilms formation

The SEM of the plain Psf membrane was noticed. It showed bacterial growth resulting in development of EPS. This bacterial biofilm formation corresponded with initiation of rupturing in the membrane (figure. 3.40).

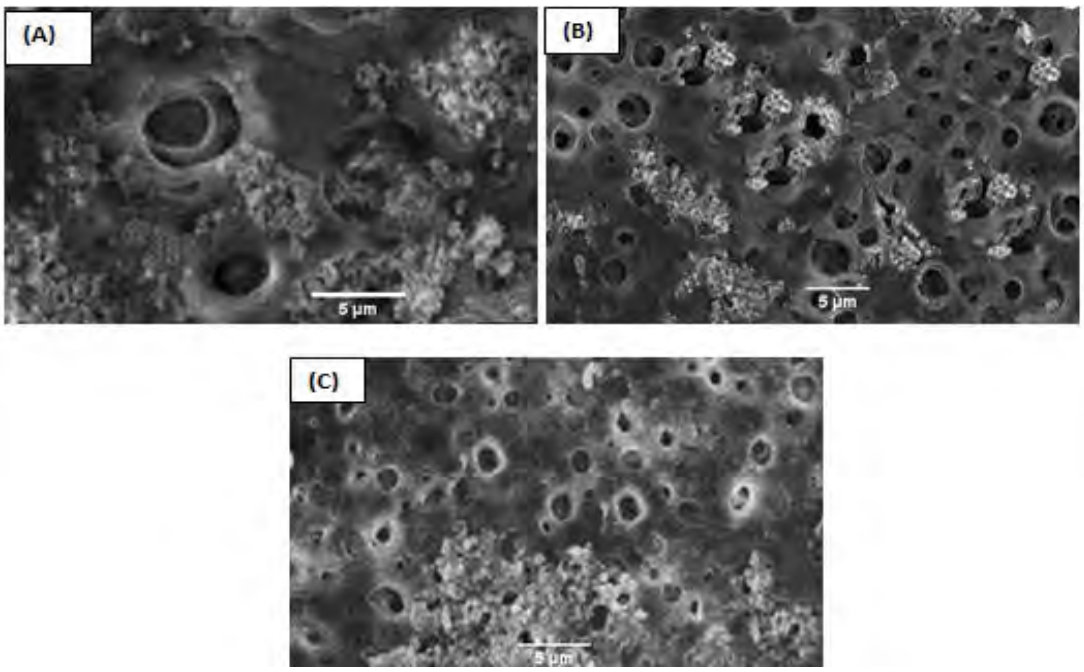

Figure3.40SEM images showing biofilm formation and damages

3.6.4.2.SEM of Ag-Psf membranes during biofilms formation

SEM spectra revealed a clear picture of the undergoing changes related to biofilm and its successive development over the period of 21 days' time. Overall, biofouling or biofilm development was found to be very less in Ag-Psf membrane (Figure 3.41) as compared to the plain Psf membrane (Figure 3.40). The biofilm formed and EPS excreted were very dense and penetrated in pore space of the membranes. In Ag-Psf membrane, the biofilm formation was less developed in terms of bacterial growth and associated EPS secretion. SEM image of Ag-Psf membrane in same conditions showed comparatively lesser development of biofilm in areas specifically impregnated with AgNPs.

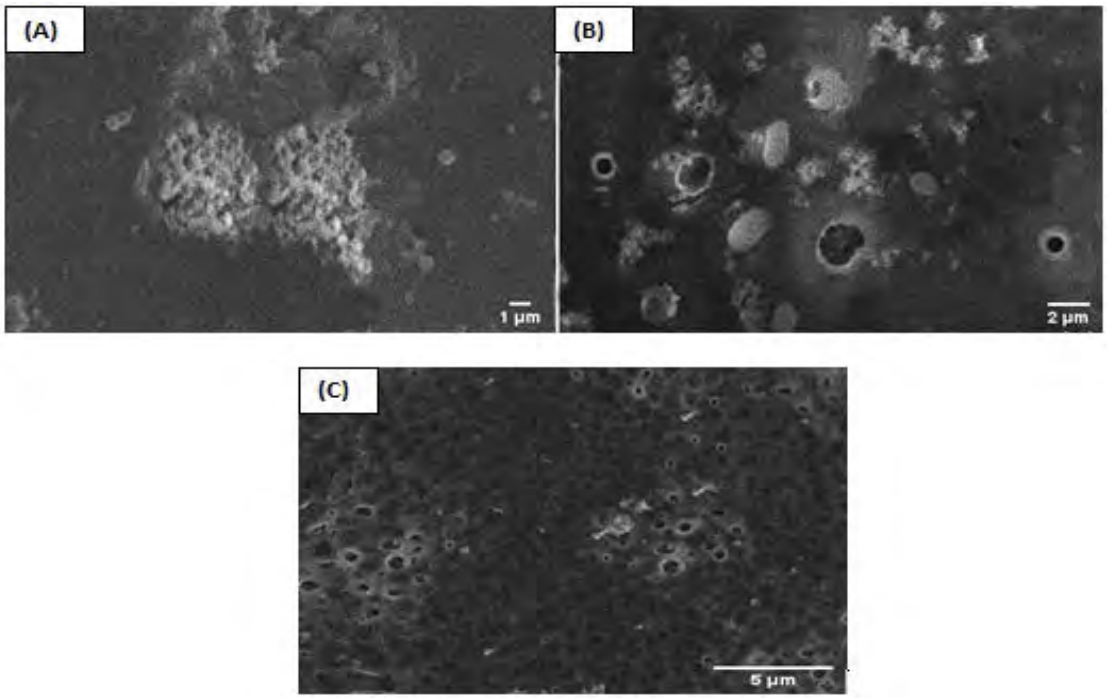

Figure3.41Anti-bio fouled Ag-Psf membrane at different resolutions (a-c)

EXPERIMENTAL V

3.7. Evaluation of anticancer and Cytotoxic ability Biogenic silver nanoparticles

3.7.1. Materials

Alamar Blue, Dulbecco's, phosphate-buffered saline, 98%NaOH, HCl (37%), nutrient agar and broth were acquired from sigma Aldrich.

3.7.2. Cytotoxicity Assay

HeLa cells line is often used for cytotoxicity evaluation. About 25000 Cells were added in 96 wells plate and incubated overnight humidified atmosphere at 37˚C and 5% CO2. After the incubation period, cells were treated with sterilized biogenic silver nanoparticles synthesized from *Fraxinus xanthoxyloides* leaf extract. About 0, 25, 50, and 75 µM of AgNPs were for 1, 3, and 5 days of incubation. At the suitable time, Alamar Blue® (10%) was supplemented to each well and incubated for 3 hours and later read by plate reader. Later DPBS was used to wash wells three times and 0.25% trypsin-EDTA was added to cells. Cell suspension was centrifuged for 10 minutes at 1000 RPM and 4^{o}C. After removing supernatant, the pellet was suspended in DMEM without phenol, 0.2 µM calcein-AM, and 16µM ethidium homodimer-1. Under cell culture conditions, this solution was incubated for 20 min and later subjected to Flow cytometry using 530 nm (610/20 band pass) for ethidium homodimer (dead cells) and 488 nm (530/30 band pass) for calcein AM (live cells)

3.7.3. Results

The cytotoxicity studies of biogenic silver nanoparticles were carried out on Human Cervix Epithelioid Carcinoma Cells. The proliferation was assessed via cell count for 5 days without AgNPs. The addition of 25 µM AgNPs expressively reduced proliferation of HeLa cells

(Figure 3.42), which was completely inhibited by increasing concentrations and proved to be cytotoxic.

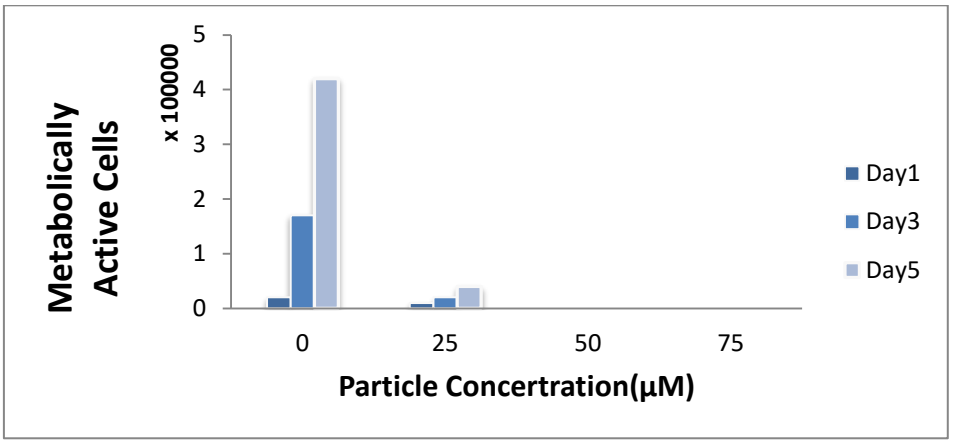

Figure3. 42HeLa cell Proliferation after Treatment with FX-AgNPs

LIVE/DEAD staining technique was used to evaluate whether reduction in proliferation was cytotoxic or inhibitory by using flow cytometry (Fig.3.43). Cellular growth without silver nanoparticles was observed for five days and majority of cells stained alive. At concentration of 25 µM, silver nanoparticles displayed a greater percentage of cells stained dead, but remaining live cells propagated by 5 days. Similar trend were observed in the Alamar Blue® proliferation assay.

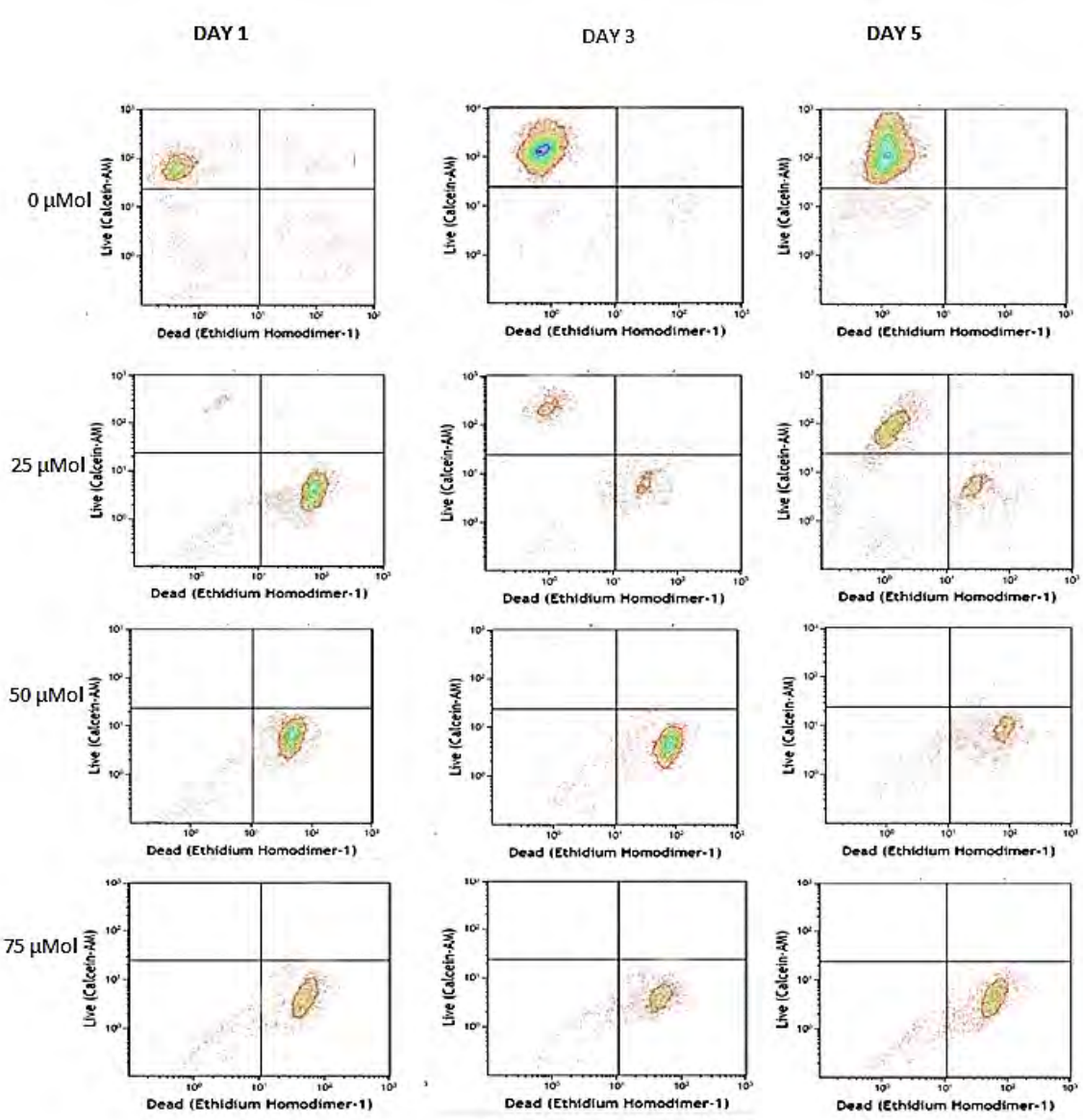

Figure3. 43Live/Dead Staining of HeLa Cancer Cells after Treatment with AgNPs

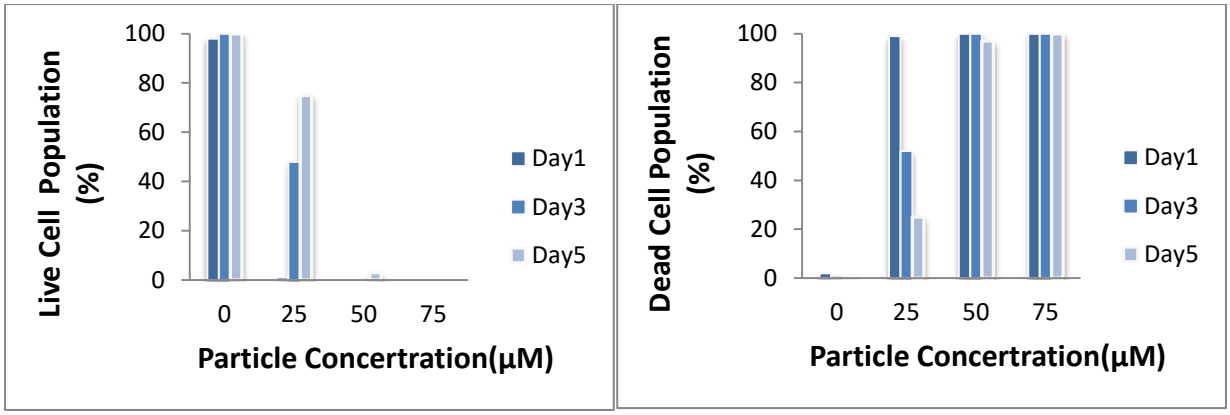

Figure3. 44. Percentage of live/dead cell Population versus AgNPs concentration

EXPERIMENTAL VI

3.8. Silver nanoparticles loaded Chitosan hydrogel as anti-infective and wound healing agent in Rat model

3.8.1. Introduction

Silver nanoparticles due to their effective biological studies, such as antibacterial, antifungal, antiviral and anti-inflammatory potential, is considered to be a promising material in chronic, non-healing and diabetic wound healing (Lara et al. 2011). As such wounds lead to limb amputation or if sepsis spread in body could lead to death. Studies revealed the role of silver nanoparticles in infection control (Zhou et al. 2011).

In our studies we used chitosan for preparing hydrogel to carry silver nanoparticles for infection control along with wound healing (Sun et al. 2003; Jiang et al. 2015). Chitosan (CS) is a highly studied (Gokarneshan 2017) natural polymer now a days, due to its biocompatible, biodegradable, non-toxic, ecofriendly, angiogenic, cellular binding capability, drug delivery (Jayakumar et al. 2010) and antimicrobial properties (Krajewska et al. 2011; Teimouri et al. 2017). Chitosan has high absorption capability; it absorbs the exudates at wound site and entraps the blood cells in it, assisting in enhanced angiogenesis. Furthermore, its porous nature allow the gaseous exchange at wound site (Bano et al. 2017). CS allows sustain release of drug as compared to the conventional hydrogels (Shu et al. 2001). Studies also explained the slower and continuous release of drug or nanoparticles from hydrogels, with increased drug stability (Satarkar and Hilt 2008; Chen et al. 2010; Venkatesan et al. 2011). Coating of nanoparticles on chitosan hydrogels will be a potential source to overcome the microbial resistance and slow healing rate due to multiple factors (Murakami et al. 2010; Mayet et al. 2014).

3.8.2. Material and Method

3.8.2.1.Materials

Chitosan, Sodium hydroxide, Ethanol and Acetic acid

3.8.2.2.Chitosan hydrogel and silver nano particles loaded hydrogels preparation:

Chitosan hydrogel was prepared through freeze gelation by following the procedure (Shahzadi et al. 2020). 2% CS solution was prepared in acetic acid and stirred till homogenous mixture was obtained. 25ml solution was poured in petri plates and kept at -20°C for 24. Later 3M chilled NaOH solution in ethanol was poured on the CS frozen solution and again kept at -20°C for 24 hours. Hydrogels were then washed with 50% ethanol thrice and then with 20% ethanol till neutralized to 7 pH. Hydrogels were then dried at room temperature.

Silver nanoparticles loaded CS hydrogels (NP-Hgl) were prepared by simple absorption method. According to the absorption capability of hydrogels, 200ul solution of silver nanoparticles was poured on to the hydrogels and let them dry for overnight at room temperature.

Figure 3.45 Chitosan hydrogel was prepared through freeze gelation

3.8.2.3.Antibacterial Studies:

Disc diffusion method was used to evaluate the antibacterial potential of silver nano particles and their activity was assessed against gram negative *E. coli* and gram positive *S. aureus* (Masood et al. 2019). The bacteria were cultured in nutrient broth and their optical density (OD) was set to 0.2 at 600 nm wavelength through spectrophotometer. As, at this OD the bacteria count is 10^5- 10^6 colony forming units (cfu) ml^{-1}, which is ideal for antibacterial activity. Later LB agar plates were prepared and 200µl of bacterial culture were spread on agar plates. Activity of silver nanoparticles, chitosan hydrogel and silver nanoparticle loaded chitosan hydrogel was tested and for silver nanoparticles a well of 0.5mm was created and 200 µl of nanoparticles were loaded and kept for 24 hours at 37°C.

3.8.2.4.Animal Trials:

For antiseptic and wound healing potential murine model was used. Albino rats of 250gm were used and three groups were made. One was sham group (control with no treatment), second group was treated with 250 µl nanoparticles loaded hydrogels and third group was

first infected with *S. aureus* and then treated with 250nanoparticles loaded hydrogels. All the hydrogels were sterilized with UV radiation 254nm for 20 min. Rats were first anesthetized with ketamine (100 mg/kg body weight) and xylazine (10 mg/kg body weight) and their dorsal sides were shaved and cleansed with PBS. Later the full thickness wound of 15mm was created by removing the skin and nanoparticles loaded hydrogels were placed on second group. On third group wound was inoculated with s.aureus and covered with nanoparticles loaded hydrogels. To create infection, *A. aureus* bacteria were first cultured in L.B broth and CFU was maintained to 10^5 and then 100 μl were inoculated on the wound through sterile cotton swabs.

3.8.3. Results:

3.8.3.1.Antibacterial studies:

The antibacterial potential of silver nanoparticles, chitosan hydrogel and nanoparticles loaded hydrogels were determined by using disc diffusion method as shown in Figure 3.46. The results revealed that the silver nanoparticles have highest antibacterial activity than nanoparticle loaded hydrogel and chitosan hydrogel. The zone of inhibition recorded for silver nanoparticles against e.coli was 22mm ± 1mm and 24mm ± 1mm against *S.aureus* which is highest than silver nanoparticles loaded hydrogels i.e. 17mm ± 1mm against both *E. coli* and *S. aureus*, whereas chitosan hydrogel showed resistance against both the bacteria and no visible zone.

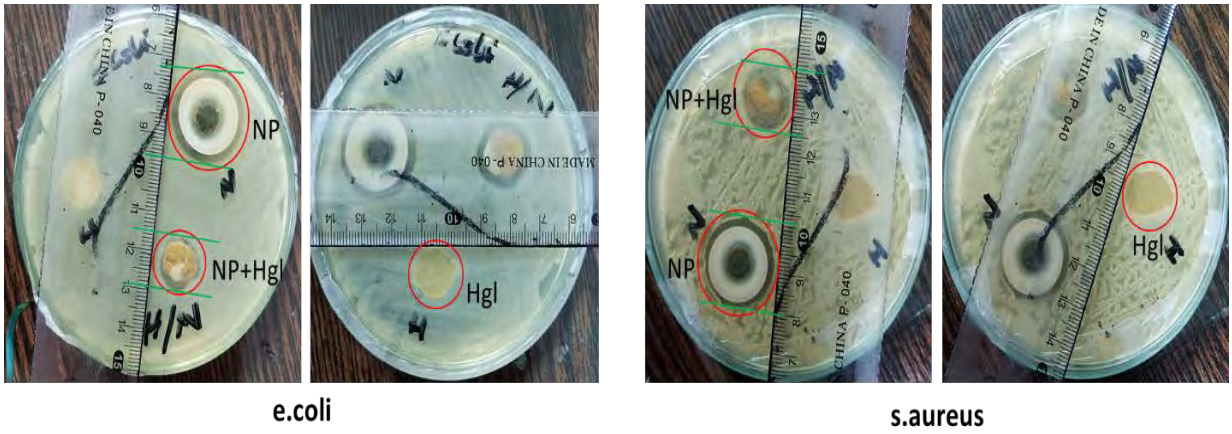

Fig 3.46: antibacterial activity of silver nanoparticles, chitosan hydrogel and silver nanoparticle loaded chitosan hydrogel

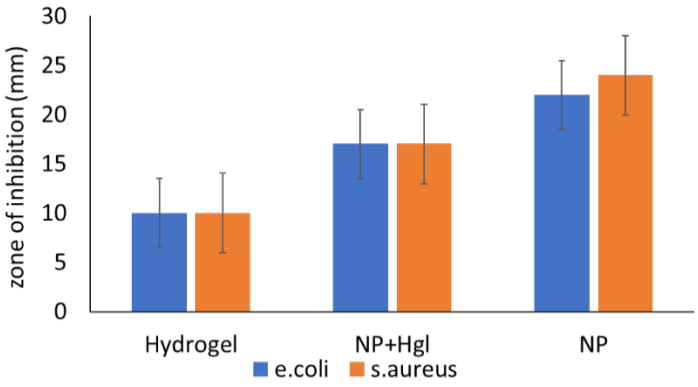

Fig 3.47 Graphical evaluation of silver nanoparticles, chitosan hydrogel and silver nanoparticle loaded chitosan hydrogel, against e.coli and s.aureus

3.8.3.2.Animal trials:

In this study we evaluated the antimicrobial effect of silver nanoparticles and wound healing activity of chitosan on in-vivo full-thickness rat model (Shown in Fig 3.48). Animals were divided in three groups, 1st group was control with no treatment showed delay healing with

scar mark in comparison to 2nd group which was treated with nanoparticles loaded chitosan hydrogel and almost fully healed in 21 days without any scars. 3rd group was infected with s. aureus and treated with nanoparticles loaded chitosan hydrogels showed complete cure from infection and healing in 34 days.

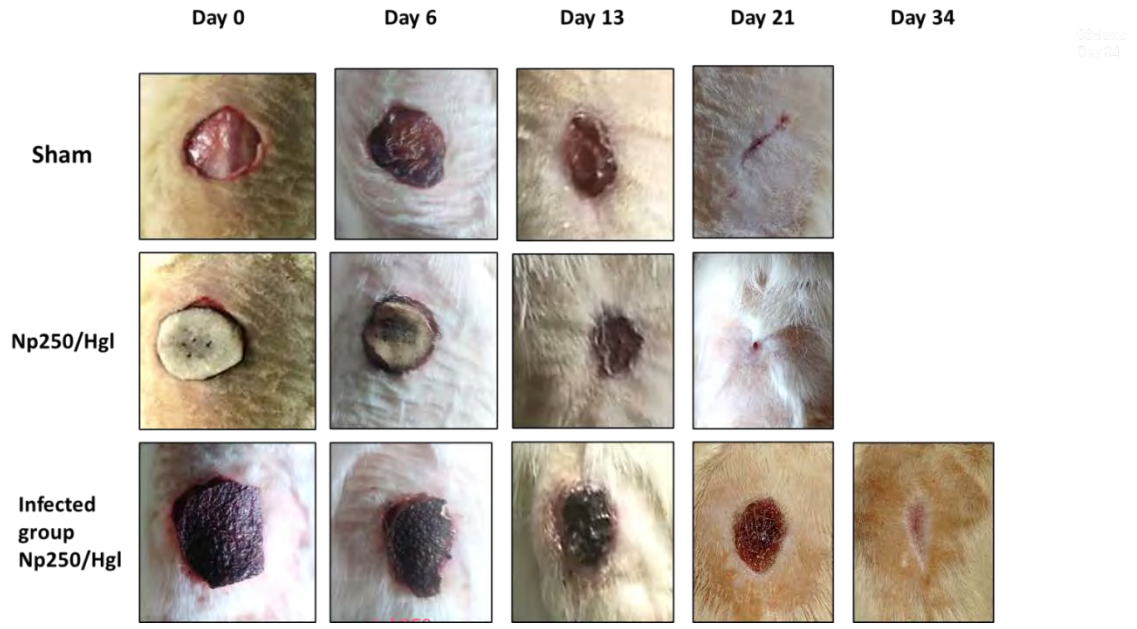

Fig 3.48: Animal trials of silver nanoparticles, chitosan hydrogel and silver nanoparticle loaded chitosan hydrogel on healthy rats and infected rat model

Discussion

Discussion

With emerging resistance to conventional antibiotics, researchers round the globe in developing countries are exploring cost effective and innovative antimicrobial agents to combat resistance issues. Over the past two decades demand of alternatives have increased many folds and led to the resurgence of finding a broad-spectrum antiseptic activity better than antibiotics. As an alternative to antibiotics treatment, Nano-technological approach uses metal nanoparticles to overcome microbial infections. These metal nanoparticles in comparison to antibiotics are more successful due to their better and efficient permeability through cell envelope and membranes. Nanoparticles possess diverse bactericidal transmission and drug delivery routes eventually reduce likelihood of resistance against them.

Metal nanoparticles could be formed in three ways generally either by chemical, physical or biological. First, two methods are not cost-effective for the industrial-scale production as compared to the biogenic one (Du et al. 2007; Cai et al. 2011; Dumur et al. 2011; Honary et al. 2012; Sarkar et al. 2012). Production nanoparticles with the help of microbes such as fungi, green algae and bacterium have been reported, these microorganisms are famously said to be as bio-Nano factories. Utilization of bio-Nano factories to produce nanoparticles is highly recommended because from this approach nanoparticles formed are sustainable, eco-friendly, and inexpensive. Enzymatic reactions by these bio-nano factories are responsible for the degradation of harmful reducing products that are formed during the synthesis of nanoparticles. The fabrication

of AgNPs with the help *of Pseudomonas* is also reported in previous researches (Kumar and Mamidyala 2011; Shivaji et al. 2011; Thamilselvi and Radha 2013; Baker et al. 2015). Similarly, the green synthesis of plant-based AgNPs has become the favorite pursuit of all researchers including biologists, chemists and technicians. The main benefit of using plant extracts for AgNPs biogenesis is that they are readily accessible, secure and non-toxic, have plenty of metabolites that can help reduce silver ions and are faster in synthesis than microbes. The growing interest in cost, time, waste etc. minimization and the implementation of sustainable processes to develop environmentally friendly and easy techniques for AgNPs manufacturing lead to the implementation of photo biological approaches(Acay and BARAN 2019)

In this study, silver nanoparticles were synthesized from three different sources, one bacterium and two plants. Initial formation of silver nanoparticles was confirmed by visual observation and UV spectrophotometer. A color change was observed when the supernatant of the *Pseudomonas aeruginosa* is incubated with the salt of silver nitrate AgNO3 (Figure 3.1). Silver ions existing in the colloidal form of nanocomposites formed broad peak due to Plasmon surface resonance. This resonance is specifically produced when the AgNPs were formed. During this investigation, UV spectrophotometer showed the highest peak at 430 nm which specifies the formation of AgNPs (Figure 3.2). However, rise in the intensity of the absorption peak with the passage of time confirms formation of more AgNPs. Majority of the studies suggested that the highest peaks were formed in the range of 400-500 nm for the formation of silver nanoparticles. For instance, the investigation lead by Jo et al reported that the

highest peak for the formation of silver nanoparticles occurred at 428 nm (Jo et al. 2016). Likewise, the highest peak was also observed at 420 nm by (Singh et al. 2015). In other reported highest peaks were observed between 415-425 nm (Kumar and Mamidyala 2011; Shivaji et al. 2011; Thamilselvi and Radha 2013; Ramalingam et al. 2014; Baker et al. 2015)

FT-IR was performed. (Figure 3.3**)** shows the FT-IR spectra of cell free culture supernatant of *P. ae ruginosa* and AgNPs with many peaks. The band at 3273 cm^{-1} indicates O–H stretching showing the presence of alcohol and phenol groups. Another band at 2981 cm^{-1} region shows C–H stretching of an aromatic compound. The peak at 2135 cm^{-1} region confirms N=C=S stretching of isothiocyanate. A 1635 cm^{-1} peak look like C=C stretching of the conjugated alkene(Yang et al. 2020).

XRD pattern was observed from 20° to 80° which confirmed successful formation of the crystalline nature of AgNPs (Figure 3.4). The average crystal size of silver nanoparticles was measured 17 nm using Scherer's formula from the (111) peak which is the most intense and sharpest peak, similar results has been described in recent studies (Yang et al. 2020)

TEM images revealed a regular as well as irregular spherical shape of silver nanoparticles with a size extending from 12 to 65 nm. (Figure 3.5)also validates the mono disperse nature of the nanoparticles and it agrees with previously reported studies.(Ali et al. 2017)

In this study silver nanoparticles were also synthesized from *Fraxinus xanthoxyloides* which is naturally present in Hindu Kush and Himalayan mountains of Pakistan. It is

also stated that methanol extract of *F. xanthoxyloides* leaves possess anti-inflammatory, analgesic capabilities (Younis et al. 2016b). In the present study, bio production and characterization of silver nanoparticles from the leaves extracts of *F. xanthoxyloides* was reported(Rafiq et al. 2020), which may prove beneficial for biological and economical features. Moreover, the produced AgNPs significantly affected bacteria growth, cell physiology and biofilm synthesis. Biosynthesis of AgNPs with plant extract is an upright method for biosynthesis due to their harmless properties and thus offers natural capping agents (Anjum and Ashraf 2020). **(Figure 3.7)** presents relation between absorbance versus wavelength. The spectra obtained from spectrophotometer confirmed successful formation of AgNPs. The pattern also revealed a strong absorption band at 430 nm, this characteristic is associated with the absorbance properties of silver at given wavelength(Bar et al. 2009). Moreover, the spectra show an up rise in the intensity of the absorption peak with the passage of time, which also confirms formation of AgNPs. A rise in the intensity of absorption line also shows that, as the time passed more silver nanoparticles are formed. A Shift in the absorbance peak was detected (Bathochromic effect) that may be due to creation of large sized silver nanoparticles (Elemike et al. 2017).The γ_{max} values in the 400-500 nm range are precise for the exterior Plasmon band of AgNPs(Ibrahim 2015).

XRD pattern was observed from 30° to 80° which confirmed successful formation of the crystalline nature of AgNPs from *Fraxinus xanthoxyloides* leaf extract (Figure 3.8**)**. The average crystal size of silver nanoparticles was measured 8nm using Scherer's formula from the (111) peak which is the most intense and sharpest peak, similar

results has been described in recent studies (Mahmoud et al. 2016; Oves et al. 2018). The small peak at 31.54° may be because of silver oxide indicated by (111*) (JCPDS No: 75-1532).

The SEM images of the as-prepared AgNPs confirmed that most of the particles are almost spherical shaped and uniformly distributed (Soman and Ray 2016).A small amount of aggregations was also seen in the SEM micrograph that may reflect variation in size of newly formed particles (Figure 3.9A and B). Variations in the sizes of AgNPs produced from different plant extracts has been reported previously (Elbeshehy et al. 2015). It has been shown that smaller size of silver nanoparticles at 7 pH is more efficient in terms of its applications. TEM images revealed a regular as well as irregular spherical shape of silver nanoparticles with a size extending from 5 to 12 nm.(Figure 3.9C and D)also validates the monodisperse nature of the nanoparticles (Venugopal et al. 2017).

Nanoparticles with PDI < 0.05 are measured as mono disperse and in our study we found it 0.02.The morphology and distribution of silver nanoparticles of the SEM and TEM images support each other (Ghiuță et al. 2018). To examine the stabilizing and reducing agents of the as-prepared silver nanoparticles, FT-IR was performed. (Figure 3.10**)** shows the FT-IR spectra of FX-leaf extract and AgNPs prepared from *Fraxinus xanthoxyloides* leaf extract with many peaks. The band at 3207 cm^{-1}could be accredited to the stretching modes of vibrations of hydroxyl (-OH) group. The spectral analysis confirms the existence of numerous functional groups that are responsible for capping of silver nanoparticles. The band at 1600 cm^{-1} is owed to the existence of amide

vibrations that could be associated to silver nanoparticles by the amine groups. The spectral peak detected at 1312 cm^{-1}might be attributed to C-H symmetric vibrations (Selvi et al. 2016; Oves et al. 2018), while the peak found at 780 cm^{-1} belongs to the AgNPs and could be assigned to stabilizing agent during synthesis of AgNPs. Moreover, the peak at 1038 cm^{-1} is a result of bending modes of hydroxyl group (-OH). Hence, FT-IR spectra verify successful formation of AgNPs. After the reduction of silver ions, following peaks showed shift 3207-3211, 2907-2927, 1575-1600, 1575-1600, 1443 -1312 and 1034-1038 cm^{-1}. This shift indicates that Hydroxyl, amide and carboxyl groups of FX-leaf extract may be involved in nanoparticle synthesis (Bankar et al. 2010; Rafiq et al. 2020)

Bischofia javanica, another plant that was used in synthesis of silver nanoparticles is an edible plant natural inhabitant of South Asian countries specially India and Pakistan. The seeds, bark, leaves and roots of *Bischofia* plant has been conventionally utilized as a source of food treatment option for sores, tooth ache and eye infections (Panda et al. 2018).

Change in the color shade indicated development of silver nanoparticles. It was due to surface Plasmon resonance (SPR) incitement of the silver nanoparticles. The spectra obtained from spectrophotometer confirmed successful formation of AgNPs. The pattern also revealed a strong absorption band at 422 nm (Figure 3.12), this characteristic is associated with the absorbance properties of silver at given wavelength (Bar et al. 2009). As the time passed more silver nanoparticles are formed as indicated by increase in

intensity of absorption. The γ_{max} values in the 400-500 nm range are precise for the exterior Plasmon band of AgNPs (Ibrahim 2015).

XRD pattern was observed from 20° to 80° which confirmed successful formation of the crystalline nature of AgNPs from *Bischofia javanica* leaf extract (Figure 3.13**)** The average crystal size of silver nanoparticles was measured 9 nm using Scherer's formula from the (111) peak, similar results has been described in recent studies (Mahmoud et al. 2016; Oves et al. 2018).

TEM micrographs shown that the most of the AgNPs were ball-shaped and very few are irregular in shape. Particles size ranges from 4 nm to 16 nm. (Figure 3.15A and B) The nanoparticles formed were monodisperse and it can be due of capping agents of extracts (Venugopal et al. 2017).

The band at 3296 cm^{-1} in the FT-IR spectrum displays O–H bond indicating presence of alcohol groups. Another band present at 2943 cm^{-1} was due to C–H stretching of alkane. 2090 cm^{-1} peak region indicates isothiocyanate N=C=S stretching. A band at 1541 cm^{-1} shows N–O stretching of the nitro compound. The band at 988 cm^{-1} shows presence of C=C stretching of alkene (Bankar et al. 2010; Yang et al. 2020)

Efficacies of sliver nanoparticles for their anti-microbial properties have been known for decades. Synthesizing AgNPs utilizing microbes and plants are well-recognized approaches for the expansion of harmless and capable control efforts for inhibition of resistant bacteria (Manikandan et al. 2017). They know to be possessed a significant activity against the MDRs (Naqvi et al. 2013). Antimicrobial activity of Biogenic silver nanoparticles synthesized from *P. ae ruginosa* was tested by well diffusion method.

From the results of present study (Figure 3.17) it is clearly predicted that Biogenic AgNPs equally inhibited the growth of different bacterial species including *Pseudomonas, K. pnemoniae* and *S. aureus* however highest susceptibility was observed in case of *K. pnemoniae* which exhibited zone of inhibition 15mm against 300 and 400 ppm concentration. Biologically synthesized AgNPs also showed more stability and higher antimicrobial activity as compared to chemically synthesized silver nanoparticles particles. (Jo et al. 2016)In another study MIC of Biogenic silver nanoparticles synthesized by aqueous extract of *F. xanthoxyloides* leaf was tested by microtiter plate assay. *In-vitro* results presented that the silver nanoparticles had potent anti-bacterial effect against all tested strains in terms of percentage that ranged between 65% -95 %(Figure3.19**).** the results of the anti-fungal activity evidently tell that growth of *C. albicans* is inhibited by 75% at concentrations as low as 50ppm using FX-NPs. The percentage inhibition increases up to 95%by increasing AgNPs concentration (Figure 3.19**)**. This may be due to apoptosis caused by accumulation of AgNPs in *C. albicans*(Hwang et al. 2012) Antimicrobial efficacy of silver nanoparticles synthesized from *Bischofia javanica* leaf extract was also evaluated. The minimum zone of inhibition (6mm) was recorded for 100ppm of *C. albicans* for the highest concentration of *K. pneumoniae* (300ppm) 13mm zone of inhibition was recoded. Therefore, we suppose that silver nanoparticles possess a broad range of anti-bacterial and anti-fungal activities (Martinez-Castanon et al. 2008). In all three studies performed, gram-negative bacteria showed lowest values for the MIC while the gram-positive, highest MIC values. Difference among the silver nanoparticle activities can be linked to the variance between

cell walls of Gram-positive and Gram-Negative bacterium as gram-positive bacterium possess a thicker cell wall in contrast to Gram-negative bacterium's cell wall (Thiel et al. 2007).Width of the peptidoglycan layer of *S. aureus*; a vital role of the peptidoglycan layer is to shield from anti-microbial such as antibiotics, chemicals, enzymes or toxins. This outcome was supported by the results of previous studies (Jung et al. 2008).

The synergistic effects of Biogenic AgNPs synthesized from *P. aeruginosa* in comparison to chemically synthesized AgNPs were also investigated with three conventional antibiotics against bacteria using a chequer board method and the effects evaluated by determination of the FICI**.** Most of the combinations exhibited effectiveness against respected pathogen. The Minimum inhibitory concentration of nanoparticles in combination with antibiotics was evaluated by broth micro dilution chequerboard method in accordance with CLSI standards. The results of combination assay are represented against *S. aureus, E. coli* and MRSA in Table 3.3, 3.4 and 3.5, respectively. Most of the combinations exhibited effectiveness against respected pathogen. Synergistic behavior of AgNPs + Ciprofloxacin was observed whereas the synergistic effect of AgNPs + Ampicillin and activities were higher as compared C-AgNps. Our synergistic activity results can be compared with(Hwang et al. 2012) who had synergistic activity of silver nanoparticles in combination with Ampicillin, Chloramphenicol and Kanamycin against Gram positive and Gram negative bacteria. These synergistic activities may possibly reduce the resistance of bacterial strains toward antibiotics.

Bacterial biofilms mediated chronic infections are more resistant to conventional drugs. The integral architecture of biofilms encloses and protects bacterial colonies in a matrix containing DNA and exopolysaccharide along with other cellular content (Costerton 2001; Flemming and Wingender 2010; Toyofuku et al. 2012). In chronic infections of *P. ae ruginosa*, the resistance is further enhanced by virulence factors and adaptive mechanism that assist bacteria to reduce susceptibility's against conventional antibiotic therapies (Folkesson et al. 2012; Gellatly and Hancock 2013). Previously, several studies assessed similar activity against *Staphylococcus aur eus, P roteus v ulgaris, Bacillus m egaterium an d E scherichia coli* (Shahverdi et al. 2007; Chudasama et al. 2010). However, very few studies were conducted on biofilm forming ability of multi-drug resistant strains of *P. aeruginosa*. The current study was conducted to evaluate the efficacies of successfully synthesized nanoparticles to prevent the in vitro retrieval of *P. aeruginosa* biofilms. Initially, five different strains of microorganism were screened for their biofilm formation ability through Qualitative Congo Red Assay (Figure 3.22). *P. ae ruginosa* showed strong biofilm formation capability. Antibiofilm activity of biogenic silver nanoparticles synthesized from Fraxinus Xanthoxyloides leaf extract was evaluated against strain *P. ae ruginosa* (Figure 3.23)(Rafiq et al. 2020). The biosynthesized AgNPs triggered 86.6% drop in the biofilm formation of *P. aeruginosa* as related to the control, while C-AgNPs showed 70.87 drop (Figure 3.24**)**. The promising activity of AgNPs is because of their physiochemical properties particularly size and surface characteristics prompting the antibiofilm response. The enhanced activity is due to the capping of nanoparticles which avert the accumulation of particles

followed by the increased permeability through biofilm matrix. (Bryaskova et al. 2011; Mohanty et al. 2012; Habash et al. 2014) Green silver nanoparticles integrate with bacterial cells and produce Reactive Oxygen Specie, which results in protein destruction, macromolecules damage and inappropriate appearance of the virulence factors, along with biofilm inhibition (Qayyum et al. 2017).

Nanoparticles have potential to induce toxicity in bacterial cells by generation of ROS and damaging mitochondria. Through cell viability assays, we can determine the metabolic activities, cell survival and death. The previous studies indicated that nanoparticles not only impede biofilm formation, but also reduce the metabolic activity. Living cells produce reductase enzyme which reduce XTT into formazan dye. The change in color of experimental group with respect to the control group is directly associated with the effectiveness of antibacterial agent (Figure 3.25). However, the amount of reductase enzyme can be attributed to the metabolic activity of cell. Cells exposed to nanoparticles produced fewer amounts of reductase enzyme and displayed compromised metabolic activity. The mechanism behind this activity may be the production of ROS, such as hydroxyl radicals which targets reductase enzyme and ultimately result into compromised metabolic activity. Hence, many studies have suggested that ROS are directly associated with stimulation and inhibition of cell death, genetic variability and regulation of biofilm (Gurunathan et al. 2014). Our findings showed that nanoparticles reduced the metabolic activity more than 50% which is indication of significantly higher production of ROS and decrease in reductase enzyme production. Among them, Biogenic silver nanoparticles synthesized from *Bischofia*

javanica showed optimal reduction i.e. 62% Whereas C-AgNPs displayed 50% activity (Figure 3.26). The higher activity might be due to the increase in permeability because of smaller size, as well as production of greater amount of ROS as compared to the other nanoparticles. Also increase in production of ROS might influence the AgNPs with target site interactions indirectly (Morones-Ramirez et al. 2013). It is very important to find out about the possible lytic effects of nanoparticles on RBCs if in an ex vivo model. If hemolysis occurs, it can cause serious disorders like anemia. Also the hemoglobin released from rapture can prove toxic to renal and vascular system. We studied the characteristics of nanoparticles related to their toxicity on human cells. In this regard we had performed hemolysis assay on human red blood cell (Figure 3.29). The hemolysis test measures the lysis of the red blood cells when exposed to an environmental agent. (Roacho-Pérez et al. 2020) as the results (Figure 3.30) indicate lysis was dose dependent but still far less as compare to control (Katva et al. 2018).

In our investigation we used polysulfone is a polymeric constituent for the fabrication of the Ag integrated membranes because polysulfone is most widely used polymer for the manufacturing of ultrafiltration membranes due to its stabilization at the higher temperature i.e. at 387 K and covers the widespread extensive range of pH (0-14).They also encounter and resist the chlorine oxidation (Sutedja et al. 2017)

Two types of polysulfone Psf membranes were fabricated (Figure 3.31a and b) i.e. with and with the help of aluminum casting knife by the phase inversion technique. The synthesized membranes were characterized by FT-IR and Scanning electron microscopy (SEM) FT-IR spectra reveals major peaks corresponding to different

groups, peak corresponding to ester (benzoate) group was observed at 1241cm-1 , while at 1150 cm-1 and 1488 cm-1, peaks for sulfone and CH3 group were observed. These groups are chief components of Psf membranes and are also in accord with Sigma-Aldrich standard spectrographs (Figure 3.33 and 3.34). As shown in (Figure 3.32) The Membrane's cross section image from scanning electron microscopy showed that the Plain and AgNPs impregnated Psf membranes were found to be 110 µm thick. It also indicated that the integration of AgNPs did not impede the development of porous structure. The pore size as was determined in the Psf membrane was around 0.5 to 5µm. (Figure3.41)

Antimicrobial activities of both the membranes were evaluated i.e. control membrane without nanoparticles and hybrid membranes with the incorporation of the silver nanoparticles. Hybrid membranes impregnated with the silver nanoparticles possess the considerable antimicrobial effect against the tested organisms as compared to the control membrane. This aspect of our study coincides with the other studies conducted by (Yan et al. 2009; Zodrow et al. 2009; Koseoglu-Imer et al. 2013). We used the four different concentrations of silver nanoparticles that were unified in the polysulfone membranes i.e. 0.22, 0.33, 0.66 and 1.66 wt% by silver (Figure 335-3.38). At 0.22 weight by silver nanoparticle concentration, the highest number of bacterial colonies were observed beside this lowest bacterial counts were observed at the highest concentration of silver nanoparticles i.e.1.6 wt% by silver (Table 3.7). A similar trend was observed in other studies conducted by (Yang et al. 2007; Koseoglu-Imer et al. 2013; Lee et al. 2013). The mechanism of antimicrobial efficacies of silver nanoparticles

against the microorganisms could be explained by Zhu et al. Their report suggested that the interaction of the thiol (−SH) group of cysteine with the silver ions is mainly accountable for the antibacterial actions of silver ions. The thiol group of cysteine is mainly located in the cell wall of the bacterium. S−Ag complex is formed and reaction taking place between the silver ions and the cysteine is highly responsible for the hampering of the enzymatic function. This hindering of the enzymatic function is proved to be fatal for the existence of the bacterium. (Zhu et al. 2010)

Water biofilms possess a large number of bacterial diversity particularly biofilms from water distribution systems and activated sewage sludge treatment plants. Drinking water and surface water also acquired biofilms (Flemming 2002). Microbial organisms residing in the complex biofilm matrix which is known as extracellular polymeric substances EPS which protects and makes them more resistant towards disinfectants and antibiotics (Flemming and Wingender 2010) Therefore, water biofilms are of keen interest as they are capable of affecting its aesthetic quality which included the color, odor and its taste. Besides this microorganisms are also detaching and releasing in the treated bulk water from these biofilms (Långmark et al. 2005). Microbial organisms present in these water biofilms included *Pseudomonas, F lavobacterium, C ryptosporidium, C ampylobacter jejuni, L egionella, G iardia, A eromonas s p., Mycobacteria s p., Sal monella s p., Shi gella sp., E. c oli*, *Staphylococcus, and C andida* (Schwartz et al. 2003; Burmølle et al. 2006; Simões et al. 2007; Warsinger et al. 2018). These microorganisms also possess the antibiotic resistance in them so they are regarded as multidrug-resistant bacteria (MDR) (Burmølle et al. 2006). Therefore, initially, we performed the screening of the multidrug-

resistant bacteria for their biofilm formation capability. For the said purpose, initial screening was performed by two assays. The qualitative assay was done by performing a Congo red plate assay while two quantitative assays were performed by biofouling assay and antibiofilm formation assay.

Initial qualitative test which was conducted for the detection of biofilm formers screening was Congo red plate assay test (Figure 3.22). Dark reddish to blackish color colonies were formed by *Candida al bicans, E.coli, S.aur eus, and Pseudomonas. aeruginosa,* among all of them *P. aeruginosa* is considered as a most potential candidate for the biofilm formation as it produces black color crystalline colonies. According to the previous reports it is stated that biofilm formation was evaluated in *Pseudomonas aeruginosa* in 30 clinical isolates out of which 27 were biofilm producers by CRA plate method. They produce black dry crystalline colonies and also do the extensive production of exopolysaccharides it is a strong biofilm former as 90% of the strains were biofilm former (Rewatkar and Wadher 2013). In another reported study on CRA plates, a positive correlation was found between the colony morphology and biofilm formation on Congo Red Assay for *S. aureus* strains. Black dry crystalline colonies were observed on CRA plates for *S. aureus* and which shows that it is a strong biofilm former (Knobloch et al. 2002). These findings coincide with our results. According to another reported study in which uropathogenic biofilm formers were isolated from UTIs catheters. Percentage of isolated biofilm formers *Pseudomonas* (100%), *S. aureus* (100%), *E.coli* (60%) and *K.pneumoniae* (37% non-biofilm former and 63 are biofilm formers) .These results were similar with our findings *P. aeruginos , E. coli* and *K. pneumoniae* while in our *results*

K. pne umoniae is a non-biofilm former. In another reported study 21.73 % *Candida albicans* were responsible for biofilm formation (Niveditha et al. 2012).In our research at the third number, *Candida albicans* produced the highest biofilm (Saxena et al. 2014).

Drinking water distribution and treatment plants possesses bacterial colonization i.e. 95 % of bacterial colonization is present on the inner surface of the DWTPs and DWDs in sessile form and only 5 % is present in the form of planktonic cells (Flemming 2002). In this regard, we are trying to evaluate the functional capability of our synthesized polysulfone membranes under the influence of biofilm formation. For this purpose, biofouling assay was performed under two different condition one is sessile and other is planktonic with both types of the membranes. Less number of bacterial count was observed on the membranes impregnated with nanoparticles it could be due to the release of the silver ions in the surrounding medium in which the membrane coupons are submerged besides this the membrane serving as control have high amounts of the viable cellular growth this might due to the fact that control membrane possesses no such material that exhibits the antimicrobial and bacterial activity. Our results coincide with a similar study conducted by the Zordow et al,(Zodrow et al. 2009). Our study is conducted under the time frame of 7, 14 and 21 days. At the initial reading, the number of bacterial growths was reduced as the silver are highly available and present in their active state for the inactivation of the bacterial growth. At the initial reading the number of bacterial counts were lowest as compared to the second and the third readings were the bacterial counts were increased with respect to the day of reading that is at day 14 less bacteria counts were observed while at day 21 highest amount of bacterial counts were

observed in both the settings that is planktonic and sessile. The number of sessile cells is lowest, and the number of planktonic cells is high during all three intervals of our readings (Figure 3.39-3.40). This trend our research is supported by the research conducted by the De Prijck et al and his coworkers. The first expected action of silver ions against the bacterium is the consecutive leaching of the silver ions from the membrane surface and in this way; it will kill the bacterium that is adhered to the surface of the membrane. This mode of action is famously known as the contact-dependent killing. The another possible explanation of this could be that the number sessile cells are released from the membrane surface where they are attached and released into the surrounding environment where it is responsible for the reduction of planktonic cells also that why the number of planktonic and sessile cells are high in Psf membranes without silver nanoparticles (De Prijck et al. 2007).

Overall, biofouling or biofilm development was found to be very less in Ag-Psf membrane (Figure 3.41) as compared to the plain Psf membrane (Figure 3.40). SEM image of PsF impregnated with AgNPs exhibited a presence of damaged bacterium near AgNPs on membrane (Figure 3.41b and c). A cloudy appearance was obvious in a region where possibly a reaction was happening due to nanoparticles in comparison to other parts of membrane. Overall results showed that the silver nanoparticles impregnated Psf membrane did not allow dense growth of biofilm as compared to Plain Psf membrane.

In modern era, Patients suffering from cancer undergo a lot of treatment strategies and require intensive care. With the advent of nanotechnology, researchers have been

working on development of new nano based anticancer agents.(Acharya et al. 2021) Because of the exceptional physical and chemical properties, silver nanoparticles proven as a potential therapeutic agent in the area of Nano medicine (Burdușel et al. 2018)The unique characteristics of nanoparticles preferably allow targeting of the specific cells. This cell specific targeting increases the effectiveness of numerous treatments and allow gene-targeted therapy (Singh et al. 2019). In order to support the development of anti-cancer therapy, we investigated possible anticancer and cytotoxic potential of Biogenic silver nanoparticles in Human Cervix Epithelioid Carcinoma Cells Alamar Blue Assay and Flow cytometry. The biogenic silver nanoparticles exhibit exceptional anticancer activity, and showed reduction in cellular proliferation (Mohanta et al. 2017) The addition of 25 µM AgNPs expressively reduced proliferation of HeLa cells (Figure 3.43), which was completely inhibited by addition of higher concentrations and appeared to be cytotoxic (Figure 3.44) Reduction in number of cells for 25 µM after 1 day of incubation indicated that AgNPs slowed down growth of cancer cells in a dose-dependent manner. (Salomoni et al. 2015)

LIVE/DEAD staining technique was used to evaluate whether reduction in proliferation was cytotoxic or inhibitory by using flow cytometry (Fig.3. 43). At concentration of 25 µM, silver nanoparticles displayed a greater percentage of cells stained dead, remaining live cells propagated by 5 days. Similar trends were observed in the Alamar Blue® based proliferation assay. These result displayed small cytotoxic effect at 25 µM concentration. At higher concentrations almost all cells stained dead from 1 to 5 days, displaying a complete cytotoxic effect. Although it is not clear but anticancer activity of

biogenic AgNPs can be due to interaction with intracellular macromolecules such as proteins and DNA and also because of the acidic lysosomal environment promoted release of reactive oxygen species and ultimately causes apoptosis (Ibrahim et al. 2020; Ramalingam et al. 2020)

Chronically infected wounds are major clinical burden, as in mostly cases limb amputation is the final option of clinicians, to save the patient's lives (Zhou et al. 2011). In our studies we have developed hydrogels to overcome such problems and their antimicrobial potential was evaluated through in-vivo studies. These hydrogels were flexible, highly spongy and have good absorption capability, with enhanced antimicrobial and wound healing activity.

Silver ions or silver nanoparticles are well known for their antimicrobial activity and have most promising results amongst wide range of metal nanoparticles (Coma 2013). Silver nanoparticle studies have reported its interference with the thiol group of bacterial respiratory enzymes which results in bacterial inhibition (Gong et al. 2007). The in-vitro antibacterial studies of silver nanoparticles have shown high impact against E. coli and S. aureus inhibition. Whereas after loading the silver nanoparticles on hydrogel it showed effective bactericidal activity against both bacteria, but this activity is lower than silver nanoparticles itself. Although chitosan hydrogel itself have antibacterial activity due to its cationic nature but chitosan hydrogel showed resistivity against the bacteria by forming a very thin zone around the hydrogel (Figure 3.46). It could be hypothesized that chitosan allow persistent release of nanoparticles and that's

why produce smaller zone in comparison to silver nanoparticles solution, as they can penetrate easily through the porous agar.

In animal study we selected s.aureus to develop infected full thickness wound model, as it is known that s. aureus or gram positive bacteria during the first few days of injury, rapidly colonizes on the wound surface (Kianvash et al. 2017) and cause infection that leads to limb amputation or even causes death. In animal trials silver nanoparticles and chitosan showed synergistic effect as chitosan hydrogel is porous in structure which assist in easy release of nanoparticle, and absorbs wound exudates which allow easy attachment with the tissue (Jadhav et al. 2016; Priya et al. 2016).

In rat models it could be clearly observed that chitosan has enhanced the wound healing than the sham rat's model and eliminated the risk of skin contraction or scar formation after healing, which could be clearly seen in control or sham group. Furthermore silver nanoparticles loaded hydrogels completely eliminated the bacterial infection in infected group and reduces the risk of sepsis leading to death which is the major issue in septic wounds. By the 21st day no infection was found in third group (Figure 3.48) and it could be said that nanoparticles loaded hydrogels, allowed the rats to heal naturally and also on other hand no hindrance in wound healing was observed due to silver nanoparticles. So the combination of silver nanoparticles and chitosan hydrogels has shown prospective future aspects in chronic wound healing.

Conclusion and Future Recommendations

Conclusion

1. Current study reports successful and novel synthesis of silver nanoparticles from *Fraxinus xanthoxyloides* and *Bischofia javanica* leaf extract
2. Comparatively Silver nanoparticles synthesized from *F. x anthoxyloides* were better in terms of size as they have minimum size (ave. size) 8.9±3.2 nm as compared to Silver nanoparticles synthesized from *B. j avanica* which have average size of 10±2.5 nm while Silver nanoparticles synthesized from *P. aeruginosa* have average size of 20±9nm
3. Silver nanoparticles showed considerable antimicrobial activity against bacteria and fungi and Gram-negative bacteria were more susceptible were more susceptible toward AgNPs as compared gram-positive bacteria
4. Silver nanoparticles causes significant reduction in biofilm formation and metabolic activity of *P. aeruginosa*
5. Hemolytic activity of silver nanoparticles was dose dependent, maximum hemolysis was 7% as compared to control.
6. Silver nanoparticles impregnated polysulfone membrane displayed reduction in biofilm formation as compared to plain Psf membrane
7. The biogenic silver nanoparticles exhibit exceptional anticancer activity, and showed reduction in cellular proliferation at 25 µM, further increase in concentration proved cytotoxic.

8. The combination of silver nanoparticles and chitosan hydrogels has shown potential in chronic wound healing

Future Prospects

1. The research projected the nanoparticles synthesizing ability of Bacteria and plants, nonetheless, it may well be enhanced by;

    a. Discovering effective microorganisms which are resilient towards metals by making catalytic proteins elicited through genes.

    b. Assessment of transforming and stabilizing abilities of plant and microbial based biomolecules and understanding of intricate mechanisms involved in the conversion metal ions into nano-composites.

    c. Adjusting the reaction conditions like PH, temperature and amount of substrate and enzyme.

2. Antibacterial and antifungal of Application of NPs agents projected their valuable role against pathogens, though; their effect on cells, tissues and organs has to be estimated for everyday application.

3. Nanoparticles demonstrated anti-cancer activity; however, comparable studies can be carried on normal cell lines. Moreover, the thorough fate of synthesized nanoparticles can be evaluated inside the cell.

4. Antibiofilm application of NPs suggests their revolutionary role in environmental viewpoint, nevertheless, and overall effects have to be fully understood.

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ORIGINAL ARTICLE

Inhibition of microbial growth by silver nanoparticles synthesized from *Fraxinus xanthoxyloides* leaf extract

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Abstract

Aims: Conventional antibiotics have been failed to treat infectious diseases due to emergence of multidrug resistance (MDR) in some common pathogens. The current study aimed to formulate new antimicrobials from greener sources. In the midst of these efforts, nanotechnology is a newly emerged field, in which the synthesis of new nanoparticles through novel and efficient means is on the rise.

Methods and Results: The current work has been carried out to assess the potential of *Fraxinus xanthoxyloides* (FX) leaf extract in biosynthesis of silver nanoparticles (FX-AgNPs). This method is economical and simple one-step approach to synthesize AgNPs. Characterization of FX-AgNPs has been done by UV-Visible spectroscopy, scanning electron microscope (SEM), X-ray diffraction (XRD), transmission electronic microscope (TEM) and Fourier transforms infrared spectroscopy (FT–IR). The formation of FX-AgNPs has confirmed through UV-Visible spectroscopy (at 430 nm) by change of colour owing to surface Plasmon resonance. Based on the XRD pattern, the crystalline property of FX-AgNPs has established. Functional groups existing in *F. xanthoxyloides* leaf extract are confirmed by FT-IR spectrum. SEM and TEM authenticated morphology of the AgNPs. The newly synthesized nanoparticles were evaluated for their antimicrobial potential. Minimum inhibitory concentration was determined against *Escherichia coli,* methicillin-resistant *Staphylococcus aureus* (MRSA) strains, *Pseudomonas aeruginosa and Candida albicans* by microtiter plate assay. The lowest inhibition (69%) observed against *MRSA* was at a concentration of 50 ppm FX-AgNPs and maximum inhibition (81%) observed was against *P. aeruginosa*. The biosynthesized AgNPs triggered up to 68·6% reduction of the *P. aeruginosa* biofilm when compared to the control.

Conclusion: It can be concluded that nanoparticles could be a better alternative of antibiotics with greater efficacies and represent a valuable milestone to fight against infections caused by MDR pathogens.

Significance and Impact of the Study: This study imparts a useful insight into the development of a new antimicrobial agent from a novel source.

Introduction

Antibiotics have brought revolution in human well-being since their discovery in the late 1920s. They eased the pain of illnesses by overcoming infectious diseases and saved millions of lives. With the passage of time, extensive and careless use of antibiotics exerted selective pressure on susceptible pathogens and resulted in the appearance of antibiotic-resistant pathogens. Conventional antibiotics have been failed to deal with microbial

infections due to emergence of resistance to multidrugs of the choice (Frieri *et al.* 2017). The methicillin-resistant *Staphylococcus aureus* (MRSA) strains are now found worldwide and most of the countries have difficulties to combat nosocomial infections associated with *S. aureus* (Gharieb *et al.* 2020). Numerous options substitute to the conventional antibiotics exists for treating infections, including bacteriophage, predatory microscopic organisms, bacteriocins and antimicrobial peptides (Joerger 2003), but current advancements in the nanotechnology to design nanoparticles with selected physicochemical properties have been paved the way for a novel mark of defence against antibiotic-resistant micro-organisms (Huh and Kwon 2011). For example, unlike to antibiotics, nanoparticles can have tendency to amend the metabolic activity of bacteria starting from contact with bacterial surface and ultimately end with cell death (Chatzimitakos and Stalikas 2016). Nanoparticles cross bacterial outer membrane and manipulate the metabolic pathways of the cell by altering its structural physiology. Then bind with vital components of the cell such as enzymes, ribosomes, DNA and lysozymes, which leads to oxidative stress, enzyme inhibition, electrolyte disparity, altered membrane permeability, protein deactivation and finally ends up with cell rupture and death. These features of NPs have incited their use to cure deadly infectious diseases (Yang *et al.* 2009; Xu *et al.* 2016). Among nanoparticles, prepared from various metals such as Au, Ag, Ce, Pt, Pd and Zn (Ahmed *et al.* 2016), AgNPs are well known for antimicrobial activity against various pathogenic and antibiotic-resistant micro-organisms. Beside antimicrobial activity, AgNPs have a broad range of beneficial properties including anti-diabetic, anti-cancerous, antioxidative and cytotoxic activities (Patil Shriniwas 2017; Ahmed *et al.* 2018; Patra *et al.* 2018).

Production and assemblage of nanoparticles via chemical or physical means have substantial adverse effect on the environment as major of their impurities are hard to decontaminate and often consume more energy (Parveen *et al.* 2016; Sathishkumar *et al.* 2016; Khan and Lee 2020). In contrast, nanoparticles created from plant extracts is the most reasonable option because of their eco-friendliness, biocompatibility and reduced toxicity (Logeswari *et al.* 2013; Verma and Mehata, 2016; Khan *et al.* 2020). The biological synthetic method is also economical, ecologically sound and easily can be produced in bulk amount (Mittal *et al.* 2013).

Many plants such as *Luffa acutangula* (Taruna et al., 2016), *Bauhinia tomentosa* (Ramar *et al.* 2018), *Theobroma cacao* (Thatikayala *et al.* 2019), *Aloe vera* (Ahmadi *et al.* 2018) and *Bridelia retusa* (Vinayagam *et al.* 2018) have shown potential to reduce silver nitrate and form AgNPs. In search of broad activities, efficiency, cost reduction and eco-friendliness, researchers are continuously investigating new plants for the production of novel nanoparticles.

In connection with this effort, this study reports the synthesis of silver nanoparticles through the reduction of aqueous Ag + with *F. xanthoxyloides* leaf extract, a plant naturally present in Hindukush and Himalayan mountains of Pakistan. In northern parts of Pakistan, bark, leaves and roots of *Fraxinus* plant have been conventionally utilized for the treatment of malarial and pneumonia infections. A list of chemical components has been extracted from *Fraxinus* plant comprising of secoiridoids, phenyl ethanoids, lignans, flavonoids and coumarins. Certain biologically active ingredients were obtained from *Fraxinus* plant, that is, Catechin Fraxetin, Esculetin, Syringin, Oleoside 11methyl ester, Calceolarioside B, Tannic acid and Quercetin Rutin (Sarfraz *et al.* 2017). It is also stated that methanol extract of *F. xanthoxyloides* leaves possess anti-inflammatory, analgesic (Younis *et al.* 2016b), anti-leishmanial (Younis *et al.* 2016c) and hepatoprotective capabilities (Younis *et al.* 2016a).

The purpose of this study is to synthesize cost-effective, ecofriendly and entirely biogenic silver nanoparticles with strong antimicrobial potential.

Materials and methods

Leaves were plucked from the *Fraxinus xanthoxyloides* plants present in the Bio Lawn of Quaid-I-Azam University, Islamabad and were brought to the laboratory. Silver nitrate of analytical grade, potato dextrose agar and Luria Bertani (LB) broth and agar were bought from Sigma Aldrich Germany. Multidrug-resistant bacterial strains, that is, Gram negative *Pseudomonas aeruginosa* ATCC 27853 and Gram-positive bacteria *S. aureus* ATCC 29213 and *MRSA* ATCC 33591 and one fungal strain *Candida albicans* ATCC 3147 were obtained from the laboratory of Microbiology, Quaid-I-Azam University Islamabad.

Preparation of plant leaf extract

The obtained *F. xanthoxyloides* leaves were 25 g in weight, which were subjected to washing thrice by distilled water, after that they were exposed to Millipore water so that all the dirt and other pollutants could be removed from these. Shredded leaves were added into 100 ml of Millipore water in 200 ml Erlenmeyer flask, followed by boiling for 15 min. Whatman (No. 1) filter paper was utilized for filtration of the plant extract (Farooqui *et al.* 2010; Nagati *et al.* 2012).

Synthesis of AgNPs from Extract of *F. xanthoxyloides* leaves

To synthesize AgNPs, a solution of 0·001 M $AgNO_3$ was prepared in 100 ml of Millipore water. The fabrication of AgNPs was confirmed through turning light brown colour to dark brown when 10 ml of the leaf extract (*F. xanthoxyloides*) was transferred to 90 ml of 1 mmol l^{-1} silver nitrate solution (Fig. 1). The silver ion reduction was determined by measuring UV-Vis spectra at different intervals ranged from 200 to 800 nm (Perugu *et al.* 2016).

Characterization of AgNPs

The effective biosynthesis of silver nanoparticles was then validated through the use of different instruments. The optical density of FX-AgNPs was measured by UV-Visible Cary 7000 spectrophotometer (Agilent Technologies, Santa Clara, CA, USA). The crystallinity of processed samples was checked through Rigaku Ultima IV X-ray diffractometer working at 45 kV voltages. Morphological structure of silver nanoparticles was determined through scanning electron microscope (SEM, JSM-JEOL USA). Transmission electron microscope (FEI Tecnai G2 Spirit Twin TEM Instrument) was used to assure the size of particle. Polydispersity index (PDI) was calculated by the Equation 1

$$\mathrm{PDI} = \frac{\sigma}{\chi} \tag{1}$$

where σ and χ represent the standard deviation and mean size diameter, respectively. Fourier transform infrared spectrum was obtained by FT-IR instrument (Vector 22, Bruker Germany).

Antimicrobial activity of FX-AgNPs

Minimum inhibitory concentration of FX-AgNPs

All tested strains were cultured overnight, bacterial strains at LB agar and *candida* on PDA. Fresh cultures of tested micro-organisms were used, that is, *P. aeruginosa, S. aureus, MRSA and C. albicans*. Broth culture of the micro-organisms was centrifuged for 10 min at 10 000 ***g*** and 4°C for obtaining the pallet. The pallet was washed with phosphate buffer saline (PBS). Supernatant from all the falcons was discarded and pellets were suspended in the fresh media, that is, LB broth. Optical density (O.D) of different bacterial isolates was adjusted at 625 nm and concentration of bacterial and fungal suspension was kept at 10^8 and 10^6 CFU, respectively (Balouiri *et al.* 2016). The 100 μl of inoculum and nanoparticles with four different concentrations such as 50, 100, 150 and 200 ppm were added in each well of a 96-well microtiter plate. The microtiter plate was covered and incubated for 24 h at 37°C. Next day, OD was taken at 492nm for *C. albicans* (Scorneaux *et al.* 2017) and 600 nm for bacterial strains by Thermo Multiskan EX Microplate Photometer (Thermo Fisher Scientific).

Antibiofilm Assay

For testing the antibiofilm activity of FX-AgNPs, the microtiter plate assay was performed following (Masum *et al.* 2019) with some modifications. Congo red assay was used for testing the ability of bacteria and *candida* for biofilm formation (Saxena *et al.* 2014). Cell suspension of *P. aeruginosa* was cultured in fresh LB broth in a shaker incubator. Optical density (OD) was adjusted at 625 nm and concentration of suspension was kept at 10^8 CFU (Balouiri *et al.* 2016). Each well of microtiter plate was inoculated with 100 μl of inoculums. The plates were then incubated for 24 h at 37°C. Wells were replaced with fresh media after 10–15 h of incubation of planktonic cells. After 24 h, plates were rinsed twice with PBS to eliminate the planktonic cells from plate. 100 μl of 50 ppm nanoparticle suspension was pipetted in it and incubated for 24 h at 37°C. Each well was then emptied from the liquid media and rinsed softly with sterilized double distilled H_2O. Additionally, to stain the biofilm in plate, a 100 μl of crystal violet (0·1%, w/v) dye was poured to well at ambient temperature and incubated for 45 min. The unattached dye was removed from the plate using double distilled H_2O. Wells without nanoparticles and inoculum were designated as negative control and wells without nanoparticles but with bacterial suspension were considered as positive control. The OD was accessed at 570 nm wavelength using a microtiter plate reader.

Statistical Analysis

All the experiments were performed in triplicates. IBM SPSS 25 was used for analysis of results. The concentrations of nanoparticles for each micro-organism were taken as independent variable while the percentage of inhibition was taken as dependent variable and one-way ANOVA was applied. One sample *t* test was performed to know the level of biofilm reduction statistically.

Results

Synthesis and Characterization of FX-AgNPs

Gradual change in the colour from light brown to dusky blackish brown was experienced when plant extract was mingled with the salt of silver nitrate (Fig. 1). Change in the colour shade indicated development of silver nanoparticles. It was due to surface Plasmon resonance

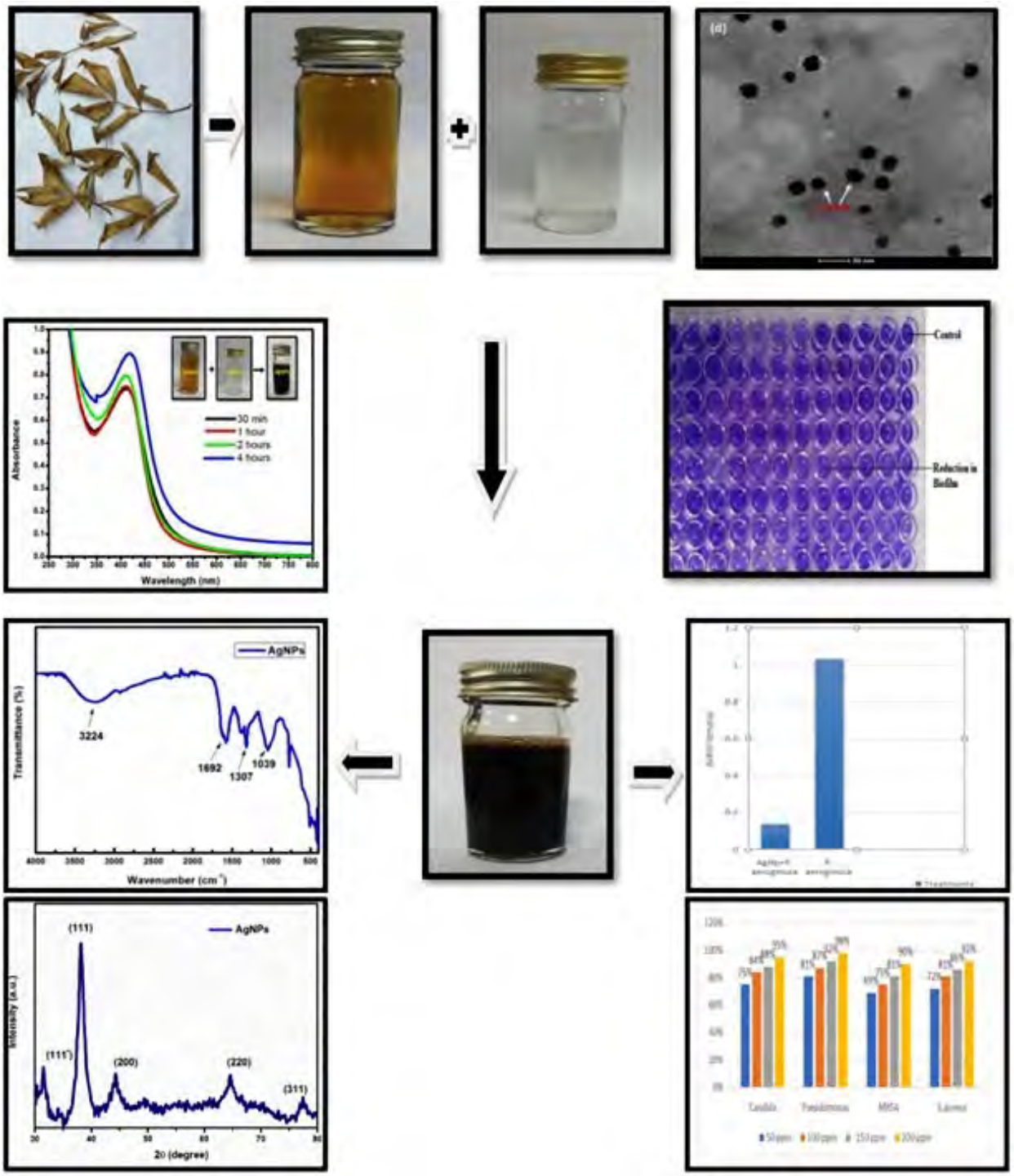

Figure 1 Graphical illustration for green synthesis of AgNPs from extract of *Fraxinus xanthoxyloides leaf* and its antimicrobial effect.

(SPR) incitement of the silver nanoparticles. The optical properties of biosynthesized AgNPs were examined in the wavelength range of 250–800 nm UV-Vis spectrophotometry. Sharp absorption peak could be seen slightly above 430 nm (Fig. 2). The crystallinity of synthesized silver nanoparticles from FX leaf extract was checked by XRD. The XRD pattern showed diffraction peaks at 77·51°, 64·94°, 38·15° and 44·27° can be indexed to the planes

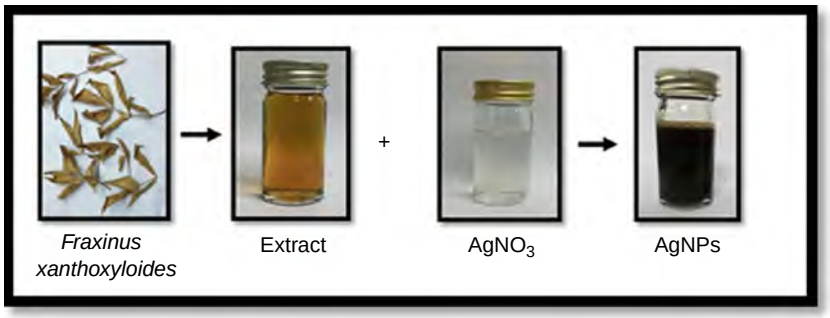

Figure 2 Graphical representation of FX-AgNPs biosynthesis.

(311), (220), (111) and (200), respectively (Fig. 3). To explore the morphology and microstructure of newly prepared particles, scanning and transmission electron microscopy was done to analyse the shape and dispersion of the Ag nanoparticles. Figure 4a,b portrays spherical morphology with dispersed nature of the AgNPs. The particle size of the AgNPs was calculated by ImageJ software with a typical diameter of 72 nm. TEM micrographs revealed that the most of the AgNPs possess regular ball-shape while some of them have irregular shape and predominantly spread (Fig. 4c). It confirms the spherical morphology of Ag nanoparticles and is in accordance with SEM images (All steps are shown sequentially in Fig. 5).

In addition, Fig. 6 shows the FT-IR spectrum of prepared nanoparticles, and FX-leaf extract, which revealed the functional group responsible for stabilizing and reducing silver nanoparticles. FT-IR spectra of AgNPs prepared from *F. xanthoxyloides* leaf extract showed different absorption peaks at 3207, 2907, 1600, 1312, 1038 and 764 cm^{-1}. FX-leaf extract showed peaks at 3211, 2927, 1575, 1443 and 1034 cm^{-1}, respectively.

Antimicrobial properties of FX-AgNPs

Minimum inhibitory concentration

Four concentrations of FX-AgNPs, that is, 50, 100, 150 and 200 ppm were used against tested micro-organisms. AgNPs displayed noticeable activity at each concentration. The

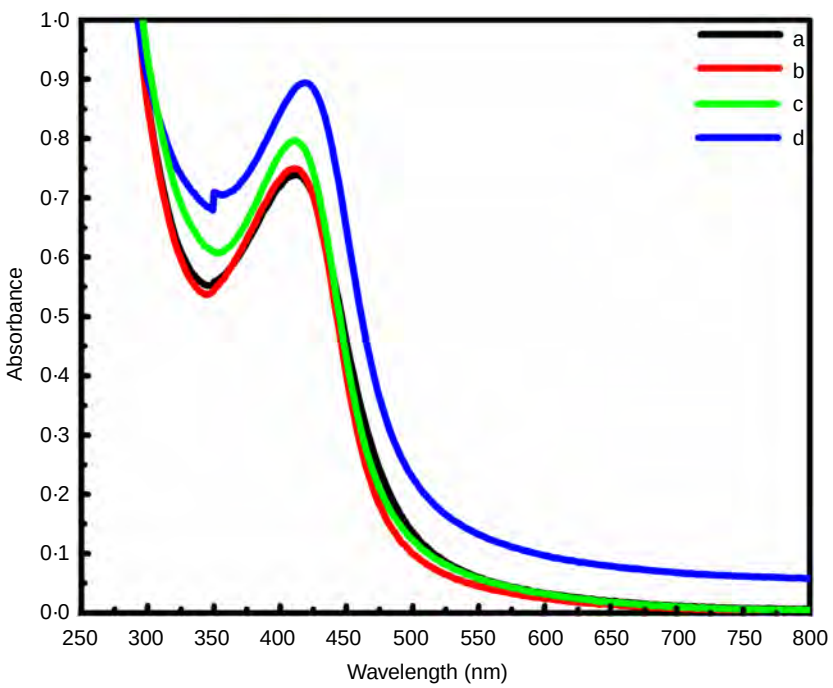

Figure 3 UV-visible absorption spectra of silver nanoparticles synthesized from *Fraxinus xanthoxyloides leaf* extract at different time intervals; (a) 30min, (b)1h, (c) 2h and (c) 4h.

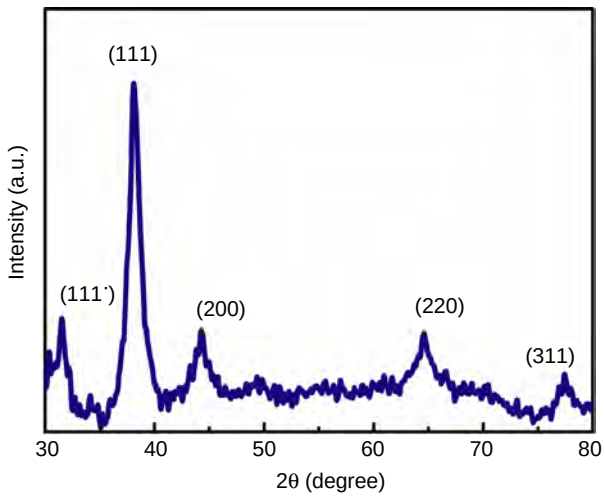

Figure 4 X-ray diffraction pattern of AgNPs synthesized from *Fraxinus xanthoxyloides leaf* extract. XRD peaks observed from 30° to 80° confirmed successful formation of the crystalline AgNPs

maximum microbial reduction was observed at 200 ppm and lowest reduction was observed against MRSA at a concentration of 50 ppm, that is, 69%. It has been observed that increase in the concentration of nanoparticle increase the inhibition of test micro-organism significantly. The *P* value for 50 ppm was recorded 0·155, for 100 and 150 ppm $P < 0{\cdot}05$ and for 200 ppm $P < 0{\cdot}01$ (Fig. 7).

Antibiofilm assay

Biofilm of *P. aeroginusa* was quantified after incubation at 30°C without agitation. Inhibition was observed against 50 ppm of FX-AgNPs in comparison with control (Fig. 8). The OD_{570} value of *P. aerogenousa* was 1·03 without AgNPs and with AgNPs had an OD value 0·137. Hence, the green synthesized AgNPs triggered 68·6% drop in the biofilm as compared to the control (Fig. 9). The *t*-test showed a *P* value of 0·416 for reduction of biofilm formation.

Discussion

The biological synthetic method is convenient, eco-friendly and can produced in bulk amount easily (Mittal *et al.* 2013). Green AgNPs have been described to be capable therapeutics with substantial antimicrobial activity. Though numerous nanoparticles have been effectively produced from plants and micro-organisms but exploration of new nanoparticles with specific biological, physiological and chemical properties is still at the forefront of research. Nanoparticle synthesis through plant extracts is the utmost feasible way because of their eco-friendly, biocompatible and least toxic nature (Logeswari *et al.* 2013; Verma and Mehata 2016).

Fraxinus xanthoxyloides is naturally present in Hindukush and Himalayan mountains of Pakistan. Certain biologically active ingredients have been obtained from *Fraxinus* plant, that is, Catechin Fraxetin, Esculetin, Syringin, Oleoside 11-methyl ester, Calceolarioside B, Tannic acid and Rutin (Sarfraz *et al.* 2017). It is also stated that methanol extract of *F. xanthoxyloides* leaves possess anti-inflammatory, analgesic capabilities (Younis *et al.* 2016b). In the present study, bio production and characterization of silver nanoparticles from the leaves extracts of *F. xanthoxyloides* was reported, which may prove beneficial for

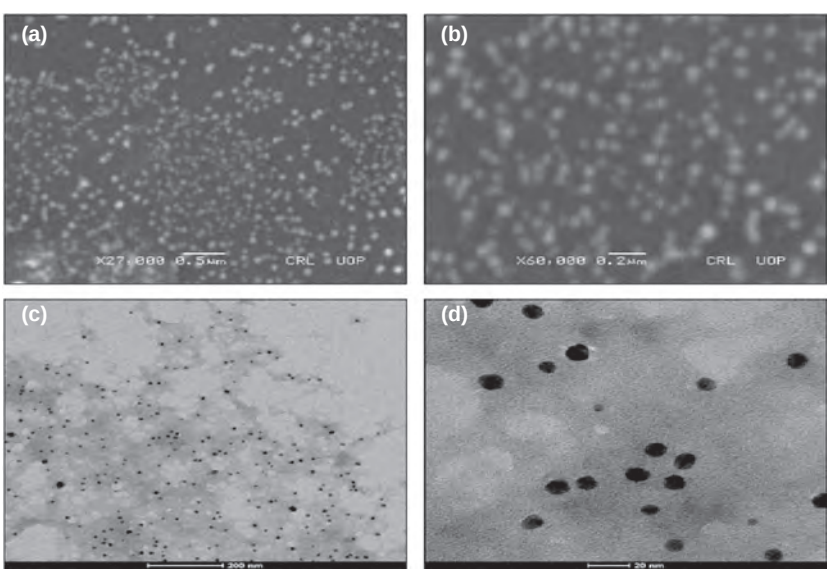

Figure 5 (a) SEM image of FX-AgNPs; scale bar is 0·5μ m, (b) SEM image of FX-AgNPs illustrates morphology of nanoparticles; scale bar is 0·2μ m, (c) TEM image of FX-AgNPs; scale bar is 200nm and (d) TEM image shows spherical shape of FX-AgNPs; scale bar is 20nm.

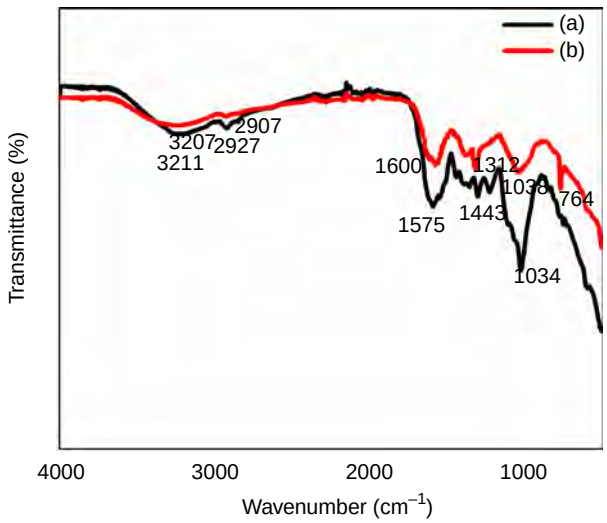

Figure 6 Fourier-transform infrared spectra of (a) *Fraxinus xanthoxyloides* leaf extract (b) FX-AgNPs.

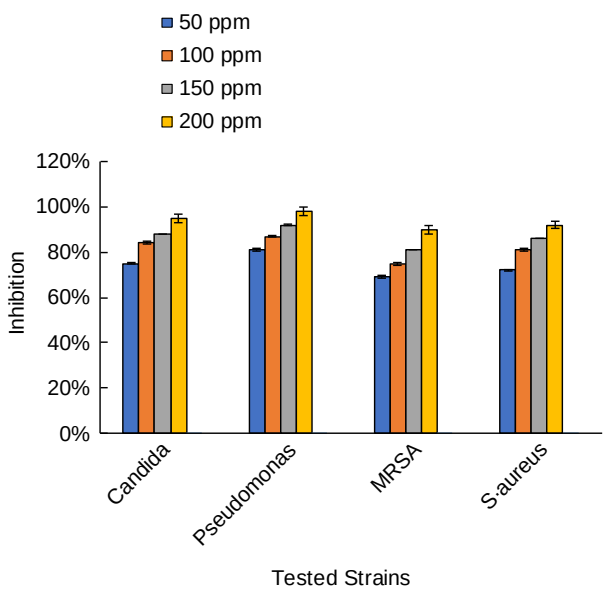

Figure 7 Antimicrobial activity of FX-AgNPs against MDR strains at different concentration ranges from 50 to 200 ppm

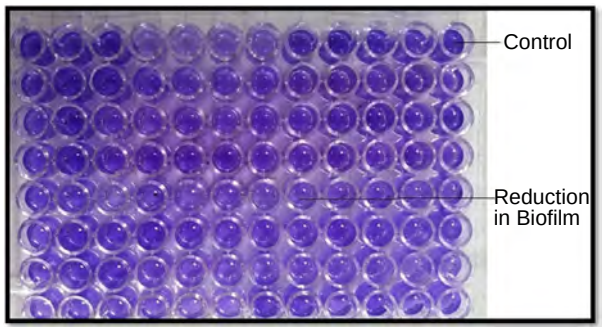

Figure 8 Effect of FX-AgNPs on biofilm formation of *Pseudomonasaeruginosa*, light purple stained well indicate reduction in biofilm in comparison to dark purple stained control wells.

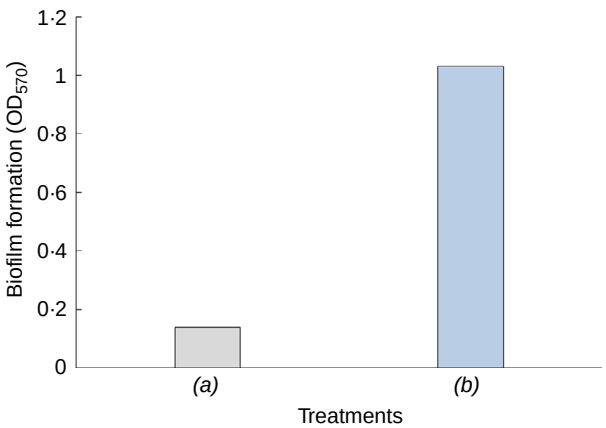

Figure 9 Biofilm formation of *Pseudomonas aeruginosa* (a) test sample with FX-AgNPs (b) control without FX-AgNPs. Biofilm reduction of 68% was observed in test sample as compared to control.

biological and economical features. Moreover, the produced AgNPs significantly affected bacteria growth, cell physiology and biofilm synthesis. Biosynthesis of AgNPs with plant extract is an upright method for biosynthesis due to their harmless properties and thus offers natural capping agents (Anjum and Ashraf 2020).

Figure 3 presents the relation between absorbance versus wavelength. The spectra obtained from spectrophotometer confirmed successful formation of AgNPs. The pattern also revealed a strong absorption band at 430 nm, this characteristic is associated with the absorbance properties of silver at given wavelength (Bar *et al.* 2009). Moreover, the spectra show an up rise in the intensity of absorption peak with the passage of time, which also confirms formation of AgNPs. A rise in the intensity of absorption line also shows that, as the time passed more silver nanoparticles are formed. A shift in the absorbance peak was detected (Bathochromic effect) that may be due to creation of large-sized silver nanoparticles (Elemike *et al.* 2017). The γ_{max} values in the 400–500 nm range are precise for the exterior Plasmon band of AgNPs (Ibrahim 2015).

XRD pattern was observed from 30° to 80° which confirmed successful formation of the crystalline nature of AgNPs from *F. xanthoxyloides* leaf extract (Fig. 4). The

average crystal size of silver nanoparticles was measured 8nm using Scherer's formula from the (111) peak which is the most intense and sharpest peak, similar results have been described in recent studies (Mahmoud *et al.* 2016; Oves *et al.* 2018). The small peak at 31·54° may be because of silver oxide indicated by (111*) (JCPDS no: 75-1532).

The SEM images of the as-prepared AgNPs confirmed that most of the particles are almost spherical shaped and uniformly distributed (Soman and Ray 2016). A small amount of aggregations was also seen in the SEM micrograph that may reflect variation in size of newly formed particles (Fig. 5a,b). Variations in the sizes of AgNPs produced from different plant extracts have been reported previously (Elbeshehy *et al.* 2015). It has been shown that smaller size of silver nanoparticles at 7 pH is more efficient in terms of its applications. TEM images revealed a regular as well as irregular spherical shape of silver nanoparticles with a size extending from 5 to 12 nm. Figure 5c,d also validates the monodisperse nature of the nanoparticles (Venugopal *et al.* 2017).

Nanoparticles with PDI $< 0{\cdot}05$ are measured as monodisperse and in our study we found it 0·02. The morphology and distribution of silver nanoparticles of the SEM and TEM images support each other (Ghiu ă *et al.* 2018). To examine the stabilizing and reducing agents of the as-prepared silver nanoparticles, FT-IR was performed. Figure 6 shows the FT-IR spectra of FX-leaf extract and AgNPs prepared from *F. xanthoxyloides* leaf extract with many peaks. The band at 3207 cm^{-1} could be accredited to the stretching modes of vibrations of hydroxyl (–OH) group. The spectral analysis confirms the existence of numerous functional groups that are responsible for capping of silver nanoparticles. The band at 1600 cm^{-1} is owed to the existence of amide vibrations that could be associated to silver nanoparticles by the amine groups. The spectral peak detected at 1312 cm^{-1} might be attributed to C–H symmetric vibrations (Selvi *et al.* 2016; Oves *et al.* 2018), while the peak found at 780 cm^{-1} belongs to the AgNPs and could be assigned to stabilizing agent during synthesis of AgNPs. Moreover, the peak at 1038 cm^{-1} is a result of bending modes of hydroxyl group (−OH). Hence, FT-IR spectra verify successful formation of AgNPs. After the reduction of silver ions, following peaks showed shift 3207–3211, 2907–2927, 1575–1600, 1575–1600, 1443–1312 and 1034–1038 cm^{-1}. This shift indicates that hydroxyl, amide and carboxyl groups of FX-leaf extract may be involved in nanoparticle synthesis (Bankar *et al.* 2010).

Efficacies of sliver nanoparticles for their antimicrobial properties have been known for decades. Synthesizing AgNPs utilizing microbes and plants are well-recognized approaches for the expansion of harmless and capable control efforts for inhibition of resistant bacteria (Manikandan *et al.* 2017). *In-vitro* results presented that the silver nanoparticles manufactured by aqueous extract of *F. xanthoxyloides* leaf had potent antibacterial effect against all tested strains in terms of percentage that ranged between 65 and 95% (Fig. 7). Therefore, we suppose that silver nanoparticles possess a broad range of antibacterial and antifungal activities (Martinez-Castanon *et al.* 2008). Gram-negative bacteria have the lowest values for the MIC while the Gram-positive have highest MIC values. Difference among the silver nanoparticle activities can be linked to the variance between cell walls of Gram-positive and Gram-negative bacterium as Gram-positive bacterium possess a thicker cell wall in contrast to Gram-negative bacterium's cell wall (Thiel *et al.* 2007). Width of the peptidoglycan layer of *S. aureus* plays a vital role in shielding from antimicrobials such as antibiotics, chemicals, enzymes or toxins. This outcome was related to the results of previous studies (Jung *et al.* 2008).

The attained results of the antifungal activity evidently tell that growth of *C. albicans* is inhibited by 75% at concentrations as low as 50 ppm using FX-NPs. The percentage inhibition increases up to 95% by increasing AgNPs concentration (Fig. 7). This may be due to apoptosis caused by accumulation of AgNPs in *C. albicans* (Hwang *et al.* 2012).

The biosynthesized AgNPs triggered 68·6% drop in the biofilm formation of *P. aeruginosa* as related to the control (Fig. 8). The promising activity of AgNPs is because of their physiochemical properties particularly size and surface characteristics prompting the antibiofilm response. The enhanced activity is due to the capping of nanoparticles which avert the accumulation of particles followed by the increased permeability through biofilm matrix. (Bryaskova *et al.* 2011; Mohanty *et al.* 2012; Habash *et al.* 2014) Green silver nanoparticles integrate with bacterial cells and produce reactive oxygen species, which results in protein destruction, macromolecules damage and inappropriate appearance of the virulence factors, along with biofilm inhibition (Qayyum *et al.* 2017).

Overall, the outcome of FX-AgNPs associations with micro-organisms is mainly relying on microbial species, nanoparticle doses and growth medium. Moreover, there is vast opportunity in this new, capable and exciting field and henceforth, a vibrant portrait of the medicinal and ecological significances of AgNPs would require further detailed insightful studies in context of nanoparticle-microbe interaction.

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Authors' contribution

Aftab Rafiq as a first author developed main idea and frame work for research. Khadija Zahid assisted in experimental section. Abdul Qadir assisted in characterizations and Nadeem Khan assisted in manuscript preparation. Dr Zafar Mahmood Khalid as supervisor provides intellectual support and Dr Naeem Ali as corresponding author provided laboratory facilities and guidance.

Conflict of Interest

The authors of the study declare that there are no known competing interests associated with this study or the data contained within.

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