ANTIMICROBIAL ACTIVITY OF METAL NANOPARTICLES ON SEELCTED MICROBIAL STRAINS

Project Report submitted in partial fulfillment of the requirement for the degree of

Bachelor of Technology

Biotechnology

BY

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To

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CERTIFICATE

We hereby certify that the work presented in this report entitled " **Antimicrobial activity of metal nanoparticles on selected microbial strain**" in partial fulfillment of the requirements for the award of the degree of Bachelor of Technology in Biotechnology submitted in the department of Biotechnology and Bioinformatics, Jaypee University of Information Technology Waknaghat is an authentic record of our own work carried out over a period from August 2016 to April 2017 under the supervision of Dr. Abhishek Chaudhary (Assistant Professor, BT).

The matter embodied in the report has not been submitted for the award of any other degree or diploma.

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This is to certify that the above statement made by the candidate is true to the best of my knowledge.

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OBJECTIVE

The objectives of this project are:

1. Green synthesis of metal nanoparticles from *Catharanthus roseus*
2. Optimization of physical parameters (temperature, time, concentration etc.)
3. Characterization using various analytical techniques (UV-Vis, DLS, and TEM)
4. Drug loading of CuNPs (Ciprofloxacin)
5. Study Antimicrobial activity: Antibacterial and Antifungal (Disc diffusion and Well Diffusion)

ABSTRACT

While metal nanoparticles are being progressively used in many areas of the economy, there is mounting interest in the biological and environmental protection of their production. The current work is dedicated to the possibility of metal nanoparticle synthesis using leaf extract of *Catharanthus roseus* which is resourceful, reasonable, and environmentally safe method for producing nanoparticles with definite properties. Further the characterization of the nanoparticle is done using characterization methods viz. DLS, TEM, UV-Vis Spectroscopy, etc. The zeta potential of Cu nanoparticles was found to be +30mV which confirmed the stability of synthesised NPs and with the confirmation of the stability DLS resulted in confirming hydrodynamic size 45nm of nanoparticles. Further the peaks of UV-Vis spectroscopy between 800-850nm showed the presence of anisotropic nanoparticles. Finally, TEM images revealed the size and morphology of the synthesised NPs which were polydispersed in the medium. The bactericidal effect of copper nanoparticles was studied based on diameter of inhibition zone against *E. coli* (ATCC 25922 and *E. coli* DH5α) which showed the enhanced effect of nanoparticle with ciprofloxacin when compared to only CuNPs. Further antifungal test using agar well diffusion against *A. flavus* ensured the enhanced antifungal effect of CuNPs when compared to the copper salt alone.

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NPs	Nanoparticles
CuO	copper oxide
$CuSO_4.5H_2O$	Copper penta hydroxide sulphate
CuNPs	Copper nanoparticles
Cip	Ciprofloxacin
$NaBH_4$	Sodium borohydride
E. coli	Escherichia coli
A. flavus	Aspergillus flavus
MIC	minimum inhibitory concentration
ZnO	Zinc oxide
AgNPs	Silver nanoparticles
ZnNPs	Zinc nanoparticles
mm	Millimetre
nm	Nanometre
cm	Centimetre
PBST	Phosphate Buffer Saline Tween-20
DLS	Dynamic Light Scattering
UV-Vis	Ultra Violet Visible
XRD	X-ray Diffractometer
FTIR	Fourier Transform Infrared
TEM	Transmission Electron Microscope
JUIT	Jaypee University of Information Technology
mV	Millivolt
ATCC	American type culture collection

CHAPTER 1

INTRODUCTION

INTRODUCTION

Nanotechnology is a rapid progressing field, it's a boon to the world. It is a science of small things its manipulation, which are less then 100nm in size, where 1nm is equal to 10^{-9} m. It has been proved that particles with such size have different properties as compared to its bulk form which results in improved applications if we know how to control the structures and compositions of these particles in different fields i.e. electronics, food industry, textile industry, medicine etc. If we look around in nature many plants and animals have adopted special features at nanoscale for instance the eyes of a moth have small bumps which help it to see better in dark and dim light as compared to humans. To see these structures there are different tools available which helps in characterization of the nanoparticles [1]. The growing demand of nanoparticles in different fields result in the different synthesis methods for nanoparticles which are cost effective as well as no toxic to environment etc.

1.1 Synthesis of nanoparticles

The synthesis of nanoparticles depends on the physical and chemical route which is costly as well as use of these nanoparticles gets limited due to the toxic compounds. To overcome the limitations of the chemical and physical synthesis of nanoparticles green synthesis is used for nanoparticle synthesis which uses the plant metabolites as reducing agents for the nanoparticles synthesis and microbes which is not harmful to the environment and its proven the synthesis by biological routes saves energy and less harmful waste is produced. The different synthesis methods used are the top down and bottom up approach, the bottom up approach include the formation of nanostructures from very small particles for instance in the range of angstrom whereas the top down approach includes the formation of structures starting from micrometre for example and moving down towards the nanoparticles. In our project, we used the 'green synthesis' approach [2].

1.1.1 Green synthesis

Different biological routes are used for the synthesis of nanoparticles. One of the biological methods used is green synthesis viz use of plant extract for the reduction and stabilization of the nanoparticles. Different research group have been synthesized different metal NPs such as Au, Ag, Fe, Cu, Pt and Pd, NPs using green synthesis approach and prove their potential in various fields. In our project, we have reported use of leaf extract of *Catharanthus roseus* for synthesis of CuNPs and its antimicrobial activity. This method has been pursued as an alternate, resourceful, and environmentally safe. The well-known property of plants to reduce metal ions helps in the formation of nanoparticles and this method is more flexible and the size and shape of the particle can be controlled just by changing the medium pH, temperature, concentrations etc. [3]. The various plant metabolites play a very important part in bio reduction of the metal ions. The mechanism of the production of nanoparticles by this method includes three phases. First, the activation phase, the reduction of metal and nucleation occurs; second, the growth phase (Ostwald ripening), convoyed by increase in the thermodynamic constancy of particles; and third, termination phase which regulate the final shape of the particles. The shape of the nanoparticles depends on the duration of the growth phase as it increases [4,5].

1.2 Applications

Nanoparticles possess different properties such as the special optical property, conductance, uniformity and a large surface area to volume ratio which makes them to be used in different fields and the small size of the nanoparticle is a boon for its use in different areas. It offers the tools and technology platforms for the study and alteration of biological systems, and biology offers inspiration models and bio-assembled components to nanotechnology. The broad range of applications of nanoparticles for medicine, tissue engineering, manufacturing and material, environment, energy and

electronics etc [6]. one of the most common applications discussed in this project thesis in the antimicrobial effects of CuNPs.

1.2.1 Antimicrobial property

The nanoparticles prepared by the biological method has application in the field of medicine as a drug delivery [7] and synthesising by plant extract as done in this project results in enhanced property due to the synergetic effect of the plant extract (*Catharanthus roseus*) and the metal (Cu). The plant extract act as a capping agent around the nanoparticle synthesised. Metals have been used as antimicrobial agent since ages but their mode of action is still unclear, different metals cause distinct injuries to the microbial cells due to oxidative stress, membrane damage or protein dysfunction or metal ions may bind to the DNA of the cell preventing its replication.

Copper is widely used material due to its optical, catalytic, biomedical and antifungal/antibacterial applications. The copper is toxic to microorganism such as bacteria and non-toxic to animal cells, due to which it is an effective bactericidal. It is safe for human beings such as food packaging application and in water treatment applications [8].

In our thesis, we did the study of the antimicrobial effect of Cu nanoparticles synthesised by *Catharanthus roseus.* Followed by antimicrobial assays to check there activity against E. coli (ATCC 25922) by disc diffusion, E. coli (DH5α) by well diffusion and A.flavus by well diffusion techniques. The **Agar Well Diffusion** and **Disc Diffusion Assay** is one of the method for quantification of the ability of the samples inhibiting the fungal and bacterial growth. This works on the principle of diffusion further resulting in the formation of Zone of Inhibition on the plates. Quantification is done by measuring the diameter of the zone of inhibition. These techniques are easy, cost effective and accurate and can be easily modified and repeated. Use of Mueller-Hinton Agar in the antibacterial test for better diffusion of the samples and use of Potato Dextrose Agar for antifungal study against A. flavus for its better growth was carried out in this project.

The particle size of NPs elects its applications in drug industry, but size has its individual pros as well as cons, for instance undesirable penetration can cause injury, reducing the specificity of the nanoparticles towards a cell. This was resolved by using certain antibiotics as used in this project ciprofloxacin along with nanoparticles to help increasing specificity at the desired place. This functionalization of nanoparticles resulted in enhanced activity of that drug against microorganisms decreasing the dose of the drug [9]. It performs a double action, by inhibiting topoisomerase IV and DNA gyrase, which decelerates the resistance development. As an antibiotic drug model, ciprofloxacin was loaded onto the nanocarrier via H-bonding interactions and by functionalization of nanoparticles controlled delivery of the drug can also be achieved [10].

1.3 Characterization Techniques

After the synthesis of nanoparticles, the most important part is the characterization of nanoparticles using variety of analytical methods, including UV-vis spectroscopy, X-ray diffractometry (XRD), Fourier transform infrared spectroscopy (FTIR), X-ray photoelectron spectroscopy (XPS), dynamic light scattering (DLS), scanning electron microscopy (SEM), transmission electron microscopy (TEM), and atomic force microscopy (AFM). As for the characterization of copper nanoparticles in our project we used UV-Vis Spectroscopy, Transmission Electron Microscopy, and Dynamic Light Scattering as discussed below:

1.3.1 TEM (Transmission Electron Microscopy)

Transmission electron microscopy is for the conformation of size and morphology of nanoparticles. It gives a 2-dimensional image of the particles using electrons in a thin beam to focus on electromagnetic plate whose wavelength is much smaller than that of light, further generating projection image at magnification of 10,000,000x, it is widely used to observe the crystal structure of the particles, it can give even the minute details

of the internal structures. In our project, we used TEM (FEI instrument, of 20KV) at IIT Mandi.

1.3.2 UV-Vis Spectroscopy

It is an absorption spectroscopy in the region between the ultra-violet and visible spectra (400-750nm), UV-vis spectroscopy is applied to molecules in solution and is best useful for quantitative analysis. It works on the principle of Beer-Lambert law determining the concentration of an analyte by absorbance at a specific wavelength. The light sources are deuterium discharge lamp for UV and a tungsten-halogen lamp for visible and instrument accordingly swap the lamps during the measurements. The light from the lamp is dispersed before it reaches the sample. For our CuNPs's characterization we used UV-Vis spectrophotometer from JUIT, Department of Bioinformatics and Biotechnology and obtained absorbance vs wavelength curve of nanoparticles, confirming the formation of the nanoparticles.

1.3.3 DLS (Dynamic light scattering)

The DLS (MALVER Nano ZS) Instrument was used from IIT Mandi for characterization of our NPs. This technique is useful for the determination of the size distribution of nanoparticles in a solution, working on the principle of Brownian motion. The fluctuation of light is dependent on the size of particles as smaller particles are pushed further by the molecules and they tend to move rapidly. Analysis of these fluctuations result in the velocity of the Brownian motion and hence the particle size. The diameter measured in DLS is called the hydrodynamic diameter and refers to how a particle in the fluid are diffused. DLS technique is well-established technique for the measurement of the size of molecules and particles in the submicron region.

CHAPTER 2

REVIEW OF LITERATURE

REVIEW OF LITRATURE

Nanotechnology is a science which is still in its infancy is an interdisciplinary science that uses all other various branches of science such as material sciences, chemistry, physics, biology and engineering. Long before the term nanotechnology was used Richard Feymann said *"There is plenty of room at the bottom"* at a conference and addressed how scientists would be able to work on individual atoms and molecules. After the extended research on this idea, the term nanotechnology was coined and since then is an emerging discipline in field of research and development [11]. After so many years of sole research on nanotechnology there have been surprising revolutionizing benefits to the mankind. Human benefit sectors like medicine, environmental science, food industry, information technology and the list is still rapidly expanding. All this have been possible because of the nanoscale particles that can be exploited in applications where larger size molecules cannot be used. These nanoscale particles are used extensively whether in tech say researches or in day to day products such as batteries, sensors etc. [12].

In the meanwhile, the NPs are being extensively used in almost every sector of the economy because of their rising need. With this growing need, there is also the emerging need of environmental safety and efficient methods of production of these nanoparticles. The common processes of nanoparticle synthesis are physical and chemical methods. The major issue associated with these processes are that they are usually hazardous to the environment, living organisms, expensive and labour intensive. Hence creating an obvious need for the synthesis of these nanoparticles via a cleaner route. An alternative to these conventional methods of synthesis is the green synthesis which utilises environment friendly material like parts of plants, microorganisms such as bacteria, fungi and enzymes. Nanoparticles which are produced using green technology are varied, more stable and of the appropriate required size. All this is true since these nanoparticles are synthesized using one step reaction which is not the case in the conventional methods. Also, the green synthesis allows less consumption of energy making it an efficient environmental friendly mode of synthesis. Green nanotechnology encourages both academic researches as well as researches in industrial and commercial

fields [13-14]. The nanoparticles produced by this technology have already been used in electronic devices, pharmaceutical industries, light emitters, optical devices, textile industries etc. In the present project, our major focus is the synthesis of these metal nanoparticles by green nanotechnology using the bio molecules present in plant extract.

In this report, we demonstrated the synthesis by using plant extract of *Catharanthus roseus* leaf. There is a vast application of this plant in medicinal field due to its different properties, *Catharanthus roseus* (Apocynaceae) (*C. roseus*) is considered as medicinal plants. It grows to 1 m tall at subtropical region. It holds known antibacterial, antifungal, antiviral, antioxidant, wound healing [15-17]. *Catharanthus roseus* consists of vinca alkaloids which inhibit the cell proliferation upon interaction with the tubulin which results in microtubule dysfunction and hence metaphase arrest, mitotic block and apoptosis [18]. Herein, we report synthesis of copper nanoparticles, reducing the copper ions in the solution of copper sulphate by the aqueous leaf extract of *C. roseus*.

The major emphasis here is on the optimization of the anisotropic nanoparticles and their antimicrobial activity. Anisotropic nanoparticles have an unusual property that enhances their applicability. They have specific directional properties that isotropic particles lack. These properties include properties such as, electronic properties. With the increasing need of applicability of these nanoparticles there is the need of exploration of more of their properties. The utilisation of the multi functionalities of these particles, their directionality and their dimensions which play such a relevant role [19].

Plant extracts constitute phytochemicals that are responsible for the bio reduction of metal ions into nanoparticles. They have metabolites like terpenoids, alkaloids, phenolic acids and proteins that reduce metal ions into NPs. In addition to their synthesis they also provide stability to these particles. Major section of these metabolites constitutes flavanols, organic acids and quinones that help in quick reduction of these nanoparticles. Metal ions used for the synthesis of nanoparticles are effective as antimicrobial agents because these are transported into cell through the cell membrane where they accumulate and kills the microbial cell. This basically happens because of the oxidation reduction in the cell wall which causes metal ions to bind to the cellular proteins damaging the cell wall [20].

Copper has always been used as a potential antimicrobial agent since ages. Even in the ancient times copper and copper alloys were used as vessels for better quality of water storage in addition to the other medicinal preparations. The antimicrobial oligodynamic property which kills various types of microorganisms such as bacteria, fungi, algae, moulds is found to be shown by copper [21].

Due to the increase in the advent of the multi drug resistant microorganisms the researchers are focussing in the production of novel antimicrobial agents. Metal nanoparticles serve as efficient antimicrobial agents. Also, it has been found out that they enhance the effect of previously produced antimicrobials. They not only act as these agents but nanoparticles can be utilised in the detection of pathogens with the help of bio sensors [22].

CuO nanoparticles are found to have tackle bacterial infections acquired by hospitals since they have an efficient bactericidal effect. Studies have shown that gram negative bacteria tend to have a greater susceptibility towards copper oxide nanoparticles. Copper ions often intercalate with the nucleic acid and result in the inhibition of various biochemical processes in a bacterial cell, hence killing them [23].

This study describes the synthesis of Cu nanoparticles (CuNPs) using leaf extract of *Catharanthus roseus* which acts as both reducing and capping agent. Till date several groups had reported synthesis of Cu nanoparticles by green method using extracts of plant tissues, fruits, vegetables. Some of these works, which used plant materials, are described. Synthesis of copper nanoparticles from papaya extract yielded CuNPs of the size 20nm at 50-60°C and was also found to be an efficient capping agent [24].

Reduction of copper sulphate by *Nerium oleander* extract and analysis of its antibacterial activity against *E. coli*, *Staphylococcus aureus*, *Klebsiella pneumonia*, *Salmonella typhi* and *Bacillus subtilis* showing the maximum zone of inhibition against *Staphylococcus typhi* that is 21mm [25]. In another study Natural plant materials such as Magnolia leaf extract and stem latex of *Euphorbia nivulia* have been used for the synthesis of CuNPs. The stable CuNPs were synthesized using *Magnolia kobus* leaf extract on treatment with aqueous solution of $CuSO_4.H_2O$. Electron microscopy analysis revealed the average size between 37 to 110nm [26]. Synthesis of highly stable and dispersive Catechin - CuNPs developed at pH 11 with excellent antimicrobial

activity confirmed by bacterial viability test showing 90% and 85% death of *S.aureus* and *E.coli* respectively within 3hr [27]. Use of *Cassia fistula* flower extract where the size of CuNPs formed in the range of 20µm [28]. Synthesis of CuNPs using aqueous extract of guava [29], cloves [30], peel extract of *Punica granatum* [31], *Cassia auriculate* leaf extract [32], root extract of *Curculigo orchioids* [33], flower extract of *Cassia alata L*. [34], *Ocimum sanctum* [35], ginger [36] and leaf extract of *Cappris zeylanica* etc. [37]. In this study, the comparison of antimicrobial activity of CuNPs and conjugated CuNPs with ciprofloxacin is done. There are reported antibacterial activity of CuNPs against selected pathogens. Reported effectiveness of nanoparticles increases with increasing particle dose, treatment time and synthesis method. In addition, clear demonstration of particle size variation and surface area to volume ratio of green Cu nanoparticle is responsible for significant higher antimicrobial activity [38]. and the antibacterial activity against both gram positive and gram negative suggesting its possible use in water purification, air filtration, antibacterial packaging etc. [39]. The fluoroquinolone antibacterial drug ciprofloxacin (cip) has been used to cap metallic nanoparticles by a robust one-pot synthetic method under optimized conditions, using $NaBH_4$ as a mild reducing agent [40]. In the recent study the coupling of nanoparticles with drugs has been shown to produce a noticeable increase in the action of the drugs, like antibiotics which when combined with nanoparticles show increased killing of bacteria [41-43].

Synthesis of Cu nanoparticles has also been reported by certain medicinal plants. One of such plant is an Indian medicinal plant, *Tabernaemontana divaricate*. The leaves of this plant were used to produce stable, spherical nanoparticles by using 50% concentration of the plant extract. The particles were found to be the size of 48 ± 4nm by using various characterization techniques such as UV–Vis absorption spectroscopy, X-ray diffraction (XRD), Fourier transform infrared spectroscopy (FTIR), scanning electron microscopy (SEM) with energy dispersive X-ray analysis (EDX) and transmission electron microscopy (TEM). The nanoparticles synthesized were effective against the urinary tract pathogens [44]. As mentioned above that nanoparticles enhance the activity of certain antimicrobials upon conjugation with these drugs, many studies have proven to be efficient.

A study shows that conjugation of antibacterial active polyacrylate with penicillin which is covalently bound with these nanoparticles enhanced the antibacterial activity of the particles. These NPs possessed antibacterial properties against both methicillin resistant and susceptible *Staphylococcus aureus* and a greater stability towards beta-lactamase. [45]. Another study shows the conjugation of silver nanoparticles with various antibiotics to check the enhancement in their antibacterial activity against gram positive and negative bacterial strains. The AgNPs were produced by green technology by using a fungus *Trichoderma viride.* The particles formed were of 5-40nm. And the maximum absorbance peak was found to be at 420nm. The presence of silver was confirmed by energy dispersed spectroscopy. These particles were conjugated with various drugs such as erythromycin, ampicillin, kanamycin, and chloramphenicol. The antimicrobial activity against gram positive and negative bacteria upon conjugation with these AgNPs was seen to be enhanced. The highest effect was observed in the case of ampicillin against the test organisms [46].

In this report, we are using ciprofloxacin for functionalization of Cu nanoparticles synthesized using green synthesis. Ciprofloxacin is an antibiotic that belongs to the class of flouroquinones. It is an antibiotic used against infections varying from joint infections to abdominal infections. Ciprofloxacin works actively against both gram positive as well as gram negative bacteria. The main mode of action is the inhibition of bacterial cell division. The antibiotic inhibits DNA gyrase and topoisomerase type II and type IV. These enzymes are basically functional for the separation of bacterial DNA strands which is a necessary step for their cell division. Hence it inhibits the bacterial cell to multiply in the host cell [47].

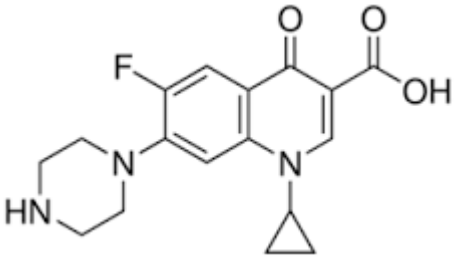

Fig 2.1 Ciprofloxacin structure

A study of 2014 described the conjugation of ciprofloxacin with ZnO nanoparticles synthesized under microwave assisted conditions. The particles formed were found to be of 18-20nm upon characterization by transmission electron microscope x-ray diffraction confirmed the hexagonal shape of the particles. Also, the conjugation of these particles was confirmed by FTIR spectra. Ciprofloxacin conjugated ZnNPs showed excellent antibacterial activity against multidrug resistant bacterial species of *Escherichia coli, Staphylococcus aureus* and *Klebsiella.* The antibacterial activity was checked by performing MIC [48]. AgNPs have also been used in conjugation with ciprofloxacin to check the enhancement in the activity of flouroquinones. AgNPs synthesized by using green synthesis by reactions of silver nitrate and aloevera extract. These NPs were treated with the solution of ciprofloxacin hydrochloride and plated against *Staphylococcus aureus* using agar plate diffusion method. An enhanced activity of the AgNPs-CIP complex was to be found [49].

By exploiting these functionalities of nanoparticles, not only the problem of multi drug resistance has been solved until an extent but also has led to the development of an efficient drug delivery system. In an efficient drug delivery system two things are of the highest importance. Firstly, the drug should be highly target specific and should be able to control the drug release. This controlled release limits the side effects caused by these antimicrobials. The nanoscale of these particles minimizes the side effects that can be caused. They also enhance the uptake of the insoluble or less soluble drugs because of their larger surface area and target the drug to the specific site of infection. Another advantage is that they are highly efficient even if taken in small doses. Using these approaches have led to greater results and hence a chance of great development in this field [50].

CHAPTER 3

MATERIAL AND METHOD

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3.1 MATERIAL USED:

CHEMICALS

1. $CuSO_4.5H_2O$
2. $AgNO_3$
3. KI
4. Drugs (ciprofloxacin)
5. Mueller Hinton agar
6. Potato dextrose agar
7. PBST

OTHER MATERIALS

1. *Catharanthus roseus* leaves
2. Distilled water

BACTERIAL STRAINS USED

1. *E. coli* (gram negative)
    - *E. coli* ATCC 25922
    - *E. coli* DH5α
2. *A. flavus* (fungal strain)

APPARATUS

1. Gloves
2. Cotton
3. Beaker
4. Conical flask
5. Eppendorffs

6. Magnetic stirrer
7. Magnetic bead
8. Glass rod
9. Hot plate
10. Micropipette
11. Tips
12. Refrigerator
13. Weighing machine
14. What man filter paper
15. Sterile swabs
16. Ethanol
17. 24 hr old culture of E. coli
18. Well puncture
19. Petri plates (autoclaved)
20. Parafilm

3.2 METHOD:

- **GREEN SYNTHESIS OF METAL NANOPARTICLES**

Following steps are included in preparation of metal nanoparticles:

a. Preparation of leaf extract
b. Preparation of metal solution of different concentration.
c. Green synthesis of nanoparticle

Preparation of leaf extract [51]

1. Fresh Leaves (*Catharanthus roseus*) were collected from the college campus itself.
2. Leaves were washed 3-4 times using distilled water and dried for 5 min.
3. The dried leaves were then weighed (10g) using weighing machine and cut into very small pieces.
4. The cut leaves were boiled in distilled water(150ml) for 30-40 min at 100°C.
5. The extract was filtered using whattman filter paper 2 times to eliminate all the impurities.
6. The extract was stored in refrigerator at 4°C for further experimentation

Fig 3.1 Catharanthus roseus leaf extract

Preparation of metal solution of different concentration.

1. Metal salt ($CuSO_4.5H_2O$) were weighed according to the concentrations (0.5M) to be prepared on weighing machine carefully using gloves.
2. The measured salt of metal was mixed in distilled water (10ml) depending on the ratios used until it was completely dissolved.
3. The solution was kept aside for further use.

Fig 3.2 Cu salt solution

Green synthesis of copper nanoparticles

1. The plant extract and the prepared metal salt solution was taken in ratio of 2:1 (20ml plant extract and 10 ml metal salt solution)
2. The extract was added to the metal solution.
3. Addition of KI (400µl of .01M) if necessary before addition of plant extract.
4. The mixture of extract and metal solution was kept on magnetic stirrer for continuous stirring (2 hr) with minimum speed at high temperature(70-80°C)
5. After the completion of reaction, the solution was kept aside for observation in colour change and stability of synthesised nanoparticles for 24hr at room temperature.
6. Further the samples were collected for characterization.

- **DRUG CONJUGATION**

The drug conjugation on the nanoparticles include following steps [52]

a) Preparation of drug solution.
b) Mixing of drug with synthesised nanoparticles.
c) Setting up the pH.
d) Incubation

Preparation of drug solution

1. The drug (ciprofloxacin) was weighed (40mg) carefully on the weighing machine.
2. The weighed drug was mixed in distilled water (10ml) for stock solution preparation.
3. Solution was kept in refrigerator at 4°C for further use.

Conjugation of drug and nanoparticle solution

1. The already green synthesised nanoparticles was mixed with the drug solution in the ratio respectively 5:1.
2. pH was set to acidic (pH 3 – pH 4).

Incubation

1. The solution of drug and nanoparticles was observed for 24hr at 37°C with continuous stirring in incubator.
2. After completion of reaction the colour change was observed and characterization was done.

- **STUDY OF ANTIMICROBIAL ACTIVITY**

    a) Antibacterial study against *E. coli* ATCC 25922 and *E. coli* DH5α.
    b) Antifungal study against *A. flavus.*

a) Antibacterial study against *E. coli* ATCC 25922 and *E. coli* DH5α

KIRBY-BAUER DISC DIFFUSION (*E. coli* ATCC 25922) [53]

1. Mueller-Hinton plate was swabbed with bacterial culture
2. After completely swabbing the plate, the plate was rotated and swabbing process was repeated.
3. The surface was allowed to dry for about 5 minutes before placing antibiotic disks on the agar.
4. Antibiotic discs were placed equidistant from each other, around the periphery using pair of forceps.
5. Incubation was done keeping the plates upside down at 37°C.
6. Observed after 24hr and measured zone of inhibition, at the widest diameter.

WELL DIFFUSION METHOD (*E. coli* DH5α) [54]

1. Mueller-Hinton plate was swabbed with bacterial culture
2. After completely swabbing the plate, the plate was rotated and swabbing process was repeated.
3. The surface was allowed to dry for about 5 minutes before punching wells using a syringe puncher in the agar plate.
4. The sample nanoparticles and control were loaded into these wells and the plates were seales without any movement.
5. Incubation was done at 37°C.
6. Observed after 24hr and measured zone of inhibition, at the widest diameter.

b) Antifungal study against *A. flavus*

WELL DIFFUSION METHOD (*A. flavus*) [55]

1. Potato dextrose agar plate was swabbed with A. flavus culture which was collected from the pure culture plate using PBST.
2. After completely swabbing the plate, the plate was rotated and swabbing process was repeated.
3. The surface was allowed to dry for about 5 minutes before punching wells using a syringe puncher in the agar plate.
4. The sample nanoparticles and control were loaded 20µl of each sample into these wells and the plates were sealed without any movement.
5. Incubation was done at 37°C for 48hr.
6. Observed after 24hr and 48hr and measured zone of inhibition, at the widest diameter.

CHAPTER 4

RESULT AND DISCUSSION

RESULT AND DISCUSSION

4.1 Optimization

4.1.1 Nanoparticles synthesised by *Catharanthus roseus*

Optimization of the green synthesis of CuNPs using leaf extract of *Catharanthus roseus*: after number of reactions the most successful reaction carried out was in 2hr, finally synthesising the required nanoparticles of copper after reduction by leaf extract. Colour change can be observed in the following pictures below showing a successful reaction. The ratio of plant extract to the Cu salt solution was optimised to 2:1 and the best concentration taken for the leaf extract was 10g of leaf in 100ml dH_2O and concentration of $CuSO_4.5H_2O$

Solution was optimized at concentration 0.5M**.** Colour change can be observed in the reaction after every 10-15min.

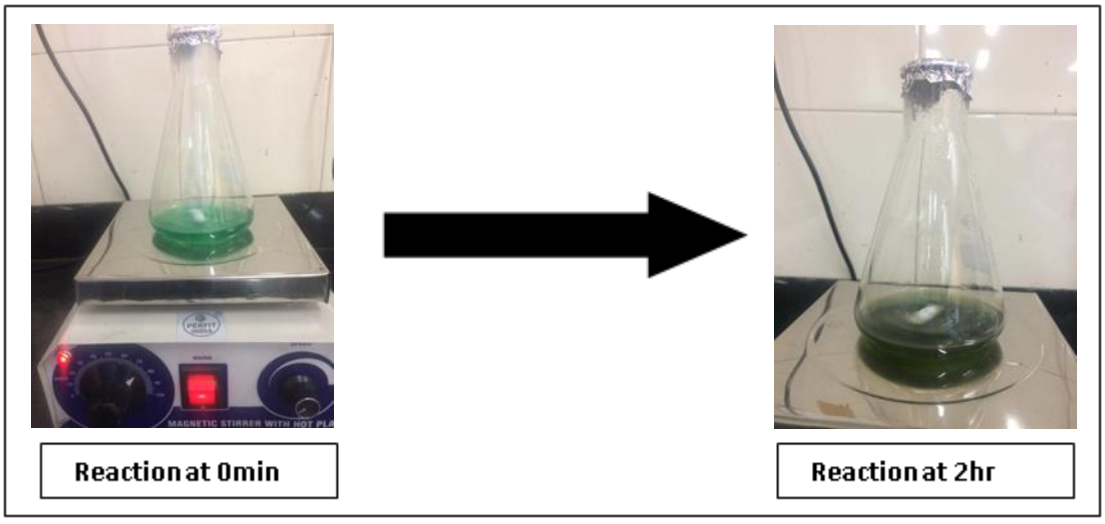


Fig 4.1 Optimization of time of reaction (CuNPs)

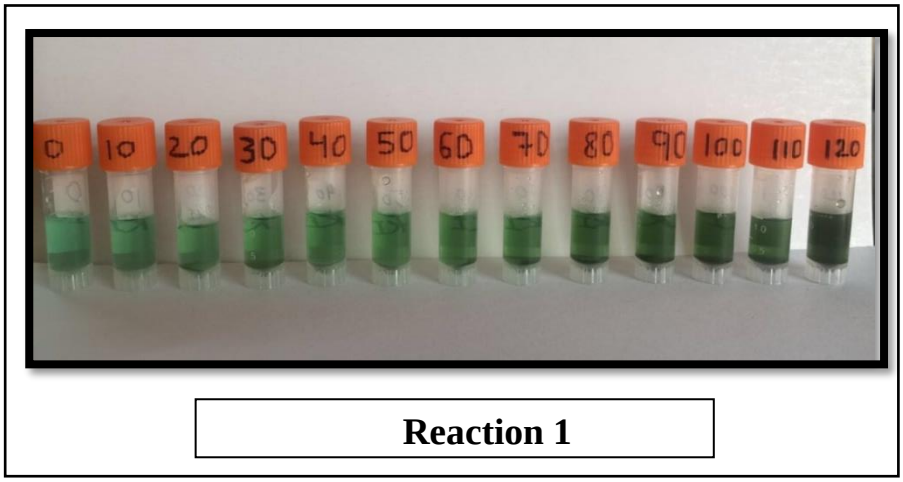
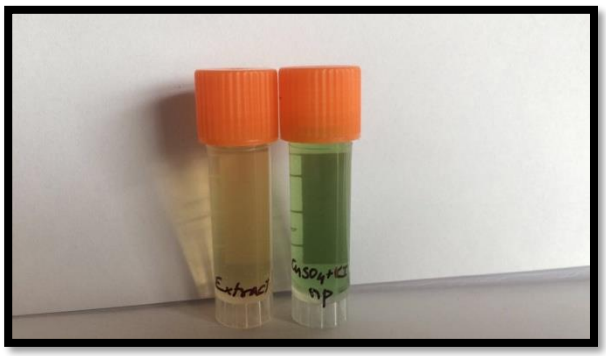


Fig 4.2 Observation of colour change of reaction after 10-15min (Reaction 1: $CuSO_4.5H_2O$)

4.2 Functionalization of nanoparticles

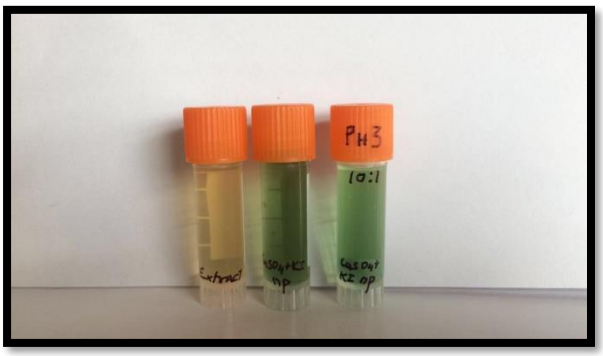
4.2.1 Nanoparticles synthesised by *Catharanthus roseus*

The results show the comparison of the functionalized nanoparticles with ciprofloxacin in the ratio 5:1 (synthesised copper nanoparticles to drug solution) with the plant extract and copper nanoparticles the change in colour results in the successful synthesis of the functionalized nanoparticles.



From left to right:

Leaf extract, synthesised nanoparticles



From left to right:

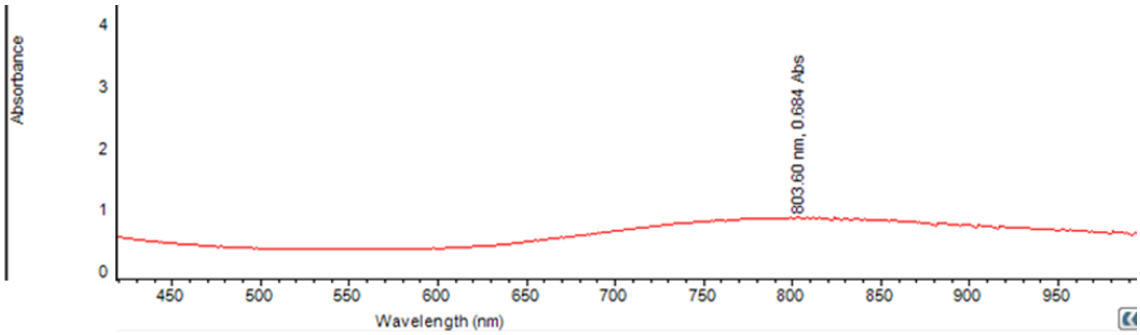
Leaf extract, synthesised nanoparticles, drug conjugated nanoparticle

Fig 4.3 Functionalization of CuNPs

4.3 UV-Vis Spectrophotometer

4.3.1 Copper Nanoparticles synthesised by *Catharanthus roseus*

The graph 4.1 shows the spectra of CuNPs in UV-Vis spectrophotometer conforming the synthesis of anisotropic nanoparticles, and the broadness of the absorption peak reveals the wide distribution of size of NPs.



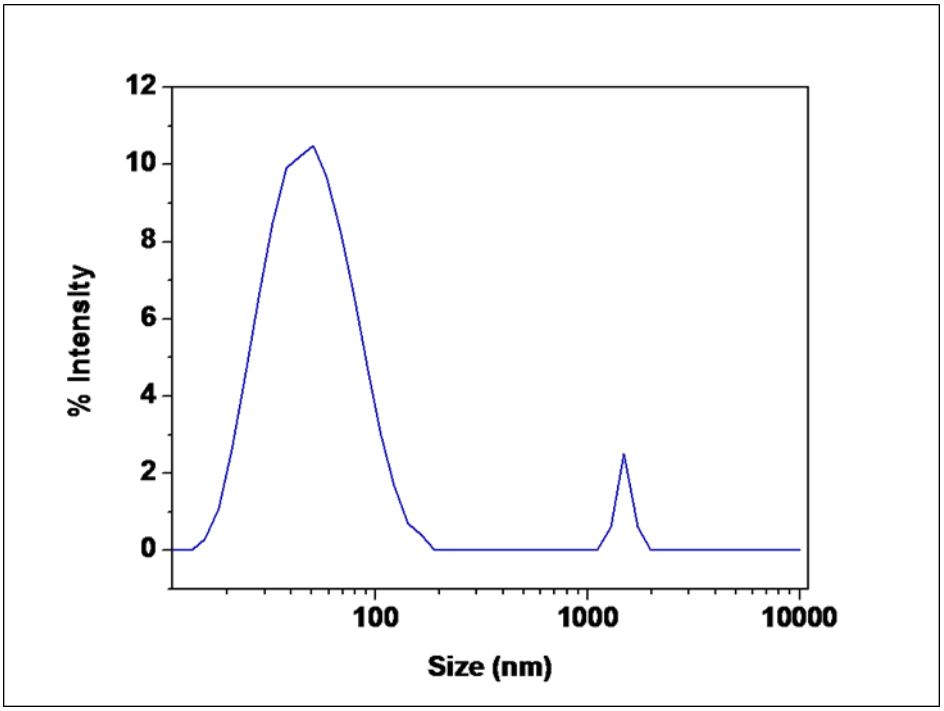
Graph 4.1 UV-Vis Spectrophotometer graph for CuNPs (with KI)

4.4 DLS (Dynamic Light Scattering)

4.4.1 Copper Nanoparticles synthesised by *Catharanthus roseus*

The results of DLS (MALVER Nano ZS) carried out in IIT Mandi showed the hydrodynamic size of the green synthesised Cu nanoparticles which is ~45nm.

Zeta Potential: The charge on the Cu nanoparticles synthesised is +30mV which confirms the stability of the nanoparticle.



Graph 4.2 DLS (Dynamic light scattering) of CuNPs

4.5 TEM (Transmission Electron Microscopy)

4.5.1 Copper Nanoparticles synthesised by *Catharanthus roseus*

The fig 4.4 showing the result of TEM (FEI instrument) of copper nanoparticles carried out in lab of IIT Mandi confirms the morphology and size of the CuNPs. The particles have an irregular shape

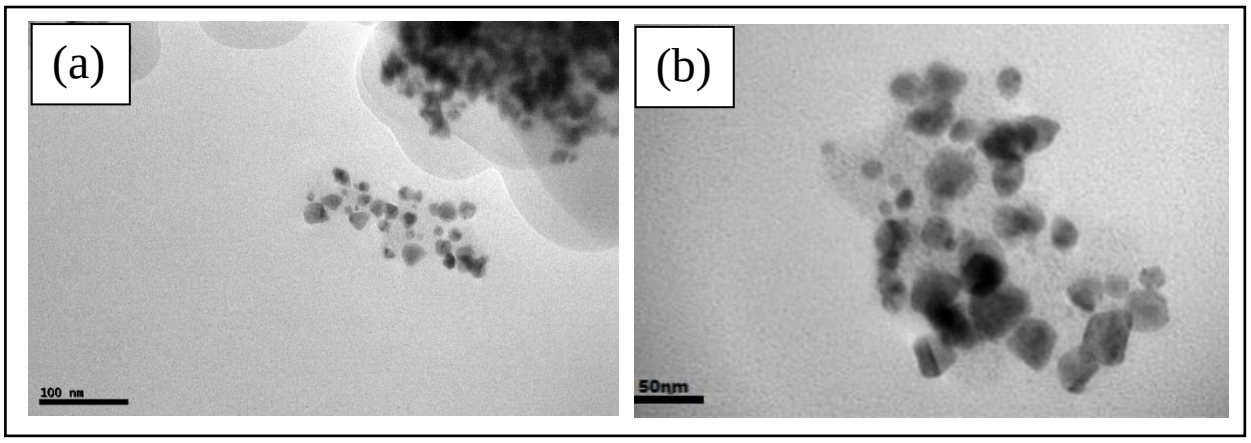


Fig 4.4 TEM micrograph of CuNPs (a) NPs at 100nm (b) NPs at 50nm

4.6 Antibacterial activity of nanoparticles

4.6.1 Disc Diffusion

4.6.1.1 Nanoparticles synthesised by *Catharanthus roseus*

The results of disc diffusion in Fig 4.5 shows the maximum zone of inhibition against E.Coli around the central disc(20mm) which is sample with nanoparticle(Cu) conjugated with drug which shows its anti-microbial activity increases when compared to disc 2 with only nanoparticles which shows the enhanced synergic effect also when compared to disc 3(17mm) containing only drug (ciprofloxacin).

Fig 4.5 Disc Diffusion

SAMPLES	**ZONE OF INHIBITION**
Only drug(ciprofloxacin)	17mm
CuNPs+ ciprofloxacin	20mm

Table 4.1: Diameter of zone of inhibition by CuNPs

4.6.2 Well Diffusion

4.562.1Nanoparticles synthesised by *Catharanthus roseus*

The results of well diffusion in fig 4.6 against E. coli DH5α show the enhanced effect of the copper nanoparticles in synergy with the ciprofloxacin drug. The diameter of the zone of inhibition is greater of the functionalized CuNPs when the diameters of the zone of inhibition of only copper, copper nanoparticles and ciprofloxacin is compared. These results show that the synergy of the ciprofloxacin with CuNPs gives enhanced bactericidal effect

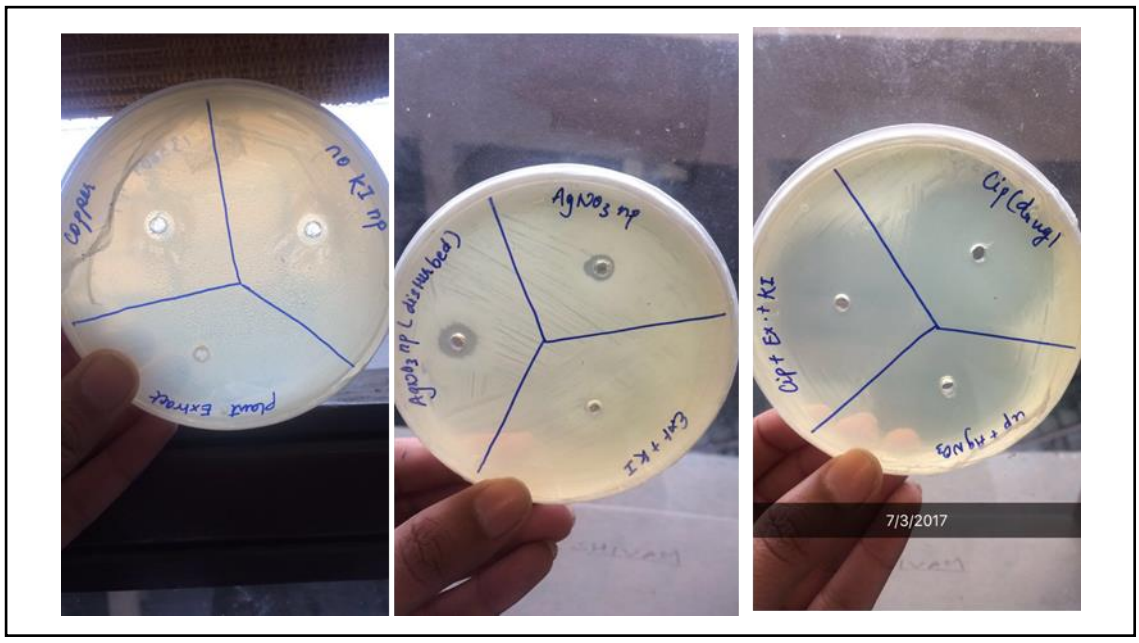


Fig 4.6 Zone of inhibition against E. coli DH5α of$CuSO_4.5H_2O$, CuNPs and CuNPs conjugated with drug

SAMPLES	ZONE OF INHIBITION
Only Copper	7mm
CuNPs (AgNO3)	7mm
Only drug(ciprofloxacin)	36mm
Drug+ np	40mm

Table 4.2: Diameter of zone of inhibition by CuNPs

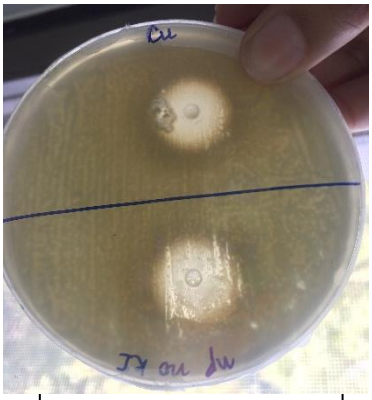
4.7 Antifungal activity of nanoparticles

4.7.1 Well Diffusion

4.7.1.1 Nanoparticles synthesised by *Catharanthus roseus*

Results in fig 4.7 show the antifungal effect of the green synthesised CuNPs against A. flavus. The enhanced fungicidal effect of the CuNPs when compared to the *Catharanthus roseus* plant extract and copper alone, comparing the zone of inhibition of the following. CuNPs showing better effects resulting its use as a better fungicidal.

A. flavus at 24hr



A. flavus at 48hr

SAMPLES	ZONE OF INHIBITION
$CuSO_4.5H_2O$	1.5cm
NP no KI	2cm

A. flavus at 24hr

A. flavus at 24hr

SAMPLES	ZONE OF INHIBITION
$CuSO_4.5H_2O$	2cm
NP $CuSO_4$+KI	2.8cm
Plant extract (*Catharanthus roseus*)	-

Fig 4.7 Antifungal activity of CuNPs against A. flavus

CHAPTER 5

SIGNIFICANCE OF THE PROJECT

SIGNIFICANCE OF THE PROJECT

In the recent year nanoparticles use have increased in all fields biology, medicine etc. due to its excellent applications. Several methods for its synthesis included the use of toxic chemicals consuming high energy hence to overcome this drawback, new biological method emerged as discussed in our project, use of plant extract for the synthesis of nanoparticles and their further potential use in different fields as antimicrobial, drug delivery etc. one of the major advantage of the plant mediated synthesis of nanoparticles is the presence of other active ingredients like antioxidant, flavonoids etc. and in case of our project the presence of the anti-cancerous property of *Catharanthus roseus* with synthesised CuNPs which could be of great use further apart from antimicrobial in drug delivery system. The CuNPs show effectual antimicrobial property due to its large surface area which provides a better interaction with the microorganisms and further antimicrobial property can be increased by decreasing the particle size. The 45nm CuNPs demonstrated the antimicrobial effects against *E. coli* (ATCC 25922 and *E. coli* DH5α) and *A. flavus* in our project. Further the use of functionalized CuNPs with ciprofloxacin can be used to combat the multi drug resistance in microorganisms.

Through our project, we aim to increase awareness for the biosynthesis of the nanoparticles which is one of the cost effective and environmental friendly method unlike other processes which use toxic chemicals. These nanoparticles can be used as multimodal agents by exploiting their properties in various applications. By exploring the antimicrobial activity of these nanoparticles there can be huge development in the area of medicine by using them commercially into ointments and medicines.

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