EVALUATION OF ANTIBACTERIAL ACTIVITY OF ZINC OXIDE NANOPARTICLES SYNTHESIZED VIA GREEN ROUTE AGAINST *BACILLUS SUBTILUS*

Project Report submitted in partial fulfillment of the requirement for

The degree of

Bachelor of technology

Biotechnology

BY

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Under the supervision of

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To

DEPARTMENT OF BIOTECHNOLOGY AND BIOINFORMATICS

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CERTIFICATE

I hereby certify that the work presented in this report entitled "**Evaluation of Antibacterial Efficacy of Zinc Oxide Nanoparticles synthesized via Green route against *Bacillus Subtillus*"** in partial fulfillment of the requirement for the award of the degree of bachelor of technology in Biotechnology submitted in the department of Biotechnology and Bioinformatics, Jaypee University of Information and Technology Waknaghat is an authentic record of my own work carried out over a period from august 2017 to may 2018 under the supervision of Dr. Gopal Singh Bisht (Associate professor)

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

Signature of Candidate
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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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Dated:

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OBJECTIVE

The objectives of this project were

1. Green synthesis of zinc oxide metallic nanoparticles from *terminalia chebula* plant extract by bio-reduction method.
2. Characterization using various analytical techniques (UV-Vis, FTIR, PI and XRD spectroscopy).
3. Evaluation of antibacterial efficacy against *Bacillus subtilis* (antimicrobial susceptibility tests AST and disc diffusion).

ABSTRACT

As the field of nanotechnology emerges, there is a lag in research surrounding the topic of green synthesis. It ascribe to the use of biological routes such as those involving microorganisms, plants etc. for the synthesis of nanoparticles. Through which it has assisted in the development of new and largely unexplored area of research based on the biosynthesis of nanomaterials. This report highlights the synthesis of metallic nanoparticles using plant extract concurring with the principle of green approach. Bio-synthesis of nanoparticles makes use of environmental friendly, non-toxic and safe reagents, where conventional synthesis of nanoparticles requires costly energy consumption and use of harsh chemicals The current work is dedicated to synthesize zinc oxide nanoparticles from plant extract of *terminalia chebula* which can be used as an alternative to chemical methods and to examine their antibacterial efficacy against gram positive bacteria. Various techniques have been developed to produce ZnO nanoparticles such as sol-gel method, direct precipitation method, combustion synthesis method. However, those methods should be performed under strict conditions and it is not favorable for low cost production. Among these methods, Bio-reduction method is considered as simple, fast and cost effective because it requires less time to produce metal oxide powders and doesn’t involve the use of catalyst. The Characterization for these nanopaticles were done through UV-VIS, FTIR, XRD, DLS, and PL. Results of antimicrobial susceptibility test indicating the antimicrobial potential of zinc oxide nanoparticles against gram positive bacteria.

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LIST OF ACRONYMS

NPs	Nanoparticles
ZnO	Zinc Oxide
ZnO NPs	Zinc oxide Nanoparticle
TC	*Terminalia chebula*
BS	*Bacillus Subtilus*
$Zn(NO_3)_2.H_2O$	Zinc nitrate hexahydrate
NaOH	Sodium hydroxide
mm	Millimeter
nm	Nanometer
cm	Centimeter
DLS	Dynamic light scattering
AST	Anti microbial susceptibility Test
JUIT	Jaypee University Of Information And Technology

CHAPTER 1

INTRODUCTION

INTRODUCTION

1.1 Nanoparticles

To study matters at nanoscale level, nanotechnology is an arising branch. Ultimately, the aim is to handle matters at the atomic level to help humanity. One emerging discipline in nanotechnology is biomedical applications [1]. Nanotechnology has been applied in fields of medicine and surgery. A lot of research has been done in these fields due to which a huge bulk of research articles has been generated so far and it is increasing day by day. Different aspects of nanotechnology that have been touched by development in this field are advanced drug delivery systems, molecular imaging and diagnostics. Areas of interest include nerve regeneration, wound healing and surgeries. Nanotechnology is also widely utilized in plastic surgeries [2].

Nanoparticles combined with other therapeutic agents has enhanced therapeutic efficacy of treatment strategies. Nanotechnology-based drugs have also been utilized to target drugs towards specific diseases. Food and Drug Administration has approved Poly-lactic-co-glycolic acid to be used as biocompatible and biodegradable polymer in production of nanotechnology based drug therapeutics. That is why, a huge number of special drug delivery systems and devices have been developed so far [3].

Various nanobiotechnological tools have been developed so far for non-cancer applications [4]. The introduction of nanotechnology had a revolutionary effect on many aspects of 21st century life. Nanotechnology has provided a chance to discover new possibilities as conventional technologies are unable to make an impact in cancer therapy. In particular, nanotechnology entities can be used to deliver conventional therapeutics having poor solubility and short half-lives [5].

The bacteria continue to grow adaptive counter measures against current antibiotics, due to which problem of antibiotic resistance is increasing day by day. In latest years, researchers have shown nanoparticles as a remarkable substitute to antibacterial reagents due to their inherited antibacterial activity in few biomedical applications, which includes tissue engineering, imaging, drug and gene delivery. Furthermore, nanomaterial research predicts a possible relationship between the magnitude of its delivered toxicity and morphological characteristics of nanomaterial [6].

1.2 Metals

Metals

Metals are vital components that are required by human beings. Metals like iron, copper, manganese and zinc are involved in various physiological processes in human body [7]. Metal oxides are exponentially being used in wide range of applications and are the largest class of commercially produced nanomaterials. This presents unparalleled human exposure. Thus, understanding nanoparticle induced cellular stress can surely help in designing strategies to combat them. Various studies have been carried out to understand the effects of metallic oxide nanoparticle exposure on human's alveolar cells, which are highly susceptible to aerosolized matter. There is a huge dismissal in the information generated and comprehensive cluster of this information is also lacking [8]. Metal oxides are the most frequently utilized nanoparticles [9].

Mainly, metal oxide nanoparticles are getting more attention because of their distinctive physicochemical properties and comparatively cheap production costs. Extensive production is a straight forward and remunerative pathway to minimize this issue [10]. Due to various uses nanoparticles are being produced at a high rate to meet demand of nanotechnology industry. But not much has been done on evaluation of their toxic potential [11].

Metallic nanoparticles are widely used as antifungals and antibacterials in dermatological products and cosmetics [12]. The study and synthesis of metallic nanoparticles are of consistent and important interest, with its uses in materials science, catalysis, and medicine. The characteristics of metallic nanoparticles depend mainly on their shape, particle size, and interparticulate distances. It is, therefore, necessary for the synthesis of metallic nanoparticles to be controlled for particular sizes and shapes. The fast growth in this research field has fascinated acute interest from researchers with divergent expertise, and various procedures towards the synthesis of monodisperse nanoparticles have been noted [13].

1.4 Green synthesis

Green synthesis deals with production of nanoparticles by natural reducing agents like plants, enzymes and proteins. These reducing agents influence the morphology of nanoparticles. Green synthesis is non-toxic to environment as no harmful chemicals are required [14]. Conventional methods of nanoparticle production are now being replaced by green synthesis methods due to their non toxic nature [15]. The green fabrication of nanoparticles is energy efficient, nontoxic, secured and simpler pathway [16].

Nanoparticles assist in many industrial and domestic purposes which is shown by their rapidly growing production volume. This fruitful victory comes along with their involvement in the environment and the probability of potentially harmful effects in natural systems. Over many years, substantial growth relating to the understanding of fate, sources, and effects of nanoparticles has been built. Prognosis of environmental concentrations depends on modeling approaches; could lately be committed by sustained concentrations in the field. Accordingly, the consequences of nanoparticles on terrestrial and aquatic systems have got growing attention. Whereas the argument on the importance of nanoparticle-released metal ions for their toxicity yet exists, it is a repeated phenomenon that inert nanoparticles can interact with biota by physical pathways like biological surface coating. This among others obstructs with the development and behaviour of exposed organisms. Furthermore, co-occurring contaminants react with nanoparticles. There is multiple proof proposing nanoparticles as a sink for organic and inorganic co-contaminants. Secondly, in the presence of nanoparticles, repeatedly an increased impact on the test species induced by the co-contaminants has been noted [17].

Table 1.1: Various method to NPs synthesis

Physical Method	**Chemical method**	**Biological Method**
Mechanical method	Co-precipitation method	Synthesis using plant extracts
Vapour deposition	Sol-gel method	Synthesis using enzymes
Sputter deposition	Microemulsions	Synthesis using agriculture waste
Electric arc deposition	Hydrothermal synthesis	
Ion beam technique	Sonochemical synthesis	
Molecular beam epitaxy	Microwave synthesis	

Table 1.2: Various nanoparticles that have been synthesized via bioreduction

S.No	**Nanoparticle**	**Reducing agent**	**Reference**
1.	Silver	*Phoenix dactylifera*	[18]
2.	Silver	*Acalypha hispida*	[19]
3.	Copper oxide	*Vaccinium arctostaphylos L*	[20]
4.	Nickel oxide	*Monsonia burkeana*	[21]
5.	Gold	*Ziziphus zizyphus*	[22]
6.	Gold	*Punica granatum*	[23]
7.	Silver	*Psidium guajava L*	[24]

Fig* 1.4 *Terminalia chebula plant

CHAPTER TWO

REVIEW OF LITERATURE

Review of the literature

Khan et al 2014: They synthesized zinc oxide nanoparticles by sol gel technique and studied the antibacterial potential of zinc oxide nanoparticles in biofilm producing bacteria. Synthesized nanoparticles had smooth surface and size less than 36 nm. Their antibiofilm production effect was studied in dentures. The synthesized nanoparticles inhibited formation of biofilms on these surfaces [25].

Dwivedi et al: They evaluated the antibacterial effect of zinc oxide nanoparticles against *P. aeruginosa.* The method selected for studying antibacterial potential was disk diffusion method. The suggested mechanism of action of zinc oxide nanoparticles behind antibacterial effect was formation of reactive oxygen species. This supports the fact that zinc oxide nanoparticles act by formation of reactive oxygen species against bacteria [26].

Seil and Webster 2011: They incorporated zinc oxide nanoparticles into polymer. Incorporation of metallic nanoparticles into poly vinyl chloride was done using sonication technique. These conjugates were further evaluated for their antibiofilm formation activity against *S. aureus* (ATCC 25923). The nanoparticle polymer complex inhibited about 50 % growth of biofilm producing bacterium [27].

Lee et al 2014: They also studied the antibacterial potential of zinc oxide nanoparticles against biofilm producing bacteria. Their results showed that zinc oxide nanoparticles have higher potential then zinc salt alone in reduction of biofilms [28].

Vijaykumar S et al: They synthesized zinc oxide nanoparticles via *Glycosmis pentaphylla* plant's leaves. Nanoparticles were found to have crystalline nature. Synthesized zinc oxide nanoparticle were pure and had average size less than 36 nm [29].

Rehana D et al 2017: They synthesized zinc oxide nanoparticles using various plants like *Moringa oleifera, Tamarindus indica, Murraya koenigii, Azadirachta indica* and *Hibiscus rosa-sinensis.* Chemically synthesized zinc oxide nanoparticles were also compared with nanoparticles synthesized by green method. Nanoparticles synthesized using *T.indica* had significant antioxidant and antidiabetic potential [30].

Azizi S et al 2017: They synthesized zinc oxide nanoparticles using microwave. In vitro cytotoxicity studies were also performed in 3T3 cells. Results showed that the technique utilized for green synthesis was easy and effective approach for production of zinc oxide nanoparticles [31].

Hashemi S et al 2016: They utilized leaves of olive for production of zinc oxide nanoparticles from zinc nitrate salt. Nanoparticles formed were found to be stable. They suggested that various plant metabolites like phenols, proteins and flavonoids play vital roles in providing stability to zinc oxide particles [32].

Moghaddam AB et al 2017: They carried out the antibacterial activity and cytotoxicity study of zinc oxide nanoparticles. The toxic potential of zinc oxide nanoparticles was evaluated in vero cells. ZnO particles were found to be non-toxic below 190 µg/ml concentrations and gave good antibacterial results against bacteria [33].

CHAPTER THREE

MATERIAL AND METHODOLOGY

3 MATERIAL AND METHODS

3.1 Synthesis of ZnO

ZnO nanoparticles were synthesized using leaf extract of plant *terminalia chebula* extract. It was prepared by boiling the leaf at elevated temperature on the magnetic stirrer for around 1 hour. It was filtered and centrifuged. The supernatant 30 ml was added to 10 ml 0.1M zinc hexahydrate in dropwise manner till white precipitate was observed. The pH (12) was maintained by NaOH.

3.2 Materials used

- plant extract
- zinc hexahydrate
- methanol
- distilled water
- sodium hydroxide

3.3 Methodology

Preparation of aqueous leaf extract of *Terminilia Chebula*

- 20gm of *Terminalia Chebula* leaves are collected and washed with Tap water and with distilled water (two times).
- Cut them into small pieces.
- 200 ml of distilled water added.
- Boil until the dark red color is obtained.
- Filtered (blotting sheet).
- Centrifuge (7000 RPM 10 min).
- Supernatant taken (plant extract).
- Stored at 4°C refrigerator.

***Fig* 3.1: Plant extract preparation**

preparation of plant extract

↓

dropwise addition of zinc nitrate solution

↓

stirred till reduction

***Table* 3.1: Preparation of ZnO nanoparticles**

***Fig* 3.2: Addition of extract to salt solution.**

- 10 ml of 0.1 M solution of zinc nitrate added to flask.
- Plant extract added (30 ml).
- Stir and heat at 60°C.
- Color change was observed.
- Formation of ZnO nanoparticles is depicted by appearance of white or creamish ppts.
- Stir for 2 hours.
- Taken the extract for centrifugation for 7000 RPM for 10 mins.
- Washed with distilled water two times and with methanol.
- Whitish precipitates where formed on the bottom walls of the tubes.
- Pellet dried in oven at 60°C overnight.
- Crushed using mortar pestle and sent for characterization.

Characterization:

Characterisation of nanoparticles was done using different analytical techniques like

- FTIR spectroscopy
- X-ray diffraction
- Photo luminescence (PL) analysis.

Antibacterial activity

Antibacterial activity was performed by disk diffusion method on Luria Broth agar plates. Initial inoculum (equivalent to 0.5 Mc farland's standard; *Bacillus subtilis*) was swabbed on plates. Disks were punched and different dilutions of zinc oxide nanoparticles were added into wells. Plates were kept in incubator at 37 degree Celsius. Results were interpreted after 24 hours [34].

CHAPTER 4

RESULTS

RESULTS AND DISCUSSION

4.1 FTIR spectra:

4.1a. FTIR Spectrum of plant extract:

FTIR spectrum was recorded and later on plotted using origin software. FTIR spectra of plant extract show broad peak around 3400cm^{-1} due to OH present in plant and peak at 1640cm^{-1} due to amide bond stretching
vibrations.

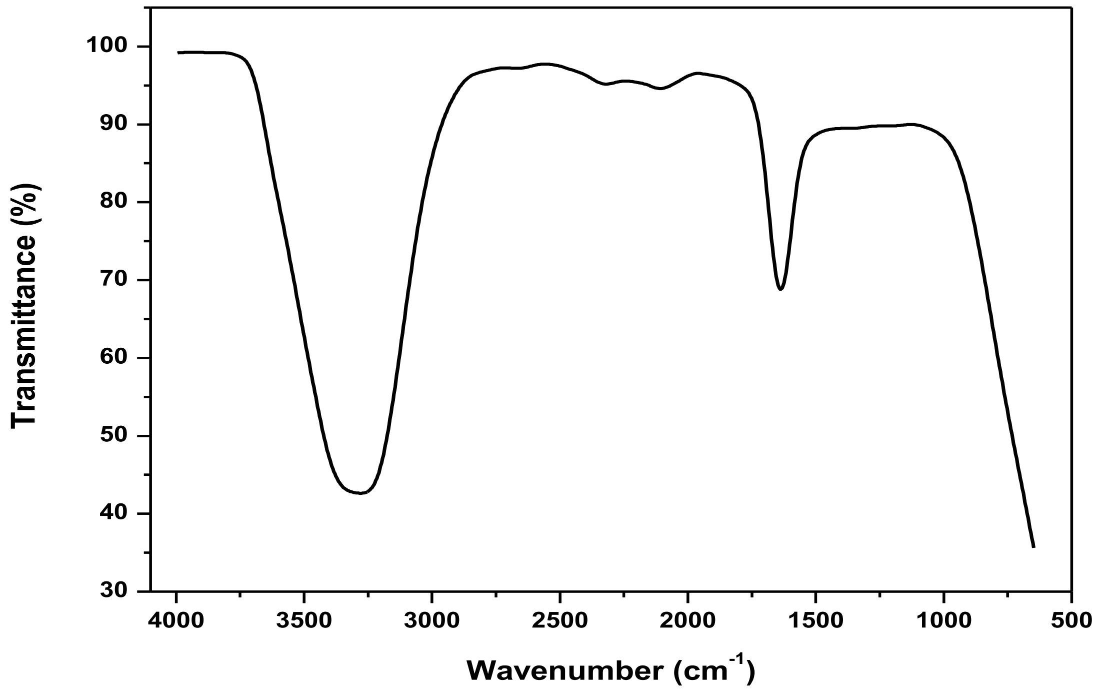


***Fig* 4.1a: FTIR spectrum of plant extract**

4.1b: FTIR Spectrum of synthesized ZnO powder

FTIR spectrum was recorded and later on plotted using origin software. FTIR spectra of ZnO powder show peak below 1000cm^{-1} due to formation of metallic nanoparticles. Results are in accordance with study by [35].

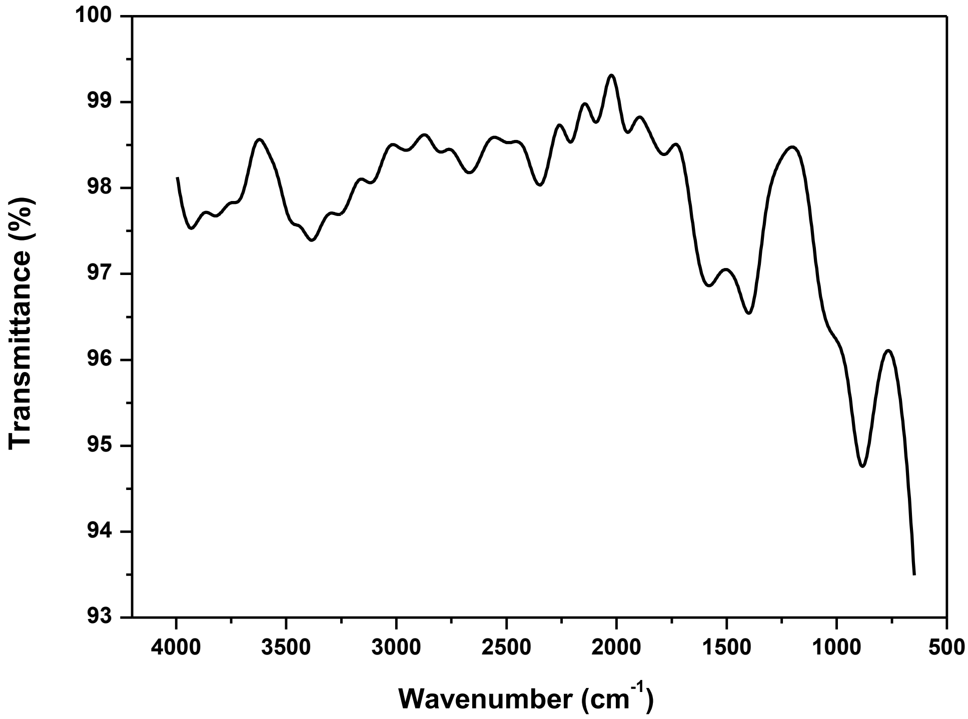


Fig 4.1b: FTIR spectrum of zinc oxide

4.2 XRD analysis

4.2a Purity

Purity of zinc oxide was confirmed from XRD spectrum. Zinc oxide gives three characteristic [**36**], these peaks were also observed in our sample suggesting the pure nature of compound.

4.2b Size of crystallites

XRD analysis results were further used to calculate crystallite size using Scherer equation. The average size of crystallites was found to be around 16.05nm.

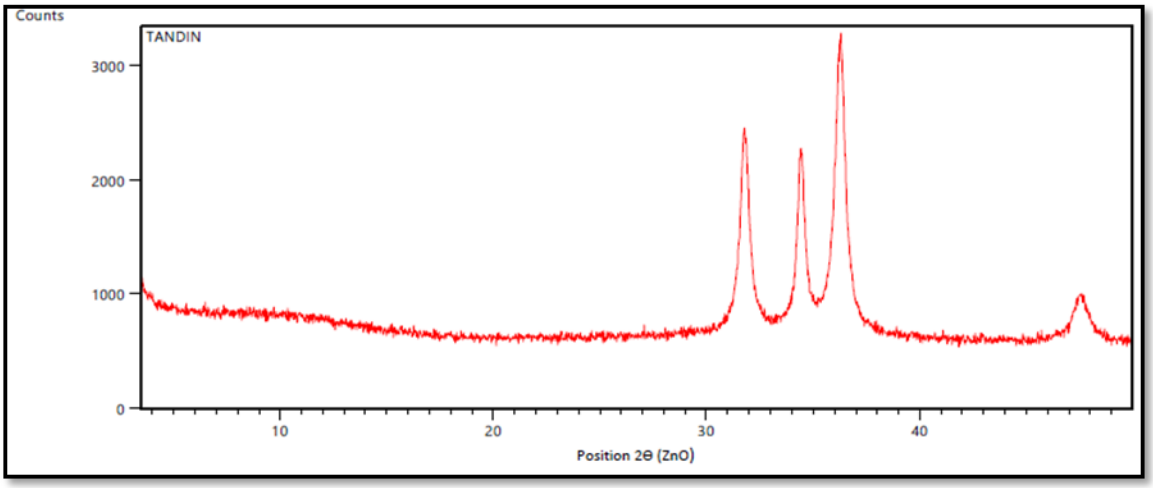
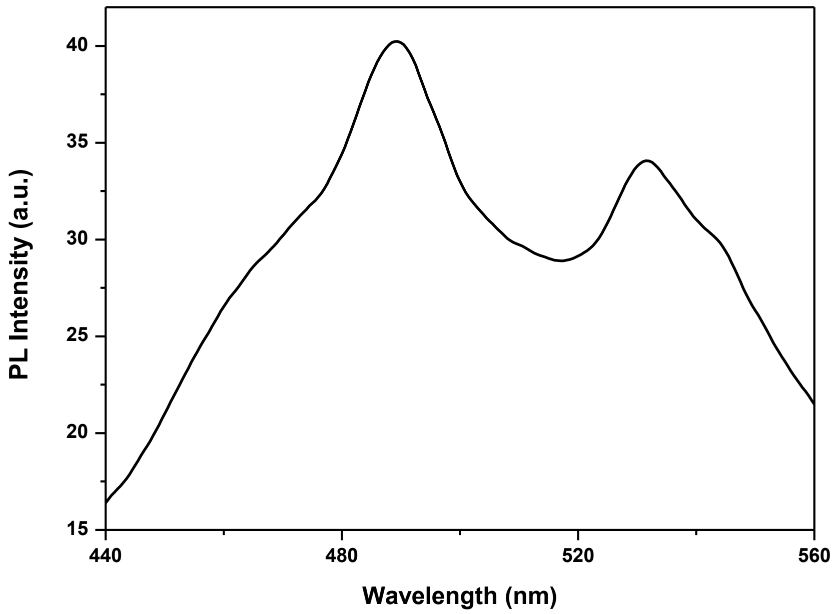


Fig 4.2: XRD of ZnONPs

4.3 Photoluminescence spectrum analysis:

Photoluminiscence spectrum was recorded and later plotted using origin software. Photoluminescence spectrum analysis gave emission peak at 489.38, very close to emission peak reported by PB Taunk et al 2015 [37]. This suggests particles have photoluminiscent property.

Figure 4.3 Photoluminescence spectra



4.5 Antimicrobial susceptibility test on *bacillus subtilis*

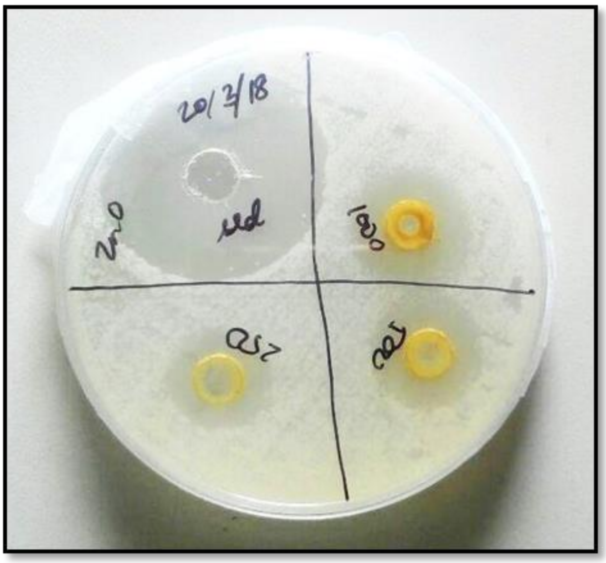


***Fig* 4.4: Antibacterial activity against *bacillus subtilis* and ZnO NPs conjugated with ampicillin.**

***Table* 4.4: diameter of zone of inhibitory by ZnO NPs**

Samples	**Zone of inhibition**
Only zinc	9mm
Zn ONPs	9mm
Antibiotic (Ampicillin)	39mm
Drug up (antibiotic)	43mm

CHAPTER 5

CONCLUSION

Conclusion: Results of our study show that zinc oxide nanoparticles can be easily synthesized via green synthesis route. FTIR results show that the plant metabolites play a vital role in reduction of zinc salt to zinc oxide. XRD analysis result suggests that synthesized nanoparticles were crystalline in nature and possessed average crystallite size of 16nm. Further PL spectrum suggests that these nanoparticles have photoluminiscent property and results of antimicrobial study support the fact that these nanoparticles have the potential to inhibit the gram positive bacteria.

CHAPTER 6

SIGNIFICANCE OF THE PROJECT

Significance of project

Various nanoparticles like gold, silver, copper, iron and cobalt have been synthesized by green synthesis. But not much work has been done on green synthesis of zinc oxide nanoparticles. Therefore in this report we have synthesized zinc oxide nanoparticles via *Terminalia chebula* leaf extract. This report is beneficial to researchers who work on green synthesis of nanoparticles and wish to explore zinc oxide nanoparticles to control antibacterial infections due to gram positive bacteria in future.

CHAPTER 7

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