Antimicrobial activity of titanium dioxide, zinc oxide and copper oxide nanoparticles

A Dissertation Report for

Course code and course Title: MBO 381 Dissertation

Credits: 08

submitted in partial fulfilment of Master's Degree

M.Sc. in Marine Biotechnology

by

KRUPA VARAPRASAD

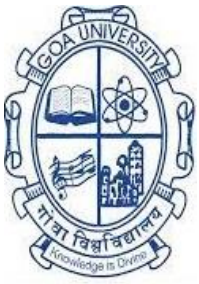
(21P050016)

Under the Supervision of

DR. DHERMENDRA K TIWARI

School of Biological Sciences and Biotechnology

Biotechnology Discipline



Goa University

April 2023

DECLARATION BY STUDENT

I hereby declare that the data presented in this Dissertation report entitled, "**Antimicrobial activity of titanium dioxide, zinc oxide and copper oxide nanoparticles**" is based on the results of investigations carried out by me in the M.Sc. Marine biotechnology at the school of biological sciences and biotechnology, Goa University under the Supervision/Mentorship of Dr. Dhermendra K Tiwari and the same has not been submitted elsewhere for the award of a degree or diploma by me. Further, I understand that Goa University or its authorities will be not be responsible for the correctness of observations / experimental or other findings given the dissertation.

I hereby authorize the University authorities to upload this dissertation on the dissertation repository or anywhere else as the UGC regulations demand and make it available to any one as needed.

O. Krupa Varaprasad
21P050016
M.Sc. Marine biotechnology
School of biological sciences and biotechnology

Date:
Place: Goa University

Examined by:

Seal of the Sch

COMPLETION CERTIFICATE

This is to certify that the dissertation **"Antimicrobial activity of titanium oxide, zinc oxide and copper oxide nanoparticles"** is a bonafide work carried out by **Mr. Krupa Varaprasad** under my supervision/mentorship in partial fulfilment of the requirements for the award of the degree of **M.Sc.** in the Discipline **Marine biotechnology** at the **school of biological sciences and biotechnology**, Goa University.

Dr. Dhermendra K Tiwari
UGC- Assistant professor
School of Biological sciences and Biotechnology

Date: 12/5/23

Dr. Savita S. Kerkar
Discipline of Biotechnology
School of Biological Sciences and Biotechnology

Dean of School of Biological Sciences & Biotechnology
Goa University, Goa 403206
Office Ph. 8669609246

Date:
Place: Goa University

SBSB: Biotechnology Taleigao Plateau, Goa 403205 GOA UNIVERSITY
School Stamp

ACKNOWLEDGEMENT

I express my deep sense of gratitude and heartful appreciation to dissertation guide Dr. Dhermendra K Tiwari, UGC-Assistant professor, school of biological sciences and biotechnology for his timely guidance, wholehearted cooperation, motivation, encouragement, constructive suggestion and variable advice to improve as a person. Thank you, Sir, for pushing me to think in a scientific direction, for improving my reasoning skills and for your time and experience that shaped this dissertation.

I would also thank to the DBT New Delhi, Dean of the School of Biological Sciences, HOD, faculty members and non-teaching staff members of our school of biological sciences and biotechnology for providing me with the facilities to conduct my work, their suggestions and support throughout the academic year. I wholeheartedly would like to thank our laboratory assistant Ms. Sandhya and Ms. Jaya, laboratory attendants Mr. Serrao, Mr. Ashish and Mr. Sameer for providing the necessary requirements, helping to manage work and most importantly for being supportive and encouraging. I am also thankful to LDC Sanjana and popkar for the smooth processing of the academic documents.

I am also grateful to Dr. Lakshangy charya, Department of Microbiology, school of biological sciences and biotechnology for allowing me to use probe sonicator and I owe a special thanks to the research scholar Preeti gawas.

A Big thanks to all my friends and classmates for always being helpful, supportive and for creating healthy, fun filled memorable environment.

Last but not the least, a special thanks to my parents for always being there behind the scenes, for their guidance, blessings, support and providing need and for being the bridge to reach my goals.

Thank you all

INDEX

LIST OF FIGURES

Figure 15	MTT assay of *Bacillus* on treatment with various concentrations of TiO_2, ZnO and CuO NPs
Figure 16	Extent of lipid peroxidation measured by TBARS on treatment with various concentrations of TiO_2, ZnO and CuO NPs against *Bacillus*
Figure 17	Extent of lipid peroxidation measured by TBARS on treatment with various concentrations of TiO_2, ZnO and CuO NPs against *E. coli*

LIST OF TABLES

ABSTRACT

In this work we investigated the antimicrobial activity of metal oxide nanoparticles, Titanium dioxide (TiO_2), zinc oxide (ZnO), and copper oxide (CuO). Nanoparticles are known to exhibit unique physical and chemical properties that can enhance their antimicrobial properties, making them promising candidates for applications in various fields, including medicine, agriculture, and food industry. The antimicrobial activity of the nanoparticles was evaluated against pathogenic gram-positive and gram-negative bacteria using standard microbiological assays.

We investigated antimicrobial effect against *E. coli* (gram positive) and *Bacillus* (gram negative*)*. TiO_2, ZnO and CuO nanoparticles showed greater antimicrobial effect at 400 µg/ml concentration against both *E. coli* and *Bacillus*. MIC results showed that TiO_2 and CuO NPs showed 45% decrease in the growth of *Bacillus* when compared to untreated sample, whereas ZnO NP showed only 25% decrease at 400µg/ml concentration. and against *E. coli* all three nanoparticles showed similar kind of effect. CFU/ml results showed that, at 200 µg/ml concentration TiO_2 NP showed 60% decrease in the growth of *E. coli* when compared to untreated sample where as ZnO and CuO NPs showed 30% and 50 % decline. At 400µg/ml concentration, ZnO showed 97% decline in the growth of *Bacillus* when compared to untreated sample, followed by CuO which showed 95% decline in the growth of *E. coli*. Least effect was shown by TiO_2 NPs where only 45% decline was seen. Growth curve characteristic gave the results, at 200 µg/ml concentration TiO_2 and CuO NPs showed 15% and 13% decline in the growth of *E. coli*. The study also investigated the mechanism of action of the nanoparticles by examining their effects on the cell membranes and intracellular components of the microorganisms. MTT and TBARS assay was performed. MTT results showed that at 400 µg/ml concentration, the number of viable cells present in the sample was low. TBARS assay showed that, with the increase in the concentration of NPs the lipid peroxidation activity also increased. CuO and ZnO NPs showed greater lipid peroxidation activity against both the bacterial strains *E. coli* and *Bacillus* at higher concentration(400µg/ml) of nanoparticles. Overall, the findings of this study suggest that TiO_2, ZnO, and CuO nanoparticles have promising antimicrobial properties that can be exploited for various applications.

Keywords: Nanoparticles, Titanium dioxide nanoparticles, zinc oxide nanoparticles, Copper oxide nanoparticles, Antibacterial Nanoparticles MIC, MTT assay, TBARS assay

CHAPTER 1

INTRODUCTION

INTRODUCTION

Antimicrobial resistance has become a major global health problem, and infections caused by multidrug-resistant microorganisms are increasing. In recent years, nanotechnology has become a promising tool in the fight against microbial infections. Bacterial resistance to antibiotics and especially the spread of ESCAPE pathogens is a concern. The abbreviation ESCAPE stands for Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa, and Enterobacter spp. These ESCAPE pathogens can easily acquire antibiotic resistance through horizontal gene transfer. (Vaishampayan & Grohmann, 2021)

Nanoparticles are very small materials with unique physical and chemical properties that allow them to interact with microorganisms in new ways. One of the most promising applications of nanoparticles is their antimicrobial activity, which has been demonstrated against a wide range of microorganisms, including bacteria, viruses, and fungi. The use of nanoparticles as antimicrobial agents could revolutionize the field of treatment and prevention of infectious diseases. One such possibility is the use of nanotechnology to develop antimicrobial agents. These properties make nanoparticles potential candidates for the development of new antimicrobial agents. The application of nanotechnology in medicine has opened up a new field of research, and the antimicrobial effect of nanoparticles is one of the most promising fields of research. The use of nanoparticles as antimicrobial agents has several advantages over traditional antibiotics. For example, nanoparticles have a broad spectrum of action against various microorganisms and can also penetrate biofilms that are resistant to conventional antibiotics.

In particular, NPs have shown broad-spectrum antibacterial properties against both Gram-positive and Gram-negative bacteria. (Wang et al., 2017). Despite the potential benefits of using nanoparticles as antimicrobial agents, several challenges remain to be addressed. For example, the synthesis of nanoparticles requires specialized equipment and expertise, and the toxicity of nanoparticles is not known. Therefore, further research is needed to fully understand the antimicrobial activity of nanoparticles and to develop safe and effective nanoparticles for clinical use.

NANOPARTICLES AND THEIR SYNTHESIS:

Nanoparticles can be synthesized by 2 different methods. One is the bottom-up approach and the other is the top-down method. The bottom-up method has many subdivisions like green synthesis, spinning, sol-process & sol-gel process, laser pyrolysis, aerosol-based process, chemical vapor deposition (CVD), and atomic or molecular condensation. The top-down approach also has subdivisions like mechanical milling, etching(chemical), electro-explosion (thermal or chemical), sputtering (kinetic), and laser ablation (thermal). The green synthesis method of nanoparticles is also referred to as a biological method in which organisms like Bacteria, Actinomycetes, Yeast, Fungi, Algae, and Plants are used to synthesize different nanoparticles. Some important methods for the synthesis of Gold, silver, copper, and zinc nanoparticles are mentioned in the table below.

NANOPARTICLE TYPE	**SYNTHESIS**
GOLD	Chemical reduction of salts; Ultraviolet irradiation; Photochemical reduction of Au; Aerosol technology Biological synthesis.
SILVER	Electrochemical techniques; gamma irradiation; Photochemical reduction; Laser ablation; Electron irradiation; Biological synthesis.
ZINC OXIDE	Sol-gel process; Vapour phase oxidation; Sono-chemical reduction; Microemulsion; Thermal vapor transport and condensation; Biological synthesis.
COPPER	Thermal reduction; Metal vapour synthesis; Radiation methods; Microemulsion techniques; Laser ablation; Chemical reduction; Biological synthesis.

Various chemical, physical and biological synthesis methods have been used to prepare metal nanoparticles. Most of these methods are still under development, and issues include nanoparticle stability and aggregation, control of crystal growth, morphology, size, and size distribution. In addition, the isolation of the produced nanoparticles for further use is still an important issue. Metal nanoparticles produced by plants have been shown to be more stable than nanoparticles produced by other organisms. Plants (especially plant extracts) can reduce metal ions faster than fungi or bacteria. In addition, plant extracts are definitely better than plant biomass or living plants to use a simple and safe green method for large-scale and industrial production of well-dispersed metal nanoparticles (Iravani, "Green Synthesis of Metal Nanoparticles Using Plants.")

OXIDATIVE STRESS IN BACTERIA:

Oxidative stress occurs when prooxidants outnumber antioxidants, i.e., ROS accumulate in the bacterial cell and exceed the cell's ability to readily scavenge ROS. This can occur during the host's immune response or due to antimicrobial therapy. Oxidative stress can be endogenous or exogenous. Host-pathogen interaction causes exogenous oxidative stress in bacteria, while intracellular redox reactions, antibiotics, and aerobic respiration promote endogenous oxidative stress. Oxygen is the final electron acceptor of aerobic respiration and leads to the formation of water after complete reduction. However, when oxygen comes into contact with flavoproteins such as oxidases and monooxygenases and becomes incompletely reduced, it causes the generation of ROS instead of water. Endogenous ROS such as superoxide anion (O^{2-}) and hydrogen peroxide (H_2O_2) are produced as by-products of aerobic respiration when oxygen interacts with the reduced FAD cofactor of flavoenzymes. ROS cause a number of damages to bacterial cells. They cause double-strand breaks in DNA oxidizing pools of dCTP and dGTP, which causes misfolding of DNA bases. In addition, ROS peroxidise lipids and carbonyl proteins (Vaishampayan & Grohmann, 2022)

Different types of NPs produce different ROS compounds by reducing oxygen molecules. The four types of ROS are superoxide radical (O^{-2}), hydroxyl radical (·OH), hydrogen peroxide (H_2O_2) and singlet oxygen (O^2), which have different dynamics and activities (Wang et al., 2017).

DISSOLVED METAL IONS:

Metal ions are slowly released from the metal oxide and absorbed through the cell membrane, followed by direct interaction with functional groups of proteins and nucleic acids such as mercaptone (-SH), amine (-NH) and carboxyl (-COOH). However, the effect of metal ions on the pH value of lipid vesicles during the antibacterial process of the metal oxide suspension is small and has a weak antimicrobial effect (Wang et al., 2017).

MECHANISM OF ACTION:

The mechanism of antimicrobial activity of NPs is generally described as one of three models: induction of oxidative stress, release of metal ions or non-oxidative mechanisms. These three types of mechanisms can occur simultaneously. According to existing studies, the main processes underlying the antibacterial activity of NPs are: 1) disruption of the bacterial cell membrane 2) ROS generation 3) penetration of the bacterial cell membrane (Wang et al., 2017).

TIO_2 NPs: The mechanism of TiO_2 NP toxicity to organisms can be described by three aspects:

1. ROS produced by TiO_2 NPs as a result of induction of electron-hole pairs in light.
2. Cell wall damage and cell wall lipid peroxidation due to NP-cell attachment by electrostatic force due to the large surface area of TiO_2 NPs.
3. Cytoplasm leaks out and TiO_2 NP adheres to intracellular organelles and biological macromolecules after damaging cell membranes. (Hou et al., 2019)

CUO NPs:

The toxic mechanisms of CuO NPs are mainly in two aspects:

1. Oxidative stress induced by intracellular CuO NPs and dissolution of CuO NPs.
2. Extracellular Cu^{2+} cross the cell membrane and enter the cytoplasm via endocytosis and copper transport proteins. (Hou et al., 2017)

ZNO NPs:

Direct contact of ZnO-NPs with cell walls resulting in destruction of bacterial cell integrity, release of antimicrobial ions, mainly Zn^{2+} ions, and generation of ROS. However, the mechanism of toxicity is different in different environments because the dissolved Zn species can change depending on the components of the environment in addition to the physicochemical properties of ZnO-NP.

UV Illumination Effect of ZNO NPs:

ZnO can absorb UV light very well and is more responsive to UV light, so its conductivity is dramatically improved, and this property greatly activates the interaction of ZnO with bacteria. Its photoconductivity persists long after the UV light is turned off and is attributed to the surface electron depletion region associated with strongly adsorbed negative oxygen species (O^{-2}, O^{2-2}) on the surface. UV light causes that loosely bound oxygen to rapidly desorb from the surface. This reduces the surface electron depletion region and improves photoconductivity. Photocatalysis is described as a light-induced oxidation process that can damage and inactivate organisms. Under UV irradiation, ZnO-NPs in aqueous solution exhibit phototoxicity, which can generate ROS compounds such as hydrogen peroxide (H_2O_2) and superoxide ions (O^2-). Such species are very important in bio applications. The active species created are able to penetrate cells and thus prevent or kill microorganisms. This process inspired the use of the photocatalytic activity of ZnO-NPs in bio nanotechnology and bio nanomedicine for many antibacterial applications. Therefore, the increase in the bioactivity of ZnO was considered to be the result of free radicals generated because ZnO absorbs UV light (Sirelkhatim et al., 2015).

The aim of this thesis is to investigate the current knowledge about the antimicrobial effect of nanoparticles, their mechanism of action, and their possible applications in medicine and industry.

CHAPTER 2

REVIEW OF LITERATURE

REVIEW OF LITERATURE

In recent years, the emergence of multidrug-resistant bacteria has become a serious concern for public health. This has led to a renewed interest in alternative approaches to combat microbial infections. One such approach is the use of nanoparticles (NPs) as antimicrobial agents. Nanoparticles have shown promising results in inhibiting the growth of various microorganisms, including bacteria, fungi, and viruses. They have the potential to overcome the limitations of traditional antimicrobial agents, such as toxicity and drug resistance.

In this review, we will explore the current state of research on the antimicrobial activity of nanoparticles. We will begin by discussing the properties of nanoparticles that make them effective antimicrobial agents, including their size, shape, and surface chemistry. We will then review the different types of nanoparticles that have been studied for their antimicrobial activity, such as metal and metal oxide nanoparticles.

Additionally, we will examine the mechanisms of action of nanoparticles against microorganisms, including oxidative stress, membrane damage, and interference with cellular processes. We will also explore the potential applications of nanoparticle-based antimicrobial agents in various fields, such as medicine, food production, and water treatment.

Overall, this review aims to provide an overview of the current understanding of the antimicrobial activity of nanoparticles and their potential as a new class of antimicrobial agents.

(S. Roy et al., 2010) evaluated the activity of TiO_2 nanoparticles with different antibiotics against methicillin-resistant S. aureus (MRSA). They found that TiO_2 nanoparticles enhanced the antimicrobial activity of beta-lactams, cephalosporins, aminoglycosides, glycopeptides, macrolides, glycosamides, and tetracycline against MRSA. In another experiment, their results showed that the antimicrobial resistance of MRSA to various antibiotics was reduced in the presence of TiO_2 nanoparticles.

According to Haghigh et al. investigated the antifungal effect of TiO_2 nanoparticles on fungal biofilms (fluconazole-resistant standard strains of Candida albicans (C. albicans). According to these results, the synthesized TiO_2 nanoparticles improved antifungal activity against the fluconazole-resistant strain of C. albicans biofilms. The authors suggested that TiO_2 nanoparticles can effectively inhibit fungal biofilms, especially those formed on the surface of medical devices (*Antifungal20191204-3616-Jvjrqn-Libre.Pdf*, n.d.)

Silver nanoparticles affect bacterial cells by catalytic generation of ROS and by direct damage on cell membrane. Beside silver nanoparticles, silver ions can also generate ROS. The biocidal mechanism of silver nanoparticles was explored against 4 types of bacteria: *Vibrio cholera, Pseudomonas aeruginosa, Scrub typhus and Escherichia coli.* High Angle Annular Dark Field microscopy technique is used to observe the accumulation of silver nanoparticles on bacterial membrane (Cioffi & Rai, 2012).

Scientists reported the size-dependent antibacterial activity of CuO nanoparticles. They investigated the antibacterial activity of CuO nanoparticles against two Gram-positive bacteria (S. aureus and B. subtilis) and two Gram-negative bacteria (Pseudomonas aeruginosa and *E. coli*). According to these results, CuO nanoparticles showed inhibitory activity against both groups of mentioned bacteria. The authors concluded that the bactericidal activity of these nanoparticles depended on their size, stability and concentration added to the growth medium. The authors concluded that metal nanoparticles limit bacterial growth by passing through nanometre-sized pores in the cell membranes of most bacteria (Azam, 2012).

Experiment was carried out using 3 different nanoparticles (ZnO, CuO and Fe_2O_3) where the nanoparticles were treated against both Gram negative (*Escherichia coli*) and Gram positive (*Staphylococcus aureus*) bacteria to check the antimicrobial activity. Well-diffusion method and Minimum bactericidal concentration experiments were carried out to check the antimicrobial activity. Scientists had observed that ZnO nanoparticles have great bactericidal potential whereas Fe_2O_3 exhibited the least effect. The order of antibacterial activity was demonstrated as ZnO > CuO > Fe_2O_3(Azam et al., 2012).

ZnO reduces the viability of bacteria; however, the exact mechanism of its antibacterial activity is still not well understood. One proposed possibility is the formation of hydrogen peroxide as the main driver of antibacterial activity. It is also believed that the accumulation of particles on the surface of bacteria due to electrostatic forces may be another mechanism of the antibacterial effect of ZnO particles (*Doi*, n.d.). Besides, ROS generated on the surface of particles, release of zinc ions, membrane dysfunction and nanoparticle internalization can also be considered as possible causes of cell damage (Rao et al., 2013).

Sawai and his colleagues investigated the antibacterial activity of MgO against *E. coli* and *S. aureus*. They suggested that the presence of active oxygen such as superoxide on the surfaces of MgO nanoparticles was one of the most important factors affecting their antibacterial activity (Sawai et al., 2000).

One study evaluated the microbial toxicity of metal oxide nanoparticles in laboratory tests against Escherichia coli, *Bacillus* subtilis and Streptococcus aureus. The tested nanoparticles were CuO, NiO, ZnO and Sb_2O_3. The metal oxide nanoparticles were fully dispersed in the culture medium and the microorganisms were grown on Luria-Bertani agar plates containing different concentrations of metal oxide nanoparticles. Bacteria were counted using colony forming units (CFU). CFU were reduced in medium containing metal oxide NPs and a dose-response relationship was characterized. Among the tested nanoparticles, CuO nanoparticles were found to be the most toxic, followed by ZnO (except for S. aureus), NiO and Sb_2O_3 nanoparticles (Baek & An, 2011).

One such study was conducted where, the release of metal ions from CuO, Fe_2O_3, ZnO, Co_3O_4, Cr_2O_3, and NiO NPs in aqueous media was investigated and their contribution to the inhibition of photobacteria phosphoric bioluminescence by metal oxide NPs was investigated. Ion release from metal oxide NPs in aqueous media was found to be complex, depending on both dissolution and adsorption processes of metal oxide NPs. The relationships between the antibacterial activity of metal oxide NPs and their released metal ions can be divided into three categories: (1) the antibacterial activity of ZnO NPs was only due to the released Zn^2; (2) the antibacterial effect of CuO NPs resulted from both released Cu2 and CuO particles; and (3) the antibacterial activity of Fe_2O_3, Co_3O_4, Cr_2O_3, and NiO NPs was due to the NPs themselves. Results indicate that the release of ions and their effect on the toxicity of NPs should be considered when evaluating the toxicity of metal oxide NPs (Wang et al., 2016).

In one study, researchers investigated the antimicrobial activity of TiO_2 nanoparticles (NPs) synthesized by the sol-gel method. Upon synthesis, the TiO_2 NPs were characterized by X-ray diffraction, scanning electron microscopy, and UV-visible absorption spectroscopy. The antimicrobial activity of calcined TiO_2 nanoparticle samples was investigated in daylight with Gram-positive bacteria (Staphylococcus aureus, Streptococcus pneumonia and *Bacillus* subtilis), Gram-negative bacteria (Proteus vulgaris, Pseudomonas aeruginosa and Escherichia coli test pathogen of fungal test pathogen). The synthesized TiO_2 NPs were found to be effective against Streptococcus pneumonia, Staphylococcus aureus, Proteus vulgaris, Pseudomonas aeruginosa and Candida albicans under visible light (Priyanka et al., 2016).

This is a report on the antibacterial activity of ZnO and CuO nanoparticles synthesized by the sol-gel method. Studies were performed on Gram-positive *Staphylococcus aureus* and Gram-negative *Escherichia coli* and *Pseudomonas aeruginosa* bacteria using disk and well

diffusion methods, bioluminescence and optical density analysis. The results show a strong reduction of bacterial strains after a short exposure to the nanoparticles. Modeling made it possible to find out the sensitivity of bacteria to toxic substances at different stages of the kinetics of population evolution. It was concluded that bacterial suppression is most effective in the exponential growth phase, while its effectiveness is lower in the late and stationary phases. CuO and ZnO nanoparticles showed comparable performance in the exponential growth phase. Meanwhile, ZnO was almost inactive in the lag phase and had a lower efficiency in the stationary phase, where CuO retained significant activity. (Dadi et al., 2019)

Antimicrobial activity of metal oxide nanoparticles against Gram-positive and Gram-negative bacteria was studies using ZnO, CuO and Fe_2O_3 NPs. Study concluded that among the three nanomaterials ZnO showed greater antimicrobial activity with both Gram-positive and Gram-negative strain. (Azam et al., 2012)

In one study, Agar well diffusion method was used to study the antimicrobial activity of CuO NPs. *Escherichia coli* (Gram-negative), *Staphylococcus aureus* (Gram-positive) strains were used to determine the activity. *E. coli* showed greater ZOI (Zone of inhibition) than *Staphylococcus aureus.* (Rajamma et al., 2020)

To determine the antimicrobial activity of ZnO NPs against selected aquatic pathogenic microbes. well diffusion and minimum inhibitory concentration (MIC) tests were performed. (Klink et al., 2022)

Antimicrobial activities of nanoparticles were determined using different aquatic microbes following disc diffusion assay. The microbial strains used were Aeromonas hydrophila, Edwardsiella tarda, Pseudomonas aeruginosa. Among different nanoparticles, commercial nanoparticles such as ZnO and CuO were found to inhibit bacteria, but Zn, Ag, Fe_2O_3 and Al2O3 nanoparticles did not inhibit any of the tested isolates. Chemically synthesized ZnO and CuO nanoparticles were found to inhibit all bacterial isolates regardless of incubation time. (Swain et al., 2014)

Antimicrobial activities of zinc oxide nanoparticles from leaf extract of Mentha pulegium was determined by performing Disc diffusion method. Experiment was performed on 2 bacterial strains *E. coli* and *S. aureus.* Sterile discs were loaded with 25, 50, and 100µg/ml of ZnO NPs and placed equidistance from each other on the agar plates containing bacterial sample. Results concluded that ZOI (Zone of inhibition) was greater in the case of *E. coli* than *S. aureus*. (Rad et al., 2019)

Silver oxide nanoparticles are biosynthesised by combustion synthesis by using *Lippia citriodora* plant powder and checked for the antimicrobial activity. Well diffusion techniques were carried out. Antibacterial activity tests showed that S. epidermidis was more susceptible to tetracycline, which was used as a standard. S. aureus was more sensitive to Ag_2O NPs synthesized with Lippia citriodora plant extract. Similarly, in the antifungal activity of A. aureus showed more sensitivity to Ag_2O NPs. From the above data, it is evident that Ag_2O NPs synthesized with Lippia citriodora plant extract were more effective against bacterial and fungal pathogens. (Li et al., 2019)

The antibacterial activity of the ZnO nano sample was determined by the plate diffusion method on Muller Hinton Agar (MHA) medium. Green-synthesized ZnO nanoparticles are more toxic to Klebsiella pneumonia and Staphylococcus aureus bacteria. (Janaki et al., 2015)

Mechanisms leading to ROS-mediated antibiotic killing. Different classes of antibiotics activate the TCA cycle. Hyperactivation of the electron transport chain leads to the formation of superoxide radicals, which can damage iron-sulphur clusters. Destabilized ferrous iron can in turn react with hydrogen peroxide in the Fenton reaction and lead to the generation of hydroxyl radicals that damage DNA, lipids and proteins. The metabolic changes that lead to increased ROS production are thought to result from the activation of a two-component system of redox reaction (ArcA) Via CpxA, a two-component coil voltage response sensor. (Van Acker & Coenye, 2017)

ROS was measured by 2′,7′-dichloro fluorescein diacetate (H_2DCF-DA). O^{2-} concentrations were tested using a hydroxylamine oxidation analyser (Suzhou Kechromium Biotechnology Inc., Suzhou, China). (Liao et al., 2019)

In a study, ROS-mediated membrane lipid oxidation of Escherichia coli treated with ZnO nanoparticles (NPs), supported by detection and spectrophotometric measurement of malondialdehyde (MDA) by TBARS (thiobarbituric acid-reactive species) assay. The MDA equivalent (ROS equivalent) was found to be higher in the batch treated with higher concentrations of nanoparticles, compared to the untreated samples. Based on these observations, it can be noted that the inhibition of the growth curve due to the increase of ZnO NPs was due to the generation of ROS, as evidenced by the increase of equivalent MDA in the medium. (Dutta et al., 2012)

Cytotoxicity of MNP was assessed in the murine RAW 264.7 macrophage cell line using the MTT assay. Untreated RAW 264.7 cells (negative control) showed 100% formation

of formazan crystals after subsequent addition of MTT. Unlike pentamidine, GNP showed negligible toxicity even at 100 µM, resulting in a significant reduction in cell viability (Figure 2). Cytotoxicity data showed that GNPs have excellent biocompatibility in macrophage cell lines. (Want et al., 2021)

Lipid peroxidation was assessed by determination of thiobarbituric acid reactive species (TBARS). The experiment showed a 173% and 132% increase in TBARS levels in PBMC treated with GML at 10 and 50 µM, respectively. (Lopes et al., 2019)

After reviewing the literature present on antimicrobial activity of nanoparticles we can conclude that, different nanoparticles have different mechanism of action to affect the microbial cells and the effect varies with the size, morphology, surface area, mechanism of action and concentration of NPs used. Even though there are many mechanisms by which NPs affect bacterial cells, there are 2 common ways by which NPs affect the cells. First, by the release of ROS (Reactive oxygen species). Second, by the dissolution of metal ions. Action of few NPs is not yet clear. sometimes the affect can be because of both dissolution of metal ions and by the release of ROS.

Some common techniques researchers used to determine the antimicrobial activity of nanoparticles are Well-diffusion method, Disc-diffusion method, MIC (Minimum inhibitory concentration), By calculating CFU/ml of different concentrations of nanoparticles and by evaluating the growth curve kinetics. These are some of the common methods researchers used to determine the antimicrobial activity of NPs.

After determining the antimicrobial activity of nanoparticles, it is needed to evaluate the amount of cell damage that had occurred to bacterial cells. Certain biochemical assays like MTT, Lipid peroxidation (TBARS), ROS assays were performed by the researchers to evaluate the cell damage.

In this thesis, we will be working with the TiO_2, ZnO and CuO nanoparticles that were synthesized by the sol-gel method in a laboratory. We will be performing MIC (Minimum inhibitory concentration), growth curve, CFU/ml to determine the antimicrobial activity of TiO_2, ZnO and CuO nanoparticles against *Escherichia coli* and *Bacillus* strains. Later, biochemical tests will be performed to evaluate the damage caused by these nanoparticles to the bacterial cell.

CHAPTER 3

AIM AND OBJECTIVES

AIM AND OBJECTIVES

Aim: To study the antimicrobial activity of TiO_2, ZnO and CuO Nanoparticles.

Objectives:

- To understand the antimicrobial activity of different concentrations of nanoparticles
- To study the growth kinetics with varying concentration of nanomaterial
- To perform biochemical assay for antimicrobial study of nanoparticles

CHAPTER 4

MATERIAL AND INSTRUMENTATION

MATERIAL AND INSTRUMENTATION

4.1: Nanoparticles

TiO_2, ZnO and CuO nanoparticles were obtained from Sardar Patel university, Anand, Gujarat. Hard crystalline form of NPs was suspended in autoclaved milliQ water by sonication method. Stock solutions of all three NPs were prepared. Different concentrations of NPs were prepared from the stock.

4.2: Preparation of master plate for bacterial samples

E. coli and *Bacillus* master plate was prepared and revived. Both the bacterial strains were grown in Luria Bertani agar.

Compound	Quantity(g)
Tryptone	1
Yeast extract	0.5
NaCl	1
Bacto Agar	1.5

Table 1: L.B agar composition

After streaking the bacterial strains on the L.B plates, plates were kept in 37-degree incubator for 24 hrs.

For performing the experiments, L.B media(broth) was used to grow the bacterial samples.

Compound	**Quantity(gms/litre)**
Tryptone	10
Yeast extract	5
NaCl	10

Table 2: L.B media composition

4.3: Instrumentation

1. Autoclave (Equitron Autoclave SLEFA)

2. High precision balance (WENSAR)

3. Probe sonicator

4. Digital ultrasonic cleaner (Model no: LMUC-3, ULTRA SONIC POWER: 100W, FREQUENCY: 40KHz)

5. Laminar air flow chamber

6. Shaker incubator (37 degree) [Tempo Bacteriological incubator, Ti 90E INCUBATOR, BACT]

7. Microcentrifuge machine (Name: Centrifuge LAB-I-FUGE C series, Power: AC 220V 60Hzs)

8. Roto spin (intermittent incubation) TARSONS

9. UV Sepctrophotometer (UV -1800 SHIMADZU)

10. ELISA plate reader (BIO-RAD, iMark Microplate reader)

CHAPTER 5

METHODS

METHODS

5.0: To determine the antimicrobial activity of TiO_2, ZnO and CuO NPs in *E. coli* and *Bacillus* strains.

5.1: Minimum inhibitory concentration (MIC) with varying nanoparticle concentrations

Antimicrobial activity of TiO2, ZnO and CuO NPs was studied by performing minimum inhibitory concentration (MIC). Before starting the experiment, different concentrations of different nanoparticles were prepared, those are going to interact with the bacterial cells. The range of nanoparticles used was between 50- 500µg/ml. The steps involved in performing MIC are as follows:

- different nanoparticles concentration concentrations (50 µg/ml, 100 µg/ml, 200 µg/ml, 400 µg/ml) of NPs were prepared.
- *E. coli* and *Bacillus* strains were inoculated in the LB media and allowed to grow till they had reached the active state (*E. coli* – O.D: 0.5 in 6 hrs, *Bacillus* – O.D: 0.68 in 16 hrs).
- 1.5 ml of the culture was shifted to 4 sterile microcentrifuge tubes and centrifuged at 5000 rpm for 5 minutes. Supernatant is discarded and pellet is suspended with 1.5 ml of sterile LB broth.
- 250µl of this culture is added into 10 sterile microcentrifuge tubes which were marked as 0 µg/ml, [(50 µg/ml, 100 µg/ml, 200 µg/ml) *3 different nanoparticles {TiO_2, ZnO and CuO}].
- To these tubes 250µl of different concentrations of NPs were added.
  (0 µg/ml [control]is added with milliQ water).
- Tubes were subjected to intermittent incubation for 2-3 hrs.
- 20 µl of treated cells were inoculated into the respective test tubes containing 3ml sterile LB broth. [Done in triplicates for each concentration range]
- Test tubes were kept for 6-7hrs incubation (*E. coli*) and 14- 17 hrs incubation (*Bacillus*)
- Absorbance was measured at 600nm and results were obtained.

5.2: CFU/ml with various concentrations of nanoparticles (TiO_2, ZnO and CuO).

- Luria Bertani Agar plates were prepared and kept in incubator (37degree) for 24 hrs to check for any contamination. The plates with no contamination were stored for future experiments.
- The plates were marked as 0 µg/ml, [(50 µg/ml, 100 µg/ml, 200 µg/ml, 400 µg/ml) *3 different nanoparticles {TiO_2, ZnO and CuO}].
- After intermittent incubation (in MIC) the treated bacterial cells in the microcentrifuge tubes were subjected to serial dilution.
- *E. coli* treated cells were diluted to $^{10-6}$ dilution and *Bacillus* treated cells were diluted to $^{10-4}$ dilution.
- 100µl of serially diluted treated cells were dispersed onto already prepared and marked LB plates using spread plate technique
- The plates were kept in 37ºdegree incubator for 24 hours.
- After 24 hours plates were counter and results are noted.

5.3 : Growth curve kinetics with various concentrations of Nanoparticles and (TiO_2, ZnO and CuO).

- Bacterial cells were treated with different concentrations of different NPs and kept for intermittent incubation.
- After intermittent incubation, 50µl of treated cells were inoculated into 50 ml sterile LB media and incubated in shaker incubator.
- Every 1 hour, sample from all the flasks were taken out into a microcentrifuge tube inside LAF (to avoid contamination)
- Microcentrifuge tubes were placed in ice box to arrest the further growth to avoid any errors.
- Absorbance was measured at 600nm
- Eight to ten readings were recorded and graph was plotted to obtain growth curve.

BIOCHEMICAL ASSAYS:

5.4: MTT ASSAY (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)

This colorimetric assay reduces a yellow tetrazolium salt (MTT) to measure cellular metabolic activity as a indicator for cell viability. Viable cells that are present contains NADPH- dependant oxidoreductase enzyme which reduces MTT reagent to formazan that has deep purple colour. These crystals are then solubilized by DMSO and absorbance was measured at 570nm

- 1ml of overnight grown bacterial cultures were taken in individual microcentrifuge tubes and centrifuged at 8000 rpm for 5 minutes.
- Supernatant was discarded and pellet was resuspended in 1xPBS and treated with individual concentrations of different NPs for 2 hours at room temperature with continuous agitation along with control.
- 200µl of treated culture is transferred to a fresh 1.5ml microcentrifuge tubes and centrifuged at 8000 rpm for 5 minutes and the supernatant was discarded.
- 200µl of MTT solution (0.5mg/ml in 1xPBS) was then added to each tube and incubated for 3 hours to allow the formation of formazan crystals.
- The tubes were then centrifuged at 8000 rpm for 5 minutes, Supernatant was discarded.
- 200µl of DMSO was added to each tube and incubated for 30 minutes in dark condition in order to dissolve formazan crystals.
- 200µl of solution was transferred to a 96 well plate and O.D was measured at 570nm using microplate reader.

5.5: TBARS (Thiobarbituric acid reactive substances)

TBARS is used for the measurement of lipid peroxidation in cells which detects the level of MDA (malondialdehyde) the major lipid peroxidation product.

- 1ml of overnight grown bacterial cultures were taken in individual microcentrifuge tubes and centrifuged at 8000 rpm for 5 minutes.
- Supernatant was discarded and pellet was resuspended in 1xPBS and treated with individual concentrations of different NPs for 2 hours at room temperature with continuous agitation along with control.

- Treated cells were lysed by placing the tubes in ultrasonic water bath for 30 min and centrifuged at 5000 rpm for 10 min at 4 ºc.
- 200µl of lysate /supernatant was taken into a fresh microcentrifuge tube and 400µl of 10% ice cold Trichloroacetic acid (TCA) was added to each tube and incubated on ice for 15 min for protein precipitation to occur.
- The tubes were then centrifuged at 10000 rpm for 10 minutes at 4ºc and 200 µl of 0.67% thiobarbituric acid (TBA) was then added to each tube and allowed to boil for 10 minutes in a boiling water bath.
- The samples were then allowed to cool and loaded into a 96 well plate.
  O.D was measured at 532nm

CHAPTER 6

RESULTS

6.1: Evaluation of Minimum inhibitory concentration

E. coli and *Bacillus* cultures were treated with TiO2, ZnO and CuO NPs and the growth was monitored by measuring the O.D. at 600nm. The readings were plotted for TiO2, ZnO and CuO NPs against *Bacillus* (Fig.1) and *E. coli* (Fig.2)

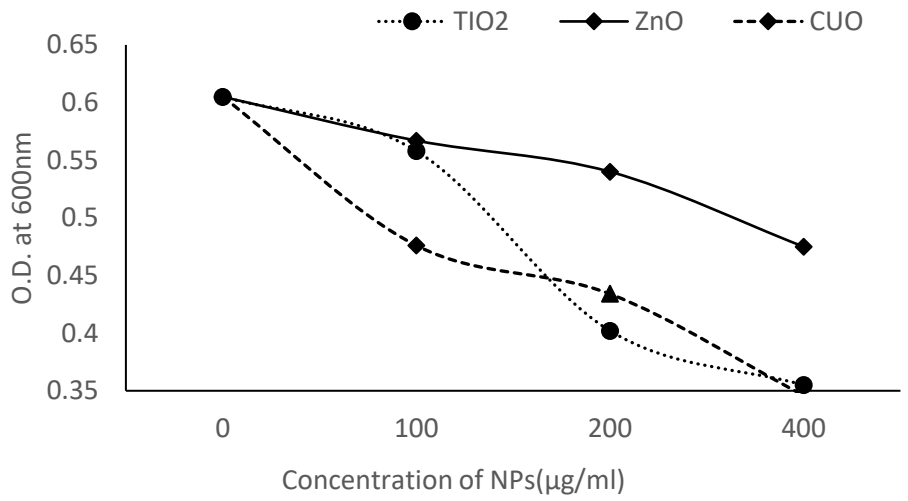


Fig.1 Minimum inhibitory concentration (MIC) of TiO_2, ZnO and CuO NPs against *Bacillus*

Various concentrations of TiO_2, ZnO and CuO NPs showed various effects on *Bacillus* strain. All the nanoparticles showed notable decline in the O.D. value at 400µg/ml concentration. Among the 3 nanoparticles TiO_2 and CuO NPs showed greater effect on *Bacillus* when compared to ZnO nanoparticles. TiO_2 and CuO NPs showed 45% decrease in the growth of *Bacillus* when compared to untreated sample, whereas ZnO NP showed only 25% decrease at 400µg/ml concentration.

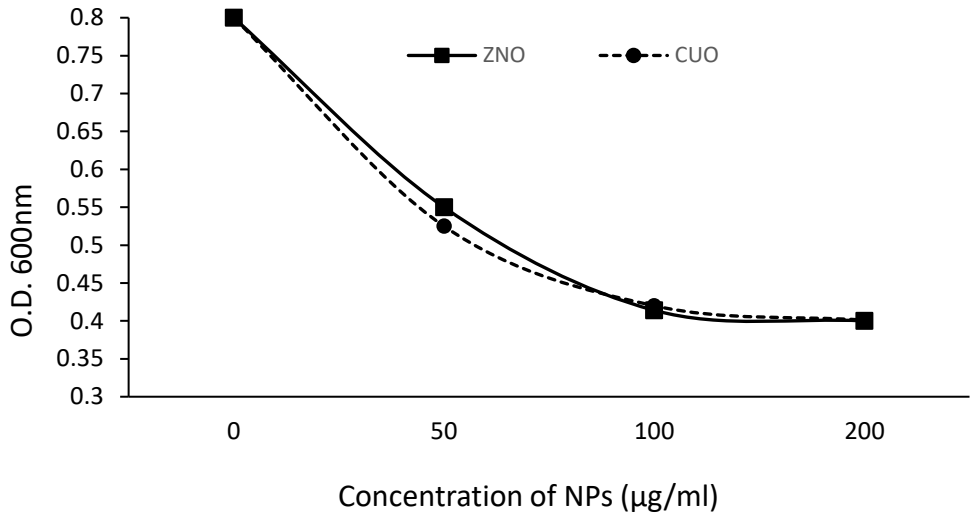


Fig.2 Minimum inhibitory concentration (MIC) of ZnO and CuO NPs against *E. coli*

.

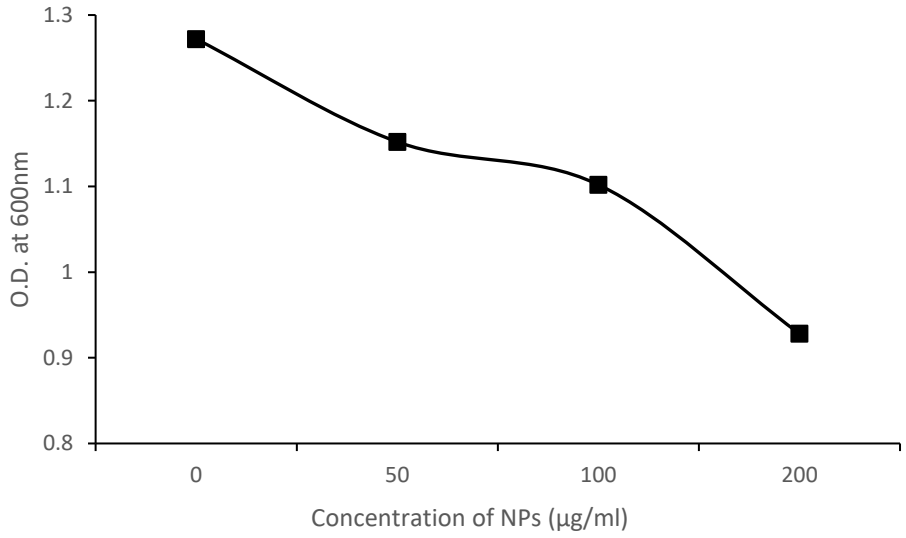


Fig.3 Minimum inhibitory concentration (MIC) of TiO_2 NPs against <u>*E. coli*</u>

TiO_2, ZnO and CuO NPs showed antimicrobial effect at the concentration 200µg/ml. ZnO and CuO NPs showed almost same effect on *E. coli (Fig.3)*. TiO_2 showed the least effect on *E. coli*. Both ZnO and CuO NPs showed 50 % decrease in the growth of *E. coli* when compared to untreated sample. TiO_2 NP showed the least effect, where it showed only 20 % decline in the growth of *Bacillus* when compared to untreated sample at 200µg/ml concentration.

6.2: Colony forming unit

Bacterial cells treated higher concentrations of NPs showed a significant loss in the number of colonies formed for both *E. coli* and *Bacillus* in comparison with the untreated control plates (Fig.5, 6, 7, 8, and 9). The amount of CFU/ml decreased with the increase in concentration of TiO2, ZnO and CuO NPs was observed (Table3 and Table 4). The CFU/ml calculated was represented in the form of a bar diagram (Fig.4 and Fig.5).

Concentration of NPs	**Amount of culture plated(µl)**	**Average number of colonies (*E. coli*)**	**CFU/ML *E. coli***
[TiO_2] 0 µg/ml	100	511	**$51.1*10^8$**
[TiO_2] 50 µg/ml	100	384	**$38.4*10^8$**
[TiO_2] 100 µg/ml	100	293	**$29.3*10^8$**
[TiO_2] 200 µg/ml	100	196	**$19.6*10^8$**
[ZnO] 0 µg/ml	100	510	**$51*10^8$**
[ZnO] 50 µg/ml	100	405	**$40.5*10^8$**
[ZnO] 100 µg/ml	100	393	**$39.3*10^8$**
[ZnO] 200 µg/ml	100	360	**$36*10^8$**
[CuO] 0 µg/ml	100	1205	**$120.5*10^8$**
[CuO] 50 µg/ml	100	925	**$92.5*10^8$**
[CuO] 100 µg/ml	100	790	**$79*10^8$**
[CuO] 200 µg/ml	100	640	**$64*10^8$**

Table 3: Observation table of colony forming unit of *E. coli*

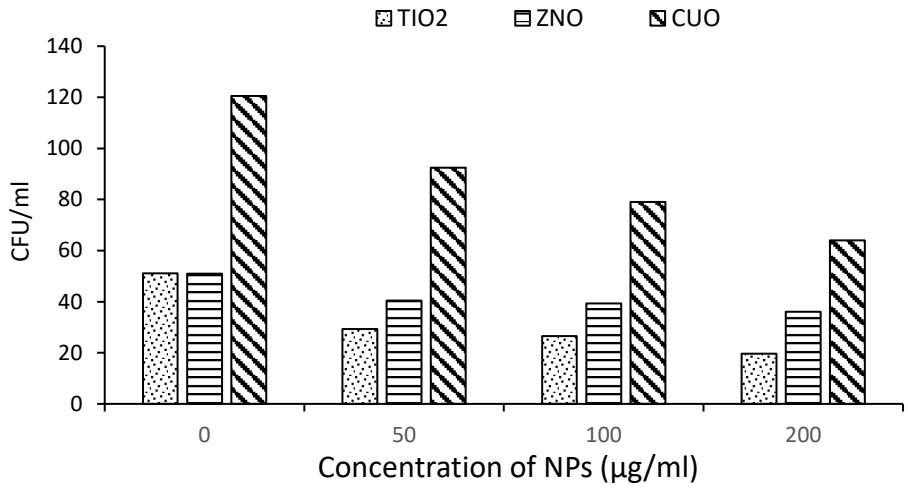


Fig.4: CFU/ml of *E. coli* against various concentrations of TiO_2, ZnO and CuO NPs

A decline in CFU/ml was observed at 200µg/ml concentration in all the three nanoparticles. (Fig.4) Among the three nanoparticles TiO_2 showed greater antimicrobial effect against *E. coli* followed by ZnO NPs. Least antimicrobial effect was shown by CuO NPs. At 200 µg/ml TiO_2 NP showed 60% decrease in the growth of *E. coli* when compared to untreated sample where as ZnO and CuO NPs showed 30% and 50 % decline.

Concentration of NPs	**Amount of culture plated(µl)**	**Average number of colonies (*Bacillus*)**	**CFU/ML *Bacillus***
[Control] 0 µg/ml	100	112	**$11.2*10^6$**
[TiO_2] 100 µg/ml	100	82	**$7.9*10^6$**
[TiO_2] 200 µg/ml	100	43	**$4.1*10^6$**
[TiO_2] 400 µg/ml	100	34	**$3*10^6$**
[ZnO] 100 µg/ml	100	9	**$0.9*10^6$**
[ZnO] 200 µg/ml	100	5	**$0.5*10^6$**
[ZnO] 400 µg/ml	100	3	**$0.3*10^6$**
[CuO] 100 µg/ml	100	12	**$1.2*10^6$**
[CuO] 200 µg/ml	100	9	**$0.9*10^6$**
[CuO] 400 µg/ml	100	4	**$0.4*10^6$**

Table 4: Observation table of colony forming unit of *Bacillus*

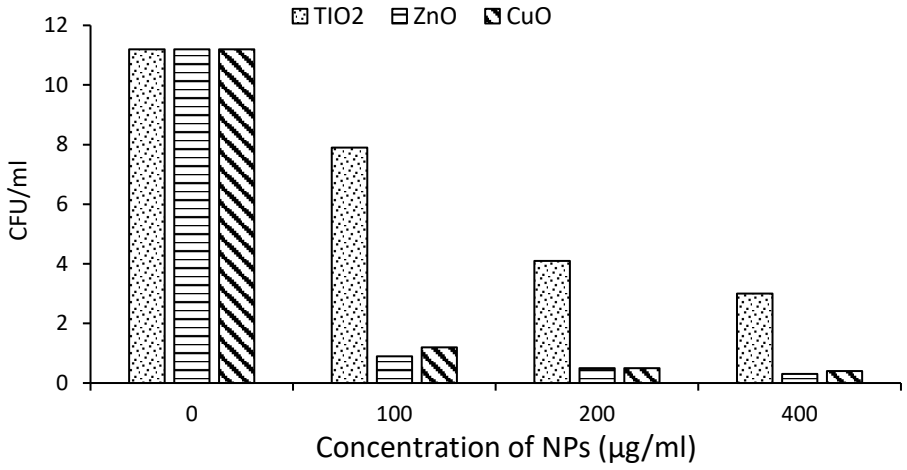
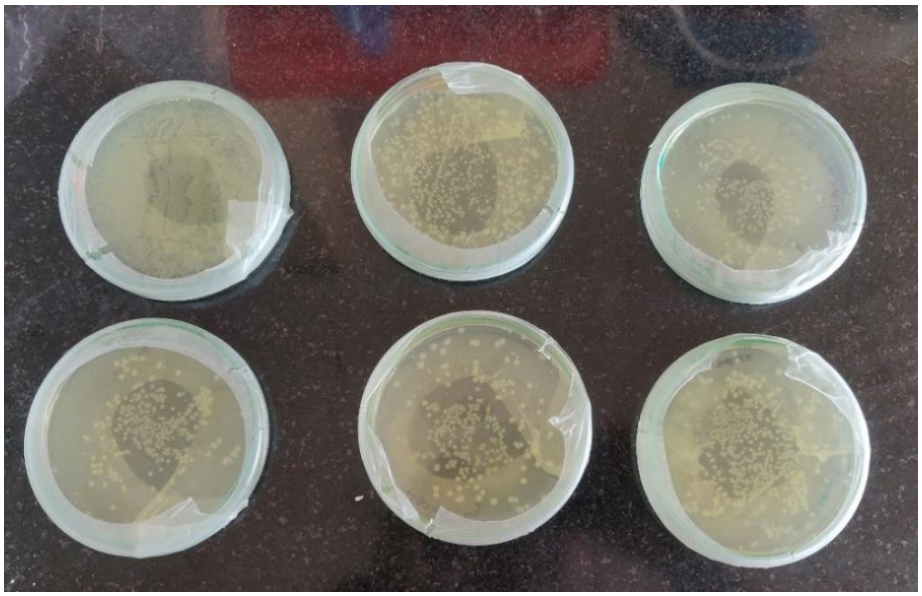


Fig.5: CFU/ml of *bacillus* against various concentrations of TiO_2, ZnO and CuO NPs

A drastical decrease in CFU/ml calculated was observed with increase in concentration of NPs. Lowest value of CFU/ml was calculated at 400µg/ml concentration, in all three nanoparticles(Fig.5). ZnO NPs showed greater antimicrobial activity followed by CuO NPs. Least effect was shown by TiO_2 NPs. At 400µg/ml concentration, ZnO showed 97% decline

in the growth of *Bacillus* when compared to untreated sample, followed by CuO which showed 95% decline in the growth of *E. coli*. Least effect was shown by TiO_2 NPs where only 45% decline was seen.

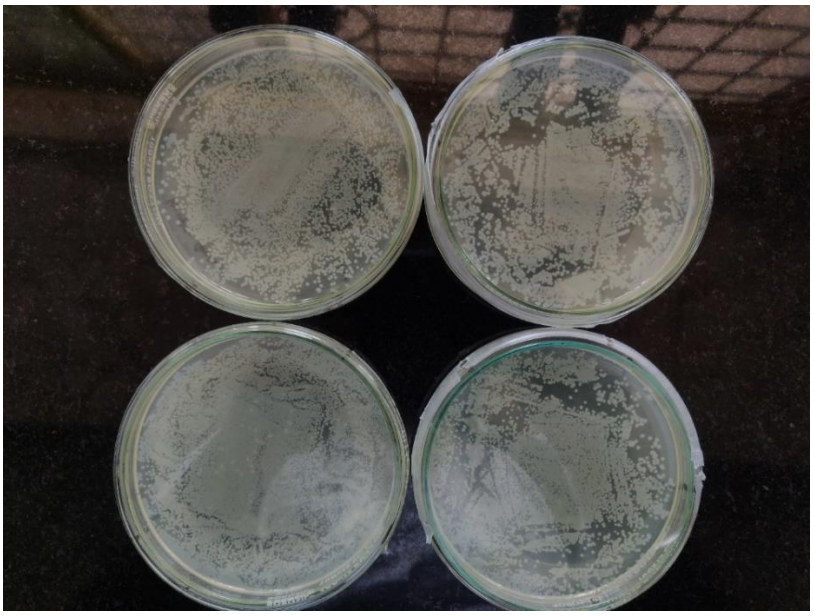
0µg/ml **100 µg/ml** **50µg/ml**



100µg/ml **200µg/ml** **400µg/ml**

Fig.6: Colonies of *E. coli* grown on LB agar plates on treatment with various concentrations TiO_2 NPs.

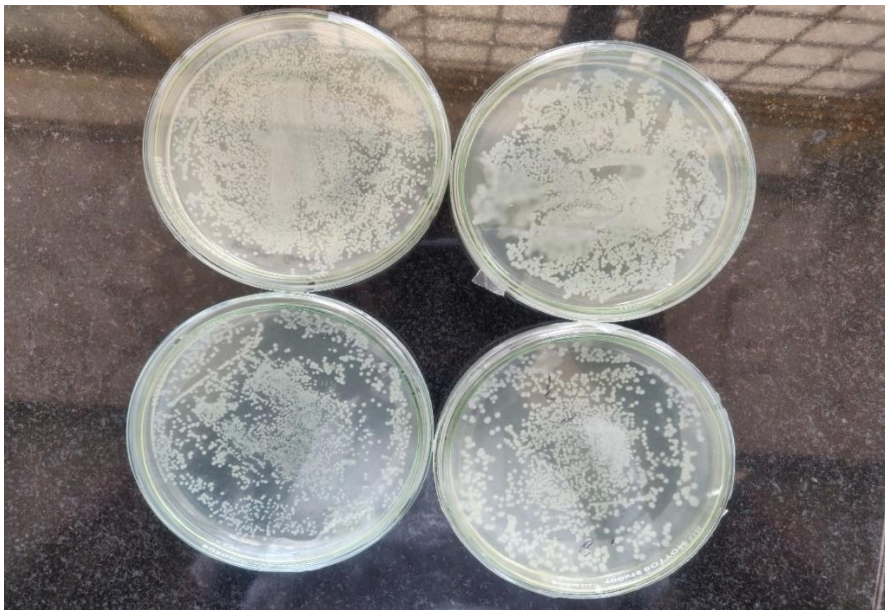
0 µg/ml **50µg/ml**



100µg/ml **200µg/ml**

Fig.7: Colonies of *E. coli* grown on LB agar plates on treatment with various concentrations ZnO NPs.

0 µg/ml **50 µg/ml**



100 µg/ml **200 µg/ml**

Fig.8: Colonies of *E. coli* grown on LB agar plates on treatment with various concentrations CuO NPs.

0 µg/ml 100 µg/ml 200 µg/ml 400 µg/ml

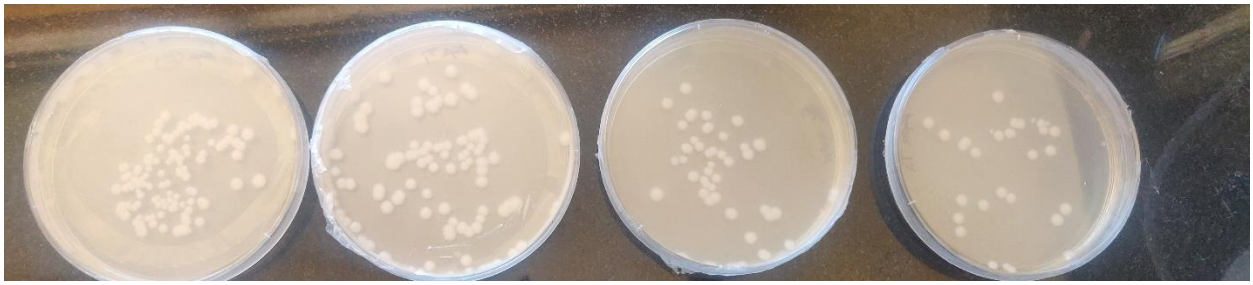


Fig.9: Colonies of *Bacillus* grown on LB agar plates on treatment with various concentrations of TiO_2 NPs

100 µg/ml 200 µg/ml 400 µg/ml

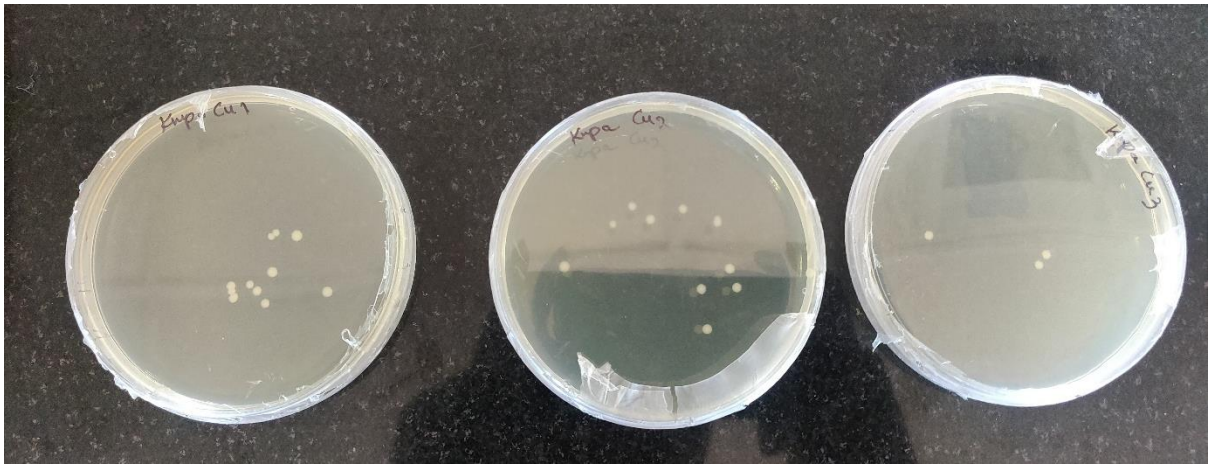


Fig.10: Colonies of *Bacillus* grown on LB agar plates on treatment with various concentrations of ZnO NPs

0 µg/ml 100 µg/ml 200 µg/ml 400 µg/ml

Fig.11: Colonies of *Bacillus* grown on LB agar plates on treatment with various concentrations of CuO NPs

6.3: Growth curve

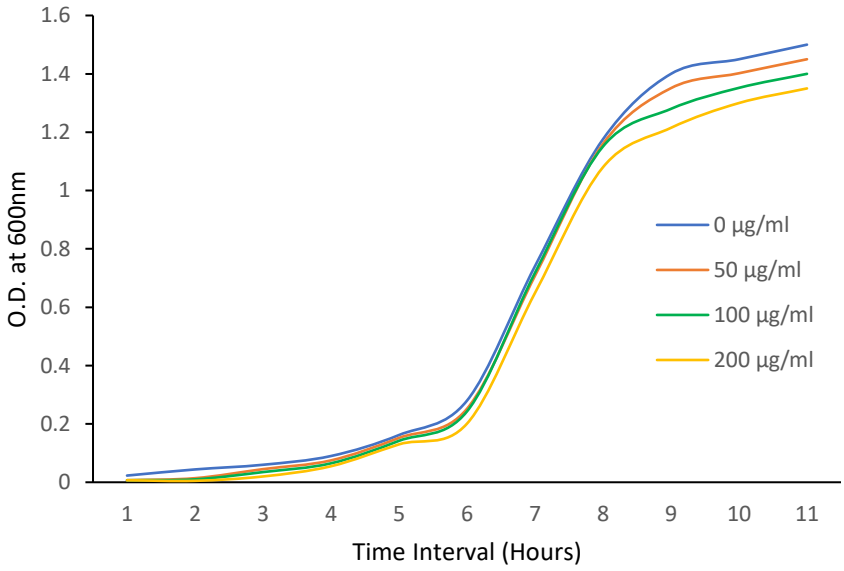


Fig.12: Growth curve characteristic of *E. coli* on treatment with various concentrations of TiO_2 NPs.

Growth curve was performed by incubating *E. coli* treated with various concentrations of TiO_2 (50, 100 and 200µg/ml) NPs. After 10 hours there was a decrease in the O.D recorded at the concentration, 200µg/ml comparatively with untreated sample(Fig.12). This confirms the antimicrobial activity of TiO_2 NPS. At 200 µg/ml concentration 15% decline in growth was observed when compared to untreated sample.

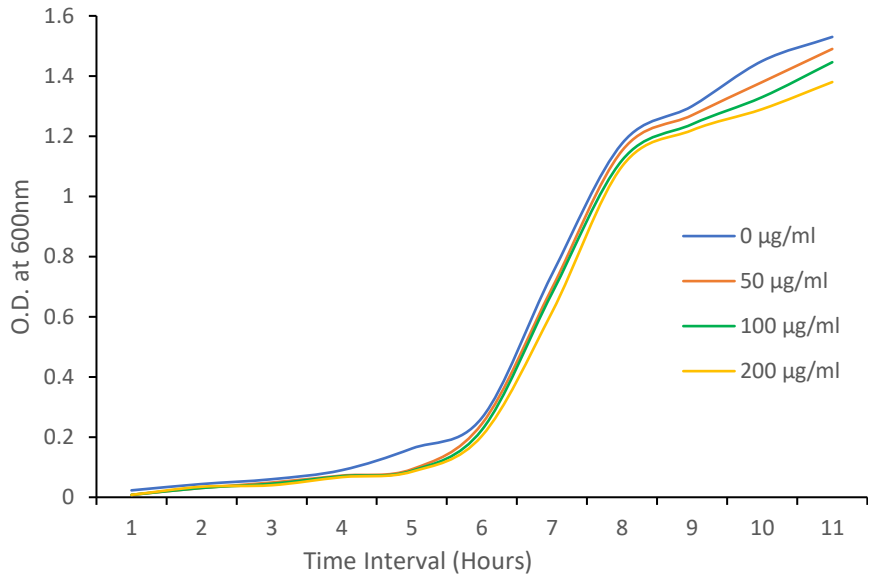


Fig.13: Growth curve characteristic of *E. coli* on treatment with various concentrations of CuO NPs.

Growth curve was performed by incubating *E. coli* treated with various concentrations of CuO (50, 100 and 200µg/ml) NPs. After 10 hours there was a decrease in the O.D recorded at the concentration, 200µg/ml comparatively with untreated sample (Fig.13). This confirms the antimicrobial activity of CuO NPs. At 200µg/ml 13% decline in the growth of *E. coli* was observed compared to untreated sample

Biochemical assays:

6.4: MTT assay

Spectrophotometric analysis of cellular activity of *E. coli* and *Bacillus*, measured by MTT revealed a decrease in microbial cell metabolism, which was observed as a decrease in absorbance with an increase in the concentration of TiO_2, ZnO and CuO NPs in the medium. (Fig.14 and Fig.15)

The negative effect on bacterial cell metabolism was greater in cells treated with higher concentrations of nanoparticles compared to lower concentrations and untreated samples.

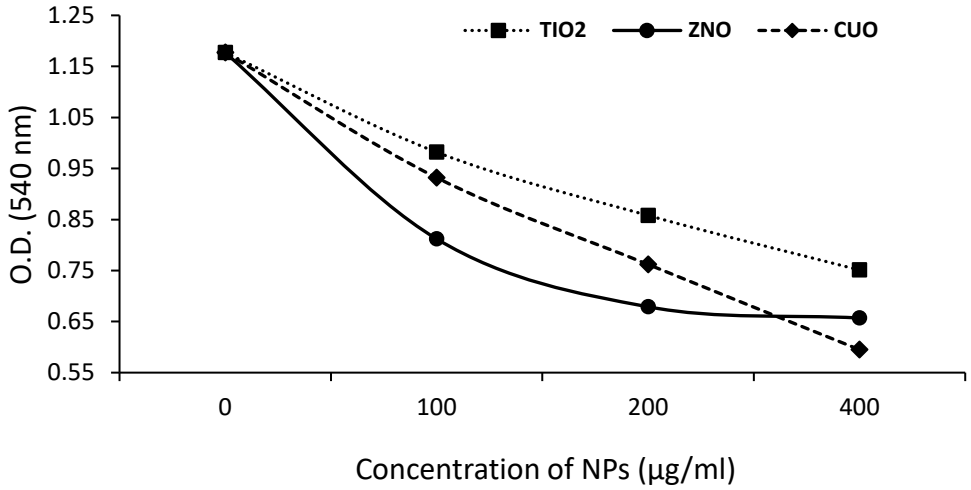


Fig.14: MTT assay of *E. coli* on treatment with various concentrations of TiO_2, ZnO and CuO NPs.

MTT assay measures the number of viable cells. O.D is directly proportional to the number of viable cells. After performing the assay, the lowest value of O.D was observed at the concentration 400 µg/ml in all three nanoparticles (Fig.14). Decrease in viable cells is seen with the increase in concentration of nanoparticles. CuO showed greater antimicrobial effect followed by ZnO and least effect was shown by TiO_2 NPs at 400 µg/ml concentration. At 400µg/ml concentration CuO showed 49% decline in the growth of *E. coli* when treated with untreated sample. Followed by ZnO which showed 45 % decline. Least effect was shown by TiO_2 NPs which showed only 34 % decline.

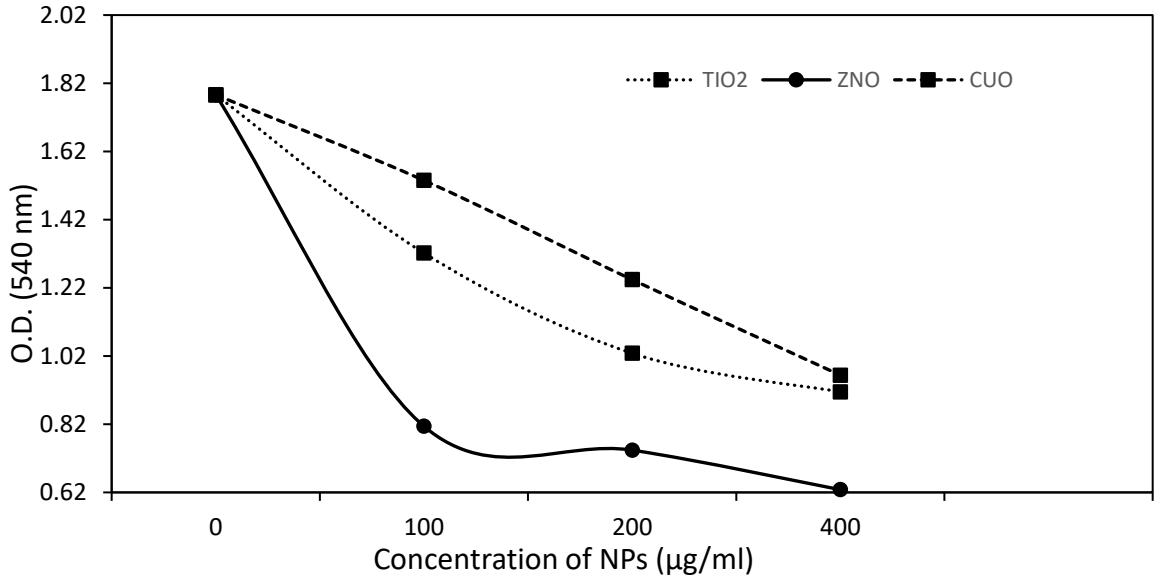


Fig.15: MTT assay of *Bacillus* on treatment with various concentrations of TiO_2, ZnO and CuO NPs.

MTT was performed by the treatment of Bacillus with various concentrations of TiO_2, ZnO and CuO NPs. At 400 µg/ml concentration, there was a decline in the O.D value recorded, this implies that at the higher concentrations of nanoparticles the number of viable cells decreased (Fig.15). ZnO showed greater effect on *Bacillus* followed by TiO_2. Least effect was shown by CuO NPs, when MTT assay was performed. At 400µg/ml concentration, ZnO NP showed 67% decline in growth of *Bacillus* when compared with untreated sample, followed by TiO_2 and CuO which showed 45% and 40% decline.

6.5: TBARAS assay

Thiobarbituric acid reactive substances that are formed as by products were the result of lipid peroxidation that is carried out in cells under division, when treated with different concentrations of NPs. This was the indication of oxidative stress that cells faced during their interaction with substances (Nanoparticles) that caused cell damage.

Upon measurement of absorbance at 540nm, the amount of TBARS produced at different concentration of nanoparticles (10,50,100.200,400µg\ml) used is detected and readings were plotted in the form of bar graph (Fig.16 and 17).

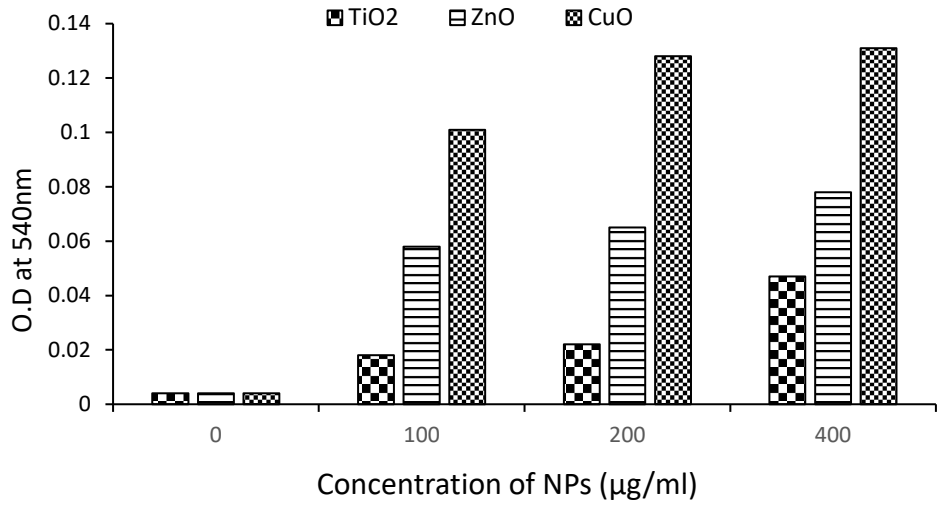


Fig.16: Extent of lipid peroxidation measured by TBARS on treatment with various concentrations of TiO_2, ZnO and CuO NPs on *Bacillus* at 540nm.

Lipid peroxidation activity was observed to be more when the *Bacillus* strain was treated with higher concentration of nanoparticles. At 400µg/ml concentration, higher value of O.D was observed which implies the greater lipid peroxidation activity (Fig.16). CuO showed greater activity against *Bacillus* followed by ZnO and TiO_2 which showed 30% and 60% decline in peroxidation activity when compared to the activity of ZnO at 400µg/ml

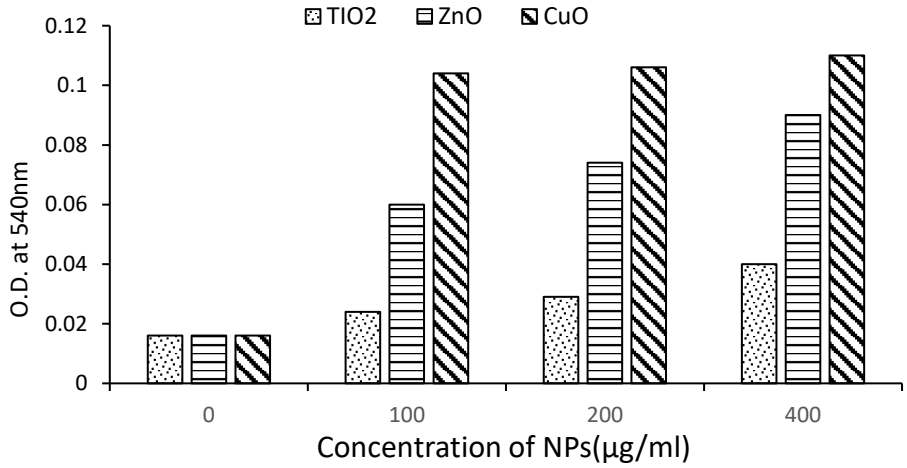


Fig.17: Extent of lipid peroxidation measured by TBARS on treatment with various concentrations of TiO_2, ZnO and CuO NPs on *E. coli* at 540nm.

Lipid peroxidation activity was observed to be more when the *E. coli* strain was treated with higher concentration of nanoparticles. At 400µg/ml concentration, higher value of O.D was observed which implies the greater lipid peroxidation activity (Fig.17). CuO showed greater activity followed by ZnO and TiO_2 which showed 10% and 30% decline in peroxidation activity when compared to the activity of ZnO at 400µg/ml concentration.

CHAPTER 7

DISCUSSION

DISCUSSION

The results of this study suggest that TiO_2, ZnO, and CuO nanoparticles have varying degrees of antimicrobial activity against different microorganisms. These findings are consistent with previous studies that have reported the antimicrobial properties of nanoparticles, particularly TiO_2 and ZnO, which have been extensively investigated for their potential applications in various fields, including medicine, agriculture, and food industry.

The antibacterial activity of TiO_2, ZnO and CuO nanoparticles can be attributed to several mechanisms, including the production of reactive oxygen species (ROS), which can damage the cell membrane and intracellular components of the microorganisms, and the disruption of the electron transport chain, leading to a loss of energy and metabolic functions, Direct contact of ZnO-NPs with cell walls resulting in destruction of bacterial cell integrity, release of antimicrobial ions, mainly Zn2+ ions, Extracellular $Cu2^+$ cross the cell membrane and enter the cytoplasm via endocytosis and copper transport proteins. The exact mechanisms of antimicrobial action of TiO_2, ZnO and CuO nanoparticles, however, are still under debate and require further investigation.

In this thesis, we have performed certain experiments to determine the antimicrobial activity of TiO2, ZnO and CuO. When MIC was performed with different concentrations of TiO_2, ZnO, and CuO NPS against Bacillus, all the three nanoparticles showed notable decline in the O.D. value recorded, at 400µg/ml concentration. Among the three nanoparticles TiO_2 and CuO NPs showed good antimicrobial effect against *bacillus.* ZnO showed the least effect. In the same way MIC was performed against E. coli. TiO_2 ZnO and CuO NPs showed similar kind of effect on *E. coli*. All the three NPs showed decline in the O.D value recorded at 200µg/ml concentration. TiO_2 and CuO NPs showed 45% decrease in the growth of *Bacillus* when compared to untreated sample, whereas ZnO NP showed only 25% decrease at 400µg/ml concentration. ZnO and CuO NPs showed 50 % decrease in the growth of E. coli when compared to untreated sample. TiO_2 NP showed the least effect, where it showed only 20 % decline in the growth of *Bacillus* when compared to untreated sample at 200µg/ml concentration.

Likewise, CFU/ml was calculated after growing the cells, treated with different concentrations of TiO_2, ZnO and CuO NPs in LB agar plate against *E. coli* and *Bacillus* cultures. Significant decrease in the CFU/ml calculated was observed at the concentrations 200µg/ml and 400µg/ml, when compared to the concentrations 50 and 100 µg/ml in both the

strains. When *E. coli* was treated with different concentration of NPs, TiO_2 NPs showed greater effect compared to ZnO NPs. When CFU /ml was performed against *Bacillus,* ZnO showed greater antimicrobial effect followed by CuO. Least effect was shown by TiO_2. At 200 µg/ml TiO_2 NP showed 60% decrease in the growth of *E. coli* when compared to untreated sample where as ZnO and CuO NPs showed 30% and 50 % decline. At 400µg/ml concentration, ZnO showed 97% decline in the growth of *Bacillus* when compared to untreated sample, followed by CuO which showed 95% decline in the growth of *E. coli*. Least effect was shown by TiO_2 NPs where only 45% decline was seen.

Growth curve of *E. coli* was monitored by treating the cells with different concentration of TiO_2 and CuO nanoparticles. It is observed that there is a slight decrease in the growth curve at 200µg/ml concentration in both the strains but not significant. In both the strains there was a decrease in the O.D value obtained at 200 µg/ml concentration. At 200 µg/ml concentration 15% decline in growth was observed when compared to untreated sample. At 200µg/ml 13% decline in the growth of *E. coli* was observed compared to untreated sample

The cell damage and cell membrane damage were checked by performing few biochemical tests like MTT assay and TBARS assay. MTT assay gives us the measure of viable cells. Viable cells react with MTT and forms a purple-coloured formazan (equivalent to viable cells). As the concentration of nanoparticles increased, a decline in colour intensity was seen. The colour intensity was least at 400µg/ml concentration compared to untreated samples and lower concentrations (100, 200µg/ml). When MTT was performed against *E. coli* ZnO NPs showed good antimicrobial activity followed by CuO NPs. When MTT was performed against *Bacillus* ZnO NPs showed good antimicrobial activity followed by TiO_2 NPs. At 400µg/ml concentration CuO showed 49% decline in the growth of *E. coli* when treated with untreated sample. Followed by ZnO which showed 45 % decline. Least effect was shown by TiO_2 NPs which showed only 34 % decline. At 400µg/ml concentration, ZnO NP showed 67% decline in growth of *Bacillus* when compared with untreated sample, followed by TiO_2 and CuO which showed 45% and 40% decline.

TBARS assay measures the amount of lipid peroxidation that had occurred in the cell. Lipid peroxidation occurs by the action of ROS on lipid cell membrane. So, the amount of lipid peroxidation occurred was directly proportional to the concentration of nanoparticles. CuO NPs exhibited greater lipid peroxidation activity in both the bacterial samples at 400µg/ml concentration followed by ZnO NPs and least activity was exhibited by TiO_2 NPs, when

compared to untreated samples and lower concentrations of NPs (100, 200µg/ml). CuO showed greater activity against *Bacillus* followed by ZnO and TiO_2 which showed 30% and 60% decline in peroxidation activity when compared to the activity of ZnO at 400µg/ml. CuO showed greater activity *followed* by ZnO and TiO_2 which showed 10% and 30% decline in peroxidation activity when compared to the activity of ZnO at 400µg/ml

The results of the experiments MIC, CFU/ml, growth curve, MTT assay and TBARS assay concludes that ZnO and CuO NPs showed good antimicrobial activity against *Bacillus,* when compared to TiO_2 NPs. ZnO and TiO_2 NPs showed good antimicrobial activity against *E. coli* when compared to CuO at higher concentrations (200 and 400µg/ml)

Overall, the results of this study highlight the potential of nanoparticles as antimicrobial agents, particularly for applications where conventional antibiotics and antimicrobial agents have limitations, such as drug-resistant microorganisms and biofilms. However, it is important to note that the use of nanoparticles as antimicrobial agents also raises concerns about their potential toxicity and environmental impact, which require careful consideration and evaluation. Further research is needed to fully understand the mechanisms of antimicrobial action of nanoparticles and to develop safe and effective strategies for their use in various applications.

CHAPTER 8

REFERENCES

References

1. Azam, A. (2012). Size-dependent antimicrobial properties of CuO nanoparticles against Gram-positive and -negative bacterial strains. *International Journal of Nanomedicine*, 3527. https://doi.org/10.2147/IJN.S29020
2. Azam, A., Ahmed, A. S., Oves, M., Khan, M. S., Habib, S. S., & Memic, A. (2012). Antimicrobial activity of metal oxide nanoparticles against Gram-positive and Gram-negative bacteria: A comparative study. *International Journal of Nanomedicine*, *7*, 6003–6009. https://doi.org/10.2147/IJN.S35347
3. Baek, Y.-W., & An, Y.-J. (2011). Microbial toxicity of metal oxide nanoparticles (CuO, NiO, ZnO, and Sb2O3) to Escherichia coli, *Bacillus* subtilis, and Streptococcus aureus. *Science of The Total Environment*, *409*(8), 1603–1608. https://doi.org/10.1016/j.scitotenv.2011.01.014
4. Cioffi, N., & Rai, M. (2012). *Nano-antimicrobials: Progress and prospects*.
5. Dadi, R., Azouani, R., Traore, M., Mielcarek, C., & Kanaev, A. (2019). Antibacterial activity of ZnO and CuO nanoparticles against gram positive and gram negative strains. *Materials Science and Engineering: C*, *104*, 109968. https://doi.org/10.1016/j.msec.2019.109968
6. *Doi:10.1016/j.pnsc.2008.01.026 | Elsevier Enhanced Reader*. (n.d.). https://doi.org/10.1016/j.pnsc.2008.01.026
7. Dutta, R. K., Nenavathu, B. P., Gangishetty, M. K., & Reddy, A. V. R. (2012). Studies on antibacterial activity of ZnO nanoparticles by ROS induced lipid peroxidation. *Colloids and Surfaces B: Biointerfaces*, *94*, 143–150. https://doi.org/10.1016/j.colsurfb.2012.01.046
8. Haghighi, F., Roudbar Mohammadi, S., Mohammadi, P., Hosseinkhani, S., & Shipour, R. (2013). Antifungal activity of TiO2 nanoparticles and EDTA on candida albicans biofilms. Infect.Epidemiol.Med., 1(1), 33-38. Retrieved from www.scopus.com
9. Hou, J., Wang, L., Wang, C., Zhang, S., Liu, H., Li, S., & Wang, X. (2019). Toxicity and mechanisms of action of titanium dioxide nanoparticles in living organisms. *Journal of Environmental Sciences*, *75*, 40-53. https://doi.org/10.1016/j.jes.2018.06.010
10. Hou, Jing, Xiangxue Wang, Tasawar Hayat, and Xiangke Wang. "Ecotoxicological Effects and Mechanism of CuO Nanoparticles to Individual Organisms."

Environmental Pollution 221 (February 1, 2017): 209–17. https://doi.org/10.1016/j.envpol.2016.11.066.

11. Iravani, Siavash. "Green Synthesis of Metal Nanoparticles Using Plants." Green Chemistry, Jan. 2011. The Royal Society of Chemistry, https://doi.org/10.1039/c1gc15386b.
12. Janaki, A. C., Sailatha, E., & Gunasekaran, S. (2015). Synthesis, characteristics and antimicrobial activity of ZnO nanoparticles. *Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy*, *144*, 17–22. https://doi.org/10.1016/j.saa.2015.02.041
13. Klink, M. J., Laloo, N., Leudjo Taka, A., Pakade, V. E., Monapathi, M. E., & Modise, J. S. (2022). Synthesis, Characterization and Antimicrobial Activity of Zinc Oxide Nanoparticles against Selected Waterborne Bacterial and Yeast Pathogens. *Molecules (Basel, Switzerland)*, *27*(11), 3532. https://doi.org/10.3390/molecules27113532
14. Lai, J., Huang, W., Chen, C., Tsao, I., Chien, F. C., Singh, B., & Liu, B. R. (2022). Effect of Size and Concentration of Copper Nanoparticles on the Antimicrobial Activity in Escherichia coli through Multiple Mechanisms. *Nanomaterials*, *12*(21). https://doi.org/10.3390/nano12213715
15. Li, R., Chen, Z., Ren, N., Wang, Y., Wang, Y., & Yu, F. (2019). Biosynthesis of silver oxide nanoparticles and their photocatalytic and antimicrobial activity evaluation for wound healing applications in nursing care. *Journal of Photochemistry and Photobiology B: Biology*, *199*, 111593. https://doi.org/10.1016/j.jphotobiol.2019.111593
16. Liao, S., Zhang, Y., Pan, X., Zhu, F., Jiang, C., Liu, Q., Cheng, Z., Dai, G., Wu, G., Wang, L., & Chen, L. (2019). Antibacterial activity and mechanism of silver nanoparticles against multidrug-resistant Pseudomonas aeruginosa. *International Journal of Nanomedicine*, *14*, 1469–1487. https://doi.org/10.2147/IJN.S191340
17. Lopes, L. Q. S., de Oliveira, P. S. B., de Souza Filho, W. P., de Almeida Vaucher, R., Giongo, J. L., Sagrillo, M. R., & Santos, R. C. V. (2019). Glycerol monolaurate nanocapsules for biomedical applications: In vitro toxicological studies. *Naunyn-Schmiedeberg's Archives of Pharmacology*, *392*(9), 1131–1140. https://doi.org/10.1007/s00210-019-01663-w
18. Priyanka, K. P., Sukirtha, T. H., Balakrishna, K. M., & Varghese, T. (2016). Microbicidal activity of TiO2 nanoparticles synthesised by sol–gel method. *IET Nanobiotechnology*, *10*(2), 81–86. https://doi.org/10.1049/iet-nbt.2015.0038

19. Rad, S. S., Sani, A. M., & Mohseni, S. (2019). Biosynthesis, characterization and antimicrobial activities of zinc oxide nanoparticles from leaf extract of Mentha pulegium (L.). *Microbial Pathogenesis*, *131*, 239–245. https://doi.org/10.1016/j.micpath.2019.04.022
20. Rajamma, R., Gopalakrishnan Nair, S., Abdul khadar, F., & Baskaran, B. (2020). Antibacterial and anticancer activity of biosynthesised CuO nanoparticles. *IET Nanobiotechnology*, *14*(9), 833–838. https://doi.org/10.1049/iet-nbt.2020.0088
21. Rao, M. C., Ravindranadha, K., & Rose Mary, T. (2013). Development of ZnO Nanoparticles for Clinical Applications. *J. Chem. Biol. Phys. Sci.*, *4*(1), 469–473. Scopus.
22. S. Roy, A., Parveen, A., R. Koppalkar, A., & Prasad, M. V. N. A. (2010). Effect of Nano—Titanium Dioxide with Different Antibiotics against Methicillin-Resistant Staphylococcus Aureus. Journal of Biomaterials and Nanobiotechnology, 01(01), 37–41. https://doi.org/10.4236/jbnb.2010.11005
23. Sawai, J., Kojima, H., Igarashi, H., Hashimoto, A., Shoji, S., Sawaki, T., Hakoda, A., Kawada, E., Kokugan, T., & Shimizu, M. (2000). Antibacterial characteristics of magnesium oxide powder. *World Journal of Microbiology and Biotechnology*, *16*(2), 187–194. https://doi.org/10.1023/A:1008916209784
24. Sirelkhatim, A., Mahmud, S., Seeni, A., Kaus, N. H. M., Ann, L. C., Bakhori, S. K. M., Hasan, H., & Mohamad, D. (2015). Review on Zinc Oxide Nanoparticles: Antibacterial Activity and Toxicity Mechanism. Nano-Micro Letters, 7(3), 219–242. https://doi.org/10.1007/s40820-015-0040-x
25. Swain, P., Nayak, S. K., Sasmal, A., Behera, T., Barik, S. K., Swain, S. K., Mishra, S. S., Sen, A. K., Das, J. K., & Jayasankar, P. (2014). Antimicrobial activity of metal-based nanoparticles against microbes associated with diseases in aquaculture. *World Journal of Microbiology and Biotechnology*, *30*(9), 2491–2502. https://doi.org/10.1007/s11274-014-1674-4
26. Vaishampayan, Ankita, and Elisabeth Grohmann. "Antimicrobials Functioning through ROS-Mediated Mechanisms: Current Insights." *Microorganisms* 10, no. 1 (December 28, 2021): 61. https://doi.org/10.3390/microorganisms10010061.
27. Van Acker, H., & Coenye, T. (2017). The Role of Reactive Oxygen Species in Antibiotic-Mediated Killing of Bacteria. *Trends in Microbiology*, *25*(6), 456–466. https://doi.org/10.1016/j.tim.2016.12.008

28. Wang, D., Lin, Z., Wang, T., Yao, Z., Qin, M., Zheng, S., & Lu, W. (2016). Where does the toxicity of metal oxide nanoparticles come from: The nanoparticles, the ions, or a combination of both? *Journal of Hazardous Materials*, *308*, 328–334. https://doi.org/10.1016/j.jhazmat.2016.01.066
29. Wang, L., Hu, C., & Shao, L. (2017). The antimicrobial activity of nanoparticles: Present situation and prospects for the future. *International Journal of Nanomedicine*, *12*, 1227–1249. https://doi.org/10.2147/IJN.S121956
30. Want, M. Y., Yadav, P., Khan, R., Chouhan, G., Islamuddin, M., Aloyouni, S. Y., Chattopadhyay, A. P., AlOmar, S. Y., & Afrin, F. (2021). Critical Antileishmanial in vitro Effects of Highly Examined Gold Nanoparticles. *International Journal of Nanomedicine*, *16*, 7285–7295. https://doi.org/10.2147/IJN.S268548

Document Information

Analyzed document	Krupa.docx (D166412197)
Submitted	2023-05-10 02:38:00
Submitted by	Priti Gawas
Submitter email	biotech.priti@unigoa.ac.in
Similarity	0%
Analysis address	biotech.priti.unigoa@analysis.urkund.com

Sources included in the report

SA **karthik magnetic.doc**
Document karthik magnetic.doc (D24662447) 1