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Study on antimicrobial activity of ZrO_2 & CeO_2 nanoparticles

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DECLARATION STUDENT

I hereby declare that the data presented in this Dissertation report entitled, "**Study on antimicrobial activity of ZrO_2 & CeO_2 nanoparticles**" is based on the results of investigations carried out by me in the Biotechnology at the School of Biological Sciences and Biotechnology, Goa University under the supervision of Dr. Meghanath Prabhu and the same has not been submitted elsewhere for the award of a degreeor diploma by me. Further, I understand that Goa University or its authorities will not be responsible for the correctness of observations / experimental or other findings given the dissertation.

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This is to certify that the dissertation report "**Study on antimicrobial activity of ZrO_2 & CeO_2 nanoparticles**" is a bonafide work carried out by Mr. Tanmay Naik Desai under my supervision in partial fulfilment of the requirements for the award of the degree of Masters of Science in the Discipline of Biotechnology at the School of Biological Sciences and Biotechnology, Goa University.

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PREFACE

The realm of nanotechnology has unleashed a paradigm shift in various fields, and its impact on biomedicine is particularly noteworthy. Among the manifold applications of nanotechnology in healthcare, the utilization of nanoparticles as potent antimicrobial agents stands out as a promising frontier. This preface delves into the captivating world of nanoparticles and their remarkable antimicrobial prowess.

In recent years, the escalating threat posed by antimicrobial resistance has necessitated the exploration of novel therapeutic strategies. Conventional antibiotics are encountering dwindling efficacy against an increasingly diverse array of pathogens. In this milieu, nanoparticles emerge as a beacon of hope, offering a multifaceted approach to combat microbial threats. Their unique physicochemical properties, including high surface area-to-volume ratio, tunable surface chemistry, and ability to penetrate microbial membranes, render nanoparticles as formidable contenders in the fight against infections.

This compendium delves into the multifaceted facets of nanoparticle-based antimicrobial strategies, elucidating their mechanisms of action, synthesis methodologies, and potential applications across diverse biomedical contexts. From metallic nanoparticles like silver and copper to organic nanoparticles such as liposomes and dendrimers, the diversity of nanoparticle platforms underscores their versatility in antimicrobial interventions.

Moreover, this compilation explores the intricate interplay between nanoparticles and microorganisms, unraveling the molecular mechanisms underpinning their antimicrobial activity. Insights into nanoparticle-microbe interactions not only deepen our understanding of microbial pathogenesis but also pave the way for the rational design of next-generation antimicrobial agents.

Furthermore, this preface underscores the imperative of translational research in harnessing the full therapeutic potential of nanoparticle-based antimicrobials. Challenges such as biocompatibility, nanoparticle stability, and regulatory hurdles necessitate concerted efforts from interdisciplinary teams to expedite the translation of bench side discoveries to bedside interventions.

In essence, this compendium serves as a testament to the transformative impact of nanotechnology on combating microbial infections. As we navigate the intricate landscape of antimicrobial resistance, nanoparticle-based strategies offer a glimmer of hope in our quest for innovative therapeutic solutions. Through collaborative endeavours and relentless pursuit of knowledge, we embark on a journey towards a future where nanoparticles stand as stalwart guardians against microbial adversaries.

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I extend my heartfelt gratitude to my family for their continuous support and encouragement during the completion of this dissertation. Their unwavering belief in me has been a source of strength throughout this journey. Additionally, I am grateful to God for enabling me to overcome challenges and reach this significant milestone. Their presence, prayers, and blessings have been invaluable to me during this process.

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Abbreviations

℃	Celius
mg	milligram
mm	millimetre
ml	millilitre
MH	Muller Hinton
ROS	Reactive Oxygen Species
g	Grams
%	Percentage
SEM	Scanning Electron Microscope
UV	Ultra violet
µl	microlitre

Abstract

In this work we investigated the antimicrobial activity of metal oxide nanoparticles. Zirconium oxide (ZrO_2) and Cerium oxide (CeO_2) nanoparticles and their composite with other metal oxides. Nanoparticles are known to exhibit unique physical and chemical properties that can enhance their antimicrobial activity making them promising candidates for applications for applications in various field, including medicine, agriculture, and the food industry. The antimicrobial activity of the nanoparticles was evaluated against different pathogenic bacterial strains using the broth assay method.

We investigated the antimicrobial activity against *Staphylococcus aureus, Pseudomonas aeruginosa, E. coli, Proteus vulgaris, Salmonella typhi, Shigella* sp., and *Klebsiella* sp. ZrO_2 showed good antimicrobial activity against *Staphylococcus aureus, Pseudomonas aeruginosa*, *E. coli* and *Salmonella typhi* and *Klebsiella* sp.

For, ZrO_2-(10%) Mn(X) composite, showed good activity for *Shigella* sp and *Klebsiella* sp. whereas for ZrO_2-(11%) V_2O_5 composite, showed very high antimicrobial activity against *Shigella* sp, and moderate against Proteus *vulgaris* and *Pseudomonas aeruginosa*. Both ZrO_2-(5%) Ag_2O and ZrO_2-(10%) CuO showed high antimicrobial activity against all the bacterial strains. Whereas CeO_2 and ZrO_2-(15%) CeO_2 showed no activity or very less activity against the pathogenic bacterial strain at 2mg/mL concentration. Moderate antimicrobial activity was observed for CeO_2-(5%) CuO and very high antimicrobial activity was observed for CeO_2-(5%) Ag_2O nanoparticles composites.

For ZrO_2-CuO, these nanoparticles were synthesized from 2 different methods that is impregnation and hydrothermal methods. We tested their antimicrobial activity both in

absence and presence of visible light and UV treatment. Against *Staphylococcus aureus Proteus vulgaris, Salmonella typhi, Shigella* sp these nanoparticles were tested. Here,

ZrO_2-CuO synthesized by showed good antimicrobial activity in the absence of any visible light and UV treatment. But for visible light irradiation and UV treatment, ZrO_2-CuO nanoparticles synthesized by hydrothermal showed better antimicrobial activity in presence of visible light irradiation and UV treatment.

Overall, ZrO_2 and CeO_2 nanoparticles showed promising antimicrobial activity that can be used for various applications.

Chapter I
Introduction

Introduction

Background:

In the current world, there is an increase in drug-resistant bacteria, and at the same time, there is a decrease in efforts to find new drugs to use against these drug-resistant pathogenic bacterial strains. There is a need to develop novel strategies to combat these pathogens swiftly and effectively. An alternative to antibiotics is the use of nanoparticles (Kashef et al., 2017).

Nanoparticles are tiny materials with special chemical and physical characteristics that enable them to interact with microbes. The antibacterial action of nanoparticles has been shown against various pathogens, including viruses, bacteria, and fungi, making it one of the most promising uses for these particles. The science of treating and preventing infectious illnesses might undergo a revolution by applying nanoparticles as antimicrobial agents. Using nanotechnology to create antibacterial agents is one such option. These characteristics render nanoparticles as viable contenders for the creation of novel antibacterial agents. Nanotechnology in medicine has created new research opportunities, and one of the most interesting areas of study is the antibacterial effect of nanoparticles. Nanoparticles have various applications in this field mass spectroscopy, analytical chemistry, therapeutic applications, and drug delivery (Kemp et al., 2009).

The antimicrobial activity of nanoparticles is due to their ability to generate reactive oxygen species (ROS), ion-releasing ability and interaction with bacterial membranes. ROS is produced when nanoparticles interact with water and release hydroxyl radicals. Light irradiation enhances this process by ROS generation by exciting the electrons in the

nanoparticles. Then these electrons interact with oxygen molecule to produce superoxide radicals. (Nisar et al., 2019).

Zirconium dioxide (ZrO_2) and Cerium dioxide (CeO_2) nanoparticles have demonstrated remarkable antimicrobial potential due to their unique physicochemical properties (Nisar et al., 2019) making them ideal candidates for combating microbial infections. Additionally, their photocatalytic activity under light irradiation presents an intriguing avenue for enhancing their antimicrobial efficacy.

We intend to investigate if ZrO_2 and CeO_2 nanoparticles have broad-spectrum antimicrobial activity and if their composite with other nanoparticles can increase the antimicrobial activity. And irradiating nanoparticles with light affects the antimicrobial activity of these nanoparticles.

Aim:

Study on antimicrobial activity of ZrO_2 & CeO_2 nanoparticles

Objectives:

Assessing the antimicrobial activity of Zr and Ce oxide and their composite nanoparticles against Gram-positive and Gram-negative pathogenic bacteria.

Investigating the influence of Zr nanoparticle synthesis methods on the antimicrobial activity against Gram-positive and Gram-negative pathogenic bacteria in the presence and absence of visible light and UV light.

Hypothesis: - This study aimed to investigate the antimicrobial activity of ZrO_2 and CeO_2 nanoparticles. ZrO_2 and CeO_2 have demonstrated antimicrobial activity against several microbial strains. This hypothesis is based on previous literature suggesting antimicrobial activity shown by metal oxide nanoparticles. The composite nanoparticles will have a higher antimicrobial activity than the single nanoparticles. Irradiation with visible light and treatment with UV light will increase the antimicrobial activity of these nanoparticles.

Scope

The research will focus on finding the best nanoparticle composite with the best antimicrobial activity, which will best method to synthesize them so they be as small as possible. The effect of light on the antimicrobial activity of nanoparticles. All this study will help us to find cost-efficient method for synthesis of these nanoparticles and finding best way to utilize it antimicrobial activity. So, we can make practical application on this.

Future studies must be done on the find exact mechanism for this nanoparticle's antimicrobial activity. And study it’s cytotoxicity so we can use them in medical treatment and potentially replace antibiotics.

Chapter II Literature Review

Literature Review

Synthesis of CeO_2 Nanoparticle

CeO_2 nanoparticle has been synthesized and its properties are controlled by a variety of processes, including solvothermal, hydrothermal, aqueous precipitation, reversed micelles, thermal breakdown, and flame spray procedures. These nanoparticles, which have hydrophilic or hydrophobic qualities, can be produced bare or coated with protective materials. Biocompatible CeO_2 nanoparticles that are appropriate for biological applications have been methodically generated in either pure water or with the use of protecting agents such as glucose, cyclodextrin, polyethylene glycol, dextran, polyacrylic acid, and so on. Their solubility, size, surface condition, charge, structural arrangement, and morphology are all strongly influenced by the synthesis techniques used, which in turn affects the catalytic activities of the products. The characterization of nanoparticles can be done using UV-vis spectroscopy, TEM, FTIR, and energy-dispersive X-ray detector.

Synthesis of ZrO_2 Nanoparticle

The synthesis of ZrO_2 nanoparticles can be done through various methods which are the hydrothermal method, sol-gel method, Ultrafast laser, and coprecipitation method. Each method is used to produce specific morphologies and crystalline structures. One of the approaches is to synthesize mixed metal ZrO_2, in this different synthesis routes are employed. For instance, the cubic form of zirconium oxide (ZrO_2) nanoparticles can be synthesized through a reaction involving xylene, zirconium oxychloride, and N-acetyl-N, N, N-trimethylammonium bromide, followed by refluxing and calcination. Similarly,

mixed monoclinic and cubic phase nanoparticles are prepared using zirconium oxychloride, 1-hexanol, and oxalic acid, with subsequent washing and drying processes. Additionally, ultrafast laser ablation and wire explosion processes offer alternative routes to obtain metallic zirconium nanoparticles and zirconium nitride nanoparticles, respectively. The sol-gel method, hydrothermal synthesis, and coprecipitation method provide versatile routes for synthesizing zirconium nanoparticles with organic precursors or in combination with other metal oxides, leading to tailored morphologies and enhanced properties. These diverse synthesis approaches offer researchers a wide range of options to tailor zirconium metal oxide nanoparticles for various applications, from catalysis to nanocomposite materials (Arshad et al., 2022).

Antimicrobial Activity of Nanoparticle

Nanoparticle exhibits antimicrobial activity through these mechanisms which are damaging the DNA, cell membrane disturbance and enzyme inactivation. All this is due to the nanoparticle activity of producing Reactive oxygen species. ROS causes oxidation of lipids and proteins, disrupting the integrity and functions of the cell membrane, formations of protein-protein cross-links impair enzymatic activities, cause oxidative damage to nucleic acids which all leads to cell malfunctioning which ultimately causes cell death (Kashef et al., 2017).

The four forms of reactive oxygen species (ROS) are singlet oxygen (O_2), hydrogen peroxide (H_2O_2), hydroxyl radical ($\cdot OH$), and superoxide radical (O^{-}_{2}). Each type of ROS has a distinct level of activity and dynamics. Restructuring, defect sites, and oxygen vacancies in the crystal are the primary sources of ROS formation. The generation and elimination of reactive oxygen species (ROS) in bacterial cells are normally balanced. On

the other hand, when ROS generation is high, the cell's redox balance Favors oxidation. Oxidative stress is created by this imbalanced situation, harming the constituent parts of bacterial cells (Wang et al., 2017).

The electrons (e−) in the valence band are stimulated and transition to the conduction band when metal oxide nanoparticles (Nanoparticles), like zinc oxide and titanium oxide, absorb light irradiation energy greater than or equal to the band gap. This results in a corresponding hole in the valence band (H+) and produces highly reactive reactants (electrons and holes) on the surface of and inside the catalytic material. After interacting with H_2O or OH−, H+ sticks to ZnO's surface where it is oxidized to form the hydroxyl radical (·OH). Similar to this, the hydroxyl radical is converted to the superoxide radical (O_{-2}) following electrical contact with O_2 and adhesion to the ZnO surface (Jian Yu et al 2015).

The impact of nanoparticles on bacterial cell barriers, particularly cell walls and membranes, is a significant factor in determining how well NP-mediated antimicrobial activity works (Roy et al., 2023). Nanoparticle adsorption mechanisms are significantly impacted by the differences in composition between the cell walls of Gram-positive and Gram-negative bacteria. Gram-negative bacteria establish a strong barrier that only allows macromolecules to get through because their cell walls are made of lipopolysaccharides (LPS), lipoproteins, and phospholipids. Teichoic acid and peptidoglycan layers (Hyldgaard et al., 2014), on the other hand, are present in Gram-positive bacteria, coupled with more porous structures. This allows foreign molecules, such as nanoparticles, to penetrate and damage cell membranes, ultimately leading to death (Imran et al., 2022).

Nanoparticles exhibit greater activity against Gram-positive strains due to their structural composition and higher negative charge on the cell wall surface. The antimicrobial effects

of nanoparticles, like zinc oxide and nanodiamonds, vary based on bacterial cell composition and specific interactions. For instance, zinc oxide's action is influenced by bacterial cell components like LPS, affecting nanoparticle adhesion and ion flow regulation across cell membranes (Kumar et al., 2017). Moreover, nanoparticles can form covalent bonds with bacterial cell wall proteins, disrupting key enzymes and metabolic processes, and ultimately leading to cell death (Slavin et al., 2017).

Nanoparticle-induced alterations in bacterial membranes and walls result in structural changes, cytoplasmic leakage, and impaired cellular functions, including respiration and cell communication, offering insights into the mechanisms underlying nanoparticle-mediated bacterial inactivation. Overall, the interplay between nanoparticles and bacterial cell barriers shapes the varied and intricate mechanisms driving antimicrobial activity.

Diffusion plays a pivotal role as nanoparticles infiltrate bacterial cells and generate reactive oxygen species (ROS) within. For instance, graphene oxide-iron oxide nanoparticles, and silver nanoparticles create hydroxyl radicals and superoxide ions, penetrating cells to inactivate bacteria effectively, particularly in the case of MRSA. These ROS, including hydrogen peroxide and hydroxyl radicals, exhibit prolonged lifespans allowing them sufficient time to diffuse through bacterial cell membranes.

Nanoparticles also exert their antimicrobial action through adsorption, where metal ions from nanoparticles bind to bacterial cell membranes, disrupting their function. Silver ion nanoparticles cause protein coagulation, while gold nanoparticles interact with membranes based on surface charges, affecting the bilayer's electric features. Additionally, smaller Ag nanoparticles can pass through membrane pores, enhancing their antimicrobial potency.

Applications of Nanoparticles

Various medical applications harness the antimicrobial prowess of nanoparticles to combat infections and enhance healing across diverse areas. Implantable devices, ranging from heart valves to catheters, utilize nanoparticles coatings to prevent bacterial colonization and infections. By incorporating titanium oxide or nano polymers, these coatings thwart bacterial adhesion, preventing inflammation and enhancing the biocompatibility of the implants. Additionally, sustained nanoparticles release from neurosurgical catheters significantly curtails bacterial growth, reducing infection risks.

Wound dressings, crucial for wound healing, benefit from nanoparticles integration. Nanoparticle silver and polymeric mixtures imbue wound dressings with high antibacterial potency against various pathogens, accelerating wound closure and minimizing infection risks. Nanoparticles-infused dressings simulate skin characteristics, fostering fibroblast growth, and epithelial tissue formation, and reducing scarring while exhibiting anti-inflammatory and antibacterial effects.

In orthopaedic procedures like joint replacements, PMMA-based bone cement infused with silver nanoparticles proves promising in diminishing arthroplasty-related infections, even against antibiotic-resistant strains like MRSA. Dental materials, such as brackets coated with CuO and ZnO nanoparticles, effectively inhibit bacterial growth but may affect aesthetics. Further innovations involve nanoparticle-enhanced root canal treatments and maxillofacial prostheses that use nano-titanium dioxide for antibacterial effects under light exposure.

Beyond conventional antibiotic approaches, nanoparticles offer a dual advantage by exhibiting bactericidal properties while aiding in drug delivery. They facilitate targeted drug transport, enabling controlled antibiotic release for treating conditions like

osteomyelitis. nanoparticles-based drug delivery systems, like CS/fucoidan nanoparticles or CS-coated alginate nanoparticles, showcase enhanced drug permeability and accumulation, offering a blend of antibacterial and therapeutic benefits with reduced side effects.

Chapter III Methodology

3.1. Nanoparticles and their characterization:

3.1.1Nanoaparticles

The various types of nanoparticles (Table 3.1) were kindly provided by Dr. Rohan Kunkalekar, from the School of Chemical Sciences at Goa University.

Table 3.1. Different types of nanoparticles produced from Zr and Ce (oxides and composite nanoparticles) and their respective synthesis methods.

Metal	Nanoparticles	Synthesis Method
Zirconium (Zr)	ZrO2-(11%) V_2O_5	Coprecipitation
	ZrO_2-(10%) Mn(X)	Coprecipitation
	ZrO_2	Coprecipitation
	ZrO_2-(15%) CeO_2	Coprecipitation
	ZrO_2-(10%) CuO	Coprecipitation
	ZrO_2 -(10%) Ag_2O	Coprecipitation
	ZrO_2-CuO	Impregnation
	ZrO_2-CuO	Hydrothermal
Cerium (Ce)	CeO_2	Coprecipitation
	CeO_2-(5%) Ag_2O	Coprecipitation
	CeO_2-(5%) CuO	Coprecipitation

Note: (X) indicates known oxidation state of Mn

3.2 Characterization:

3.2.1: UV-Visible spectrum analysis

Two milligrams of nanoparticles were added to 20ml distilled water making the final concentration 0.1mg/ml. UV-visible spectrum of this suspended nanoparticles was measured between the range of 200nm – 800nm using Shimadzu UV-2450 UV Visible Spectrophotometer.

3.2.2: SEM analysis

Three milligram of nanoparticle samples were weighed on a 1×1 cm glass slide and analysis were done by ZESSIS EVO 18 scanning electron microscope (SEM)Prior and FEI Apreo LoVac field emission electron microscope.

3.3 Pathogenic Bacterial Cultures

The pathogenic bacterial cultures, including *Staphylococcus aureus, Pseudomonas aeruginosa, E. coli, Proteus vulgaris, Salmonella Typhi, Shigella* sp., and *Klebsiella* sp., were kindly provided by Prof. Savita Kerkar from the School of Biological Sciences and Biotechnology at Goa University. These cultures were sub cultured on Nutrient Agar and then in Mueller Hinton (MH) Broth for future experiments.

3. Antimicrobial activity of nanoparticles

3.1 Preparation of inoculum

Above mentioned (section 3.3) pathogenic bacterial cultures were sub-cultured by inoculating a loopful of culture taken from nutrient agar slants in 5ml of MH Broth. These tubes were kept for incubation at 37°C for 18 hours.

3.2 Antimicrobial Activity of Nanoparticles using Broth Assay

To the 1ml sterile MH broth media, 2 mg of nanoparticles and 10 µl of the bacterial inoculum were added respectively. The test tubes were then incubated overnight at 37°C in an incubator shaker, (Ti 90E Tempo bacteriological incubator) for approximately 18 hours. Post-incubation, the absorbance of each culture was measured at 595 nm using a BIO-RAD iMark ELISA plate reader. Control test tubes were maintained in the same fashion for each culture without adding nanoparticles to it.

3.2 Antimicrobial activity of nanoparticles under the irradiation of visible light

Two milligrams of nanoparticles were added to 1 ml of sterile media in a test tube. Ten microlitres of an overnight-grown culture inoculum were added to the test tube. The test tube was subsequently irradiated with visible light for 30 minutes using a Lelesil Innovative Systems chamber. Following irradiation, the test tubes were incubated in a shaker incubator at 37°C for 18 hours. The next day, the cultures were assessed for growth by measuring their absorbance at 595 nm using an ELISA plate reader. Control test tubes were maintained in the same fashion for each culture without adding nanoparticles to it.

3.3 Antimicrobial activity of UV light-treated nanoparticles

Inside a biosafety cabinet, 2 mg of nanoparticles were first added to individual test tubes containing sterile MH broth media. The tubes were kept open and then exposed to UV light irradiation within the Biosafety cabinet for 30 minutes. Following irradiation, 10µl of overnight-grown bacterial inoculum was carefully added to each test tube respectively and kept on a shaker incubator for overnight incubation. The next day, absorbance was taken at 595nm using an ELISA plate reader. Control test tubes were maintained in the same fashion for each culture but without having nanoparticles.

3.4 Live/Dead Assay for bacterial viability

This assay was performed using the L13152 Invitrogen LIVE/DEAD Baclight Bacterial Viability Kit assay. After Expt. 3.1 from above, 1 ml of each culture treated with nanoparticles was taken in clean micro centrifuge tubes. And centrifuged at 10000 rpm for 10 minutes at 25°C in Roto spin TARSONS centrifuge. Afterward, supernatant was discarded and 1 ml of 0.85% of saline was added to resuspended the pellet. Again, it was centrifuged at 10000 rpm for 10 minutes at 25°C and supernatant was discarded (this step was repeated one more time). After discarding the supernatant 500 µl of 0.85 % of saline solution was added and pellet was resuspended. Finally, 60 µl of culture suspension for each culture treated with nanoparticles was taken in sterile microcentrifuge tube.

Working solution was prepared by dissolving contents of Component A pipet (SYTO 9 dye) and Component B pipet (Propidium iodide) in common 5ml filter sterile distilled water. And 60 µl of working solution was added into microcentrifuge tube which was prepared earlier. Reading was taken using Shimadzu RF- 6000 spectrofluorometer, excitation wavelength provided at 470nm and emission spectrum was taken between 490 nm – 700 nm.

Chapter IV
Results
and
Discussion

4.1. Characterization of nanoparticles

4.1.1 SEM Analysis

Fig 4.1.1.1. SEM images of nanoparticles were examined for the structural and morphological features of nanoparticles samples. In Fig. 1 a), b), shows that the nanoparticles are roughly spherical in nature, and in Fig. 1. c), d), shows that these nanoparticles have a rod-like shape. All the nanoparticles are agglomerated in nature and their size in between 75nm to 150nm.

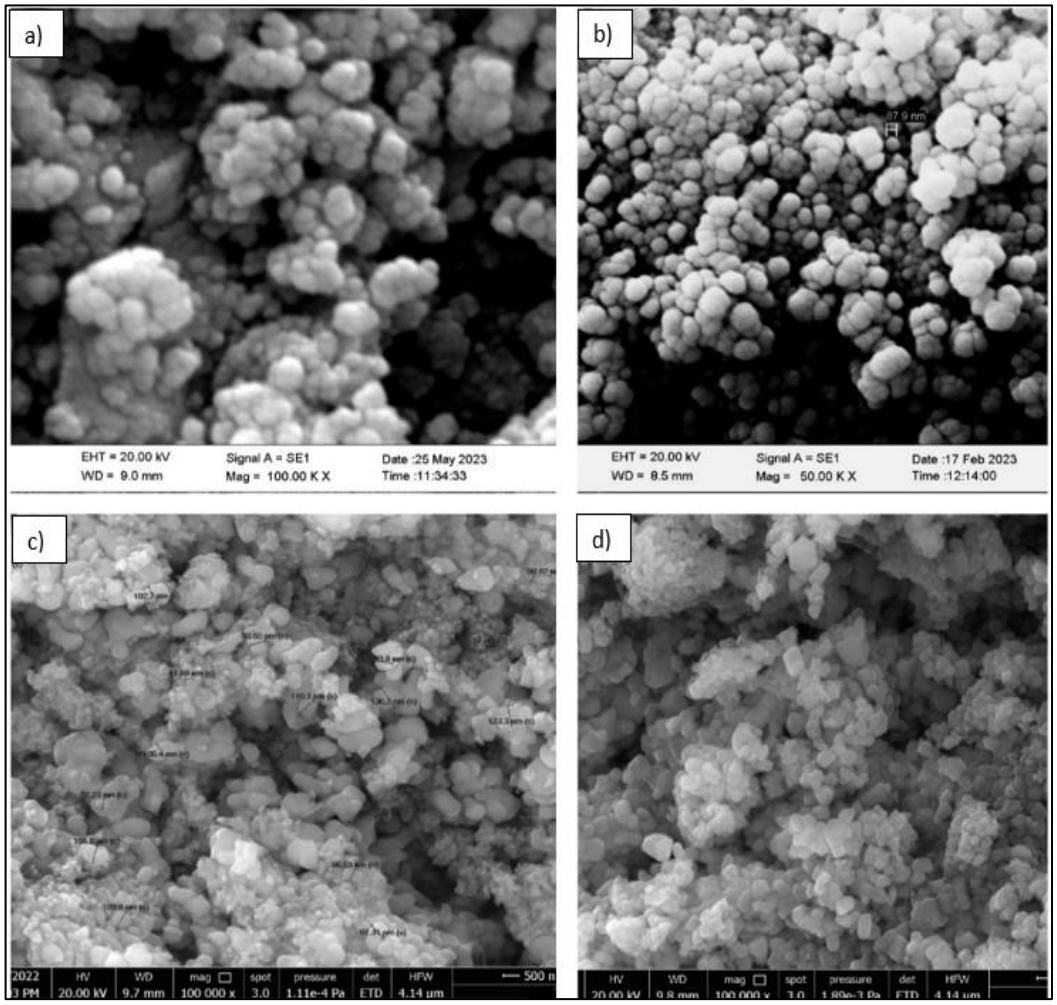

Fig. 1. SEM analysis of nanoparticles a) ZrO_2-(15%) CeO_2 b) ZrO_2-(11%) V_2O_5 c) ZrO_2-(10%) CuO d) ZrO_2

4.1.2 UV- Visible Spectroscopic Analysis

In UV-visible analysis the peak absorption by CeO_2-(5%) Ag_2O was observed at 259.5nm. This correlates with previous observations where the peak for CeO_2 nanoparticles was around 275nm to 330 nm (Kabure et al., 2021) (Fig. 2.a).

For ZrO_2-Mn(X) nanoparticles the peak was observed at 278nm for ZrO_2-Mn(X) nanoparticles in UV-visible spectrum analysis (Fig. 2.b). The peak shifted toward a shorter wavelength due to the presence of Mn doping in the nanoparticles which was also observed during the previous study reported by Chang& Doong.

The peak for ZrO_2-(5%) CuO was observed at 306nm in UV-Visible analysis (Fig.2. c). The peaks for ZrO_2 nanoparticles at an absorption range of 300nm-350nm. It can be analyzed that these are evenly distributed nanoparticles and they are nanosized. (Abbas, 2019).

The peak for ZrO_2-(10%) Ag_2O was observed at 385nm in UV-visible analysis (Fig.2. d). As this peak absorption for Ag_2O nanoparticles was observed between 370 nm to 410 nm in the previous studies done by Pandey.

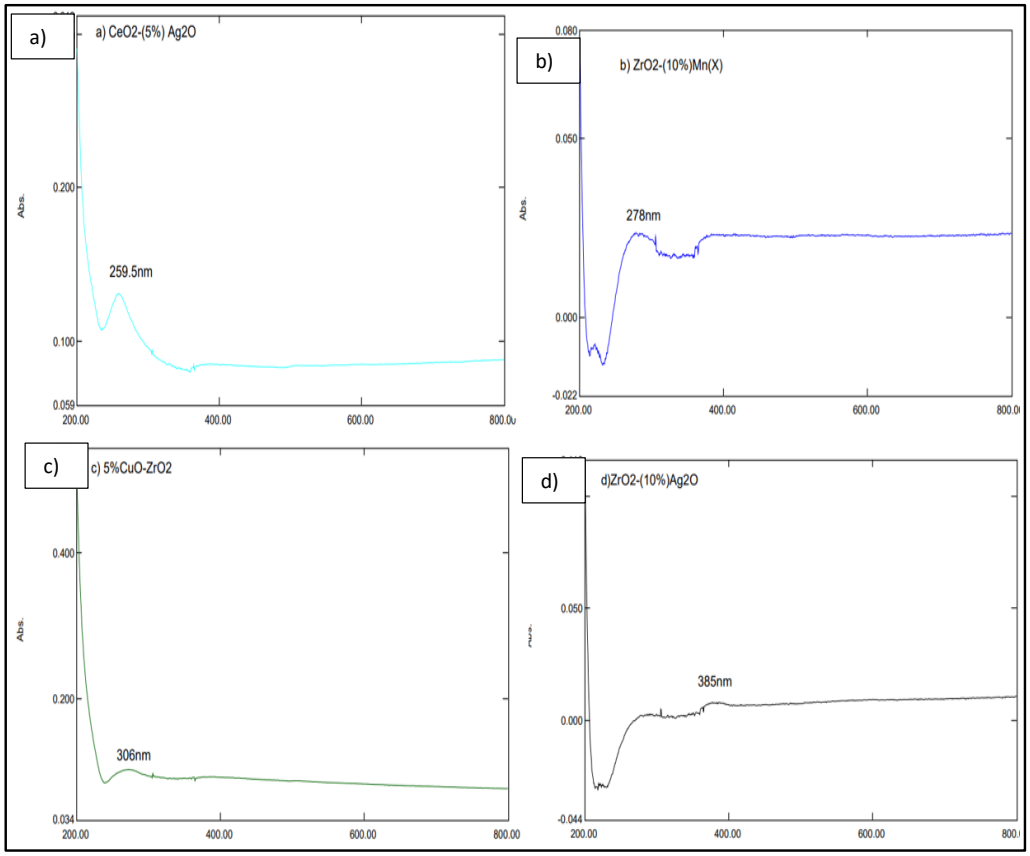

Fig 2. UV-Visible spectrum analysis of nanoparticles between the range of 200nm to 800nm a) CeO_2-(5%) Ag_2O, b) ZrO_2-(10%) Mn(X), c) ZrO_2-(5%) CuO d) ZrO_2-(10%) Ag_2O

For ZrO_2-V_2O_5 nanoparticles the peak was at 258nm. V_2O_5 nanoparticle doping might have shifted the peak absorption in a shorter UV region. (Molli et al., 2016). This might be the reason for this peak (Fig. 3. e).

The peak absorption was observed at 391nm for ZrO_2-(15%) CeO_2. For the composite of ZrO_2-CeO_2 composite, the peak absorption was observed between 370nm to 410nm in previous study (Taniguchi et al., 2010) (Fig. 3. f).

The peak absorption was observed at 266 nm for CeO_2-(10%) CuO nanoparticles. The peak absorption was observed for CeO_2 nanoparticles is between 370 nm to 400 nm in previous literature (Nurhasanah et al., 2018) (Fig. 3. g).

The peak absorption observed was at 278nm for CeO_2 nanoparticles. The peak absorption is observed in range of 370 nm to 400 nm for CeO_2 nanoparticles which confirms that it is in the nano range (Nurhasanah et al., 2018) (Fig. 3. h).

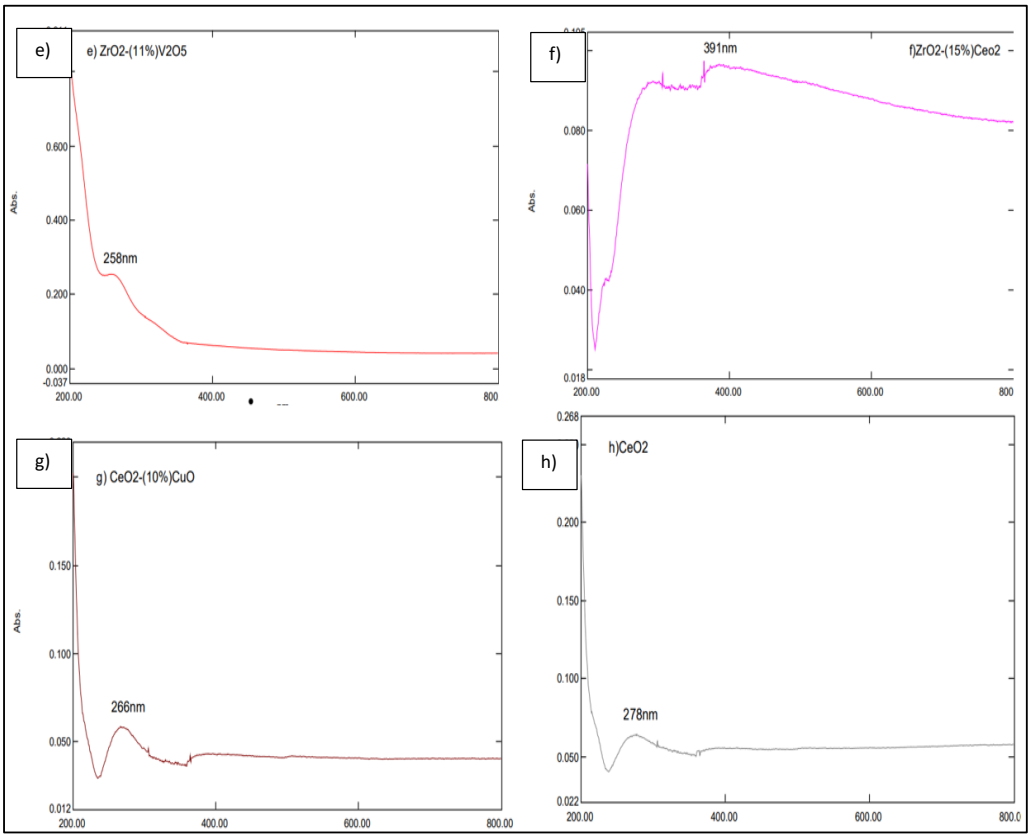

Fig. 3. UV-Visible spectrum analysis of nanoparticles between the range of 200nm to 800nm e) ZrO_2-(11%) V_2O_5 f) ZrO_2-(15%) CeO_2 g) CeO_2-(10%) CuO h) CeO_2

4.2 Antimicrobial Activity of Nanoparticles

4.2.1. Antimicrobial Activity of Zirconium oxide and Cerium oxide and their composites

ZrO_2 Nanoparticles

At 2mg/ml concentration, ZrO_2 showed antimicrobial activity against all the cultures. The highest growth inhibition was observed against *E. coli* (67.42±0.5%) followed by *Staphylococcus aureus* (52.82±0.5%) and *Salmonella typhi* (59.81±0.4%). Conversely, lower inhibition was seen against *Pseudomonas aeruginosa* (14.49±0.1%), *Proteus vulgaris* (12.26±0.9%), *Shigella* sp. (30.47±1.1%), and *Klebsiella* sp. (46.36±1%) (Fig. 4).

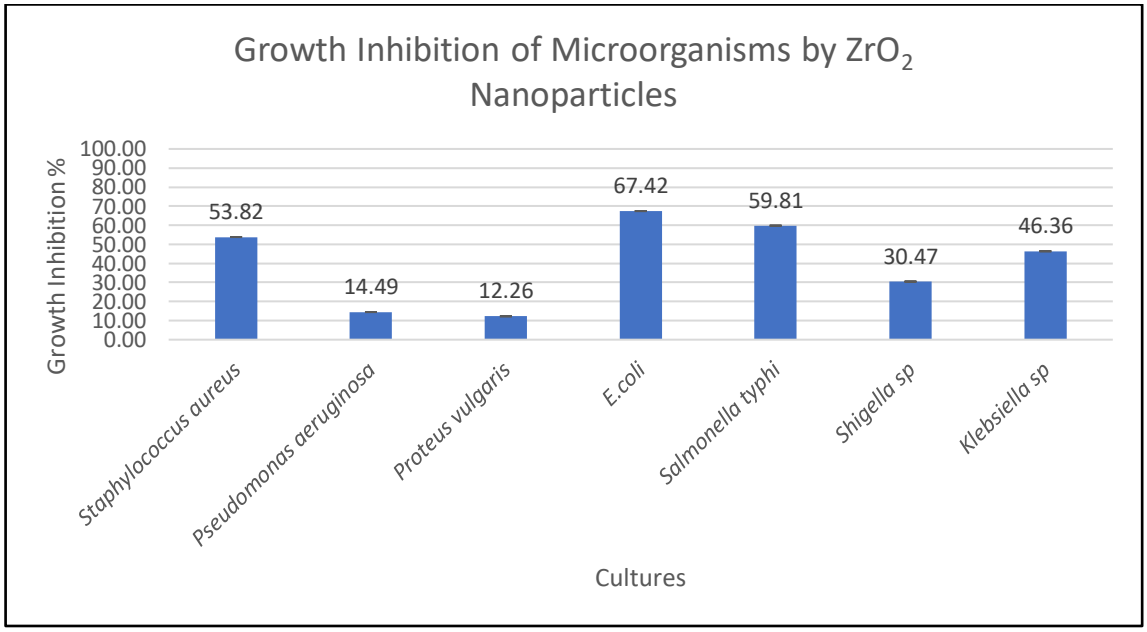

Fig. 4. Antibacterial Activity of ZrO_2 Nanoparticles: Growth Inhibition Percentage (error bar indicate std. error).

In a previous study (Skłodowski et al., 2023), the antimicrobial activity of ZrO_2 and glutamic acid functionalized ZrO_2 nanoparticle activity was tested against *Rothia mucilaginosa, Rothia dentocariosa, Streptococcus mitis*, and *Streptococcus mutans* at concentrations from 0-600µg/ml. In this study, glutamic acid functionalized ZrO_2 showed better antimicrobial activity than ZrO_2 as it was easily attached to the outer membrane of microorganisms. *Staphylococcus aureus* (52.82±0.5%) and *Salmonella Typhi* (59.81±0.4%) showed moderate sensitivity to ZrO_2, these findings can be seen in previous studies.

Pseudomonas aeruginosa, Proteus vulgaris, Shigella sp.*, and Klebsiella* sp. displayed lower susceptibility to ZrO_2, and showed lower sensitivity. These variations in sensitivity could be attributed to differences in bacterial cell wall composition, membrane permeability, and mechanisms of antimicrobial resistance. Previous studies have also reported similar trends, with certain bacterial species exhibiting reduced sensitivity to ZrO_2 nanoparticles due to intrinsic resistance mechanisms (Poole, 2012).

ZrO_2-(10%) Mn(X) nanoparticles

At 2mg/ml concentration, ZrO_2 - Mn(X) (10%) showed highest antimicrobial activity against *Shigella* sp. (52.32±0.8%), followed by *Klebsiella* sp. (48.47±0.5%), *Salmonella typhi* (30.52±0.7%), *Pseudomonas aeruginosa* (28.83±0.1%), *E. coli* (25.15±1.1%), *Proteus vulgaris* (22.21±0.2%), least growth inhibition was seen in *Staphylococcus aureus* (19.71±0.4) (Fig. 5).

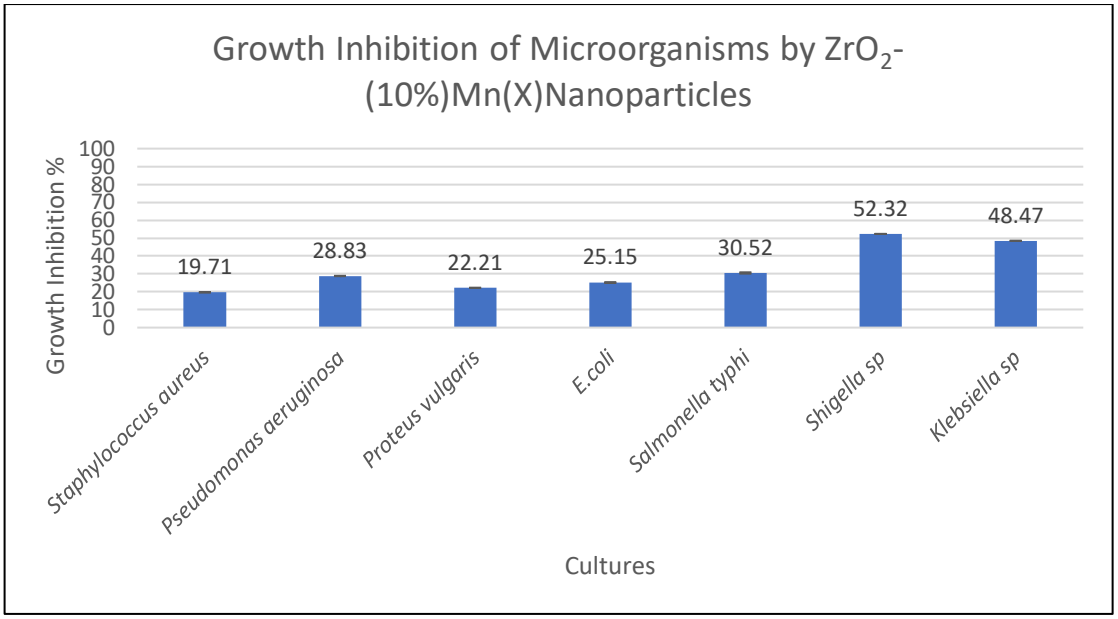

Fig. 5 Antibacterial Activity of ZrO_2-(10%) Mn(X) Nanoparticles: Growth Inhibition (error bar indicate std. error)

Shigella sp., and *Klebsiella* sp. are most sensitive for ZrO_2-Mn(X) while for ZrO_2, E. *coli* showed the highest growth inhibition. For *Staphylococcus aureus* and *E. coli,* there was a decrease in growth inhibition percentage. This suggests us that Mn(X) might have hinder the activity of ZrO_2. ZrO_2 nanoparticles often exert their antimicrobial effects by generating reactive oxygen species (ROS) that damage bacterial membranes. When mixed with other metal oxides, these additional oxides might compete with ZrO_2 for the precursors or reactants needed for ROS generation. This competition can limit the overall ROS production, thereby weakening the antimicrobial efficacy (Kalishwaralal et al., 2010).

Pseudomonas aeruginosa, Salmonella typhi and Proteus vulgaris combining ZrO2 NPs with certain metal oxides might lead to antagonistic effects. Some metal oxides might possess inherent antioxidant properties, scavenging the ROS generated by ZrO2 NPs and negating their bactericidal effect (Feng Q et al 2015).

ZrO $_2$-(11%) V_2O_5 nanoparticles

For 2 mg/ml concentration, ZrO_2-(11%) V_2O_5 showed antimicrobial activity against all the cultures. Against *Shigella* sp *(*84.48±1%) highest growth inhibition was seen. Followed by, *Proteus vulguris* (67.86±00.4%), *Pseudomonas aeruginosa* (40.41±0.1%), *Salmonella thypi* (29.03±1), *E. Coli (*26.6%), *Staphylococcus aureus (*25.6±0.1%) and least growth inhibition was seen in Klebsiella sp (24.64±0.4) (Fig. 6).

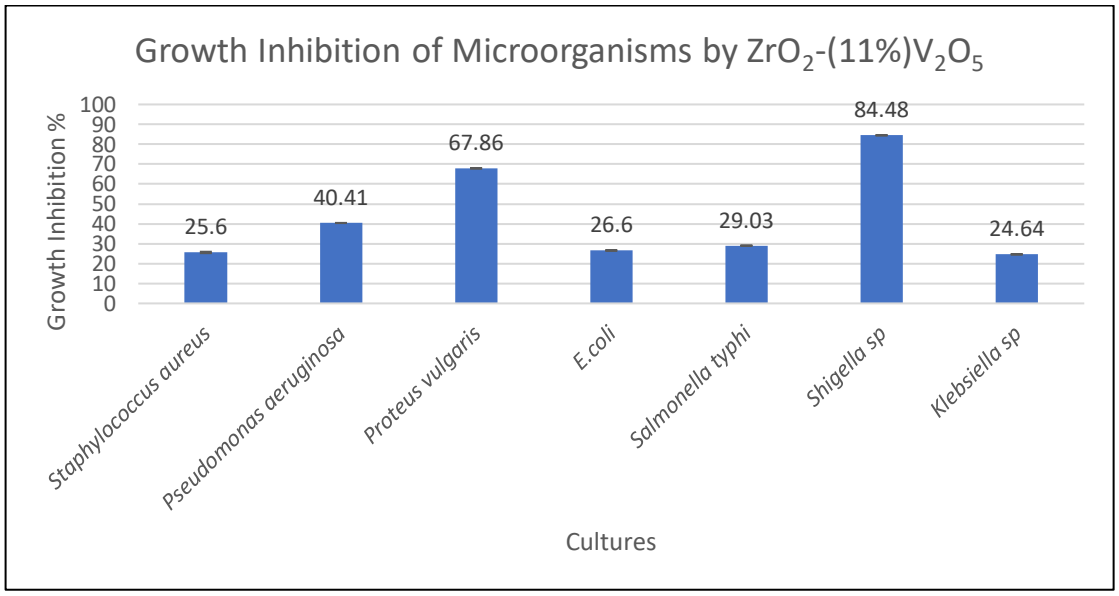

Fig. 6. Antibacterial Activity of ZrO_2-V_2O_5 Nanoparticles: Growth Inhibition Percentage (error bar indicate std. error)

Shigella sp. (84.48%) *Proteus vulgaris* (67.86%), *Pseudomonas aeruginosa* emerged as the most sensitive bacteria to ZrO_2-V_2O_5. In previous studies, V_2O_5 nanoparticles showed good antimicrobial activity for *Proteus vulgaris*, *Pseudomonas aeruginosa*. And in the same study, it was seen that *Staphylococcus aureus* and *E. coli* showed less sensitivity for V_2O_5 nanoparticles (Karthik et al., 2019).

ZrO2-V2O5 showed a moderate to good level of inhibition against most other bacteria tested**.** *Proteus vulgaris* (67.86%), *Pseudomonas aeruginosa* (40.41%), *Salmonella typhi* (29.03%), and *E. coli* (26.6%) all displayed significant growth inhibition. This broader

spectrum of activity compared to unmodified ZrO_2 (highest inhibition against *E. coli*) suggests that ZrO_2-V_2O_5 might target a wider range of cellular mechanisms in bacteria. *Staphylococcus aureus* and *Klebsiella* sp. remained least inhibited by V_2O_5 and ZrO_2-V_2O_5**.** This implies that the cell wall structure or defence mechanisms of these bacteria might be less susceptible to the combined effects of ZrO_2-V_2O_5 and V_2O_5 (Alaya et al., 2023).

ZrO_2-(15%) CeO_2 nanoparticles

ZrO_2-(15%) CeO_2 showed very low antimicrobial activity against bacterial culture. Growth inhibition was seen for *Shigella* sp (11.46%), *Klebsiella* sp (7.22%), *& Pseudomonas aeruginosa* (4.96%). It did not show antimicrobial effect on *Staphylococcus aureus*, *E. coli, Salmonella typhi, and Proteus vulgaris* (Fig. 7).

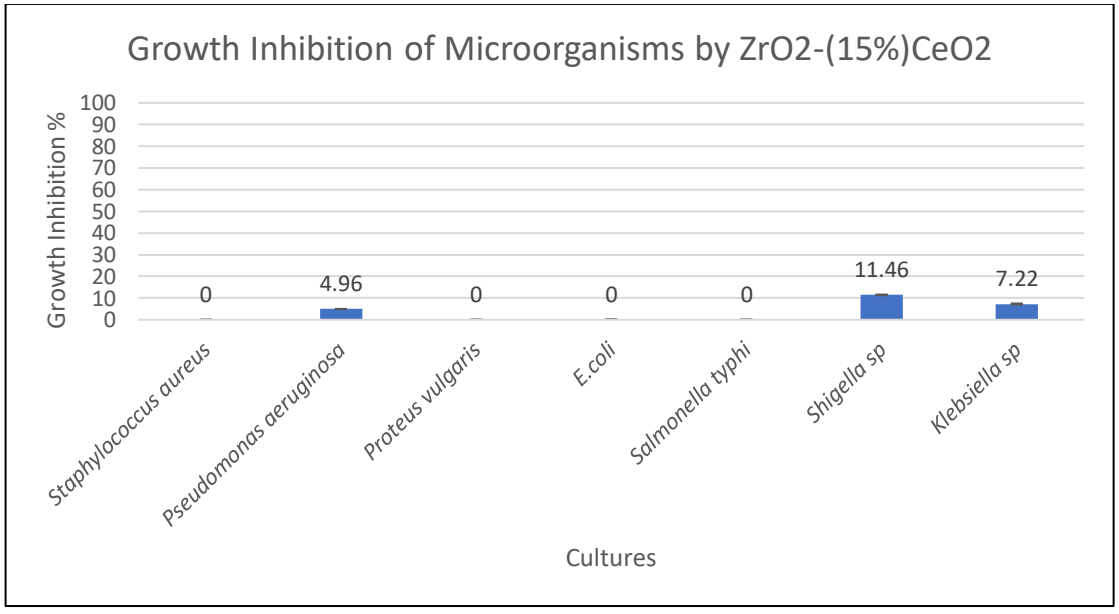

Fig. 7. Antibacterial Activity of ZrO_2-(15%) CeO_2 Nanoparticles: Growth Inhibition Percentage (error bar indicate std. error)

In a previous study, it was found that CeO_2 might modify the surface properties of ZrO_2 nanoparticles. Depending on the CeO_2 content and surface interactions, this could lead to a decrease in surface area of individual metals (Damyanova et al., 2009).

ZrO_2-(10%) CuO Nanoparticles

ZrO_2-CuO showed high antimicrobial activity against all bacterial cultures. The highest growth inhibition was seen in *Staphylococcus aureus* (80.89±1.1%), *Shigella* sp (80.73±2%), followed by *E. coli* (69.51±0.5%), *Klebsiella* sp (62.35±0.9%), *Proteus vulgaris* (55.04±1.1%), *Pseudomonas aeruginosa* (49.19±0.4) and least growth inhibition was seen in *Salmonella typhi* (48.82±1%) (Fig. 8).

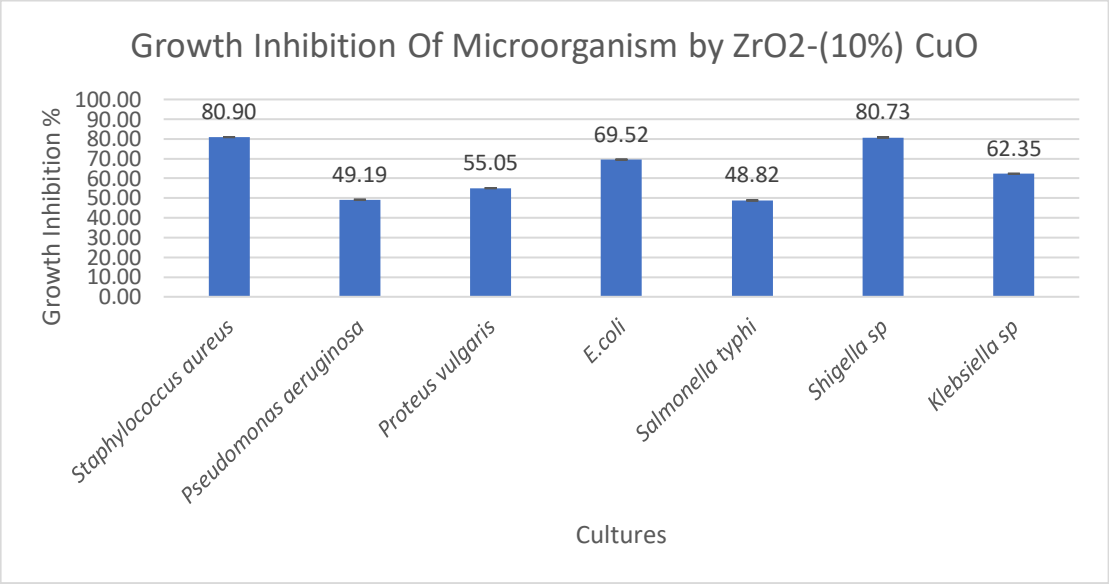

Fig. 8. Antibacterial Activity of ZrO_2-(10%) CuO Nanoparticles: Growth Inhibition Percentage (error bar indicate std. error)

CuO nanoparticles are well-established antimicrobial agents, known to disrupt bacterial membranes, deactivate enzymes, and generate ROS (Selvaraj, 2022). CuO is known to be a potent catalyst for ROS generation. Its incorporation into ZrO_2 might promote the production of ROS, leading to a more oxidative stress response that can damage bacterial membranes more effectively (Sicwetsha et al., 2021).

The combined action of ZrO_2 and CuO might target bacteria through multiple mechanisms. ZrO_2's potential for membrane disruption could be complemented by CuO's ability to

deactivate enzymes and disrupt cellular processes, leading to a more comprehensive assault on bacterial viability.

ZrO_2-(5%) Ag_2O Nanoparticles

ZrO_2-Ag_2O showed very high antimicrobial activity against all bacterial cultures. Growth inhibition was seen as *Staphylococcus aureus* (99.82±1%), *Shigella* sp (92.27±0.7%), *Pseudomonas aeruginosa (*86.89±2.1%), *Proteus vulgaris* (85.68 ±0.5%), *E. coli* (82.04±0.6%), *Salmonella typhi* (80.99±0.8%) and *Klebsiella* sp (79.7±0.8%) (Fig. 9).

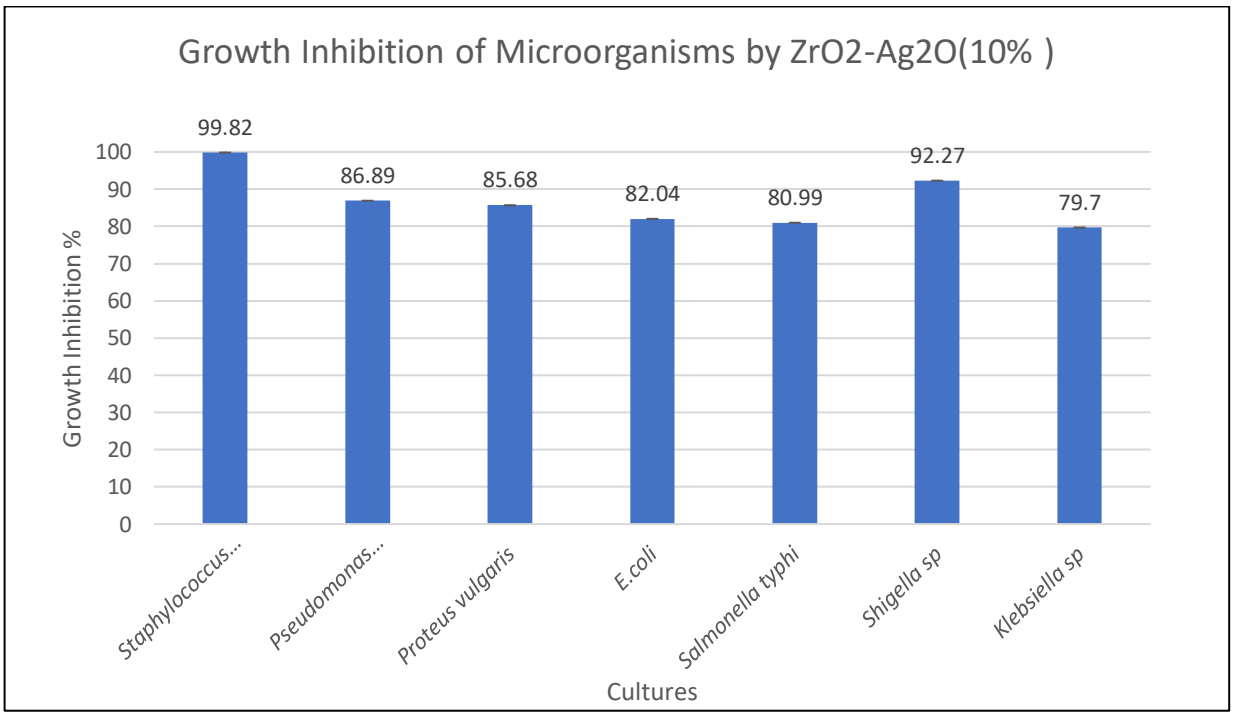

Fig. 9. Antibacterial Activity of ZrO_2-(5%) Ag_2O Nanoparticles: Growth Inhibition Percentage (error bar indicate std. error)

Silver ions (Ag+) released from Ag_2O are highly effective broad-spectrum antimicrobial agents. Their ability to disrupt various cellular functions in bacteria contributes significantly to the observed high inhibition rates (Gudkov et al., 2022).

ZrO_2 nanoparticles might act as carriers for Ag+ ions, facilitating their delivery and penetration into bacterial cells. The combination of ZrO_2's potential for membrane

disruption and ROS generation with Ag_2O's multifaceted antimicrobial actions creates a multi-pronged attack on bacteria, making it more difficult for them to develop resistance mechanisms.

ZrO_2 nanoparticles might act as carriers for Ag^+ ions, facilitating their delivery and penetration into bacteria. This targeted delivery might enhance the overall effectiveness of Ag+ compared to its use alone (Mudshinge et al., 2011).

CeO_2 Nanoparticles

CeO_2 showed low antimicrobial activity against all the bacterial cultures. Highest growth inhibition was seen again *Shigella* sp (29.64±0.5%), *Proteus vulgaris* (24.86±0.6%), *E. coli* (21.77±0.9%), *Staphylococcus aureus* (16.12±0.8%), *Klebsiella* sp (15.38±2%), *Salmonella typhi* (8.26±1.1%), *Pseudomonas aeruginosa* (7.54±1.5%) (Fig. 10).

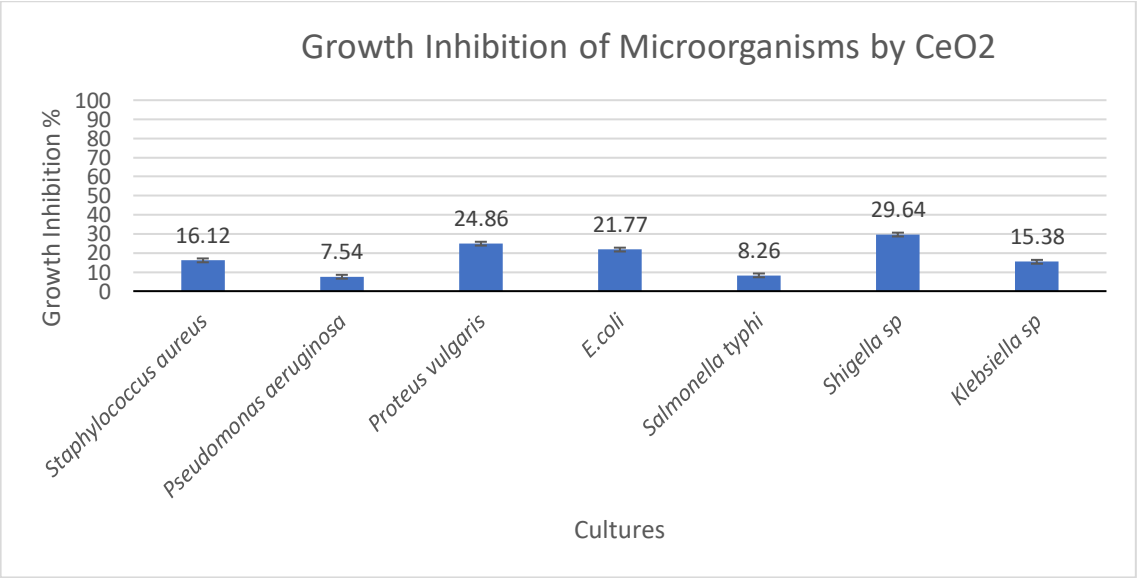

Fig. 10. Antibacterial Activity of CeO_2 Nanoparticles: Growth Inhibition Percentage (error bar indicate std. error)

The research findings indicated that CeO_2 nanoparticles exhibit low to moderate antimicrobial activity against various bacterial cultures. It could be because of lower amount of nanoparticles concentration was used (2 mg/ml concentration),

CeO_2 does not shows high antimicrobial activity. In a previous study, CeO_2 showed antimicrobial activity of 2.67 mm zone of inhibition with as high as at 10 mg/ml concentrations (Yadav et al., 2017).

CeO_2 demonstrates excellent biocompatibility, meaning it exhibits minimal toxicity to mammalian cells, we can also say that it is not toxic to bacterial cells (Manjón et al., 2018). We use CeO_2 nanoparticles to replace antibiotics and bring them to the medical sector.

CeO_2– (5%) Ag_2ONanoparticles

CeO_2-(5%) Ag_2O showed very high antimicrobial activity against all bacterial cultures. Against *Staphylococcus aureus* (89.17±0.3%), *Pseudomonas aeruginosa* (90.79±0.3%), *Proteus vulgaris* (88.07±0.6%), *E. coli* (90.24±0.7%), *Salmonella typhi* (87.56±0.8%) highest growth inhibition was seen. Followed by*, Klebsiella* sp (68.11±1%), and least was seen in *Shigella* sp (51.7±0.2%) (Fig. 11).

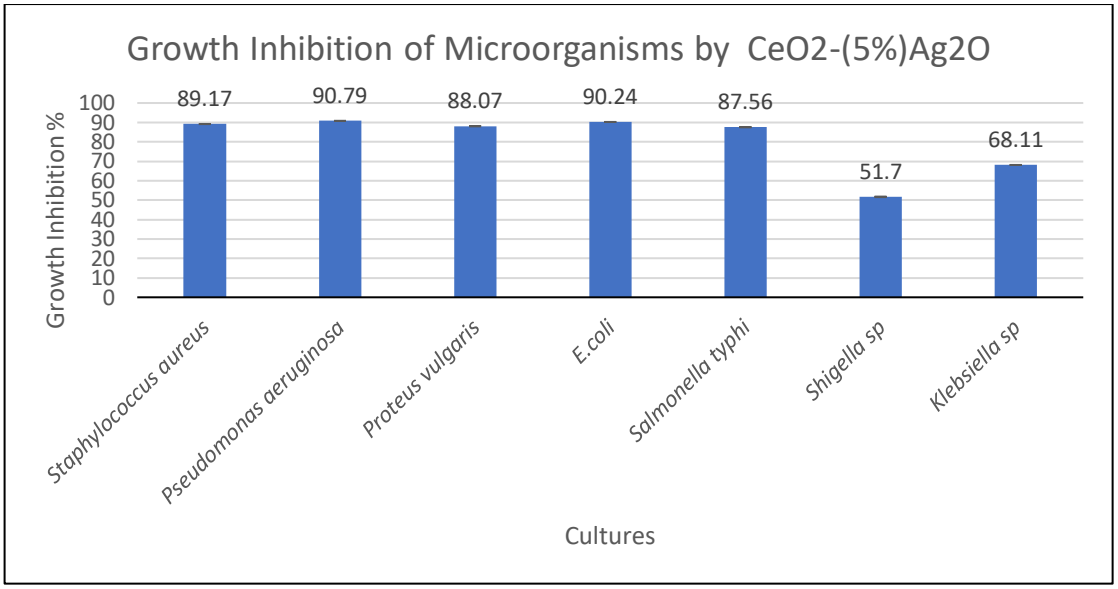

Fig. 11. Antibacterial Activity of CeO_2– (5%) Ag_2ONanoparticles: Growth Inhibition Percentage (error bar indicate std. error)

CeO_2-Ag_2O (5%) showed high antimicrobial activity as silver nanoparticles are well-known for their potent broad-spectrum antimicrobial activity. This activity is attributed to multiple mechanisms, including disrupting bacterial cell membranes and interfering with their respiratory chain (Gudkov et al., 2022).

CeO_2– (10%) CuO Nanoparticles

CeO_2-CuO showed antimicrobial activity against all the cultures. The highest growth inhibition was seen against *Klebsiella* sp (43.87±0.7%), *Shigella* sp (38.05±0.9%), *Salmonella typhi* (33.79±0.8%), *E. coli* (31.61±0.3%), *Proteus vulgaris* (31.01±1.1%), *Pseudomonas aeruginosa (*30.31±0.9%), and least was in seen *Staphylococcus aureus (*29.42± 0.4%) (Fig. 12).

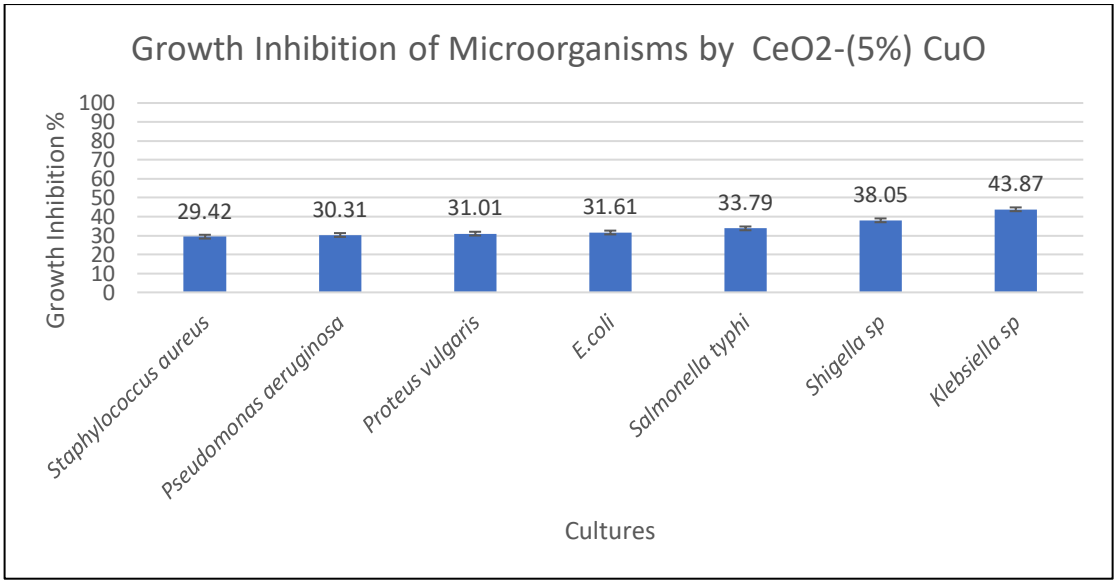

Fig. 12. Antibacterial Activity of CeO_2– (10%) CuO Nanoparticles: Growth Inhibition Percentage (error bar indicate std. error)

CeO_2-CuO composite exhibited moderate antimicrobial activity against various bacteria. Reasons behind this can be Copper ions (Cu2+) released from CuO can disrupt bacterial cell membranes, damage proteins, and generate ROS, leading to cell death.

CuO nanoparticles are also well-established antimicrobial agents, known to disrupt bacterial membranes, deactivate enzymes, and generate ROS (Selvaraj, 2022).

For ZrO_2, ZrO_2, ZrO_2-Mn(X), and ZrO_2-V_2O_5 nanoparticles showed high different antimicrobial activity against different bacterial strains. This is because some of the bacterial strain being more susceptible to this nanoparticle then others. In case of ZrO_2-(10%) CuO and ZrO_2-(5%) Ag_2O showed high antimicrobial activity against all the bacterial strains as CuO and Ag_2O nanoparticles are already well known for their antimicrobial activity. ZrO_2-(15%) CeO_2 very less activity for few cultures and no activity for the others.

For CeO_2, CeO_2 nanoparticles showed low antimicrobial activity against all the cultures. Wherein CeO_2-(5%) CuO showed moderate antimicrobial activity and CeO_2-(10%) Ag_2O showed very high antimicrobial activity.

In both the cases, for CeO_2 and ZrO_2 nanoparticles combining them with AgO nanoparticles showed the best results in-case of antimicrobial activity.

4.2.2 Antimicrobial activity of ZrO_2 in presence and absence of Visible light and UV-treatment

CuO-ZrO_2 nanoparticles were synthesized by two different methods they were hydrothermal method and impregnation method. And their antimicrobial activity was checked under visible light irradiation and after UV treatment of nanoparticles.

CuO-ZrO_2 synthesized by Hydrothermal Method

CuO-ZrO_2 nanoparticles synthesized by hydrothermal showed increasing trend in antimicrobial activity, when nanoparticles were irradiated with visible light and nanoparticles was being treated with UV light. For *Staphylococcus aureus*, 30.25±2% for untreated nanoparticles, 68.69±2% growth inhibition when irradiated with visible light, and 67.19±2% growth inhibition for UV-treated nanoparticles were seen respectively. For *Salmonella Typhi*, 10.9±2% growth inhibition for untreated nanoparticles, 40.69±2% growth inhibition for nanoparticles irradiated with visible light, and 44.9±2% growth inhibition for UV-treated nanoparticles. For *Proteus vulgaris*, 65.28±2% growth inhibition for untreated nanoparticles, 78.5±2% growth inhibition for nanoparticles irradiated with visible light, and 80.23±2% growth inhibition for UV-treated

nanoparticles. For *Shigella* sp, 24.56±2% growth inhibition for untreated nanoparticles, 60±2% growth inhibition for nanoparticles irradiated with visible light, and 57.39±2% growth inhibition for UV-treated nanoparticles were seen respectively (Fig. 13.).

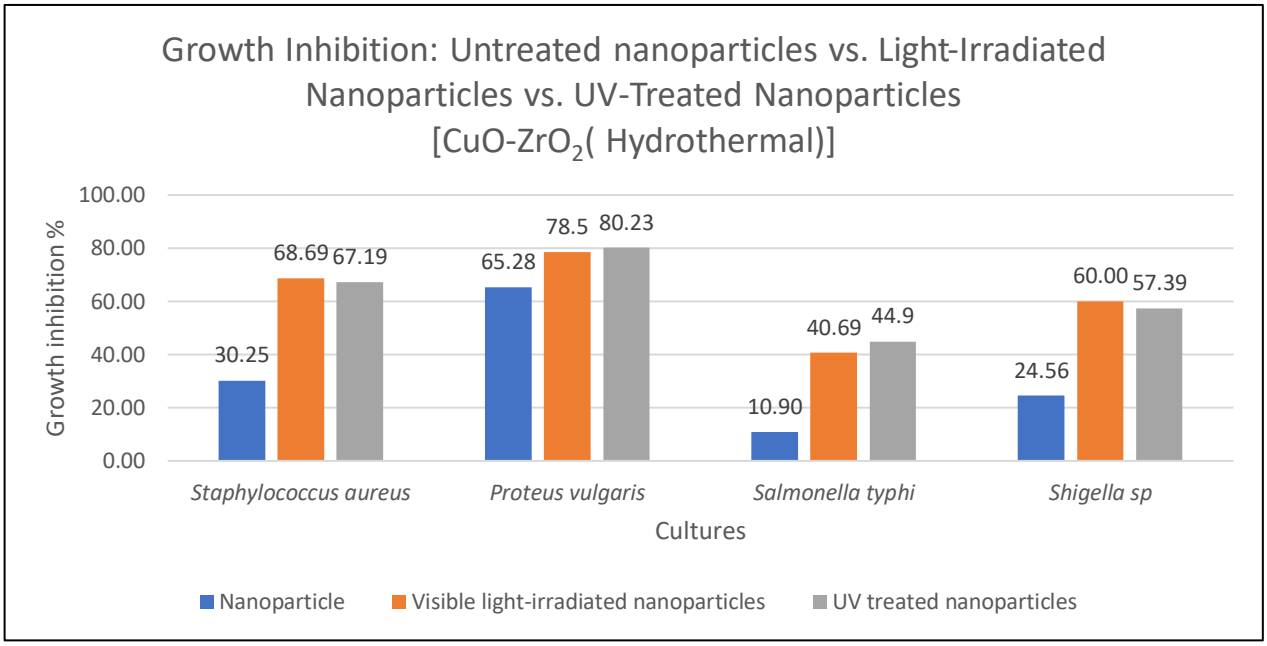

Fig. 13. Antimicrobial activity: Growth Inhibition percentage for ZrO_2-CuO Nanoparticles synthesized by hydrothermal method (Untreated nanoparticles, Visible-Light irradiated nanoparticles & UV treated nanoparticles)

CuO in the composite can generate reactive oxygen species (ROS) like singlet oxygen and superoxide radicals. These ROS are highly reactive and can damage bacterial cell membranes, proteins, and DNA, leading to cell death (Godoy-Gallardo et al., 2021).

Light irradiation might enhance the separation of photogenerated electron-hole pairs within the CuO-ZrO_2 composite. This can promote the formation of ROS and improve the overall photocatalytic antimicrobial activity (Zhu et al., 2020).

While both visible light and UV can activate CuO and ZrO_2, the specific wavelengths might influence the efficiency of ROS generation. UV light typically has higher energy and might generate a greater initial burst of ROS, but visible light with a broader spectrum could provide more sustained ROS production over time (Yadav et al., 2021).

$CuO\text{-}ZrO_2$ synthesized by Impregnation method

$CuO\text{-}ZrO_2$ nanoparticles synthesized by impregnation showed an increasing trend antimicrobial activity, when nanoparticles were irradiated with visible light and nanoparticles were being treated with UV light. For *Staphylococcus aureus*, 61.64±2% growth inhibition for untreated nanoparticles, 80.2±2% growth inhibition for nanoparticles irradiated with visible light, and 95.69±2% growth inhibition for UV-treated nanoparticles were seen respectively. For *Proteus vulgaris*, 58.89±2% growth inhibition for untreated nanoparticles, 69.24±2% growth inhibition for nanoparticles irradiated with visible light, and 81.82±2% growth inhibition for UV-treated nanoparticles growth inhibition was seen respectively. For *Salmonella* sp, 59.09±2% growth inhibition for untreated nanoparticles, 80.53±2% growth inhibition for nanoparticles irradiated with visible light, and 78.24±2% growth inhibition for UV treated nanoparticles were seen respectively, For *Shigella* sp 96.56±2% growth inhibition for untreated nanoparticles and 100% growth inhibition for both nanoparticles irradiated with visible light and UV treated nanoparticles was seen respectively (Fig. 14.).

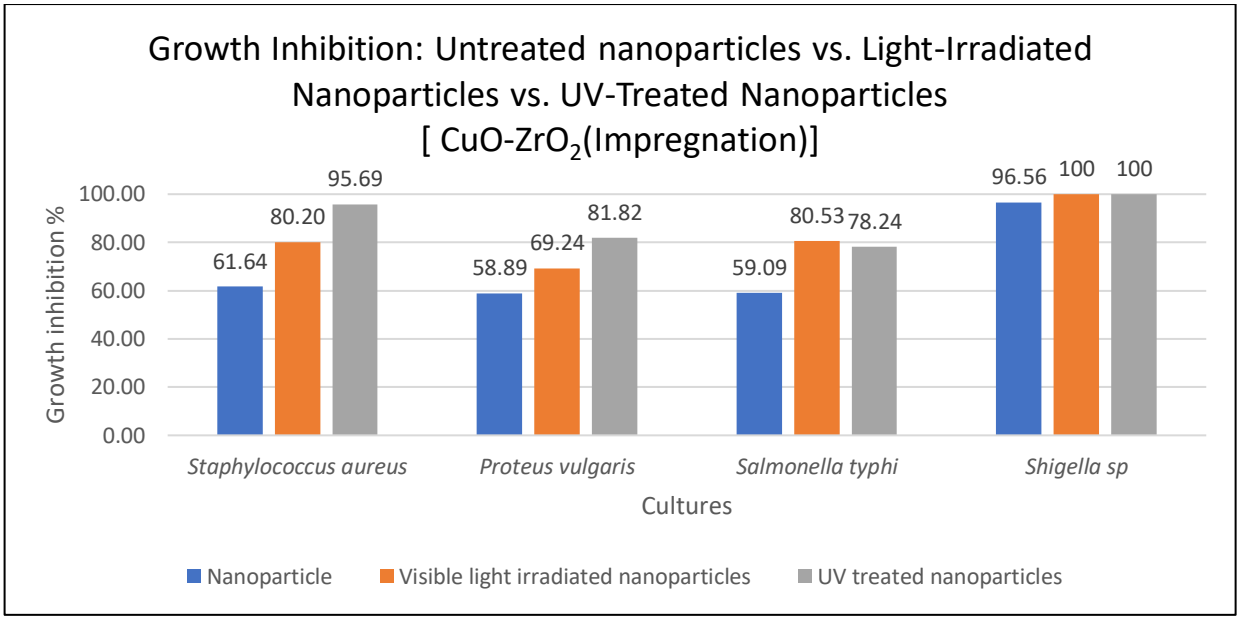

Fig. 14. Antimicrobial activity: Growth Inhibition percentage for ZrO_2-CuO nanoparticles synthesized by impregnation in both the presence and absence of visible light irradiation and UV treatment

A higher concentration of CuO in the nanoparticles translates to a greater number of active sites available for light absorption and ROS generation. light irradiation might activate CuO within the CuO-ZrO_2 composite, leading to enhanced ROS (Reactive Oxygen Species) production that can damage bacteria. UV might create defects in CuO that act as reaction centres, promoting ROS generation upon subsequent visible light exposure.

In the case of antimicrobial activity, ZrO_2-CuO nanoparticles produced from impregnation method showed good antimicrobial effect. As the impregnation method produces evenly doped nanoparticles which will have better antimicrobial activity (Karunakaran et al., 2010).

In ZrO_2 -CuO(hydrothermal) method showed good increase in antimicrobial activity under visible light irradiation and UV- treatment. As it has more photocatalytic activity, because

hydrothermal method is known to produce lattice defects in nanoparticles, as these defects help in producing ROS during light irradiation (Ma et al., 2024).

Both the nanoparticles showed increased antimicrobial activity after UV treatment and visible light irradiation. But there was not much different between in antimicrobial activity when nanoparticles irradiated with visible light and UV treated nanoparticles.

4.2.3 Live/Dead Assay for bacterial viability

Even after performing repeatedly, the standardization of the assay was not achieved. graphs for live cells for each culture were not obtained.

Conclusions

This study aimed to study the antimicrobial activity antimicrobial activity of the CeO_2 and ZrO_2 nanoparticles. Our finding suggests that ZrO_2-Ag_2O (10%) nanoparticles showed best antimicrobial activity (more than 80% for all the cultures). CeO_2-(5%) Ag_2O composite showed the best antimicrobial activity among the composite nanoparticles. It showed the highest antimicrobial activity against all bacterial pathogens (more than 80% for most of the cultures).

The nanoparticles (ZrO_2-CuO) synthesized using the impregnation method showed overall high antimicrobial activity in comparison to nanoparticles (ZrO_2-CuO) synthesized by the hydrothermal method. Whereas nanoparticles synthesized from hydrothermal showed more improvement in antimicrobial activity in the presence of visible-light and UV-treatment. Antimicrobial activity is almost the same for visible-light irradiated nanoparticles and UV light treated nanoparticles.

Appendix

Preparation of MH broth

Dissolve 2.1 g of MH broth in 100 ml of distilled water.

Composition of MH Broth

Ingredients	**g/L**
Beef, Extract Powder	2
Casein Acid Hydrolysate	17.5
Starch	1.5

Live/Dead assay standardization of cultures

1. Grew 30 mL cultures to late log phase in nutrient broth.
2. Centrifuged 25 mL of the bacterial culture at 10,000 × g for 10-15 minutes. Removed the supernatant and resuspended the pellet in 2 mL of saline or appropriate buffer.
3. Added 1 mL of the resuspended culture to each of the two centrifuge tubes. One tube contained saline (live control) and the other contained isopropyl alcohol (killed control).
4. Incubated both samples at room temperature for 1 hour, mixing every 15 minutes.
5. Centrifuged both samples at 10,000 × g for 10-15 minutes. Resuspended the pellets in 20 mL of saline or appropriate buffer and centrifuged again.
6. Resuspended both pellets in separate tubes with saline or buffer.
7. Determined the optical density at 670 nm of a 3 mL aliquot of the bacterial suspensions.

8. Adjusted the concentration of the Gram-negative suspensions (live and killed) to 2 x 10^8 bacteria/mL or the Gram-positive (live and killed) to 2 x 10^7 bacteria/mL.
9. Prepared a 2X working solution of the LIVE/DEAD BacLight staining reagent mixture.
10. Mixed five different proportions (0/100, 10/90, 50/50, 90/10, 100/0) of live and dead cells from the adjusted bacterial suspensions in cuvettes.
11. Mixed 1.5 mL of the 2X staining reagent mixture with an equal volume (1.5 mL) of each bacterial suspension. Incubated samples at room temperature in the dark for 15 minutes.
12. Measured the fluorescence emission spectrum (excitation 470 nm, emission 490-700 nm) of each cell suspension using a fluorescence spectrophotometer.
13. Calculate the ratio of the integrated intensity of the green fluorescence (510-540 nm) to the red fluorescence (620-650 nm) for each bacterial suspension.
14. Plotted the ratio of integrated green fluorescence to integrated red fluorescence versus the percentage of live cells in the culture suspension.

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