

**EVALUATION OF ANTIBIOFILM AND PHOTOCATALYTIC
POTENTIAL OF GREEN SYNTHESISED NANOPARTICLES
AGAINST MULTI-DRUG- RESISTANT BACTERIAL
PATHOGENS OF PUBLIC HEALTH IMPORTANCE**

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(21-MVP-12)**

THESIS

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Kerala Veterinary and Animal Sciences University**



**DEPARTMENT OF VETERINARY PUBLIC HEALTH
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DECLARATION

I hereby declare that this thesis entitled **“Evaluation of antibiofilm and photocatalytic potential of green synthesised nanoparticles against multi-drug-resistant bacterial pathogens of public health importance”** is a bonafide record of research done by me during the course of research and that the thesis has not previously formed the basis for the award of any degree, diploma, fellowship or other similar titles, of any other University or Society.

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LIST OF ABBREVIATIONS

%	:Percent
µg	:Microgram
µl	:Microliter
Å	:Angstrom
ABST	:Antibiotic Sensitivity Test
Ag	:Silver
Ag/ZnO	:Silver-zinc oxide
AgNO ₃	:Silver nitrate
AMR	:Antimicrobial Resistance
ANNOVA	:Analysis of variance
ATCC	:American Type Culture Collection
Au	:Gold
BP	:Baird Parker
CDC	:Centre For Disease Control And Prevention
CFU	:Colony Forming Units
CLSI	:Clinical Laboratory Standards Institute
CuO	:Copper oxide
CV	:Crystal violet
EAEC	:Enteraggregative <i>Escherichia coli</i>
EMB	:Eosin-methylene blue
ESBL	:Extended Spectrum β-Lactamase
<i>etal.</i>	:and co-workers
Fig.	:Figure
g	:Gram
g/L	:Gram per liter
GRAS	:Generally recognised as safe
h	:Hour

ICAR	:Indian Council of Agricultural Research
LED	:Light emitting diode
<i>M</i>	:Molar
MB	:Methylene blue
MBC	:Minimum Bactericidal Concentration
MDR	:Multi-drug-resistant
Mg	:Milligram
MH	:Mueller-hinton
MIC	:Minimum Inhibitory Concentration
min	:Minute
mL	:Milliliter
Mm	:Millimole
mm	:Millimeter
<i>mM</i>	:Millimolar
MRS	:de Mann-Rogosa-Sharpe
MRSA	:Methicillin-resistant <i>Staphylococcus aureus</i>
MTCC	:Microbial type culture collection
NASF	:National Agricultural Science Fund
NCs	:Nanocomposites
ng	:Nanogram
nm	:Nanometer
NPs	:Nanoparticles
NTS	:Non-typhoidal <i>Salmonella</i>
O.D	:Optical Density
°C	:DegreeCelsius
pH	:Log hydrogen ion concentration
RhB	:Rhodamine B
ROS	:Reactive Oxygen Species

rpm	:Revolutions per minute
Sec	:Seconds
spp.	:Species
SPR	:Surface plasmon resonance
UV	:Ultraviolet
UV-Vis	:Ultraviolet visible
v/v	:Volumebyvolume
Via	:Bywayof/bymeansof
Viz	:Namely
WHO	:World Health Organisation
XDR	:Extensively drug-resistant
XLD	:Xylose Lysine Deoxycholate
ZnNO ₃	:Zinc nitrate
ZnO	:Zinc oxide
θ	:Theta

1. Introduction

1. INTRODUCTION

In every corner of the world, antimicrobial resistance (AMR) is escalating at an alarming level. Both humans and animals are susceptible to infections, and the infections are more difficult to treat when caused by such drug-resistant bacteria. Eventually, AMR results in an increased mortality, prolonged hospital stays, and elevated medical expenses (Prajescu *et al.*, 2023). The transmission of drug resistance is accelerated when the antibiotics are used indiscriminately, especially with the widely practiced over-the-counter sale. The drug resistance is worsened by the fact that several bacterial strains can form biofilms (Ben Abdallah *et al.*, 2020). Antibiotic susceptibility of the biofilm cells is typically 100 to 1000 times lower than that of planktonic cells (Di Domenico *et al.*, 2022). The decrease in drug penetration through the extracellular matrix, the decrease in drug concentration, the decrease in bacterial metabolic rates, and the transfer of resistant genes within the biofilms contribute to the growth of drug resistance by the biofilms (Abebe, 2020; Yi *et al.*, 2020). Moreover, the diminishing antimicrobial pipeline warrants the search for alternative practices for routine antimicrobials (Hu *et al.*, 2020). Therefore, either drug development or drug re-purposing to improve antibacterial potential is a priority area of research in the modern era (Thornber *et al.*, 2020).

Of the proposed techniques to combat drug resistance, nanotechnology is the novel viable therapeutic approach due to its therapeutic efficacy against microorganisms. Nanotechnology provides an excellent platform to transform properties of metals to nanoparticles (NPs) with promising applications in the fields of diagnostics, antimicrobial agents, drug delivery systems, biomarkers, cell labeling, contrast agents for biological imaging, and nano drugs for the treatment of various diseases (Sahu *et al.*, 2021; Milan *et al.*, 2022). Generally, biomaterials with diameters up to 100 nm are known as NPs. Due to their extensive use in agriculture, medicines, consumer goods, energy, and most importantly as antimicrobial agents, nanomaterials have received wide research attention (Waktole and Chala, 2023). They are currently regarded as viable substitutes and/or

supplements to the existing antimicrobials (Mba and Nweze, 2021). Generally, the biomedical properties of NPs depend primarily on their modes of synthesis as well as formulation conditions such as the nature of the reducing agent, temperature, concentration, and nature of the solvent (El Shafey, 2020; Vishwanath and Negi, 2021). The most promising materials so far seem to be the metallic NPs which have been reported to exhibit diverse activities against several multi-drug-resistant (MDR) pathogens (Guo *et al.*, 2020; Mba and Nweze, 2021).

The global population is facing unprecedented environmental pollution, ascribed to the industrial dyes, antibiotics, as well as wastes from various settings contaminated with microbes (Ahmed *et al.*, 2020). Consequently, water supplies have largely been polluted with hazardous substances. An assortment of organic and inorganic dyes constitute the primary water pollutants (Shabir *et al.*, 2022). Most of these industrial dyes have been derived from well-known carcinogens like benzidine and naphthalene (Al-Niaeem *et al.*, 2022). Therefore, these dyes have the potential of being carcinogenic when they enter the biological systems as a result of the microbial breakdown (Marimuthu *et al.*, 2020). Hence, the treatment of wastewater containing dyes before being discharged into water resources is necessitated in order to avoid further adverse environmental effects.

In recent years, the use of NPs as agents for dye removal from wastewater has gained considerable relevance because of their high surface area, strong adsorption characteristics, decreased resistance to diffusion, and ability to exhibit quicker rates of equilibrium (Khan *et al.*, 2021). Over the years, the use of nanomaterials as photocatalysts has received considerable attention. The mode of operation of NPs as photocatalysts is based primarily on the modification of light-sensitive molecules when triggered by light, allowing the release of the conjugated or encapsulated medication (Salkho *et al.*, 2022); hence, the light responsive construction is simple to control and less intrusive (Zhao *et al.*, 2019). It has been reported that the NPs outperform bulk materials as potential absorbent or photocatalytic materials (Verma *et al.*, 2022; Altuner *et al.*, 2023) by its ability to use a full valence band and an empty conduction band (Falah and MacKenzie,

2020). When strong oxidising species like hydroxyl or superoxide ions are produced, the hole responds by either using electron donors from the solution or hydroxide ions. This ability confirms its effectiveness as a photocatalyst for pollutant degradation when exposed to solar radiation. The NPs could also be used as a green semiconductor photocatalyst since sunlight is a cheap and abundant source of energy (Kumar *et al.*, 2022).

In addition to the industrial dyes, numerous synthetic pharmaceutical compounds, especially antibiotics have polluted the aquatic system (Rathi *et al.*, 2021). Since they are extremely hazardous and are often used in both human and veterinary practice, antibiotics also pose a major environmental threat. Moreover, the presence of these antibiotics in water sources, even at low concentrations, would result in the emergence of AMR. Interestingly, the antibiotic residues in wastewater cannot be efficiently removed using traditional wastewater treatment methods (Wang *et al.*, 2019). However, the heterogeneous photocatalytic method using NPs has demonstrated an effective capacity to degrade several drug types and chemical compounds (Batra *et al.*, 2022). It is based on this idea of using energy provided by an identified light source (light emitting diode [LED], ultra violet [UV], or sun light), the NPs could serve as efficient photocatalyst. As the NPs are effective and simple to create, they are frequently utilised as photocatalysts for antibiotic removal (Noor *et al.*, 2022) besides being used for dye degradation and antimicrobial activity. In the earlier studies, the antimicrobial activity of the green synthesised silver (Ag) NPs, zinc oxide (ZnO) NPs and Ag/ZnO nanocomposites (NCs) were evaluated (Abishadet *et al.*, 2022; Unni *et al.*, 2022; Arya *et al.*, 2023); however, the photocatalytic potential remained unexplored.

Under these circumstances, the present study was undertaken with the following objectives:

1. Assessment of antibiofilm activity of green synthesised AgNPs, ZnO NPs and Ag/ZnONCs against MDR strains of non-typhoidal *Salmonella* spp. (NTS), enteroaggregative *Escherichia coli* (EAEC) and methicillin-resistant *Staphylococcus aureus* (MRSA).

2. Evaluation of the photocatalytic potential of green synthesised Ag NPs, ZnO NPs, and Ag-ZnO NPs.

2. Review of Literature

2. REVIEW OF LITERATURE

2.1. Antimicrobial resistance (AMR)

Antibiotic-resistant bacteria are evolving at an accelerated rate, making antimicrobial resistance (AMR) a significant public health concern that must be addressed and necessitate immediate action (Tang *et al.*, 2023). The primary contributors to the emergence of AMR are thought to be the improper and excessive use of various antimicrobial agents both in the fields of medical and agricultural practice (Puvača *et al.*, 2022). The overuse or abuse of antibiotics results in selection pressure in clinical as well as natural settings, which is detrimental to both human and animal health (Salam *et al.*, 2023). Nonetheless, the prolonged use of sub-therapeutic doses of antibiotics in the animal production system creates a perfect environment for bacteria to fix the genes involved in their resistance (Irfan *et al.*, 2022). The human gut microbiota can acquire such drug-resistant genes either from polluted environments or diets (Tiedje *et al.*, 2023). With the ongoing trends in the use of antimicrobials, the death toll due to AMR has been projected to reach nearly 10 million by the year 2050 (Micheli *et al.*, 2023; Tang *et al.*, 2023; Zhou *et al.*, 2023).

The drug-resistant genes have been reported to be transferred to human commensal and pathogenic bacteria because of environmental contamination (Kothari *et al.*, 2023). Whereas the development of extended-spectrum beta-lactamases (ESBL) genes has been linked to the resistance of bacterial pathogens to third-generation cephalosporins (Adibe-Nwafor *et al.*, 2023).

A significant factor contributing to the transmission of resistance to humans through animal products is the egregious use of antimicrobials as growth boosters in poultry or animals (Rahman *et al.*, 2022). The most prevalent drug-resistant infections in this category include *Campylobacter* spp., *Salmonella* spp., community-acquired methicillin-resistant *Staphylococcus aureus* (MRSA) and food-borne *E. coli* (Denissen *et al.*, 2022). Antibiotic use in cattle and poultry has significantly increased across all continents, and by 2050, it is predicted that 67 per

cent of the world's developing and quickly polluted nations would be using antibiotics (Rizzo *et al.*, 2019). Besides, growing human and animal populations, international trade, increased globalisation, rising demand for animal feed, and too-easy access to antimicrobial compounds in both developing and developed countries microorganisms, which pose serious threats to the environment, are often considered a major global health concern by world health leading agencies and surveillance systems (Li and Zhang, 2023).

2.1.1. Global scenario

Due to its prevalence and association with higher death rates in low- and middle-income countries (LMICs), AMR is becoming an increasingly difficult problem. Over the last decade, AMR has been acknowledged as one of the major threats to public health. Although WHO has stated that using antibiotics increases life expectancy by roughly 20 years, this benefit has been decreasing due to drug resistance (Parveen *et al.*, 2022). The WHO has also stated that AMR is one of the top ten threats affecting global health. In general, AMR affects the general public health and bacterial populations to exhibit persistence, resistance, and tolerance which are the three main contributing factors to the evolution of AMR (Algamma *et al.*, 2023). Animal-related drivers of AMR in LMICs differ from high-income nations in specific ways, aside from human and environmental variables (Olaru *et al.*, 2023). It has also been stated that AMR is predicted to become the world's leading cause of mortality by 2050 unless preventative actions are taken. In the Global Burden of Diseases (GBD) region, Sub-Saharan Africa has the highest all-age death rate that is directly linked to or related to AMR, whereas Australasia has the lowest rate of AMR-related mortality (Tang, *et al.*, 2023).

These situations have necessitated many countries to create national action plans (NAPs) in response to the need to control AMR, which call for extensive information about the AMR situation in all sectors through the development of tools that were developed following the Codex Alimentarius and WOA risk analysis recommendations and the AMR epidemiology model (Caipo *et al.*, 2023).

A prominent factor contributing to the increased rate of AMR worldwide is the ineffective techniques of wastewater management and treatment. Wastewater containing antibiotics is a major environmental hazard. Many of these antibiotics which were consumed through over-the-counter consumption of antibiotics resulted in the elimination of these along with the resistant bacteria in major water sources and backwaters resulting in their biomagnifications (McCorquodale-Bauer *et al.*, 2023).

2.1.2.Indian scenario

Due to the high prevalence of bacterial diseases in India, widespread resistance may have an adverse impact on India than it would on other nations. At the moment, India has no regulations governing the use of antibiotics in domestically reared livestock and poultry. There are no guidelines for the tolerance of antibiotic residues in chicken, but there are guidelines for seafood, which includes prawns, and other types of fish and fishery products (Kakkar and Rogawski, 2013). Given the magnitude of India's pharmaceutical industry, wastewater treatment as well as antibiotic production facilities have been linked to the introduction of resistance genes into the human microbiome (Krishnan Venkatesan *et al.*, 2023). These facilities offer a severe threat to antibiotic effectiveness. The release of antimicrobial waste into the environment is not subject to any laws, and accounts to be major public health threat (Le *et al.*, 2023).

The transition of India into a center for *Mycobacterium tuberculosis* that is extensively drug-resistant (XDR) and multi-drug-resistant (MDR), necessitates measures to slow the spread of the disease (Verma *et al.*, 2022). Additionally, as the available antibiotics would no longer be effective against such organisms, the genomic plasticity makes it possible for them to develop resistance against last-resort antibiotics such as carbapenemases and colistin through recombination or genetic acquisition (Jangir *et al.*, 2022). One such genetic component that confers resistance to carbapenemases is exhibited in the New Delhi metallo-beta-lactamase-1 (*bla*_{NDM-1}).

It is alarmingly necessary to create action plans to safeguard public health due to the prevalence of such antibiotic resistance genes (*bla*_{NDM-1}, *bla*_{OXA-48}, *mcr*-1, and *mcr*-2) in the environment and the possibility of their transmission (Zhenget *al.*, 2022).

Self-administration of antibiotics for therapeutic, preventive, and metaphylactic purposes is a common practice in livestock, which increases the danger of the establishment of AMR. Besides, a supportive socio-economic environment encourages the irrational use of antibiotics in livestock kept by peri-urban smallholder farmers in addition to the simple over-the-counter availability of antibiotics, frequently with no prescriptions, invalid prescriptions, or prescriptions from unlicensed practitioners (Adebisi, 2023).

India is regarded as the center of the worldwide AMR challenge due to its record-high levels of antimicrobial production, use, and misuse (Dixit *et al.*, 2019). However, India's story of resistance is nuanced, originating from the intersections of industrial pharmaceutical development, healthcare rationing and purchasing, regulatory infrastructure, and dynamics of disadvantage (Broom and Doron, 2020). What appears to be widespread, increasing antibiotic overuse and a need for stricter drug and prescriber regulations turns out to be a complicated societal issue. These dimensions are felt at the bedside, albeit in different ways, as Indian clinicians cope with the perilous landscapes of infectious diseases, the threat of MDR organisms, and (national) demands for more judicious practices (Broom and Doron, 2020).

Nair *et al.* (2021) asserted that the WHO's global action plan on AMR served as the main inspiration for the NAP on AMR that the Indian government created. Even though NAP-AMR successfully replicates the global action plan and sets forth ambitious targets, they inferred that the progress has been stymied by insufficient funding across states, subpar enforcement, and insufficient multi-sectoral coordination expanding the scope of efforts to upgrade water and sanitation systems and connect the AMR to ongoing vertical health activities (Biswas and Khottyal, 2022).

2.2. AMR and environmental hazards

The boundless use of antibiotics can cause a large-scale spread of AMR and can be detrimental to both human and animal populations (Jani *et al.*, 2021). Antibiotic overuse and unprocessed disposal jeopardise the environment and its inhabitants of a catastrophic health emergency (Nambiar and Asha, 2023). The potential risk of ingesting antibiotic residues in the environment alters the human microbiome and encourages the emergence and selection of bacteria resistant to antibiotics inhabiting the human body. Besides, the development of selection pressure on the environmental microbiome reservoirs antibiotic resistance in the environment which is considered to be the main health threat associated with environmental residues (Ben *et al.*, 2019). Bacteria and genes with antibiotic resistance are prevalent in the environment, which necessitates quick action for the efficient removal of drugs (Apreja *et al.*, 2022). Currently, there is no standardised regulatory framework for monitoring the purpose of enforcing environmental water quality regulations for the allowed concentrations of antimicrobials and their residues in water bodies, resulting in a widespread practice of discharging antimicrobials into urban wastewater treatment plants at potentially active quantities (Thornber *et al.*, 2020). Nonetheless, continuous exposure of bacteria to antimicrobials has been associated with the development of clinically significant multiple drug-resistant bacteria in hospital effluents and wastewater treatment plants (WWTPs) (Mutuku *et al.*, 2022) resulting in the emergence and spread of AMR which is a significant public health concern.

The WWTPs collect sewage water that contains antibiotic-resistant bacteria (ARB) and antimicrobial-resistant genes (ARGs) from a range of sources, including livestock farms, hospitals, companies, and households. These ARB and ARGs are eventually re-introduced into the aquatic environment, since current treatment procedures cannot eliminate them (Raza *et al.*, 2022).

2.3. AMR and bacterial biofilms

The AMR poses a challenge to the efficient prevention and treatment of diseases brought on by infection-causing microorganisms including bacteria, fungi, and viruses with the creation of novel resistance mechanisms in them (Burri *et al.*, 2023). Concerns in a variety of fields, including public health, medicine, and the pharmaceutical business, have made biofilm a topic of great interest (Aflakianet *et al.*, 2023). The exceptional antibiotic resistance caused by bacterial biofilms raises morbidity and death has significant impacts on the economy as well (Cámara *et al.*, 2022).

Balducci *et al.* (2023) stated that bacterial biofilms are one of the factors causing bacteria to become resistant to treatments like antibiotics that are currently available. The development of a biofilm, also known as an extracellular matrix, embedded with various bacterial cells, is an efficient method by which bacterial cells form a relationship in a particular environment and their growth is a complicated phenomenon that is influenced by several variables (Adumeneet *et al.*, 2021). There have been several attempts to understand the processes that lead to the creation of biofilms, and these endeavours have revealed pathways that can be targeted for therapy (Dutt *et al.*, 2022). Intrinsic biofilm growth has been cited as a crucial contributing component among the many aspects of AMR. Additionally, antibiotic tolerance acquired from biofilms causes significant recurrent chronic infections (Goel *et al.*, 2021). Antibiotics do not have the same effect on bacteria in biofilms as they do on planktonic bacteria in suspension (Yu *et al.*, 2022). It is suggested that the alarming developments in AMR are mainly because of the high dosages and lengthy courses of antimicrobial drugs required to treat biofilm-associated illnesses (Jang and Mosolygó, 2020).

2.4. One health perspective

Antimicrobials are employed more frequently in society, because of the rising demands for food products and changes in the environment brought on by humans (de Mesquita Souza Saraiva *et al.*, 2022). However, it appears that there are limits to the quantum of antibiotics that can be used while maintaining the

susceptibility of microbiome to antibiotics, just as there are limits to our understanding of the effects of human activities on the biosphere, which is currently restricted but developing (Paul and Das, 2022). Understanding the origins, scope, and potential solutions of this public health dilemma is essential to finding the most effective treatments. Though scores of drug-resistant pathogens have garnered media attention over the past 25 years, many clinical microbiologists now concur that MDR strains of Gram-negative bacteria constitute the biggest threat to public health (Vasanthrao and Chattopadhyay, 2023). The increasing resistance among *S. aureus* has also been on the rise in Gram-positive bacteria, with extensive ramifications for the loss of effective therapies for skin and soft tissue infections, urinary tract infections, and pneumonia (Mbhele, 2021). Although AMR-related interventions have become more varied in recent years, there are still significant gaps in the design, implementation, and reporting of AMR initiatives (Charaniet *al.*, 2023). Using knowledge from the implementation, sustainability, social, and health sciences, as well as current projects to create integrated methods for evaluating One Health initiatives (Léger *et al.*, 2021).

The ‘One Health’ strategy is described as a collaboration between numerous disciplines that seeks to address issues relating to environmental, animal, and human health (WHO, 2022). Food safety, zoonotic disease control, laboratory services, neglected tropical diseases, environmental health, and AMR are significant areas where the ‘One Health’ approach is particularly relevant (Miao *et al.*, 2022). The AMR is a challenge in global health that most exemplifies the One Health concept (Rhouma *et al.*, 2023). The AMR relates to industries like agriculture, animal husbandry, and human medicine, due to the careless and excessive use of antimicrobials by them (Koju *et al.*, 2022).

One Health local interventions are undoubtedly more practical than global health interventions, as the latter requires a global strategy and must be prepared with a thorough awareness of international and multicultural issues. Global health as well as one health are connected and may employ comparable technologies (Hernando-Amado *et al.*, 2019). Scaling up the concept of "antibiotic-resistant

environments" including contaminated rivers, beaches, and soils would allow for the ranking of hospitals, farms, and environments according to their level of AMR transmission risk and, as a result, the design of evidence-based interventions to reduce these risks, like isolating these "antibiotic-resistant entities" from nearby non-contaminated ones and reducing the flow of AMR-containing materials such as hosts, flies, and other organisms (Aslam *et al.*, 2021).

All ecological niches that support different microbial communities harbour ARGs, which are ancient and widespread components of bacteria (Chen *et al.*, 2022). The environment can be split into natural and developed from the perspective of microbial ecology, with the former being further broken down into aquatic and terrestrial ecosystems (Ghannam and Techtmann, 2021). Freshwater ecosystems (rivers, lakes, and wetlands) and marine ecosystems (oceans and estuaries) are both included in the aquatic environment (Li *et al.*, 2020). The term terrestrial environment refers to a broad range of terrestrial ecosystems found in a variety of climatic regions, including forests, deserts, grasslands, and tundra (Yan *et al.*, 2020). Hospital facilities, agricultural areas, aquaculture activities, and wastewater treatment plants (WWTPs) are examples of built environments (Kim and Cha, 2021).

The idea of 'One Health' is not new and has long been at the forefront of interdisciplinary and multisectoral conversations, there is now a greater desire for this strategy to be put into practice (Adisasmito *et al.*, 2022). The World Health Assembly supported a global action plan on AMR in 2015, and nations all around the world created frameworks defining their specific responses to the pandemic (WHO, 2022). In the LMICs, the emphasis is on problems like lax regulation of the production and sale of antibiotics, whereas in high-income jurisdictions, frameworks and action plans include objectives like advancing AMR knowledge through research and surveillance, raising public awareness of AMR through communication and education, and aiming to improve infection prevention and control, primarily working with the private sector (Pattis *et al.*, 2022).

One Health is a transdisciplinary strategy that considers the connections among three main pillars viz., people, animals, and the environment (Prata, 2022). Water is a resource that is necessary for both human and animal survival, and its cycle is influenced by the environment as well, which is worsened by contamination and climate change (du Plessis, 2023). Water contamination is a big issue, with 28 per cent of groundwater and 22 per cent of surface water bodies in the European Union being adversely impacted by diffuse pollution from agriculture (Duquennoi and Martinez, 2022). All these necessitate the formation of the One Health philosophy which should be used to regulate the contamination of water resources (Prata, 2022).

2.5. One health and environment

Though environmental pollution is not a recent phenomenon, it continues to be the biggest threat to humanity and the main factor in death and disease in the environment (Ukaogo *et al.*, 2020). Environmental contamination around the world is largely due to human activities such as urbanisation, industrialization, mining, and the exploration of stricter rules (Akhtar *et al.*, 2021). The rising public health concern over the effects that the enormous release of chemicals and consequent contamination will have on the environment and human health has been rising and unintentional exposure to various pollutants, such as heavy metals, air pollutants, and organic compounds, may have a variety of negative consequences on human bodies, leading to the onset and development of various diseases (Xu *et al.*, 2022).

Zambrano (2023) stated that the health of connected habitats may pose a threat to human health since several interconnected human, animal, and environmental habitats might influence the emergence, evolution, and spread of antibiotic resistance. Furthermore, the spread of ARGs and resistant clones among human-associated, animal-associated, and environmental microbiomes has the potential to modify local and global bacterial population genetics, affecting the structure and ultimately the productivity of microbiomes where drug-resistant bacteria can spread (Nielsen *et al.*, 2022).

The pathogens, ARGs, and chemicals can all be found naturally in the environment (Amarasiri *et al.*, 2020). Overuse of toxic chemicals, an overabundance of nutrients, pathogens, and antibiotics have been left behind by anthropogenic contamination of the air, water, and soil (Anand *et al.*, 2021). These substances also build up over time in the environment, some for very long times. They move through the water and air currents as well as on and within the bodies of living things (WHO, 2022).

2.6. Water pollution

Environmental as well as human health are significantly threatened by water contamination. Several resources for water pollution, including various pollutants like antibiotics, cosmetics, and pollutants from industry such as dyes and pigments are just a few of the increasing organic contaminants that contaminate water sources (Pouraminiet *al.*, 2023). Chemical-related water pollution has become a top priority for the industrial world as well as a major source of concern for society and public authorities (Tariq *et al.*, 2023). Numerous factors contribute to water pollution, including energy consumption, industrial waste, mining activities, sewage and wastewater, pesticides and chemical fertilisers, urban development, radioactive waste, and others (Akbar *et al.*, 2023). Majority of the human activities including domestic, agricultural, and industrial production result in the production of effluents that contain hazardous substances (Crini and Lichtfouse, 2019). Nonetheless, the wastewater from hospitals usually contains radionuclides, chemical reagents, pharmaceutical residues, and other dangerous materials (Moldovan *et al.*, 2023). There are differences in the characteristics, volume, and handling techniques of wastewater not just between nations but even within one (Mao *et al.*, 2021). Although there are rules for the treatment of wastewater on a global scale, some dangerous compounds in hospital wastewater may have a regulatory status and should be handled properly, while others may resemble home sewage in their features (Kumari *et al.*, 2020).

Conventional domestic wastewater treatment plants, which frequently use stabilisation ponds and activated sludge systems, have not been designed to treat recalcitrant organic pollutants, including emerging substances (Morin-Criniet *al.*, 2022). It is well known that the presence of many emerging contaminants can be correlated with the discharge of wastewater effluents, especially from wastewater treatment plants located in highly industrialised and urban areas (Fatimazahra *et al.*, 2023).

2.7. Approaches to water treatment

The primary goal of wastewater treatment is to eliminate the several contaminants that are present in the wastewater, including suspended particles, organic carbon, nutrients, inorganic salts, heavy metals, pathogens, and others. The protection of human health and the environment is the ultimate objective of wastewater treatment (de Oliveira *et al.*, 2020). To create an effective treatment system that will assure the safety, effectiveness, and quality of the treated wastewater, it is essential to understand the nature of wastewater (Saravanan *et al.*, 2021). In general, physical, chemical, and biological processes or combinations among these are used for wastewater treatment; operations are also used in conventional wastewater treatment to remove soluble pollutants and insoluble particles from effluents (Fitch *et al.*, 2022).

For many centuries, physicochemical treatment procedures were most commonly used to produce potable water for human use (Tóth *et al.*, 2023). With the advent of the industrial revolution and industrialisation, many novel chemicals have emerged as pollutants in water. The existing methods of water treatment including physical (screening, filtration, sedimentation, membrane filtration), chemical (chemical precipitation, adsorption, ion exchange, electrochemical treatment) and biological processes like biosorption and precipitation are ineffective in modern wastewater treatment (Dutta *et al.*, 2021).

As a result, research into water treatment techniques has been crucial to treating the growing pollution (Shahediet *al.*, 2020).

2.8. Nanotechnology as an alternative

Nanotechnology has been utilised to enhance wastewater quality and remove organic and inorganic pollutants that are challenging to remove using standard techniques (François *et al.*, 2023). The use of emerging materials like nanomaterials has the potential to influence technology in several ways (Aragawet *al.*, 2021). The use of nanotechnology in wastewater treatment is an emerging trend not only for their pollutant removal applications but also for their practicality and affordability (Aragawet *al.*, 2021; Eid *et al.*, 2023).

Iron oxide nanoparticles (IONPs) have been the subject of extensive research in numerous domains by a factor of their environmental applications, including wastewater treatment. The IONPs are widely used most likely because of their low cost, ability to be separated using external magnetic fields, high surface area, and high adsorption capability (Jabbar *et al.*, 2022). Kefeni and Mamba (2020) investigated the capacity of spinel ferrite nanoparticles (SFNPs) for pollutant adsorption and ease of magnetic field separation as an efficient contributor to wastewater treatment systems. The SFNPs were found not only easy to separate and reuse owing to their magnetic properties but also to efficiently capture free solar energy due to their tiny energy band gap (Akram *et al.*, 2023).

Wastewater treatment is a major issue in developing countries like India (Jadeja *et al.*, 2022). Bioremediation using NPs is a recently developing method for the treatment of wastewater as it is more cost-efficient and effective than traditional physical and chemical procedures. Due to their huge surface area, metal and metal oxide NPs, are being used often in bio-nanotechnology, particularly for the treatment of wastewater (Sharma *et al.*, 2023).

2.8.1. Metal oxide nanoparticles

Metal oxide NPs have drawn a lot of attention in recent years for their properties like excellent biological, energetic, environmental and catalytic potentials (Samuel *et al.*, 2022). The NPs are used extensively due to their excellent selectivity and removal of heavy metals as they have vast surface area and low

application concentrations. This functions as their ability to remove these pollutants from water (Naseem and Durrani, 2021). They are promising materials for several biomedical advantages such as high stability, straightforward preparation procedures, easy engineering to the desired size, shape, and porosity, easy incorporation into hydrophobic and hydrophilic systems, and easy functionalisation by various molecules (Nikolova and Chavali, 2020).

The importance of green synthesis or the use of plant and microbial extracts as reducing and stabilising agents for the synthesis of these NPs has been increasing recently as they are an easy, affordable, environmentally beneficial, and renewable method of fabrication (Gebre, 2023). Metal oxide NPs are extensively used as antimicrobial agents due to their potential in killing the microbes through pathways including the production of reactive oxygen species (ROS). The rate of ROS production depends directly or indirectly on the dose, metal speciation, and exposure route (Kessler *et al.*, 2022).

2.8.1.1. Zinc oxide NPs

The practical applications of zinc oxide (ZnO) have been significantly improvised by reducing their sizes to a nanoscale level (Bandeira *et al.*, 2020). Ecological-friendly, less waste-producing, safe and non-toxic ZnO NPs could be synthesised using green nanotechnology (Issaabadi *et al.*, 2022). Different characterisation techniques have shed light on the architecture of the synthesised nanoparticles (Gur *et al.*, 2022). Biocompatible green synthesised ZnO nanoparticles are extensively used as medications in the pharmaceutical and biomedical industries owing to their superior antibacterial and antioxidant properties as compared to the ones synthesised by physical and chemical methods (Velsankaret *et al.*, 2022).

The antibacterial efficiency of ZnO NPs synthesised using a supernatant of *Escherichia hermannii* strain isolated from raw milk exhibited minimum inhibitory concentration (MIC) values ranging from 10 to 40 µg/mL against MDR-*E. coli* and *Klebsiella pneumoniae* isolated from urine (Gad *et al.*, 2020). Moreover, ZnO NPs

synthesised using *Acremonium potronii* were able to effectively degrade 93 per cent of cationic methylene blue (MB) dye (Ameen *et al.*, 2021).

Additionally, ZnO NPs were synthesised using piperine in a prior investigation to assess the *in vitro* antibacterial effectiveness against MDR strains of non-typhoidal *Salmonella* (NTS). The research exhibited an MIC ($62.50\mu\text{g/mL}$) and minimum bactericidal concentration (MBC) value of $125\mu\text{g/mL}$ displayed by ZnO NPs against the MDR-NTS strains. These relatively low values may be caused by the induction of oxidative stress, destabilisation of the bacterial outer membrane, disruption of ion mobility, or the start of apoptosis (Unni *et al.*, 2022).

2.8.1.2. Silver NPs

Silver (Ag) NPs have been used extensively in the fields of biomedicine, pharmacy, and cosmetics due to their superior antibacterial properties, decreased toxicity, and increased biocompatibility when compared to other metallic NPs (Crisan *et al.*, 2021). Large surface areas, capacity to bind with bacterial biomolecules, penetration into cells, generation of ROS, and capacity to act as moderators in microbial signal transduction pathways are the several mechanisms for the antimicrobial activities of AgNPs (Khan *et al.*, 2022). The antibacterial activity of green synthesised AgNPs synthesised using aqueous extract of *Pisum sativum* L. (pea) exhibited superior antibacterial activity against *S. aureus* ATCC 25923, with MIC and MBC values ranging from $6.25\mu\text{g/mL}$ to $75\mu\text{g/mL}$ and $50\mu\text{g/mL}$ to $150\mu\text{g/mL}$ (Alarjaniet *al.*, 2022).

The dye degradation abilities of AgNPssynthesised by *Chloroxylon swietenia* were found to be more than 95%, 90%, and greater than 90% against congo red (CR), coomassie blue (CB), and crystal violet (CV) dyes within 24 h of treatment, respectively under ideal circumstances. Later, the antibacterial capacity of these AgNPs (10 mg/mL) was evaluated against *Staphylococcus nepalensis* ($3.03 \pm 0.35\text{ cm}$), *Staphylococcus gallinarum* ($2.96 \pm 0.15\text{ cm}$), *Bacillus subtilis* ($2.86 \pm 0.23\text{ cm}$), *Enterococcus faecalis* ($2.8 \pm 0.30\text{ cm}$) and *Pseudomonas stuteria* ($2.06 \pm 0.25\text{ cm}$) employing disc diffusion method (Nga *et al.*, 2022).

The photocatalytic antibiotic degradation abilities of green synthesised AgNPs fabricated using *Melia Azedarach* fruit extract, loaded on amorphous silica against ciprofloxacin reached 98 per cent (Golmohammadi *et al.*, 2023).

2.8.1.3. Ag/ZnO nanocomposites

The Ag/ZnO nanocomposites (NCs) have garnered a lot of attention in recent times due to their enhanced antibacterial activity as compared to individual NPs. The synergistic impact creates a new class of hybrid nanomaterials. Additionally, the synergistic effect of Ag and ZnO is beneficial for the mass generation of ROS such as H_2O_2 , increasing the antibacterial activity of NCs (Mousavi-Kouhi *et al.*, 2021).

The distinctive heterostructure of ZnO nanorods, crystallinity, oxygen defect, presence of metallic AgNP, and oxygen vacancy enhance the distance of photogenerated electron-hole pairs, hence increasing the photocatalytic activity (Khan *et al.*, 2021). The antibacterial efficiency of green synthesised Ag/ZnO NCs fabricated using *Verbascum speciosum* aqueous extract was evaluated against *S. aureus* and *E. coli* (Mousavi-Kouhi *et al.*, 2021). The photocatalytic dye degradation of Ag/ZnO NC synthesised using leaf extract of *Carya illinoensis* demonstrated an exceptional catalytic efficacy at pH 10. Furthermore, a pseudo-first-order kinetic model was used for the photo-degradation of rhodamine-B (RhB) dye (Ahmad *et al.*, 2022).

The free radical scavenging abilities of Ag/ZnO NCs synthesised using an aqueous extract of *Capparis zeylanica* exhibited exceptional antioxidant activity measured using a DPPH scavenging assay with exceptionally better IC_{50} values of 340 $\mu g/mL$ (Suresh *et al.*, 2023).

2.8.2. Green synthesis of NPs

The synthesis of NPs using a green approach has become a viable option for creating nanomaterials with a variety of physical, chemical, and biological properties (Sikiru *et al.*, 2022). Physical and chemical approaches have historically

been employed, but their usefulness has been constrained by the creation of hazardous byproducts, expensive instrumentation, and time-consuming experimental procedures. A prospective substitute that offers an eco-friendly, economically viable, and energy-efficient method to produce a wide variety of NPs is the biogenic synthesis of NPs (Khan *et al.*, 2022).

Most nanoscale metals which are produced chemically, have unforeseen consequences for the environment, high energy consumption, and potential health risks (Ying *et al.*, 2022). The green synthesis of NPs is a crucial method for ensuring lower toxicity levels and, consequently, permitting their unimpeded deployment in human consumer goods (Samuelet *al.*, 2022). Nonetheless, green synthesis is less expensive, produces less pollution, and enhances environmental and human health safety, it is more advantageous than standard chemical synthesis. Green synthesis of metal oxide NPs utilising microbes and plant extracts opens up a plethora of opportunities for the creation of biocompatible and inexpensive particles with potential uses in the healthcare industry (Labanniet *al.*, 2023). Applications of biological techniques for the synthesis of NPs are more advantageous since it makes use of the biomolecules that act as reducing, capping, and stabilising agents in plant extracts (Aswathi *et al.*, 2023).

2.9. NPs in environmental remediation

Employing nanotechnology to enhance the quality of the air, water, and soil by improving contamination identification and sensing might help with the creation of novel cleanup solutions (Li *et al.*, 2021). The NPs could be effectively employed in bioremediation in addition to drug delivery applications since they could decompose pollutants without harming the environment because the nanocarriers are made from natural substances (Samuel *et al.*, 2022). The reusability of magnetic NPs, and their highly efficient mechanisms in dye degradation even after five cycles were reported earlier (Nasri *et al.*, 2021).

Understanding the dynamic production and growth processes of NPs is essential for developing ways that effectively stop pollutants from forming in the

first place and reduce their emissions. The characteristic physical and chemical features of metal oxide NPs along with their significant potential have attracted significant attraction for environmental remediation, especially in the fields of heavy metals removal, the detection of toxic gases, textiles, biomedical applications, and the photocatalytic destruction of organic pollutants (Danish *et al.*, 2022).

Green synthesised AgNPs using *Aegle marmelos* aqueous leaf extract showing antimicrobial effects against *Bacillus megaterium*, *Bacillus aryabhattai*, *S. aureus*, *Serratia marcescens*, and *Pseudomonas putida* also exhibited photocatalytic dye degradation against MB dye under visible light irradiation (Sampath *et al.*, 2023).

The photocatalytic potential of ZnBi₂O₄NPs demonstrated the removal of cefixime under visible and UV spectra of absorption which exhibited 89 per cent of antibiotic degradation with solar light at 30 min of exposure, while yielding 88 per cent degradation under UV light after two hours (Baaloudjet *et al.*, 2021).

The use of biodegradable materials in place of plastics and the reduction of harmful chemicals in manufacturing processes are only a few of the innovative methods offered by nanotechnology to reduce pollution in various operations. It is also employed in the detection and removal of environmental contaminants, such as those that harm both the environment and human health over the short and long terms, such as air, soil, and water pollution (Beni and Jabbari, 2022).

3. Materials and Methods

3. MATERIALS AND METHODS

3.1. MATERIALS

3.1.1. Chemicals and reagents

The chemicals and reagents used in this study were of Analytical Grade and were procured from Merck (Germany), Invitrogen (USA), Sigma-Aldrich (India), Sisco Research Laboratories (SRL, India), Loba Chemie (India), HiMedia (India), DSS Takara Bio (India) and other reputed national and international firms.

3.1.2. Plastic ware and glassware

The plastic ware and glassware were procured from Tarsons, JSGW, and Borosil (India). The washing and sterilization of glassware were carried out according to the standard procedures.

3.1.3. Scientific Instruments and Equipment

The scientific equipment used in this study includes a probe sonicator (Hielscher Ultrasonics, Germany), digital ultrasonic sonicator (Labman Scientific Instruments, India), cyclomixer (REMI Equipments, India), microplate reader (Bio-Rad, USA), hot air oven (Roteck, India), water bath (Labline, India), laboratory centrifuge (REMI Equipments, India), UV-Vis spectrophotometer (ThermoFisher Scientific, USA), laminar airflow system (Labline, India), bacteriological incubator (Labline, India), electronic weighing machine (Shimadzu, Japan), pH meter (Eutech Instruments, India), LED light (Oppl, India), UV light (Violight, India) and micropipettes (ThermoFisher Scientific, Eppendorf, Germany).

3.1.4. Bacterial strains

The characterised multi-drug-resistant (MDR) bacterial strains of *S. enterica* Typhimurium (n= 3; ST1, ST2, ST3), *S. Enteritidis* (n= 3; S1, S2, S3), enteroaggregative *E. coli* (EAEC; n=3; E1, E2, E3), methicillin-resistant *S. aureus* (MRSA; n=3; Sa1, Sa2, Sa3) maintained in the Zoonoses laboratory of the

Department of Veterinary Public Health, College of Veterinary and Animal Sciences, Pookode as a part of the ICAR- NASF funded research project constituted for the study. Besides, standard strains of *E.coli* ATCC 25922, *E. coli* DH5 α and *Lactobacillus acidophilus* MTCC 10307 were also used in this study.

3.2. METHODS

3.2.1. Green synthesis of nanoparticles (NPs)

3.2.1.1. *Green synthesis of silver (Ag) NPs*

The green synthesis of AgNPs was carried out using the procedure described by Abishadet *et al.* (2022) using a cell-free supernatant of potential probiotic strain *L. acidophilus* MTCC 10307.

In brief, *L. acidophilus* MTCC 10307 was grown overnight in deMan, Rogosa and Sharpe (MRS) broth (100 mL; HiMedia) and incubated at 37 °C for 72 h. The bacterial biomass after incubation was separated by centrifugation (8000 xg for 15 min) to obtain the cell-free supernatant. The green synthesis of AgNPs was carried out by mixing 0.10 M silver nitrate (Sigma-Aldrich) in deionized water with the decline phase cell-free supernatant of the probiotic strain (4:1) at 37 °C in a magnetic stirrer (300 rpm) for 60 min.

A change in the colour of the reaction mixture from colourless to brown indicated the formation of AgNPs. The AgNPs thus formed were washed thrice each in methanol (Loba Chemie) and nanopure water to remove the impurities. Finally, the AgNPs were air-dried at 50 °C overnight and stored at 4 °C until further use.

3.2.1.2 *Green synthesis of zinc oxide (ZnO) NPs*

The green synthesis of ZnO NPs was carried out using an ethanolic extract of piperine (10mg/mL; Unni *et al.*, 2022).

Briefly, the ethanolic extract of piperine (30 mL; Sigma-Aldrich) was added to 0.10 M zinc acetate dihydrate (SRL) solution in deionized water (70 mL) and subjected to continuous stirring in a magnetic stirrer (300 rpm) at 60 °C for 2 h.

A colour change of the reaction mixture from colourless to milky white with the formation of yellowish-white particles in the solution indicated the synthesis of ZnO NPs. Later, the ZnO NPs formed were washed thrice each in methanol and nanopure water to remove the impurities. Finally, the ZnO NPs were air-dried at 50 °C overnight and stored at 4 °C until further use.

3.2.1.3. Green synthesis of Ag/ZnO nanocomposites (NCs)

The green synthesis of Ag/ZnO NCs was carried out as described by Arya *et al.* (2023) using the methanolic extract of the leaves and stem of *C.longa*.

Briefly, the filtrate of *C.longa* methanolic extract (20 mL) was added to an aqueous mixture of 0.10 M zinc nitrate dihydrate solution (48 mL) and 0.10 M silver nitrate solution (32 mL). The solution was then kept for continuous stirring in a magnetic stirrer (300 rpm) at 60 °C for 24 h.

A colour change of the reaction mixture from light yellow to dark brown indicated the formation of Ag/ZnO NCs. Later, the synthesised Ag/ZnO NCs were washed thrice each in methanol and nanopure water to remove the impurities. Finally, the Ag/ZnO NCs were air-dried at 50 °C overnight and stored at 4 °C until further use.

3.2.2. UV-Vis spectrophotometric characterisation of green synthesised NPs

The green synthesised NPs were validated by scanning within a range of 200 to 700 nm using a UV-visible spectrophotometer (ThermoFisher Scientific Pvt. Ltd., USA), keeping the silver nitrate (0.10 M), zinc acetate dihydrate (0.10 M) and a mixture of silver nitrate (0.10 M) and zinc nitrate (0.10 M) as respective controls for AgNPs, ZnO NPs and Ag/ZnO NCs.

As the physicochemical characterisation of the NPs (Fourier-transform infrared spectroscopy, powder X-ray crystallography, Thermogravimetric-differential thermal analysis, scanning electron microscopy, transmission electron microscopy and selected area electron diffraction) was performed in the earlier

studies (Abishadet *et al.*, 2022; Unni *et al.*, 2022; Arya *et al.*, 2023), the analysis was not repeated.

3.2.3. *In vitro* antimicrobial efficacy of green synthesised NPs

The *in vitro* antimicrobial efficacy of green synthesised NPs was evaluated against the MDR strains of EAEC (n= 3), non-typhoidal *Salmonella* spp. (NTS; *S. Typhimurium* and *S. Enteritidis*; n= 3 for each serotype) and MRSA (n=3).

3.2.3.1. *Determination of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)*

The *in vitro* antimicrobial efficacy of green synthesised NPs was evaluated by determining the MIC and MBC values against the characterised MDR field strains of EAEC, *S. Typhimurium* and *S. Enteritidis*, and MRSA by micro broth dilution technique (CLSI, 2019).

In brief, 100 µL of the individual test cultures (final concentration of 1×10^7 CFU/mL) were co-incubated with decreasing concentrations of NPs (500 to 0.244 µg/ mL) in 100 µL of cation-adjusted Mueller Hinton broth (CA-MH; HiMedia) in a 96- well flat-bottom microtiter plate. After the incubation at 37 °C for 18 to 24 h, 20 µL of the resazurin dye (0.015 per cent; HiMedia) was added to all the wells to determine the dye reduction (from purple to pink) and thereby the bacterial inhibition. The lowest concentration of NPs without visible growth was designated as MIC.

In order to determine the MBC of NPs (Miles *et al.*, 1938), 10 µL aliquots were drawn from each well revealing no visible growth in 96-well flat-bottom microtiter plate and inoculated onto Eosin-methylene blue (EMB) agar (HiMedia) supplemented with 100 µg of ampicillin (SRL), since all the three test isolates of EAEC used in this assay were ampicillin resistant. Similarly, Xylose Lysine Deoxycholate (XLD) agar (HiMedia) supplemented with 100 µg of ampicillin was used for the inoculation of NTS, as these isolates exhibited resistance to

ampicillin. Baird-Parker (BP) agar (HiMedia) was used for the inoculation of MRSA to determine the MBC values.

The lowest concentration of the NPs which revealed 99.90 per cent killing of the test cultures in the EMB, XLD and BP agars was determined to be their MBC (NCCLS, 1999).

3.2.3.2. *In vitro* antibiofilm potential of NPs against MDR test isolates

The *in vitro* antibiofilm efficacy of green synthesized NPs against the tested strains of MDR isolates viz., EAEC, NTS and MRSA were investigated at 24 h and 48 h by using the crystal violet staining assay in 96-well microtiter plates (Vergis *et al.*, 2019).

In short, the individual MDR isolates (1×10^7 CFU/mL; 100 μ L) were co-incubated with MIC (1X) levels of NPs (100 μ L) in sterile nutrient broth (containing 0.45% D-Glucose; HiMedia), keeping respective controls. The positive control consisted of individual untreated bacterial cultures (100 μ L) in nutrient broth (100 μ L), whereas the negative control consisted of sterile nutrient broth (200 μ L). In this assay, *E. coli* ATCC 25922 was employed as a known biofilm former, whereas *E. coli* DH5 α was used as a non-biofilm former. After the incubation at 37 $^{\circ}$ C, the supernatant (consisting of planktonic cells) from each well was discarded at 24 h and 48 h, and the wells were stained with 200 μ L of 0.10% crystal violet (SRL) for 30 min. Later, the stain was removed carefully, the wells were washed thrice with sterile phosphate buffered saline (200 μ L; pH 7.40), and the stained biofilms formed at the bottom of the well were solubilized with 95% ethanol.

The absorbance was measured at 595 nm (Bio-Rad iMark Microplate reader, USA) for estimating the inhibition of biofilm biomass. Further, the reduction in biofilm biomass over 24 h and 48 h for the tested NPs was estimated (in per cent) as

$$(\text{A}_{\text{Control}} - \text{A}_{\text{Test}}) / (\text{A}_{\text{Control}}) \times 100$$

wherein A_{Control} denotes the absorbance of untreated control taken at 24 h and 48 h, whereas A_{Test} denotes the absorbance of treated samples taken at 24 h and 48 h.

3.2.4. *In vitro* antioxidant activity of NPs

The *in vitro* antioxidant activity of green synthesized NPs was determined by 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS^{•+})-based free radical scavenging assays.

3.2.4.1. DPPH assay

The DPPH-based free radical scavenging assay of the green synthesized NPs was carried out as described by Zare *et al.* (2019).

Briefly, 0.10 mM of DPPH (35 μL ; 3.94 mg in 100 mL methanol; SRL) was added to different concentrations (100 μL ; 0, 20, 40, 60, 80 and 100 $\mu\text{g/mL}$ in distilled water) of individual green synthesized NPs taken in 96-well microtiter plate. The plate was then incubated aerobically at room temperature in the dark for 30 min. The NP-untreated DPPH served as control and DPPH with distilled water was employed as the blank. Ascorbic acid (SRL) at different concentrations (0, 20, 40, 60, 80 and 100 $\mu\text{g/mL}$) served as the standard antioxidant.

After the incubation, the absorbance was recorded at 517 nm and the DPPH scavenging activity was calculated as

$$(\mathbf{A_{Control} - A_{Test}}) / (\mathbf{A_{Control}}) \times \mathbf{100}$$

wherein A_{Control} denotes the absorbance of the control, whereas A_{Test} denotes the absorbance of test samples.

3.2.4.2. ABTS assay

The antioxidant activity of green synthesized NPs was assessed as a measure of 2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) radical (ABTS^{•+}), described previously with certain modifications (Dalmolin *et al.*, 2016).

In brief, a mixture of ABTS (7 mM; SRL) and potassium persulfate (140 mM; SRL) was prepared and incubated in the dark at room temperature for 15 h to generate ABTS^{•+} radical. Later, the ABTS^{•+} solution was diluted in phosphate buffer (10 mM; pH 7.40; HiMedia) to attain an absorbance of 0.70 at 734 nm. Different concentrations of individual green synthesized NPs (20, 40, 60, 80 and 100 µg/mL) were prepared, keeping ascorbic acid as the standard. The samples were then aerobically incubated at 37 °C for 20 min in the dark and the absorbance was measured at 734 nm.

The radical scavenging activity was estimated as

$$(\text{A}_{\text{Control}} - \text{A}_{\text{Test}}) / (\text{A}_{\text{Control}}) \times 100,$$

wherein A_{Control} denotes the absorbance of control, whereas A_{Test} denotes the absorbance of test samples.

3.2.5. *In vitro* photocatalytic dye degradation potential of NPs

The *in vitro* photocatalytic dye degradation potential of the green synthesised NPs was determined against methylene blue (MB), crystal violet (CV), and Rhodamine-B (RhB) dyes (Loba Chemie) on exposure to light sources (Abishadet *al.*, 2023; Hezamet *al.*, 2023).

3.2.5.1 *In vitro* photocatalytic potential of NPs for degradation of individual dye

The *in vitro* photocatalytic dye degradation potential of the green synthesised NPs was determined individually against methylene blue (MB), crystal violet (CV), and Rhodamine-B (RhB) dyes (Loba Chemie) on exposure to three different light sources *viz.*, sunlight, light emitting diode (LED), and ultra-violet (UV) light (Abishadet *al.*, 2023; Hezamet *al.*, 2023).

Briefly, the aqueous solution of individual dyes (5 ppm for MB and RhB, and 50 ppm for RhB) was mixed with individual NPs (final concentration of 0.50 mg/mL). The dye-NP mixtures were then agitated in a magnetic stirrer and kept at 300 rpm for 15 min in the dark to obtain an adsorption-desorption isotherm. Later,

the mixtures were exposed separately to sunlight (11.5408348°, 76.0209177°; 52100 lx), LED light (63400 lx, 460 nm, 50 W [Oppl, India]), and UV light (260 nm, 16 W [Violight, India]). The samples drawn at specific intervals (0, 15, 30, 45, 60, 75, 90, and 105 min) were then centrifuged for 15 min at 5000 rpm.

The absorbance was recorded at 670, 592 and 550 nm for MB, CV and RhB, respectively (Saravan *et al.*, 2020; Waghchaure *et al.*, 2022) using a UV-Vis spectrophotometer (ThermoFisher). The degradation of dye in solution (in per cent) was estimated (Muthuvel *et al.*, 2020) as

$$(\text{A}_{\text{Control}} - \text{A}_{\text{Test}}) / (\text{A}_{\text{Control}}) \times 100$$

wherein $\text{A}_{\text{Control}}$ denotes the absorbance of the control, whereas A_{Test} denotes the absorbance of test samples at specific time points.

3.2.5.2. Determination of kinetics of photocatalytic dye degradation

In order to quantify the photocatalytic dye degradation assay, different orders of kinetics were analysed *viz.*, zero-order (eq. 1), first-order (eq. 2), pseudo-first-order (eq. 3), and second-order (eq. 4), with its rate constants, as follows:

$$C_1 = -kt + C_0 \text{ (eq. 1)}$$

$$\ln C_1 = -kt + \ln C_0 \text{ (eq. 2)}$$

$$\ln \frac{C_1}{C_0} = -kt \quad \text{(eq. 3)}$$

$$\frac{1}{C_1} = kt + \frac{1}{C_0} \text{ (eq. 4)}$$

wherein the rate constant k was expressed as $\text{molL}^{-1}\text{min}^{-1}$ for zero-order, min^{-1} for first-order, and pseudo-first-order and $\text{Lmol}^{-1}\text{min}^{-1}$ for second-order reactions (Song *et al.*, 2022; Lanjwani *et al.*, 2023).

3.2.5.3. Selectivity of dyes on photocatalytic dye degradation

The selectivity of dyes on photocatalytic dye degradation was assessed by mixing all three dyes (MB, CV, and RhB) to obtain a final concentration of 5 ppm

for MB and RhB and 50 ppm for RhBppm, and mixed with Ag/ZnO NCs (since the best dye degradation potential was observed in section 3.2.5.1) to obtain a final concentration of 0.50 mg/mL of the NCs. The resultant mixture was mixed, agitated in a magnetic stirrer and kept at 300 rpm for 15 min in the dark to obtain an adsorption-desorption isotherm. Later, the mixtures were exposed to sunlight (11.5408348°, 76.0209177°; 52100 lx). The samples drawn at specific time intervals (0, 15, 30, 45, 60, 75, 90, and 105 min) were then centrifuged for 15 min at 5000 rpm. The absorbance was recorded at 670, 592 and 550 nm for MB, CV and RhB, respectively (Saravan *et al.*, 2020; Waghchaure *et al.*, 2022) using a UV-Vis spectrophotometer (ThermoFisher Scientific Pvt. Ltd., USA).

The degradation of dye in solution (in per cent) was estimated (Muthuvel *et al.*, 2020) as

$$(A_{\text{Control}} - A_{\text{Test}}) / (A_{\text{Control}}) \times 100$$

wherein A_{Control} denotes the absorbance of the control, whereas A_{Test} denotes the absorbance of test samples at specific time points.

3.2.5.4. Effect of reusability of NPs in photocatalytic dye degradation

The effect of reusability of Ag/ZnO NCs in the selectivity of dyes (section 3.2.5.3) on photocatalytic dye degradation was assessed (Tumbelaka *et al.*, 2022).

Briefly, the used Ag/ZnO NCs after selectivity assays (section 3.2.5.3) were washed (5000 rpm for 15 min) using nanopure water and were dried at 50 °C. The resultant NCs were then mixed with the combination of three dyes (MB, CV, and RhB) to obtain a final concentration of 5 ppm for MB and RhB, 50 ppm for RhB and 0.50 mg/mL for the NCs. The resultant mixture was mixed, agitated in a magnetic stirrer and kept at 300 rpm for 15 min in the dark to obtain an adsorption-desorption isotherm. Later, the mixtures were exposed to sunlight (11.5408348°, 76.0209177°; 52100 lx). The samples drawn at specific intervals (0, 15, 30, 45, 60, 75, 90, and 105 min) were then centrifuged for 15 min at 5000 rpm. The absorbance was recorded at 670, 592 and 550 nm for MB, CV and RhB, respectively (Saravan *et*

al., 2020; Waghchaure *et al.*, 2022) using a UV-Vis spectrophotometer (ThermoFisher Scientific Pvt. Ltd., USA).

The degradation of dye in solution (in per cent) was estimated (Muthuvel *et al.*, 2020) as

$$(A_{\text{Control}} - A_{\text{Test}}) / (A_{\text{Control}}) \times 100$$

wherein A_{Control} denotes the absorbance of the control, whereas A_{Test} denotes the absorbance of test samples at specific time points.

3.2.6. *In vitro* photocatalytic antibiotic degradation ability of NPs

The *in vitro* photocatalytic potential of the green synthesised NPs to degrade ampicillin, ciprofloxacin and tetracycline (SRL) was investigated on exposure to sunlight (Baaloudjet *al.*, 2021).

Briefly, the aqueous solution of individual antibiotics (20 ppm) was mixed with individual NPs to attain a final concentration of 2 mg/mL. To obtain an adsorption-desorption isotherm, the antibiotic-NP solutions were then stirred at 300 rpm for 15 min in the dark using a magnetic stirrer. Later, the mixture was exposed separately to sunlight (52100 lx). The aliquots drawn at specific intervals (0, 15, 30, 60, 90, and 120 min) were then centrifuged for 15 min at 5000 rpm to settle down the NPs. The absorbance was recorded at 269, 277 and 357 nm for ampicillin, ciprofloxacin and tetracycline, respectively, using a UV-Vis spectrophotometer.

The degradation of antibiotics in solution (in per cent) was estimated (Lin and Cen, 2022) as

$$(A_{\text{max}} - A_0) / (A_{\text{max}}) \times 100$$

wherein A_{max} denotes the absorbance of the test sample at a specific time point, whereas A_0 denotes the absorbance of control.

3.2.7. *In vitro* dose- and time-dependent photocatalytic killing kinetics of MDR bacterial strains treated with NPs

The photocatalytic antibacterial activity of green synthesised NPs was determined against the MDR test strains of EAEC, NTS and MRSA by an *in vitro* dose- and time-dependent extracellular growth kinetics with certain modifications (Vergis *et al.*, 2020).

Briefly, the log-phase cultures of each of the individual test isolates grown on nutrient broth were centrifuged at 10,000 rpm for 10 min. Later, the supernatant was discarded, and the bacterial pellet was mixed with sterile PBS (pH 7.40) to achieve 0.50 Mc Farland standard tube turbidity (approximately 1.50×10^8 CFU/mL) for each test isolate. The bacterial cultures were then diluted to attain a concentration of 1×10^7 CFU/mL using sterile CA-MH broth. The green synthesized NPs were added to the bacterial inoculum to attain a final concentration of MIC (1X), MIC (1/2X), MIC (1/5X) and MIC (1/10X), and the following groups were formed:

Group 1: 1×10^7 CFU of MDR test strains (100 μ L) + 1X MIC NPs (100 μ L);

Group 2: 1×10^7 CFU of MDR test strains (100 μ L) + 1/2X MIC NPs (100 μ L);

Group 3: 1×10^7 CFU of MDR test strains (100 μ L) + 1/5X MIC NPs (100 μ L);

Group 4: 1×10^7 CFU of MDR test strains (100 μ L) + 1/10X MIC NPs (100 μ L);
and

Group 5: 1×10^7 CFU of MDR test strains (100 μ L) + sterile CA-MH Broth (100 μ L) taken as untreated control.

To enumerate the photocatalytic antibacterial effect of green synthesized NPs on MDR-EAEC, NTS and MRSA isolates, an aliquot (10 μ L) from all the groups was drawn at 0, 30, 60, 120, 180 and 240 min. At each time point, the aliquots drawn from each respective group were serially diluted with 90 μ L of sterile PBS and 10 μ L was plated on EMB agar plates supplemented with 100 μ g

of ampicillin for MDR-EAEC, XLD agar plates supplemented with 100 µg of ampicillin for MDR-NTS and BP agar plates for MDR-MRSA (Miles *et al.*, 1938).

After the incubation period at 37 °C for 18 to 24 h, the individual MDR-EAEC, NTS and MRSA colonies were counted and the colonies were expressed as log₁₀ CFU/mL.

3.2.8. Statistical Analysis

All experiments were performed as triplicates in three independent assays. The results were represented as means ± standard deviations of the three independent experiments performed using GraphPad Prism version 5.01 (GraphPad Software Inc., California, USA).

A two-way (repeated measures) ANOVA with Bonferroni multiple comparison post-test was used to compare the differences between control and NPs treated tests for the photocatalytic assays of MDR-EAEC, NTS and MRSA. Moreover, the *in vitro* antibiofilm efficacy of biosynthesized NPs against MDR-EAEC, NTS and MRSA isolates was performed by one-way ANOVA with Bonferroni multiple comparison post-test.

A *P*-value of ≤ 0.01 was considered highly significant, while a *P*-value ≤ 0.05 was considered statistically significant.

4. Results

4. RESULTS

The present study was envisaged to evaluate the antioxidant, and photocatalytic potential of green synthesised silver (Ag) nanoparticles (NPs), zinc oxide (ZnO) NPs and Ag/ZnO nanocomposites (NCs) in the dye removal and antibiotic degradation. Further, the anti-fouling as well as photocatalytic killing kinetics of these nanomaterials were assessed against multi-drug resistant (MDR) strains of enteroaggregative *E. coli* (EAEC), non-typhoidal *Salmonella* spp. (NTS), and methicillin-resistant *S. aureus* (MRSA). Initially, the green synthesis of AgNPs, ZnO NPs and Ag/ZnO NCs was carried out and re-validated using UV-Vis spectroscopy. Later, for assessing their antimicrobial and antibiofilm activity, the green synthesised NPs were tested against MDR strains of EAEC (n=3), *S. Enteritidis* (n=3), *S. Typhimurium* (n=3) and MRSA (n=3). Besides, the antioxidant activity of the green synthesised NPs was estimated. In addition, the photocatalytic activity of the green synthesised NPs was assessed for their ability to degrade industrial dyes as well as antibiotics and further to determine the *in vitro* time- and dose-dependent reduction of the MDR test isolates to determine the antimicrobial activity. The findings of the assays are systematically presented as follows.

4.1 GREEN SYNTHESIS OF NPs

4.1.1 Green synthesis of AgNPs

The green synthesis of AgNPs in this study was performed using a cell-free supernatant of *Lactobacillus acidophilus* MTCC 10307.

The active components present in the cell-free supernatant of *L. acidophilus* MTCC 10307 reduced the silver nitrate solution (0.10 M) to AgNPs at 25°C under constant stirring for 60 min. The synthesis of AgNPs was evidenced by the formation of a brownish-white precipitate at the bottom of the conical flask. The formation of AgNPs was later validated using UV-Vis spectroscopy.

4.1.2 Green synthesis of ZnONPs

In the present study, ethanolic extract of commercial piperine was used for the synthesis of ZnO NPs.

The ethanolic extract of piperine reduced the zinc acetate dihydrate solution (0.10 M) to ZnO NPs at 60 °C under constant stirring for 2 h. The synthesis of ZnO NPs was evidenced by the change in the colour of the solution to milky white and the formation of a yellowish-white precipitate at the bottom of the conical flask. Later, the formation of ZnO NPs was confirmed using UV-Vis spectroscopy.

4.1.3 Green synthesis of Ag/ZnO NCs

The Ag/ZnO NCs were fabricated using the methanolic extract of leaves and stem of *C. longa*.

The aqueous mixture of silver nitrate solution (0.10 M) and zinc nitrate dihydrate solution (0.10 M) was reduced using the methanolic extract of *C. longa* to produce Ag/ZnO NCs at 25°C under constant stirring for 24 h. The formation of a brown-coloured precipitate at the bottom of the conical flask indicated the formation of Ag/ZnO NCs. Further, the formation of Ag/ZnO NCs was confirmed using UV-Vis spectroscopy.

4.2 CHARACTERISATION OF NPs

The formation of the green synthesised NPs was validated using a UV-Vis spectroscopy which exhibited a characteristic absorbance peak at 430 nm for AgNPs (Fig. 1A), 340 nm for ZnO NPs (Fig. 1B) and two characteristic peaks both at 430 nm and 340 nm for Ag/ZnO NCs (Fig. 1C).

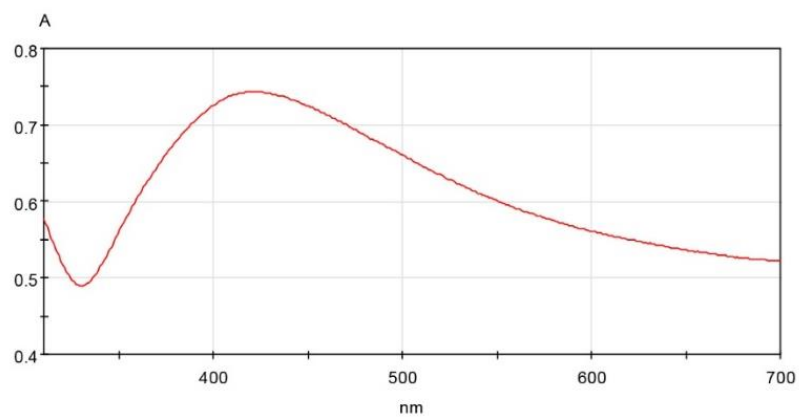


Fig. 1A. UV-Vis spectroscopic characterisation of green synthesised AgNPs

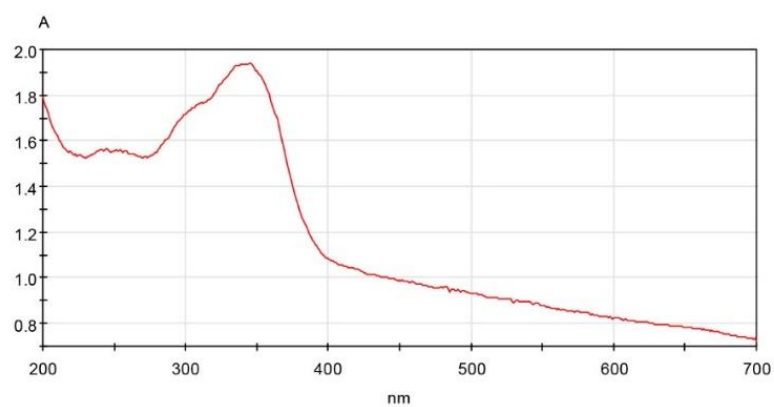


Fig. 1B. UV-Vis spectroscopic characterisation of green synthesised ZnO NPs

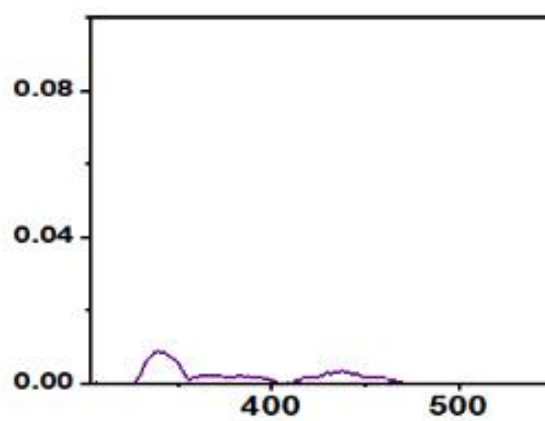


Fig. 1C. UV-Vis spectroscopic characterisation of green synthesised Ag/ZnO NCs

4.3. *IN VITRO* ANTIMICROBIAL EFFICACY OF GREEN SYNTHESISED NPs

4.3.1 Determination of MIC and MBC of green synthesised NPs against MDR-EAEC, NTS and MRSA strains

The *in vitro* antibacterial activity (MIC and MBC) of the green synthesised NPs against the MDR-EAEC, NTS(*S. Typhimurium* and *S. Enteritidis*) and MRSA strains was determined according to the CLSI (2019) guidelines.

The obtained results are presented in Table 1. The MIC values of green synthesised AgNPs, ZnO NPs and Ag/ZnO NCs against the tested MDR pathogens under the study were 7.8125, 62.50 and 31.25 µg/mL, respectively, whereas the MBC values were 15.625, 125 and 62.50 µg/mL, respectively (Table 1). In general, the obtained MBC values were twice as great as the values obtained for MIC value.

Table 1. MIC and MBC values of green synthesised NPs against MDR-EAEC, NTS and MRSA isolates

MDR-ISOLATES		MIC/MBC (µg/mL)		
		AgNPs	ZnO NPs	Ag/ZnO NCs
EAEC	E1	7.8125/15.625	62.50/125	31.25/62.50
	E2	7.8125/15.625	62.50/125	31.25/62.50
	E3	7.8125/15.625	62.50/125	31.25/62.50
<i>S. Enteritidis</i>	S1	7.8125/15.625	62.50/125	31.25/62.50
	S2	7.8125/15.625	62.50/125	31.25/62.50
	S3	7.8125/15.625	62.50/125	31.25/62.50
<i>S. Typhimurium</i>	ST1	7.8125/15.625	62.5/125	31.25/62.50
	ST2	7.8125/15.625	62.50/125	31.25/62.50
	ST3	7.8125/15.625	62.50/125	31.25/62.50
MRSA	Sa1	7.8125/15.625	62.50/125	31.25/62.50
	Sa2	7.8125/15.625	62.50/125	31.25/62.50
	Sa3	7.8125/15.625	62.50/125	31.25/62.50

4.3.2. *In vitro* antibiofilm potential of NPs against MDR test isolates

The *in vitro* antibiofilm potential of the green synthesised NPs was assessed at MIC (1X) and MIC (2X) against the biofilm-forming MDR-EAEC, NTS and MRSA strains both at 24 and 48 h.

The present study depicted that both at 24 and 48 h of co-incubation, the green synthesised AgNPs (Fig. 2), ZnO NPs (Fig. 3) and Ag/ZnO NCs (Fig. 4) evidenced significant inhibition ($P < 0.001$) in the biofilm-forming ability of all the tested MDR strains (EAEC, NTS and MRSA) on comparison with the corresponding untreated strains. The reduction (in per cent) of biofilm biomass when treated with green synthesised NPs is given in Table 2.

The reduction in the biomass value of MDR test strains was observed to be higher at 48 h as compared to 24 h (Table 2). Regardless of the NPs (AgNPs, ZnO NPs, and Ag/ZnO NCs) and MDR test strains (EAEC, *S. Typhimurium*, *S. Enteritidis*, MRSA) under study, a better antibiofilm activity was exhibited at 1X MBC dose as compared to 1X MIC dose (Table 2). Moreover, this study exhibited comparatively better biomass reduction by the AgNPs than Ag/ZnO NCs and ZnO NCs. While comparing the MDR-test strains, MDR-MRSA strains exhibited a comparatively higher reduction in the biofilm biomass than the MDR strains of EAEC, *S. Typhimurium* and *S. Enteritidis*.

To specify, the green synthesised AgNPs reduced the biofilm biomass of MDR-MRSA strains by 80 to 85 per cent at 1X MIC doses in comparison to 29 to 41 per cent for MDR-EAEC strains and 21 to 41 per cent for the NTS isolates. Nonetheless, at 1X MBC dose, a reduction of 84 to 89 per cent was observed for MDR-MRSA strains, while MDR-EAEC and MDR-NTS strains exhibited a reduction in the biofilm biomass of 43 to 64 per cent and 27 to 69 per cent, respectively (Table 2).

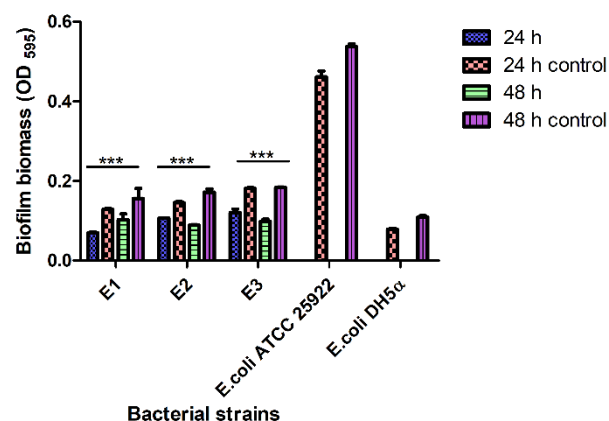


Fig. 2A. Antibiofilm potential of AgNPs at 1X MIC doses against MDR-EAEC isolates

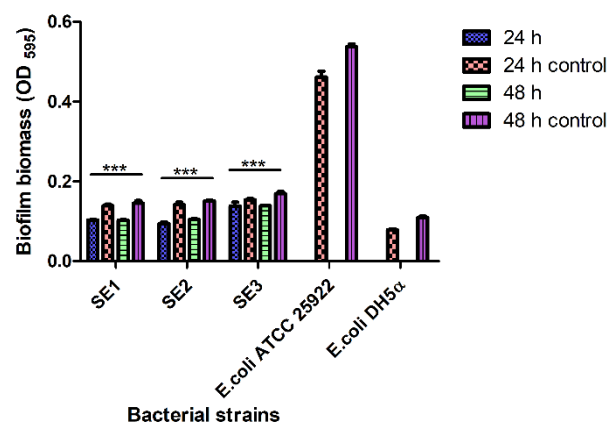


Fig. 2B. Antibiofilm potential of AgNPs at 1X MIC doses against MDR-S. Enteritidis isolates

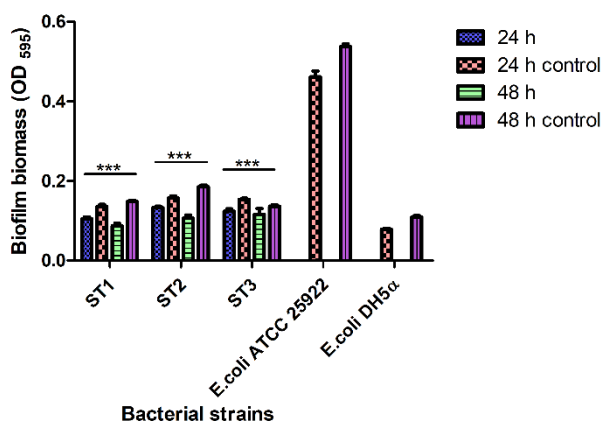


Fig. 2C. Antibiofilm potential of AgNPs at 1X MIC doses against MDR-S. Typhimurium isolates

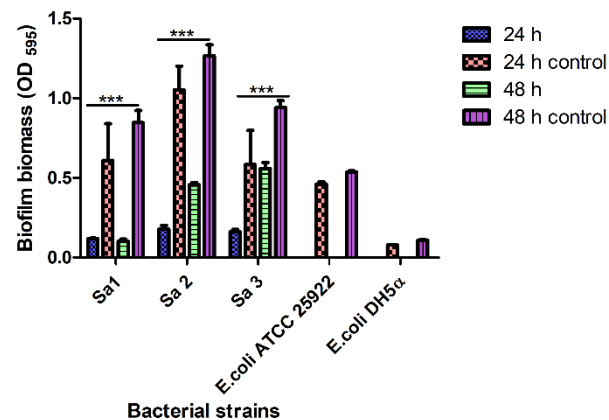


Fig. 2D. Antibiofilm potential of AgNPs at 1X MIC doses against MDR-MRSA isolates

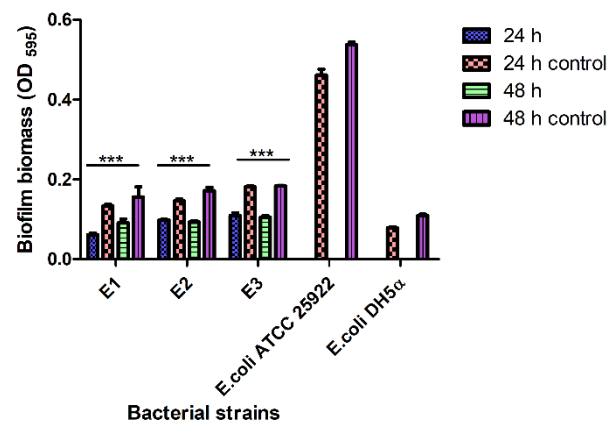


Fig. 2E. Antibiofilm potential of AgNPs at 2X MIC doses against MDR-EAEC isolates

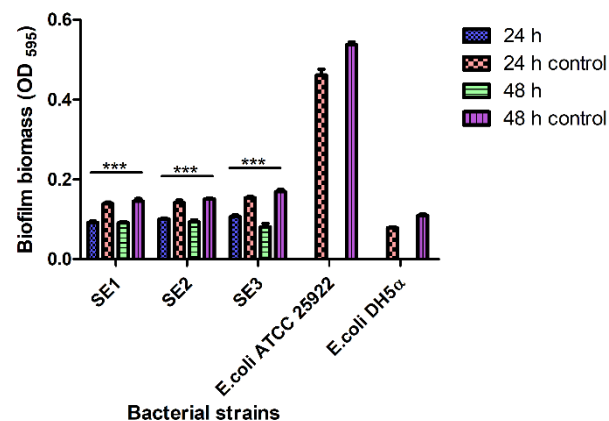


Fig. 2F. Antibiofilm potential of AgNPs at 2X MIC doses against MDR-S. Enteritidis isolates

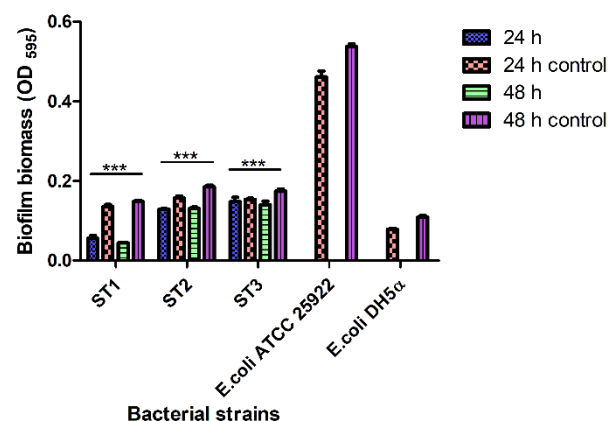


Fig. 2G. Antibiofilm potential of AgNPs at 2X MIC doses against MDR-*S. Typhimurium* isolates

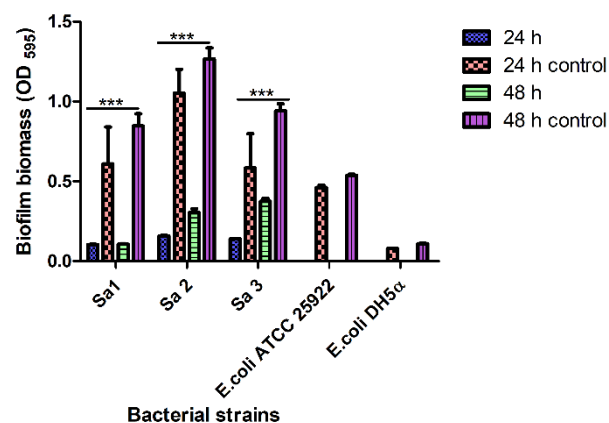


Fig. 2H. Antibiofilm potential of AgNPs at 2X MIC doses against MDR-MRSA isolates

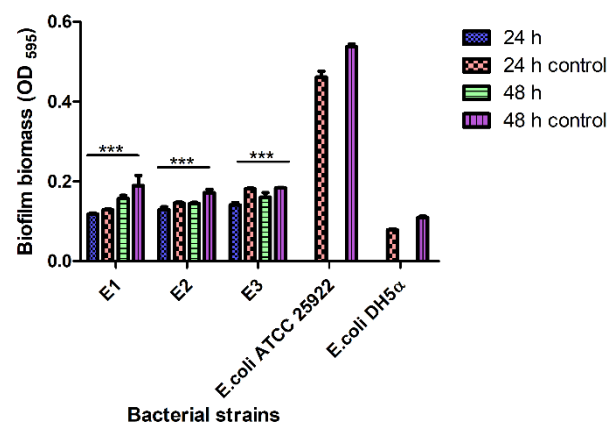


Fig. 3A. Antibiofilm potential of ZnO NPs at 1X MIC doses against MDR-EAEC isolates

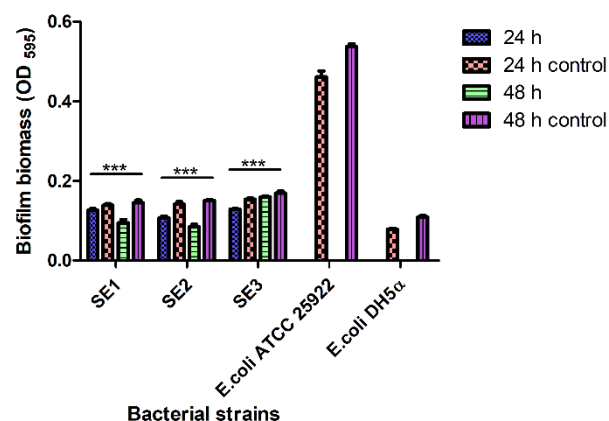


Fig. 3B. Antibiofilm potential of ZnO NPs at 1X MIC doses against MDR-*S. Enteritidis* isolates

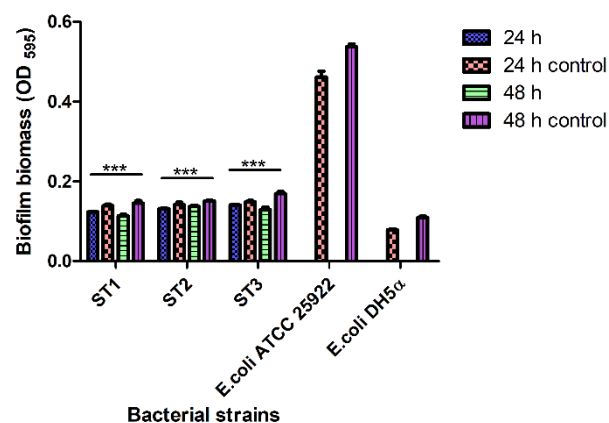


Fig. 3C. Antibiofilm potential of ZnO NPs at 1X MIC doses against MDR-*S. Typhimurium* isolates

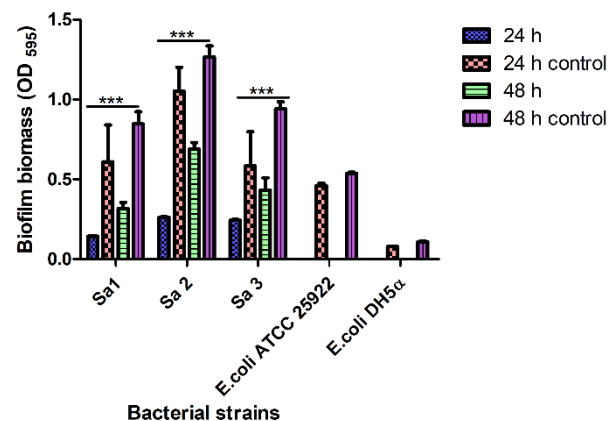


Fig. 3D. Antibiofilm potential of ZnO NPs at 1X MIC doses against MDR-MRSA isolates

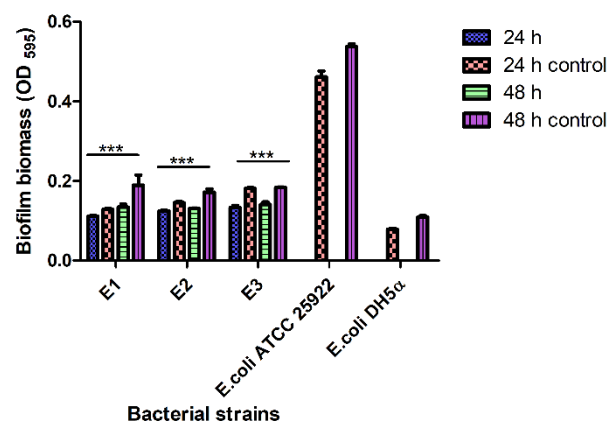


Fig. 3E. Antibiofilm potential of ZnO NPs at 2X MIC doses against MDR-EAEC isolates

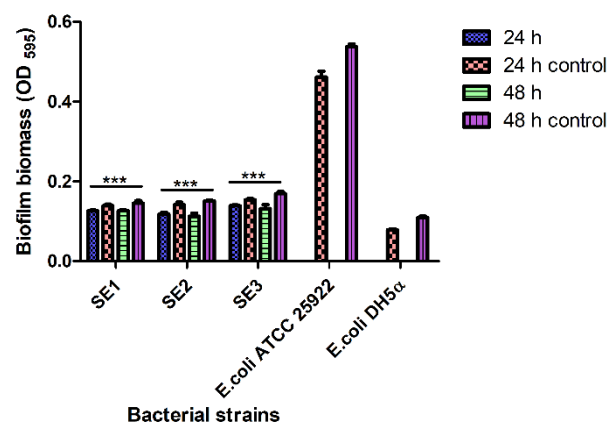


Fig. 3F. Antibiofilm potential of ZnO NPs at 2X MIC doses against MDR-S-Enteritidis isolates

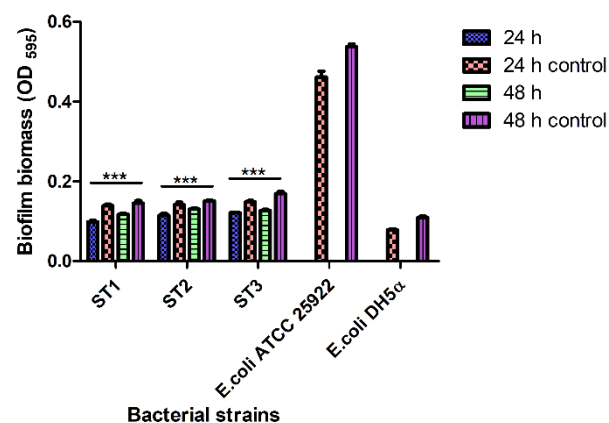


Fig. 3G. Antibiofilm potential of ZnO NPs at 2X MIC doses against MDR-S-Typhimurium isolates

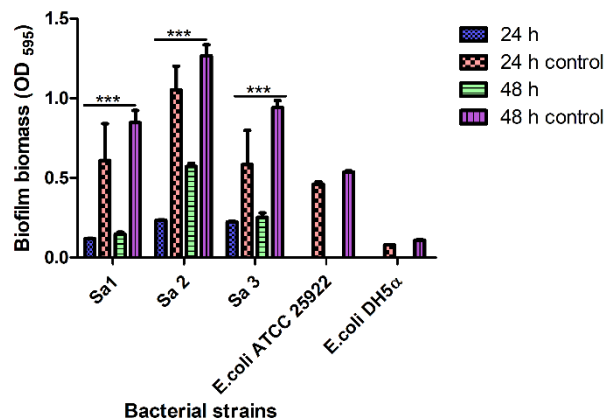


Fig. 3H. Antibiofilm potential of ZnO NPs at 2X MIC doses against MDR-MRSA isolates

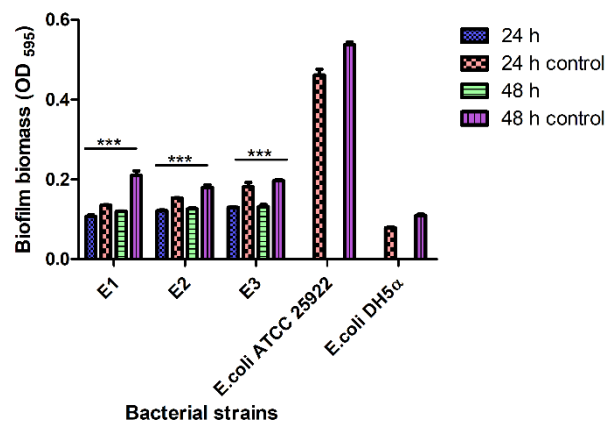


Fig. 4A. Antibiofilm potential of Ag/ZnO NCs at 1X MIC doses against MDR-EAEC isolates

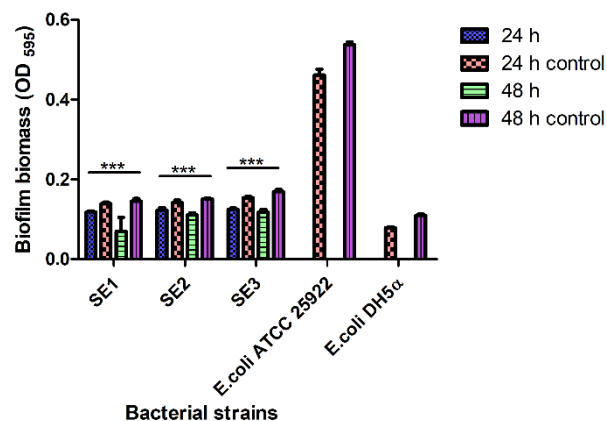


Fig. 4B. Antibiofilm potential of Ag/ZnO NCs at 1X MIC doses against MDR-S. Enteritidis isolates

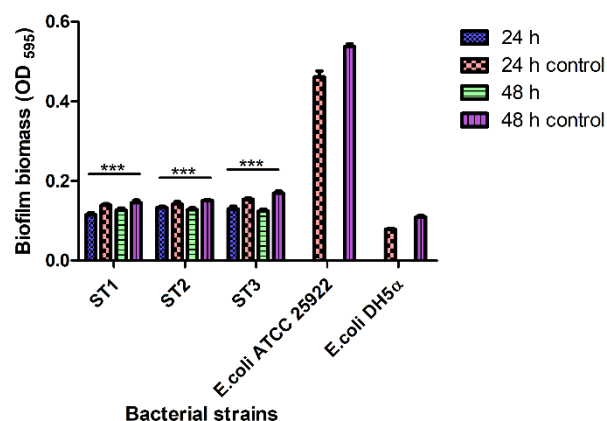


Fig. 4C. Antibiofilm potential of Ag/ZnO NCs at 1X MIC doses against MDR-*S. Typhimurium* isolates

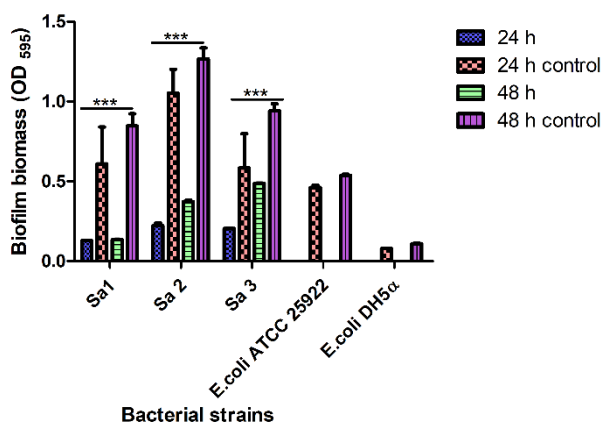


Fig. 4D. Antibiofilm potential of Ag/ZnO NCs at 1X MIC doses against MDR-MRSA isolates

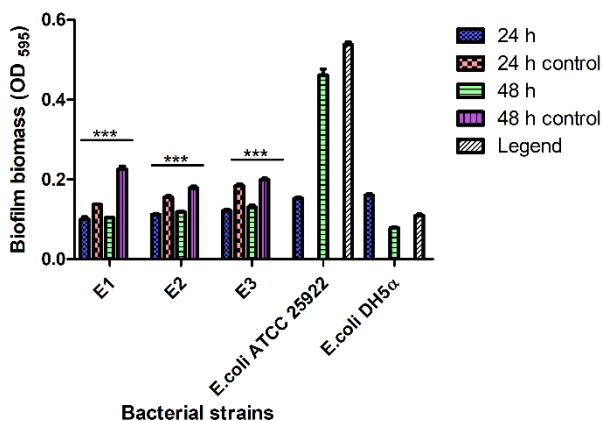


Fig. 4E. Antibiofilm potential of Ag/ZnO NCs at 2X MIC doses against MDR-EAEC isolates

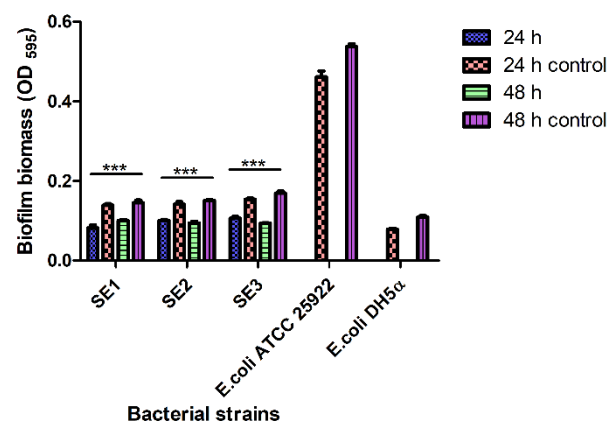


Fig. 4F. Antibiofilm potential of Ag/ZnO NCs at 2X MIC doses against MDR-*S. Enteritidis* isolates

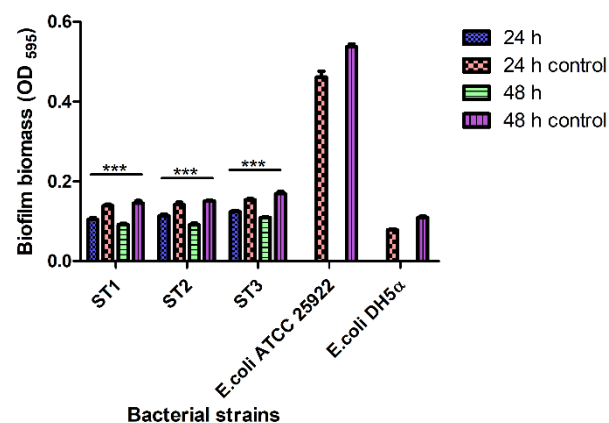


Fig. 4G. Antibiofilm potential of Ag/ZnO NCs at 2X MIC doses against MDR-*S. Typhimurium* isolates

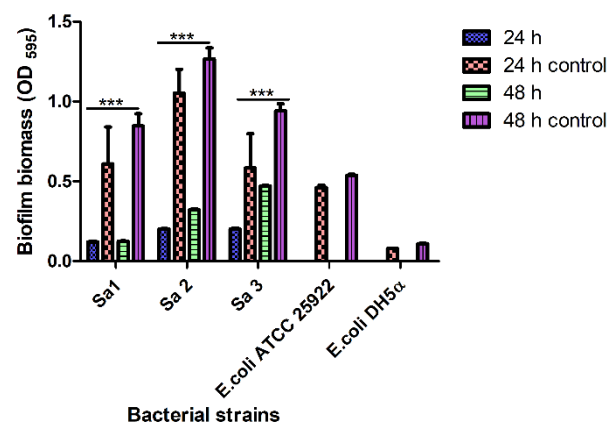


Fig. 4H. Antibiofilm potential of Ag/ZnO NCs at 2X MIC doses against MDR-MRSA isolates

Table 2. Reduction in biofilm biomass of MDR isolates of EAEC, NTS, and MRSA when exposed to MIC (1X) and MBC (1X) of green synthesised NPs measured over 24 h and 48 h

Bacterial strains	Strains	ZnO NPs				AgNPs				Ag/ZnO NCs			
		MIC (1X)		MBC (1X)		MIC (1X)		MBC (1X)		MIC (1X)		MBC (1X)	
		24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h
MDR-EAEC	E1	9.302	30.493	11.628	34.081	39.837	64.563	46.875	64.563	28.261	48.927	35.971	56.652
	E2	6.993	19.337	12.587	28.729	29.801	42.949	36.943	42.949	19.868	24.551	24.324	30.178
	E3	17.222	39.130	28.333	32.474	41.899	46.409	43.429	54.128	32.804	45.411	37.566	59.864
MDR-S. Enteritidis	S1	10.563	40.940	12.676	18.868	21.212	47.399	27.273	50.521	14.394	43.353	25.000	45.902
	S2	29.412	46.939	16.340	23.810	27.692	34.899	32.168	49.246	33.987	43.787	37.013	46.032
	S3	18.868	23.034	10.692	23.602	34.416	41.270	35.714	49.112	31.098	35.754	35.714	44.970
MDR-S. Typhimurium	ST1	13.380	25.503	19.718	25.503	30.496	48.993	52.482	69.231	28.788	45.355	34.302	49.223
	ST2	15.686	31.293	24.183	26.347	41.488	41.667	22.024	26.882	33.077	39.196	33.742	40.268
	ST3	2.113	27.329	16.197	26.708	33.553	37.640	33.721	38.579	27.273	34.921	39.674	46.032
MDR-MRSA	Sa1	51.656	67.335	66.317	77.531	85.417	85.594	87.240	89.720	83.724	84.514	85.156	90.923
	Sa2	51.592	71.555	64.534	76.885	80.548	82.993	86.033	88.163	78.235	74.821	83.820	86.497
	Sa3	58.717	72.106	74.455	76.931	75.163	80.064	81.664	84.130	72.562	76.399	77.243	80.697

4.4. *IN VITRO* ANTIOXIDANT ACTIVITY OF NPs

The antioxidant scavenging activity of the green synthesized NPs (AgNPs, ZnO NPs and Ag/ZnO NCs) was assessed by DPPH (Fig. 5) and ABTS (Fig. 6) assays, keeping ascorbic acid as standard.

A dose-dependent increase in the antioxidant capacity of the three NPs under study was observed in both assays (Figs. 5 & 6). In comparison with ZnO NPs, both AgNPs and Ag/ZnO NCs exhibited better antioxidant capacity. Nonetheless, the scavenging abilities of the green synthesised NPs were observed to be lower than the ascorbic acid standard.

Moreover, the inhibition concentration (IC_{50}) value of AgNPs was determined to be greater than 80 $\mu\text{g/mL}$ in the DPPH assay, while it was observed to be 60 $\mu\text{g/mL}$ in the ABTS assay. However, the IC_{50} value of Ag/ZnO NCs was determined to be greater than 60 $\mu\text{g/mL}$ in the DPPH assay and 80 $\mu\text{g/mL}$ in the ABTS assay.

4.5. *IN VITRO* PHOTOCATALYTIC DYE DEGRADATION ABILITY OF NPs

The *in vitro* photocatalytic dye degradation ability of the green synthesised NPs was measured against three cationic dyes i.e., methylene blue (MB), crystal violet (CV) and rhodamine B (RhB) on exposure to three different light sources *viz.*, sunlight, LED light and UV light.

4.5.1. *In vitro* photocatalytic potential of NPs for degradation of individual dye

The present study exhibited a highly significant ($P < 0.01$) dye degradation capacity for the tested NPs (AgNPs, ZnO NPs, and Ag/ZnO NCs) against all the tested dyes (MB, CV, and RhB) (Table 3; Fig. 7).

Irrespective of the light source, treatment with ZnO NPs exhibited a gradual increase in the photodegradation pattern for all the tested dyes throughout the study. However, on exposure to sunlight for 105 min, MB revealed a

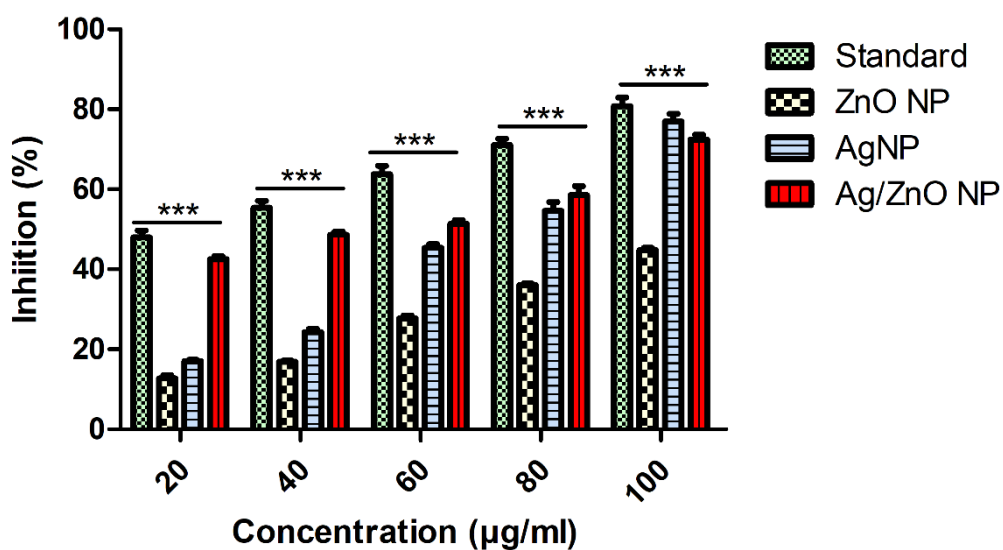


Fig.5.Antioxidant activity ofgreen synthesised ZnO NPs, AgNPs and Ag/ZnO NCs atdifferent concentrations estimated by DPPH scavenging assay

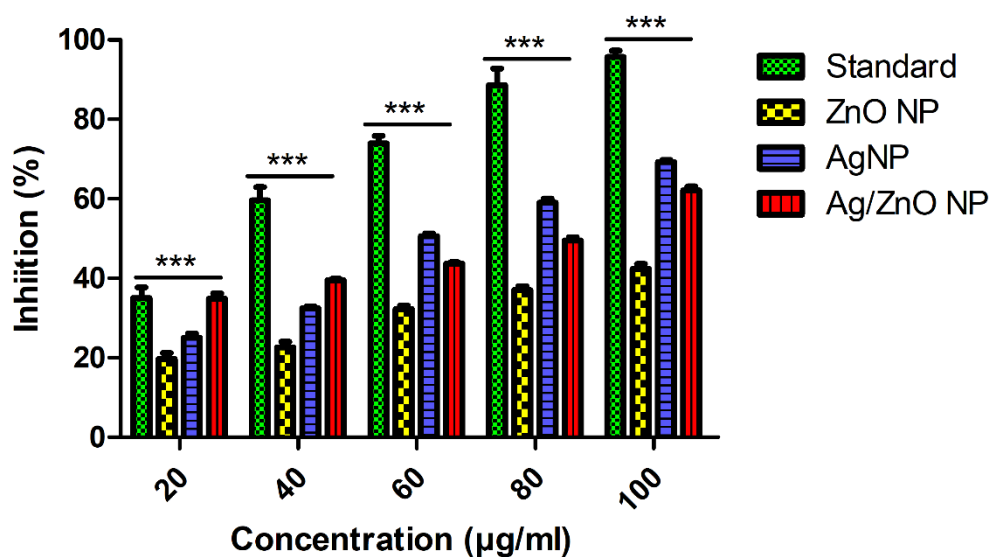


Fig.6.Antioxidant activity ofgreen synthesised ZnO NPs, AgNPs and Ag/ZnO NCs atdifferent concentrations estimated by ABTS scavenging assay

progressive increase (92.53 per cent) (Table 3a; Fig. 8A), whereas RhB exhibited a negligible degradation (7.22 per cent) pattern (Table 3a; Fig. 8B).

In contrast, AgNPs exhibited a drastic increase (20 to 80 per cent) in the photodegradation pattern for MB and CV dyes from the initial timepoint, regardless of the light source. Nonetheless, the degradation of RhB when treated with AgNPs was gradual throughout the entire duration. Surprisingly, on exposure to LED (Table 3b), degradation of RhB was found to be minimal (10.80 to 11.40 per cent).

Moreover, Ag/ZnO NCs exhibited a momentous increase in the degradation behaviour (23 to 85 per cent) for CV (Fig. 8C) and MB (Fig. 8A), irrespective of the light sources. The degradation of RhB when treated with Ag/ZnO NCs was gradual with LED and UV light (Fig. 8B), whereas a drastic increase in the photocatalytic dye degradation of RhB was observed with sunlight starting from 42 per cent at 15 min of exposure which reached 98 per cent in 105 min when compared to 58 and 38 per cent with LED and UV light, respectively (Fig. 8B).

4.5.2. Determination of photocatalytic dye degradation kinetics

The photocatalytic dye degradation kinetics were determined to assess the quantification of degradation of MB, CV and RhB. The results are presented in Table 3.

According to the reaction parameters obtained, RhB was fitted in zero-order kinetics irrespective of the light sources and NPs used except when treated with Ag/ZnO NCs with sunlight. The RhB treated with Ag/ZnO NCs on exposure to sunlight fitted to the first-order kinetics (Table 3a), while fitted to the second-order kinetics with UV light (Table 3c).

Besides, MB followed primarily second-order kinetics in all the cases (Table 3) except for treatment with ZnO NPs while exposed to UV light, wherein it followed zero-order kinetics (Table 3c). However, MB when treated with Ag/ZnO NCs and exposed to UV light exhibited first-order kinetics irrespective of the source of light (Table 3c).

Similarly, CV mainly followed second-order kinetics throughout the experiment (Table 3) except for AgNPs on exposure to sunlight, which exhibited first-order kinetics (Table 3a). Moreover, for Ag/ZnO NCs on exposure to sunlight and LED light, followed first-order kinetics (Table 3a, 3b).

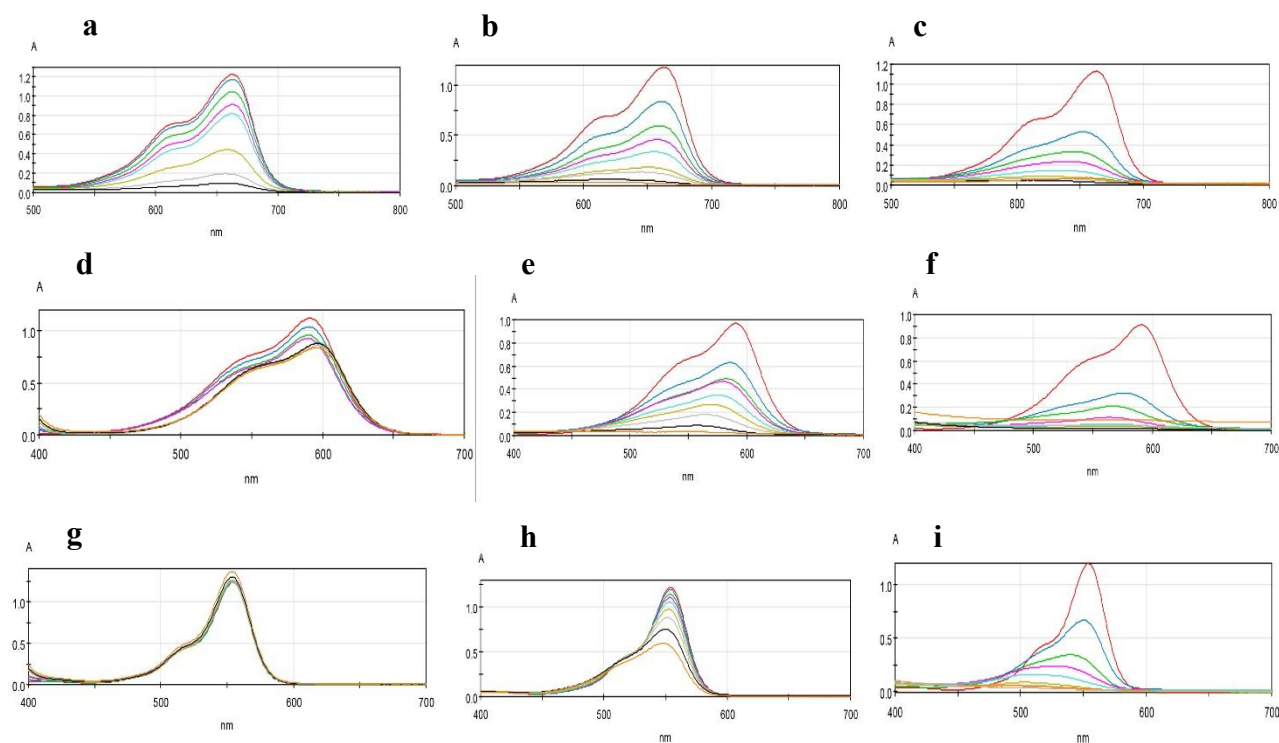


Fig.7A. UV-Vis spectroscopic patterns of dye degradation of cationic dyes (MB, CV, and RhB) on treatment with ZnO NPs, AgNPs, and Ag/ZnO NCs under sunlight

a.MB+ZnO NPs

d.CV+ZnO NPs

g.RhB+ZnO NPs

b.MB+AgNPs

e.CV+AgNPs

h.RhB+AgNPs

c.MB+Ag/ZnO NCs

f.CV+Ag/ZnO NCs

i.RhB+Ag/ZnO NCs

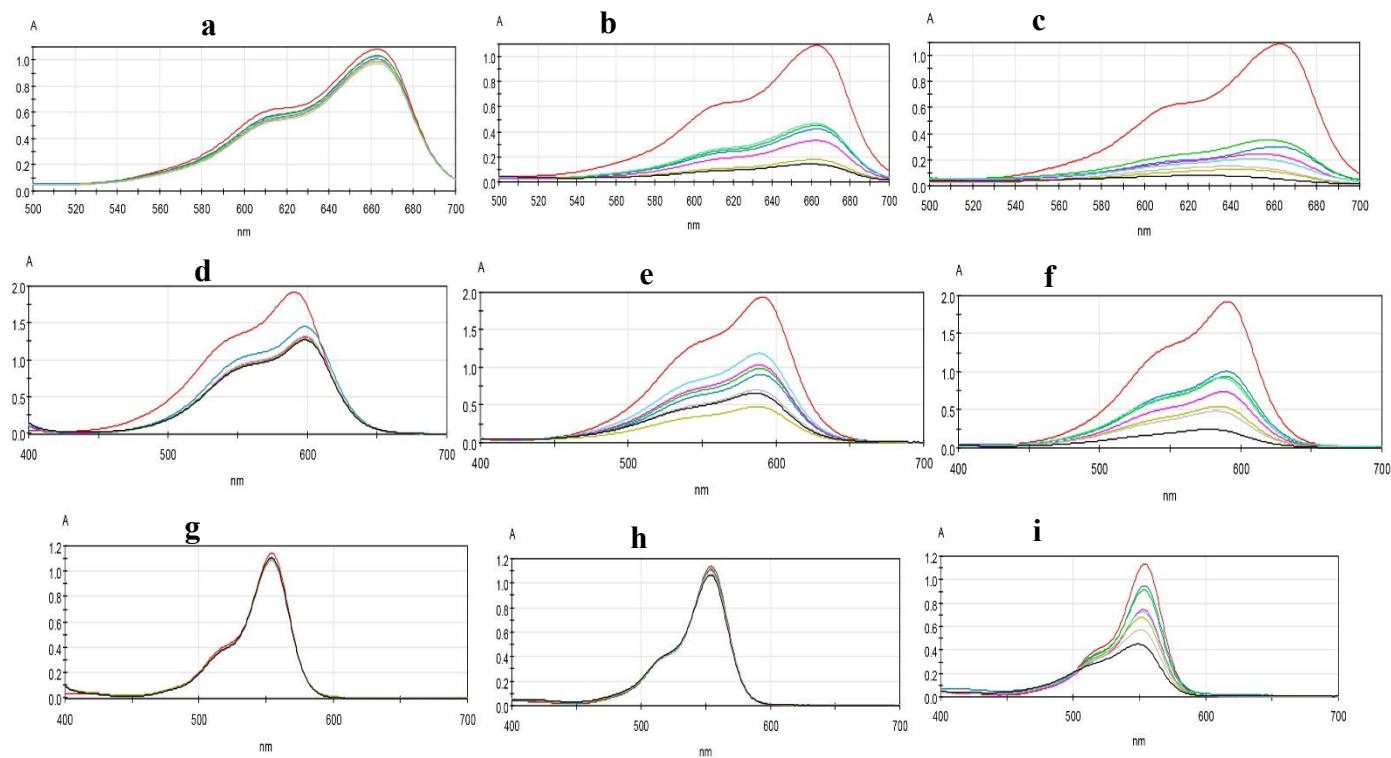


Fig.7B. UV-Vis spectroscopic patterns of dye degradation of cationic dyes (MB, CV, and RhB) on treatment with ZnO NPs, AgNPs, and Ag/ZnO NCs under LED light

a.MB+ZnO NPs

d.CV+ZnO NPs

g.RhB+ZnO NPs

b.MB+AgNPs

e.CV+AgNPs

h.RhB+AgNPs

c.MB+Ag/ZnO NCs

f.CV+Ag/ZnO NCs

i.RhB+Ag/ZnO NCs

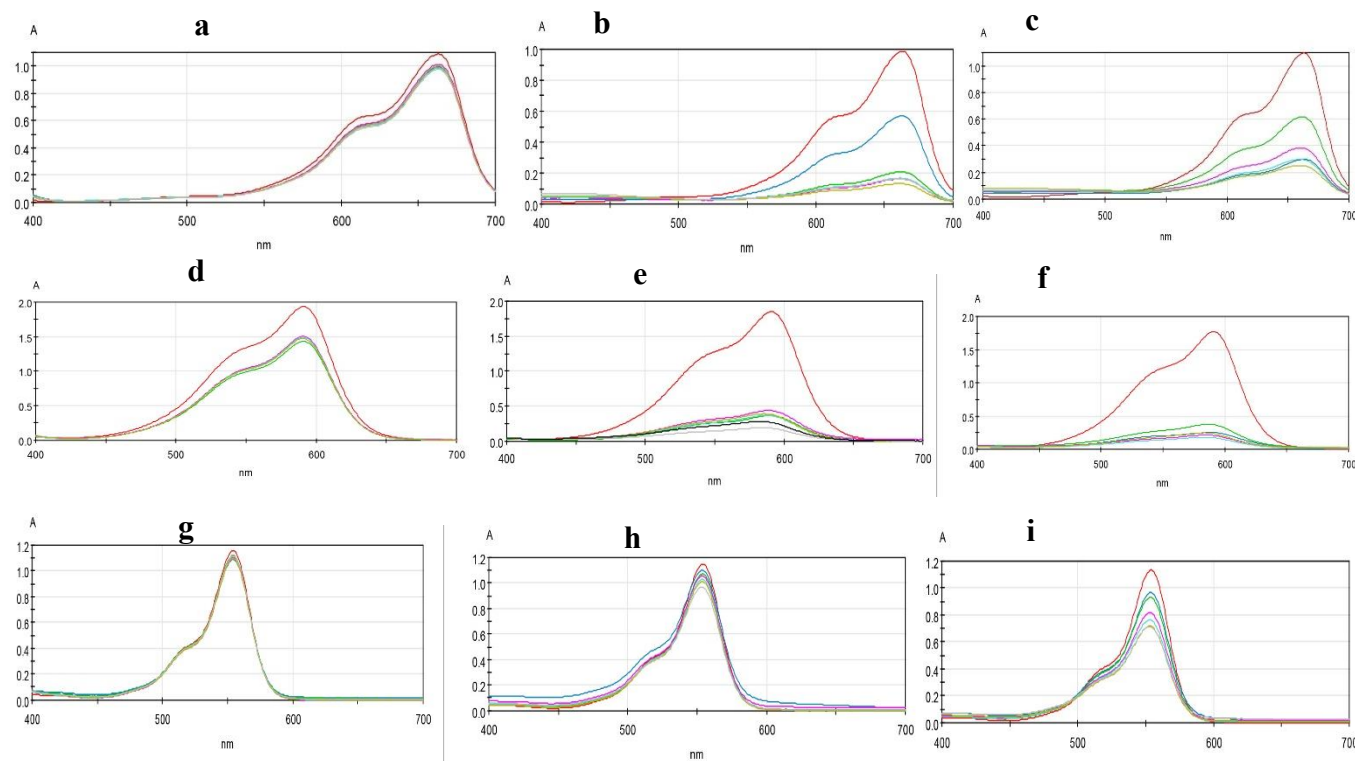


Fig.7C. UV-Vis spectroscopic patterns of dye degradation of cationic dyes (MB, CV, and RhB) on treatment with ZnO NPs, AgNPs, and Ag/ZnO NCs under UV light

a.MB+ZnO NPs

d.CV+ZnO NPs

g.RhB+ZnO NPs

b.MB+AgNPs

e.CV+AgNPs

h.RhB+AgNPs

c.MB+Ag/ZnO NCs

f.CV+Ag/ZnO NCs

i.RhB+Ag/ZnO NCs

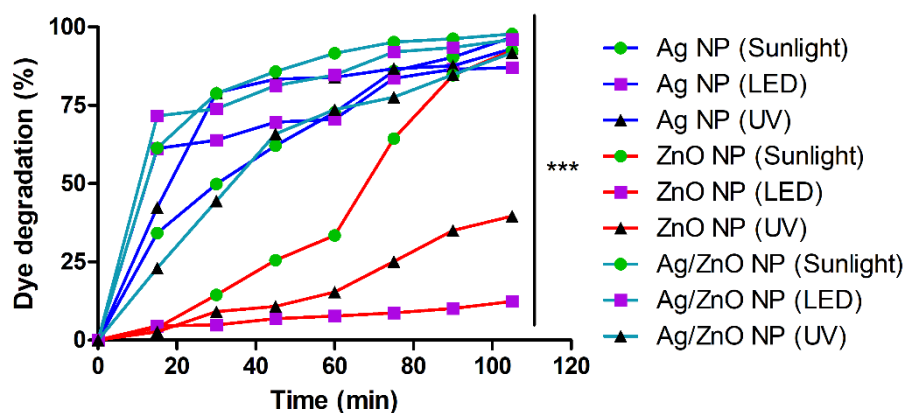


Fig.8A. Patterns of methylene blue (MB) degradation under sunlight, LED light, and UV light with AgNPs, ZnO NPs and Ag/ZnO NCs

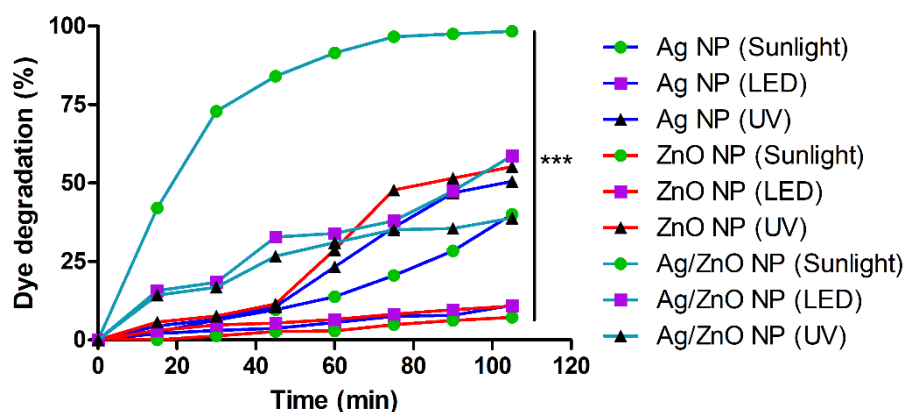


Fig.8B. Patterns of rhodamine-B (RhB) degradation under sunlight, LED light, and UV light with AgNPs, ZnO NPs and Ag/ZnO NCs

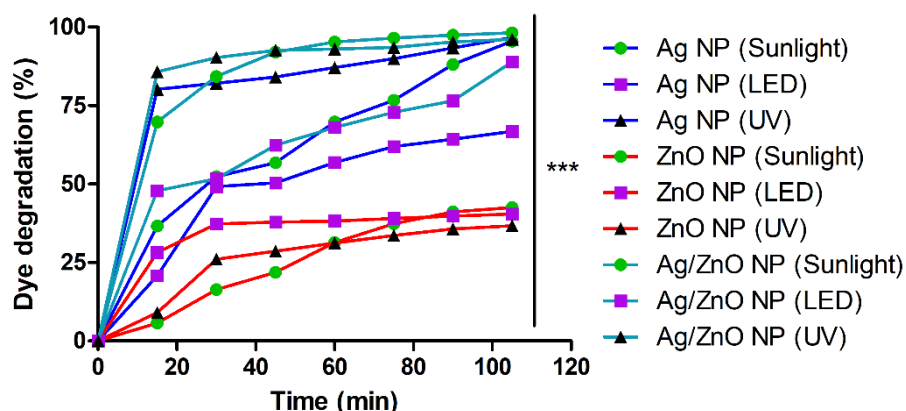


Fig.8C. Patterns of crystal violet (CV) degradation under sunlight, LED light, and UV light with AgNPs, ZnO NPs and Ag/ZnO NCs

Table3a. Determination of photocatalytic dye degradation potential and order of reaction on treatment with ZnO NPs, AgNPs, and Ag/ZnO NCs on exposure to sunlight

Time (min)	Sunlight								
	MB			CV			RhB		
	ZnO NPs	AgNPs	Ag/ZnO NCs	ZnO NPs	AgNPs	Ag/ZnO NCs	ZnO NPs	AgNPs	Ag/ZnO NCs
	Degradation (%)								
15	3.94	34.24	61.40	5.71	36.59	69.81	0	2.53	42.03
30	14.46	49.87	78.81	16.35	52.41	84.27	1.33	6.38	72.85
45	25.51	62.10	85.76	21.84	56.83	92.01	2.67	9.61	84.06
60	33.44	72.68	91.61	31.36	69.78	95.26	2.86	13.83	91.47
75	64.4	85.99	95.17	37.39	76.68	96.58	4.88	20.58	96.61
90	84.62	90.37	96.29	41.14	88.16	97.44	6.23	28.35	97.57
105	92.53	96.80	97.82	42.51	95.53	98.20	7.22	40.06	98.37
Kinetics Order	Second	Second	First	Second	First	First	Zero	Zero	First
R² value	0.944	0.944	0.9815	0.960	0.932	0.9444	0.970	0.937	0.9912
Intercept	0.001	0.001	-0.2802	0.007	-0.026	-0.6549	0.000	-0.004	0.1169
Slope	0.939	0.939	-0.0345	0.861	0.086	-0.0362	1.185	1.268	-0.0409

Table 3b. Determination of photocatalytic dye degradation potential and order of reaction on treatment with ZnO NPs, AgNPs, and Ag/ZnO NCs on exposure to LED light

Time (min)	LED light								
	MB			CV			RhB		
	ZnO NPs	AgNPs	Ag/ZnO NCs	ZnO NPs	AgNPs	Ag/ZnO NCs	ZnO NPs	AgNPs	Ag/ZnO NCs
	Degradation (%)								
15	4.56	61.24	71.60	28.23	20.83	47.84	3.01	2.03	15.67
30	4.86	63.88	73.91	37.27	49.18	51.71	4.76	3.01	18.38
45	6.91	69.63	81.32	37.88	50.40	62.35	5.41	3.73	32.75
60	7.67	70.52	84.71	38.21	56.90	68.07	6.52	5.54	33.97
75	8.67	83.63	92.07	39.07	61.95	72.89	8.19	7.51	37.99
90	10.14	86.50	93.42	39.79	64.3	76.58	9.62	7.88	47.43
105	11.56	87.03	96.06	40.48	66.8	88.96	10.80	11.04	58.72
Kinetics Order	Second	Second	First	Second	Second	First	Zero	Zero	Zero
R² value	0.944	0.922	0.9404	0.631	0.902	0.9219	0.974	0.974	0.9627
Intercept	0.001	0.059	-0.3642	0.002	0.008	0.4833	-0.001	-0.001	1.0393
Slope	0.939	0.823	-0.0268	0.657	0.626	-0.0169	1.127	1.139	-0.0054

Table 3c. Determination of photocatalytic dye degradation potential and order of reaction on treatment with ZnO NPs, AgNPs, and Ag/ZnO NCs on exposure to UV light

Time (min)	UV light								
	MB			CV			RhB		
	ZnO NPs	AgNPs	Ag/ZnO NCs	ZnO NPs	AgNPs	Ag/ZnO NCs	ZnO NPs	AgNPs	Ag/ZnO NCs
	Degradation (%)								
15	2.73	42.36	23.05	9.02	80.19	85.76	5.71	4.45	14.25
30	9.11	79.02	44.47	26.07	82.12	90.30	7.61	6.63	16.80
45	10.77	83.40	65.89	28.66	84.13	92.61	11.45	10.66	26.67
60	15.35	83.97	73.59	31.21	87.14	93.04	28.83	23.30	31.07
75	25.15	86.74	77.61	33.59	89.94	93.56	47.82	35.85	35.10
90	35.05	87.59	84.81	35.75	93.40	95.30	51.50	46.89	35.50
105	42.32	93.2	91.78	39.33	96.49	96.15	56.13	49.11	38.90
Kinetics Order	Zero	Second	First	Second	Second	Second	Zero	Zero	Second
R² value	0.943	0.913	0.9839	0.924	0.909	0.9564	0.909	0.924	0.9637
Intercept	-0.003	0.073	0.0622	0.003	0.082	1.5149	-0.007	-0.006	0.9666
Slope	1.005	1.675	-0.0227	0.551	0.394	0.118	1.225	1.208	0.0055

4.5.3. Selectivity of dyes on photocatalytic dye degradation

The selectivity of dyes on *in vitro* photocatalytic dye degradation was evaluated by treating the mixture of dyes (MB, CV and RhB) with green synthesised NPs and exposing them to sunlight.

This study depicted that a non-significant ($P > 0.05$) dye degradation capacity was exhibited by Ag/ZnO NCs on degrading MB (Fig. 9A) and CV (Fig. 9B) either individually or in combination in the presence of sunlight. Nonetheless, a significant ($P < 0.01$) dye degradation capacity was observed for Ag/ZnO NCs in degrading RhB both individually and in combination with exposure to sunlight (Fig. 9C).

At 105 min of exposure, the degradation of MB was estimated to be 97.82 and 93.37 per cent when the dyes were used individually and in combination, respectively (Table 4). Similarly, a degradation rate of 98.20 per cent and 95.79 per cent was obtained for CV (Table 4). Nonetheless, RhB exhibited degradation rates of 98.37 and 79.52 per cent when treated individually and in combination, respectively (Table 4).

Table 4. Comparative photocatalytic potential of MB, CV and RhB dyes treated with Ag/ZnO NCs when used individually and in combination with exposure to sunlight

Degradation rate (%)						
Time (min)	MB		CV		RhB	
	Individual	Combination	Individual	Combination	Individual	Combination
15	61.40	66.36	69.81	73.39	42.03	47.61
30	78.81	79.17	84.27	85.28	72.85	59.50
45	85.76	85.68	92.01	90.18	84.06	66.40
60	91.61	86.24	95.26	92.27	91.47	72.62
75	95.17	89.25	96.58	92.83	96.61	73.84
90	96.29	91.83	97.44	94.07	97.57	77.00
105	97.82	93.37	98.20	95.79	98.37	79.52

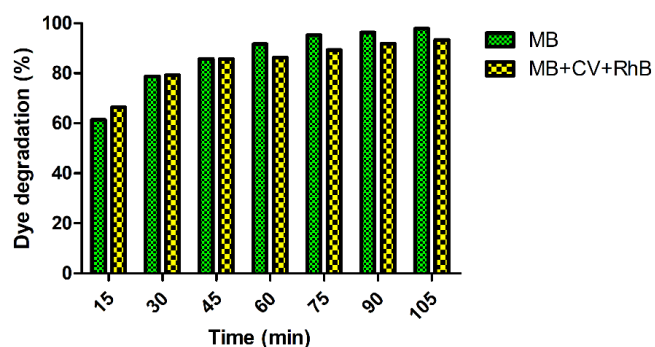


Fig. 9A. Photocatalytic degradation of MB treated with Ag/ZnO NCs when used individually and in combination with CV and RhB with exposure to sunlight

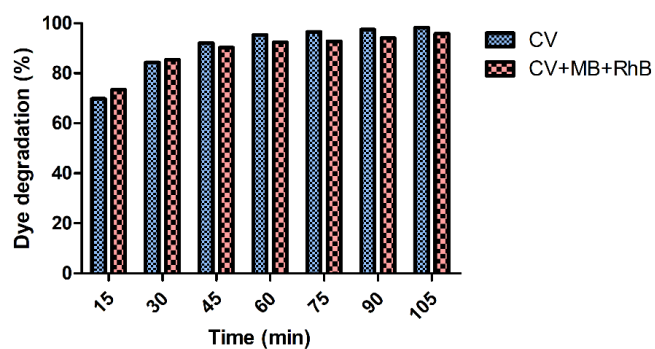


Fig.9B. Photocatalytic degradation of CV treated with Ag/ZnO NCs when used individually and in combination with MB and RhB with exposure to sunlight

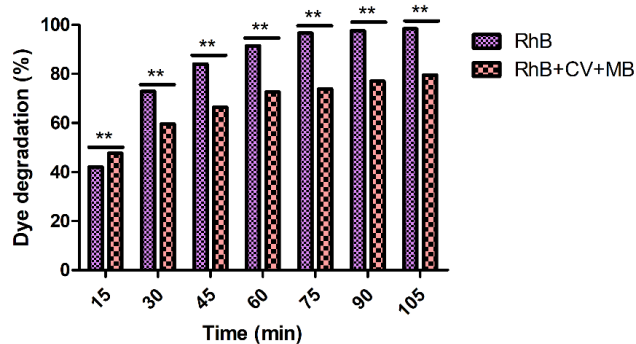


Fig.9C. Photocatalytic degradation of RhB treated with Ag/ZnO NCs when used individually and in combination with CV and MB with exposure to sunlight

4.5.4. Effect of reusability of NPs in photocatalytic dye degradation

The reusability of NPs in photocatalytic dye degradation was determined by treating the used Ag/ZnO NCs from the earlier dye degradation assays as mentioned in section 4.5.1. The degradation assay was carried out by treating the dye mixture containing CV, MB, and RhB at specified concentrations (5 ppm for MB and RhB, and 50 ppm for CV) and exposing the resultant mixture to sunlight.

The comparative dye degradation rates of fresh and reused Ag/ZnO NCs in the presence of sunlight are depicted in Table 5. In this study, diverse dye degradation patterns were observed for degrading MB, CV and RhB by Ag/ZnO NCs, when they were used either freshly or reused.

A degradation of 93.37 per cent was obtained for the fresh Ag/ZnO NCs, while 84.25 per cent was obtained while using the reused NCs in degrading MB (Fig. 10A). Similarly, a degradation of 95.79 and 90.59 per cent was obtained for CV (Fig. 10B). Nonetheless, degradation rates of 79.52 and 61.50 per cent was obtained for the fresh and reused NCs, respectively for degrading RhB (Fig. 10C).

Table 5. Comparative photocatalytic potential of fresh and reused Ag/ZnO NCs for degradation of MB, CV and RhB dyes on exposure to sunlight

Time (min)	Degradation (%)					
	MB		CV		RhB	
	Fresh NC	Reused NC	Fresh NC	Reused NC	Fresh NC	Reused NC
15	66.36	43.70	73.39	64.10	47.61	32.05
30	79.17	59.84	85.28	78.42	59.50	40.40
45	85.68	67.00	90.18	82.47	66.40	44.39
60	86.24	73.64	92.27	85.25	72.62	49.40
75	89.25	79.11	92.83	86.73	73.84	54.09
90	91.83	82.00	94.07	87.53	77.00	56.62
105	93.37	84.25	95.79	90.59	79.52	61.50

4.5. *IN VITRO* PHOTOCATALYTIC ANTIBIOTIC DEGRADATION ABILITY OF NPs

The *in vitro* photocatalytic antibiotic degradation abilities of the green synthesised NPs (AgNPs, ZnO NPs, and Ag/ZnO NCs) were measured against the selected antibiotics (ampicillin, ciprofloxacin and tetracycline) on exposure to sunlight (Fig. 11).

In this study, a highly significant ($P < 0.001$) degradation of tetracycline was observed when treated with all the tested NPs. Likewise, a significant ($P < 0.05$) degradation of ampicillin as well as ciprofloxacin was observed irrespective of all the tested NPs (Fig. 12). In addition, it was also observed that the degradation of the tested antibiotics reached a maximum at one-time point, beyond which there was no significant difference in the degradation (Fig. 12).

The degradation of ampicillin (Fig. 12A) and ciprofloxacin (Fig. 12B) when treated with AgNPs and Ag/ZnO NCs reached its maximum (nearly 65 to 75 per cent) at 90 min of exposure. Nevertheless, the degradation of ampicillin and ciprofloxacin when treated with ZnO NPs was observed to reach their maxima at 15 min and 60 min of exposure amounting to 64 and 84 per cent, respectively. Interestingly, with a degradation efficiency of 12 to 27 per cent, tetracycline exhibited a moderate degradation on treatment with AgNPs as well as Ag/ZnO NCs (Fig. 12C). Nonetheless, the degradation of tetracycline when treated with ZnO NPs produced a drastic increase, peaking at 60 min of exposure yielding 74 per cent degradation.

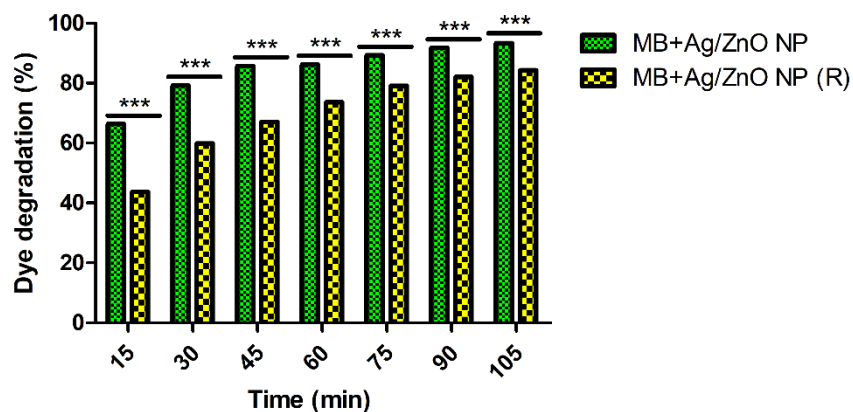


Fig.10A.Photocatalytic degradation of MB treated with fresh and reused Ag/ZnO NCs on exposure to sunlight

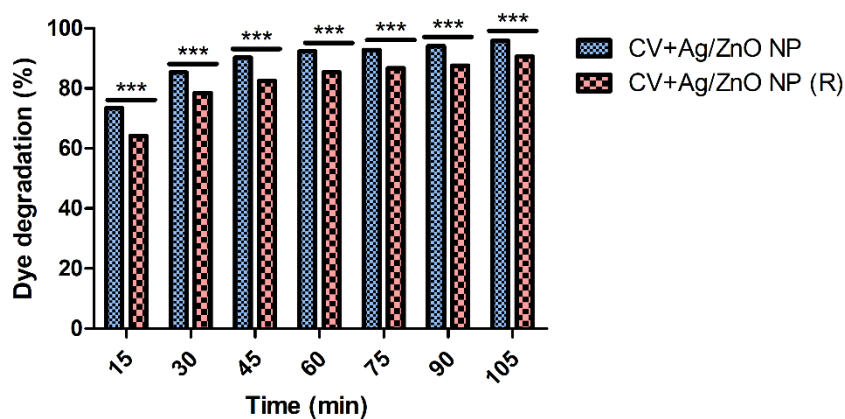


Fig.10B.Photocatalytic degradation of CV treated with fresh and reused Ag/ZnO NCs on exposure to sunlight

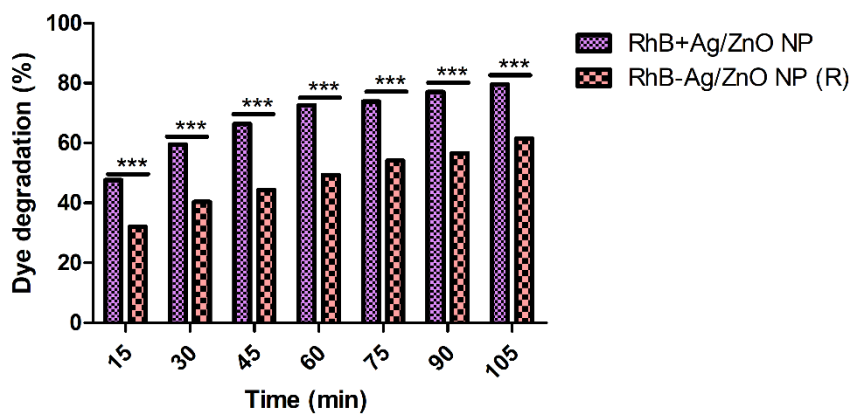


Fig.10C.Photocatalytic degradation of RhB treated with fresh and reused Ag/ZnO NCs on exposure to sunlight

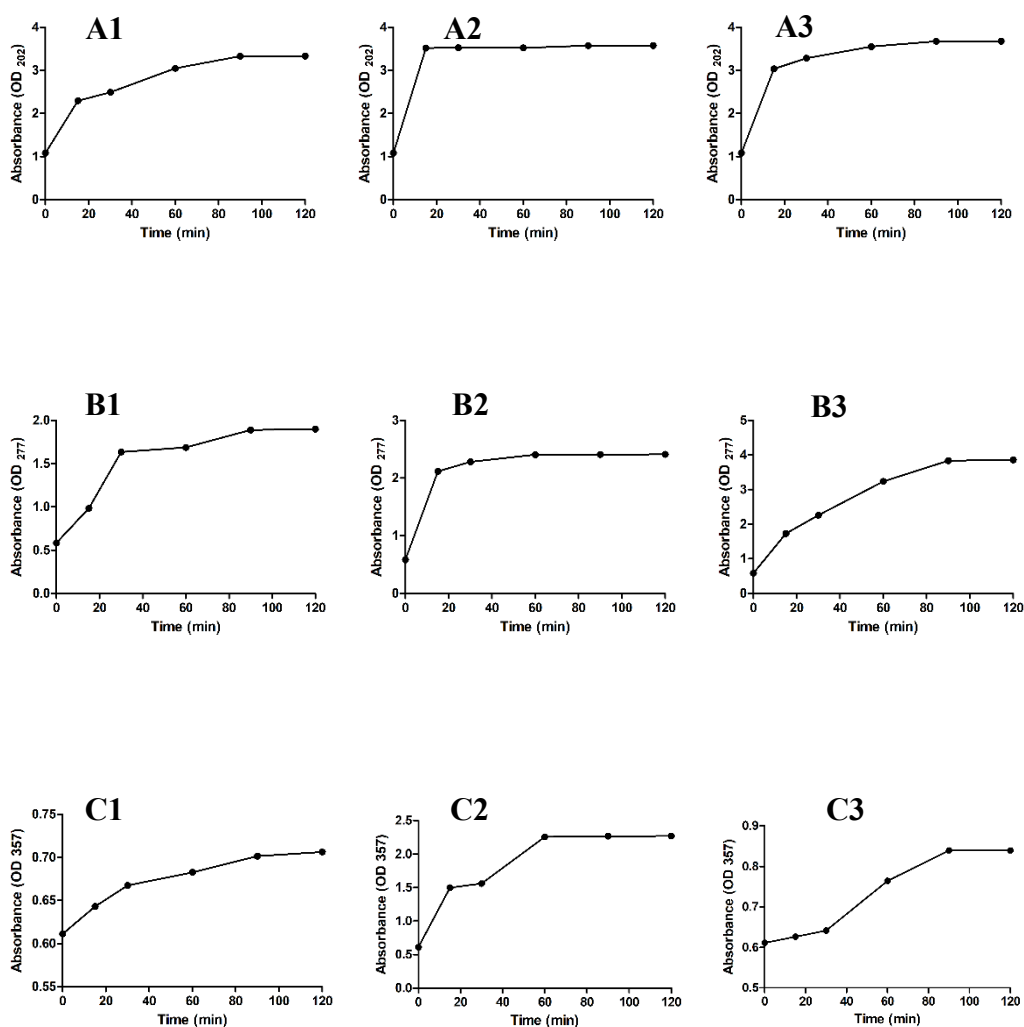


Fig. 11. Photocatalytic degradation of antibiotics treated with NPs under study on exposure to sunlight

A. Ampicillin

A1. Ampicillin+AgNPs

A2. Ampicillin+ZnO NPs

A3. Ampicillin+Ag/ZnO NCs

B. Ciprofloxacin

B1. Ciprofloxacin+AgNPs

B2. Ciprofloxacin+ZnO NPs

B3. Ciprofloxacin+Ag/ZnO NCs

C. Tetracycline

C1. Tetracycline+AgNPs

C2. Tetracycline+ZnO NPs

C3. Tetracycline+Ag/ZnO NCs

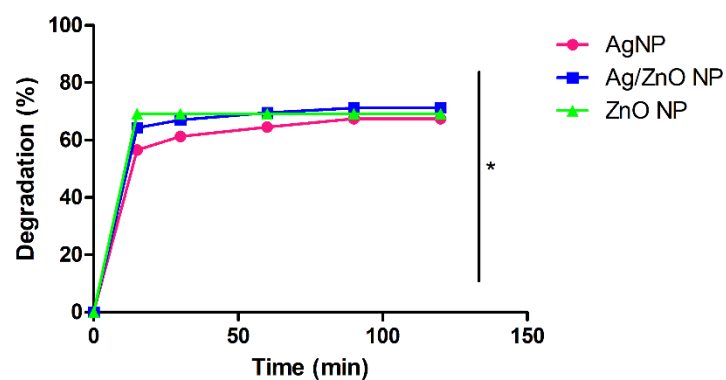


Fig.12A. Antibiotic degradation activity of green synthesized AgNPs, Ag/ZnONCs, and ZnO NPs against ampicillin on exposure to sunlight

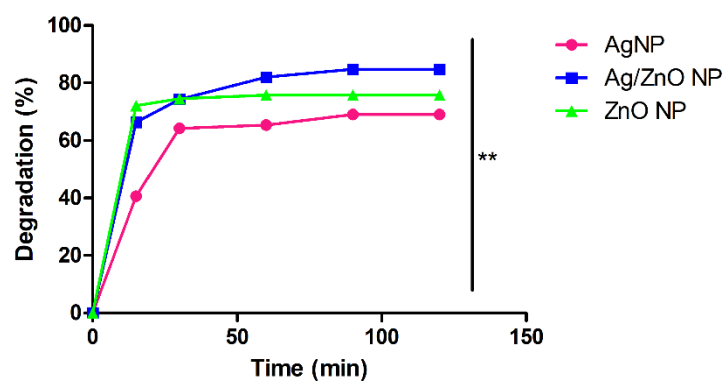


Fig. 12B. Antibiotic degradation activity of green synthesized AgNPs, Ag/ZnO NCs, and ZnO NPs against ciprofloxacin on exposure to sunlight

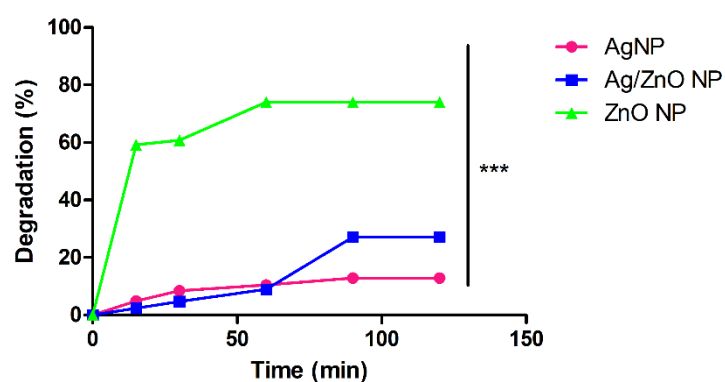


Fig. 12C. Antibiotic degradation activity of green synthesized AgNPs, Ag/ZnO NCs, and ZnO NPs against tetracycline on exposure to sunlight

Table 6. Comparative photocatalytic potential of NPs for degradation of ampicillin, ciprofloxacin and tetracycline on exposure to sunlight

Antibiotic	Test NPs	Time of maximum degradation (min)	Degradation (%)
Ampicillin	ZnO NPs	15	69.24
	AgNPs	90	71.22
	Ag/ZnO NCs	90	71.22
Ciprofloxacin	ZnO NPs	60	75.83
	AgNPs	90	69.08
	Ag/ZnO NCs	90	84.83
Tetracycline	ZnO NPs	60	74.07
	AgNPs	90	12.85
	Ag/ZnO NCs	90	27.15

4.6. *IN VITRO* DOSE- AND TIME-DEPENDENT PHOTOCATALYTIC KILLING KINETICS OF MDR BACTERIAL STRAINS TREATED WITH NPs

The time-kill kinetic assay of individual MDR-EAEC, *S. Typhimurium*, *S. Enteritidis* and MRSA strains co-cultured with the four different concentrations (1X MIC, 1/2X MIC, 1/5X MIC and 1/10X MIC) of green synthesised NPs (AgNPs, ZnO NPs and Ag/ZnO NCs) along with their respective controls on exposure to LED light were estimated in log₁₀ CFU/mL and expressed as mean $\pm$ standard errors at different time intervals are presented in Table 7 and Figs. 13 to 15.

In this assay, all the untreated test isolates (MDR strains of EAEC, *S. Typhimurium*, *S. Enteritidis* and MRSA) exhibited an increasing growth pattern at 30, 60, 120, 180, and 240 min of incubation (Figs. 13-15).

However, MDR strains of EAEC, *S. Typhimurium* and *S. Enteritidis* isolates did not exhibit discernable growth after 30 min of incubation ($P < 0.001$) when

treated with AgNPs at 1X MIC and 1/2X MIC. Interestingly, two strains of MDR-MRSA (Sa1 and Sa2) when treated with MIC and well as sub-MIC levels (1X MIC, 1/2X MIC and 1/5X MIC) did not exhibit visible growth patterns after 30 min of incubation. Moreover, when treated with 1/10X MIC of AgNPs, no growth could be visualised in the MDR-MRSA strains (Sa1 and Sa2) after 120 min of co-incubation ($P < 0.001$). Nevertheless, the Sa3 strain when co-incubated with AgNPs (1X MIC and 1/2X MIC) exhibited no visible bacterial growth after 30 min and 180 min with 1/5X MIC. No elimination of the Sa3 strain of MDR-MRSA could be observed when treated with 1/10X MIC of AgNPs (Table 7; Fig. 13).

In this study, the MDR bacterial test strains when treated with ZnO NPs (1X MIC) exhibited a progressive decline in bacterial growth after 30 min of co-incubation. Surprisingly, none of the MDR test strains (EAEC, *S. Typhimurium*, *S. Enteritidis* and MRSA) exhibited visible growth after 240 min of co-incubation ($P < 0.001$) with ZnO NPs at 1X MIC value (Table 7; Fig. 14). Regardless of the MDR-test strains, no bacterial elimination could be observed when treated with sub-MIC levels (1/2X MIC, 1/5X MIC and 1/10X MIC) of ZnONPs (Table 7; Fig. 14).

When treated with Ag/ZnO NCs, complete elimination of MDR-EAEC test strains was observed after 120 to 180 min of incubation with strain variation, even at sub-MIC levels up to 1/10X MIC (Table 7; Fig. 15). However, MDR strains of *S. Typhimurium* and *S. Enteritidis* exhibited a complete bacterial elimination at 60 min when treated with both 1X and 1/2X MIC levels, except for ST1 and ST2 strains. The ST1 and ST2 strains when treated with 1/2X MIC levels exhibited complete elimination at 120 min when treated with Ag/ZnO NCs. Further sub-MIC levels (1/5X MIC and 1/10X MIC) could not exert complete bacterial elimination even after 240 min of co-incubation with Ag/ZnO NCs (Table 7; Fig. 15). Moreover, the MDR strains of MRSA exhibited diverse

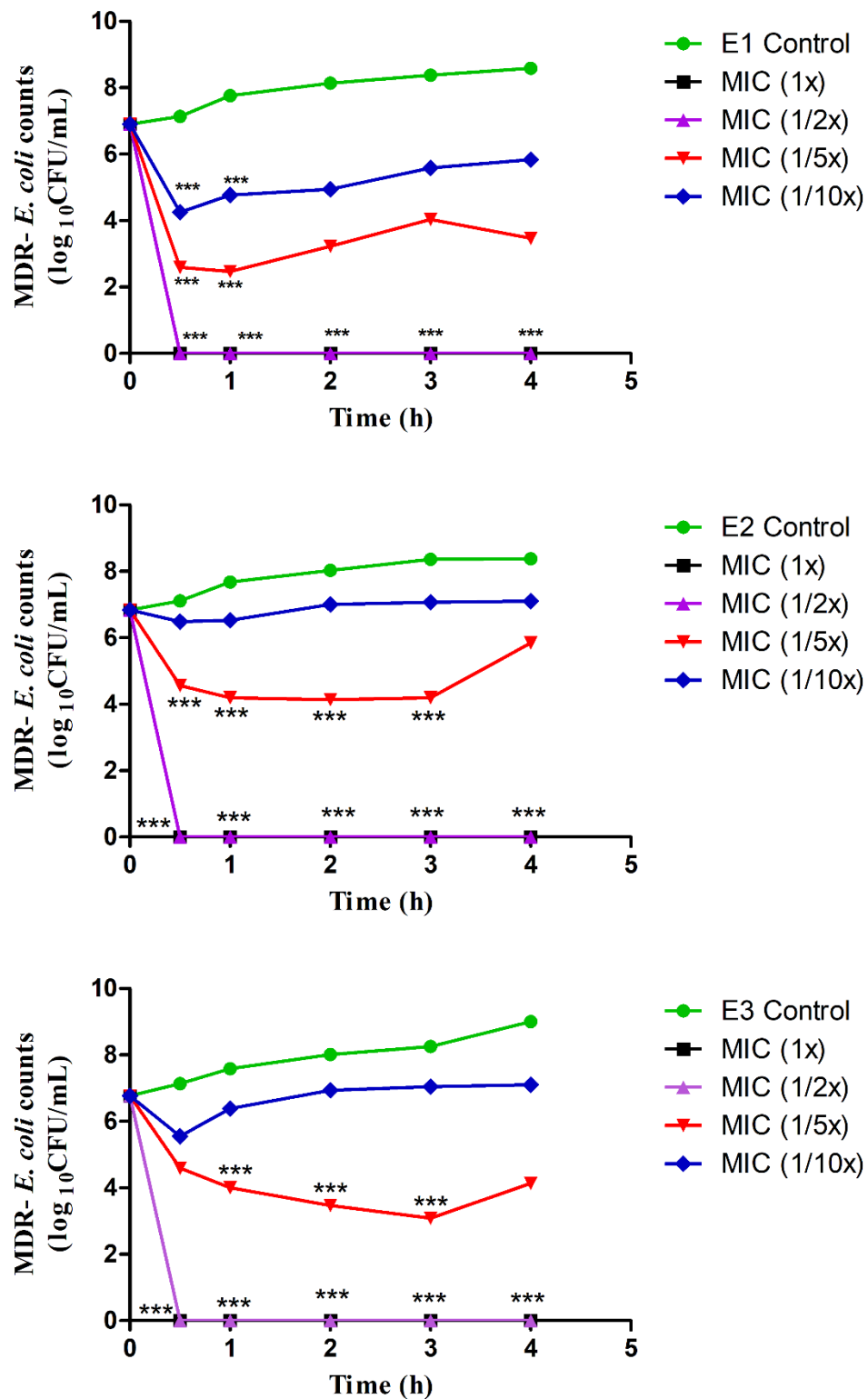


Fig.13A. *In vitro* dose- and time-dependent photocatalytic killing kinetics of green synthesised AgNPs against MDR-EAEC strains

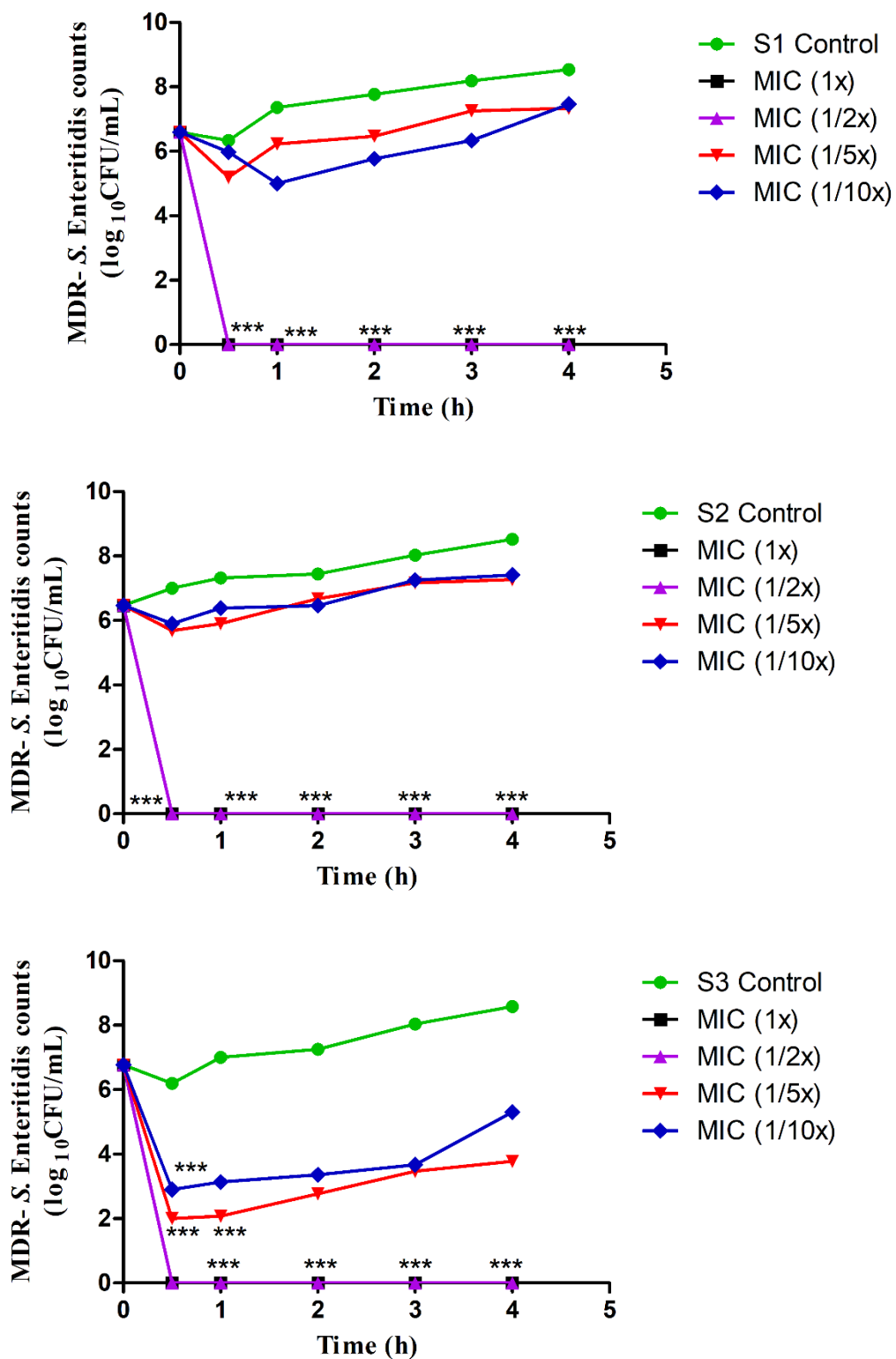


Fig. 13B. *In vitro* dose- and time-dependent photocatalytic killing kinetics of green synthesised AgNPs against MDR-*S. Enteritidis* strains

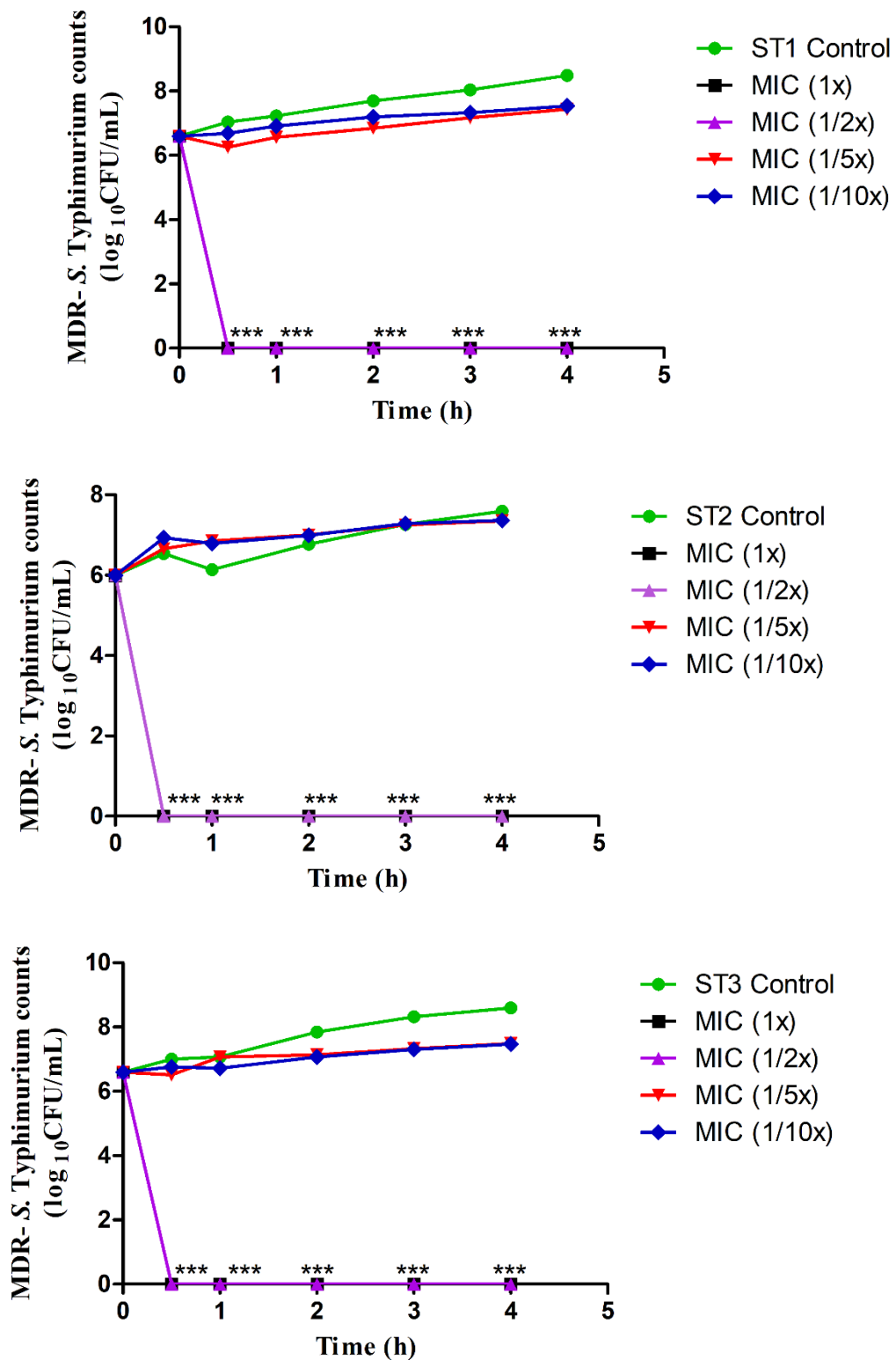


Fig. 13C. *In vitro* dose- and time-dependent photocatalytic killing kinetics of green synthesised AgNPs against MDR-*S. Typhimurium* strains

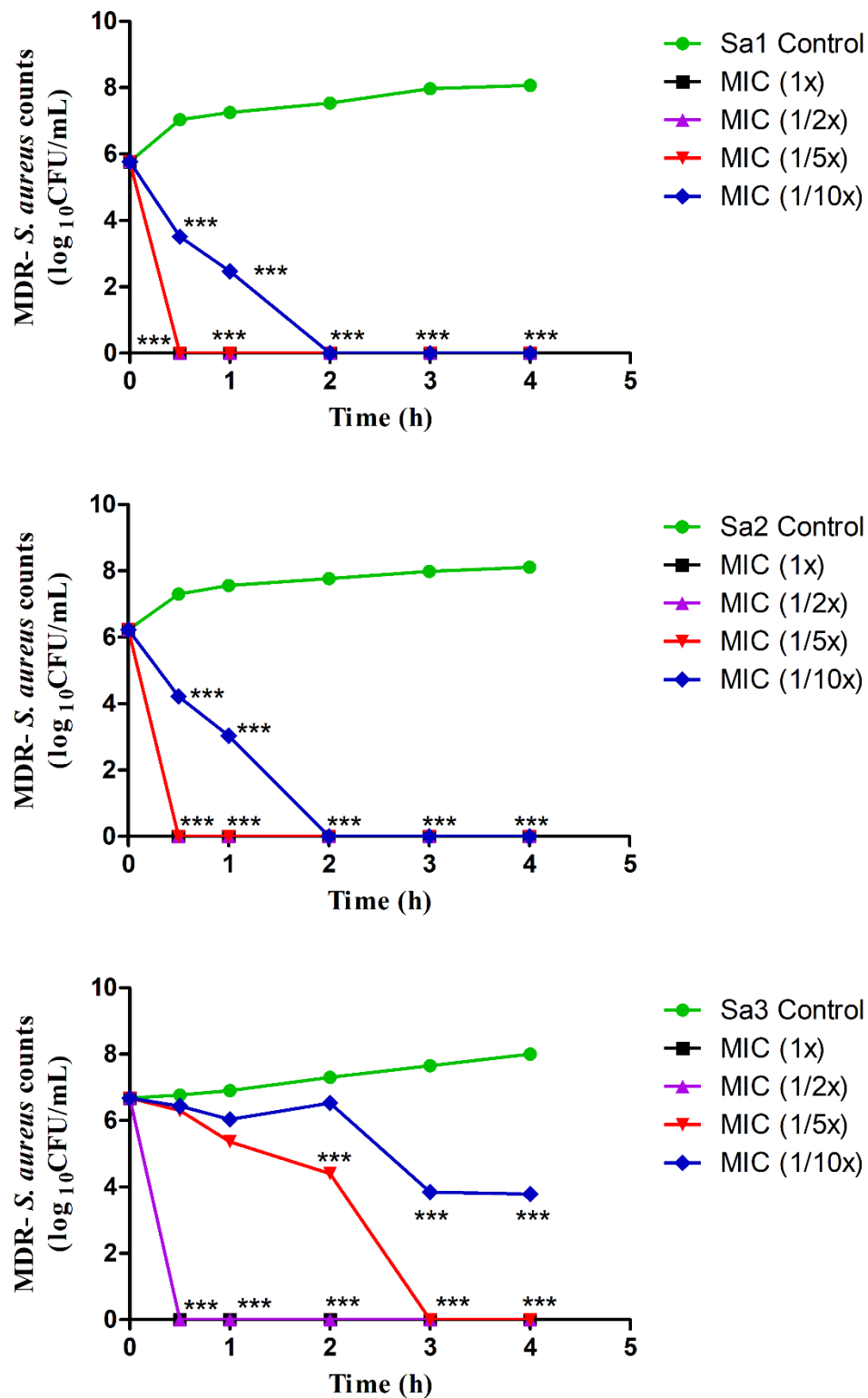


Fig. 13D. *In vitro* dose- and time-dependent photocatalytic killing kinetics of green synthesised AgNPs against MDR-MRSA strains

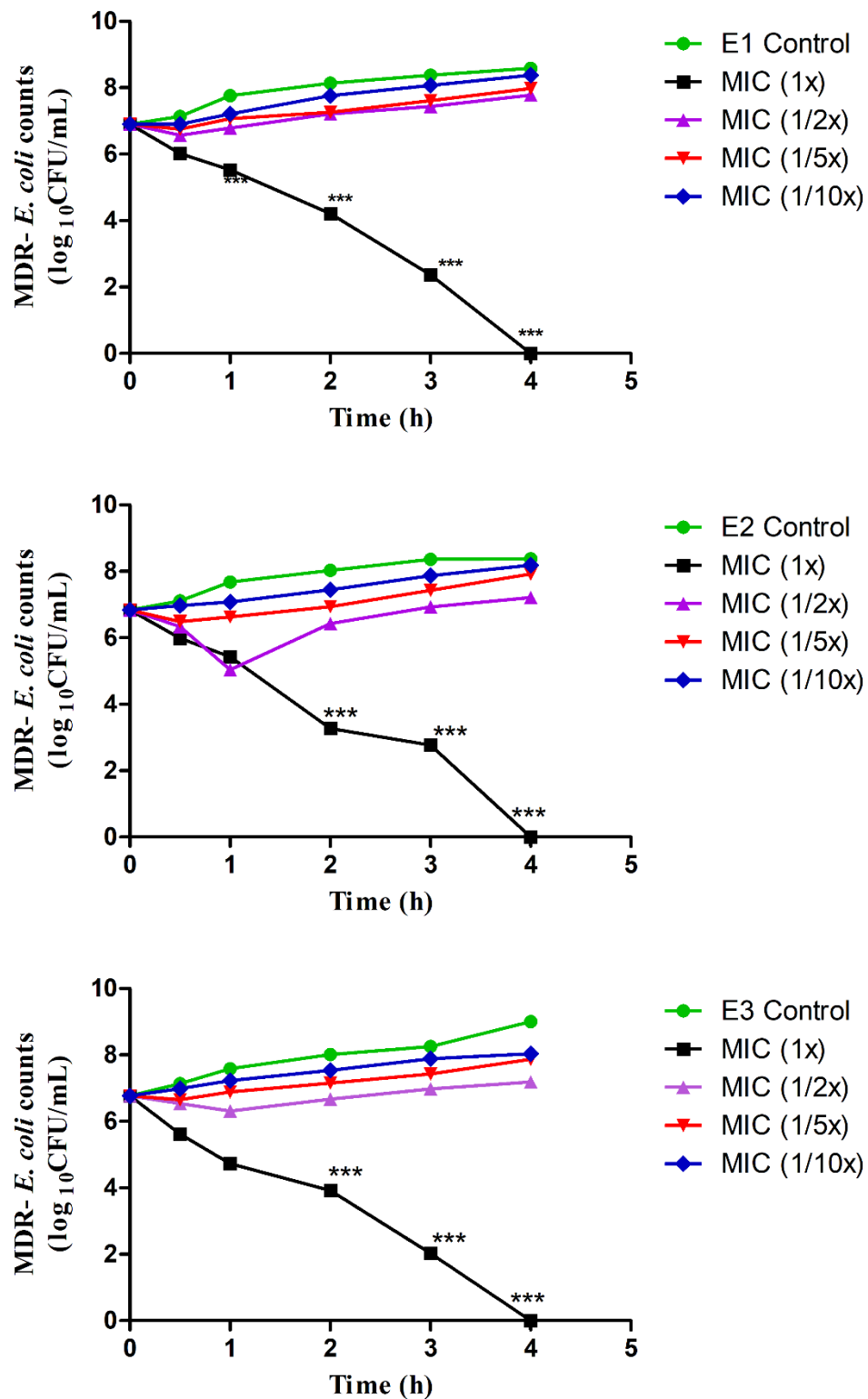


Fig. 14A. *In vitro* dose- and time-dependent photocatalytic killing kinetics of green synthesised ZnO NPs against MDR-EAEC strains

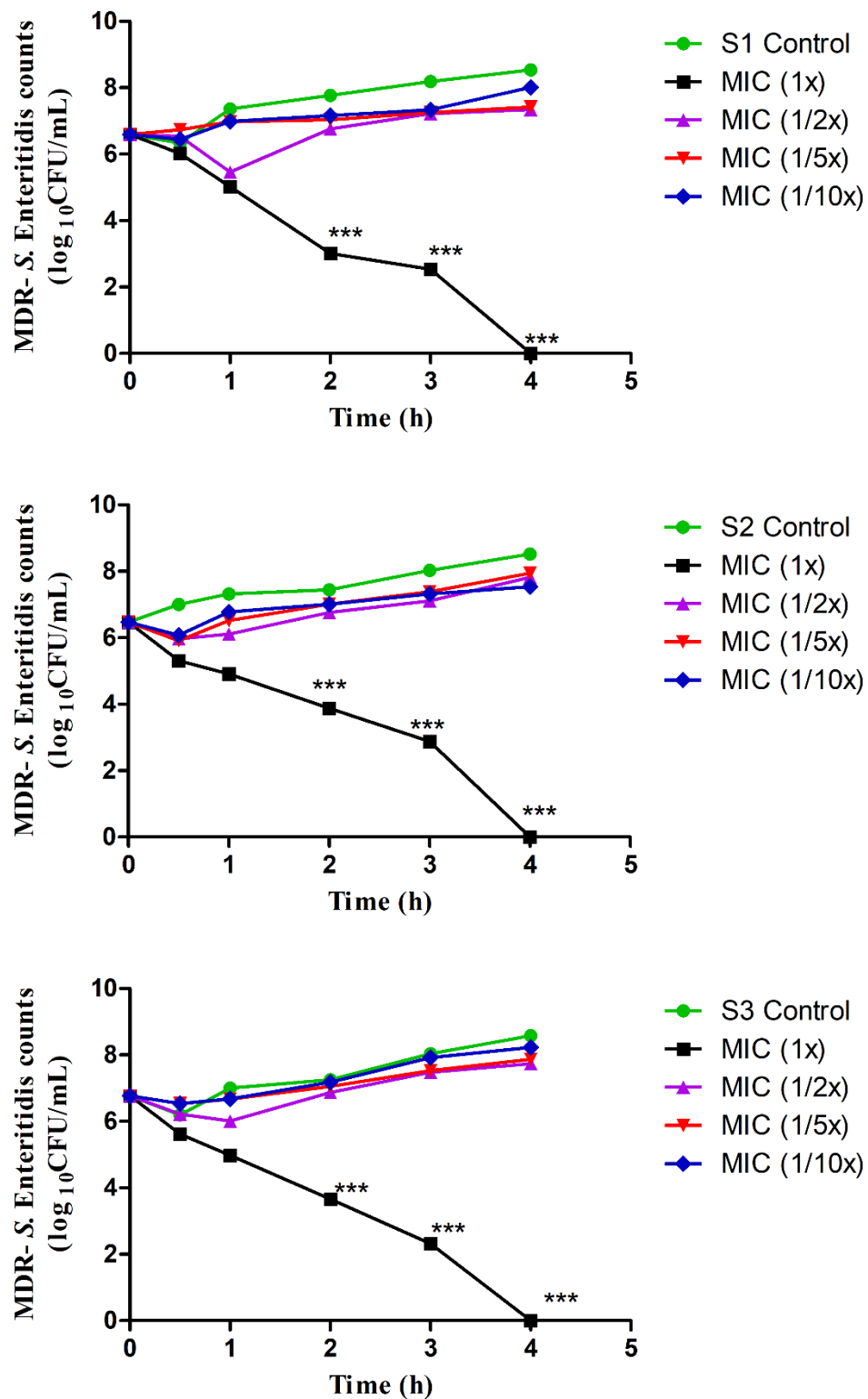


Fig. 14B. *In vitro* dose- and time-dependent photocatalytic killing kinetics of green synthesised ZnO NPs against MDR-*S. Enteritidis* strains

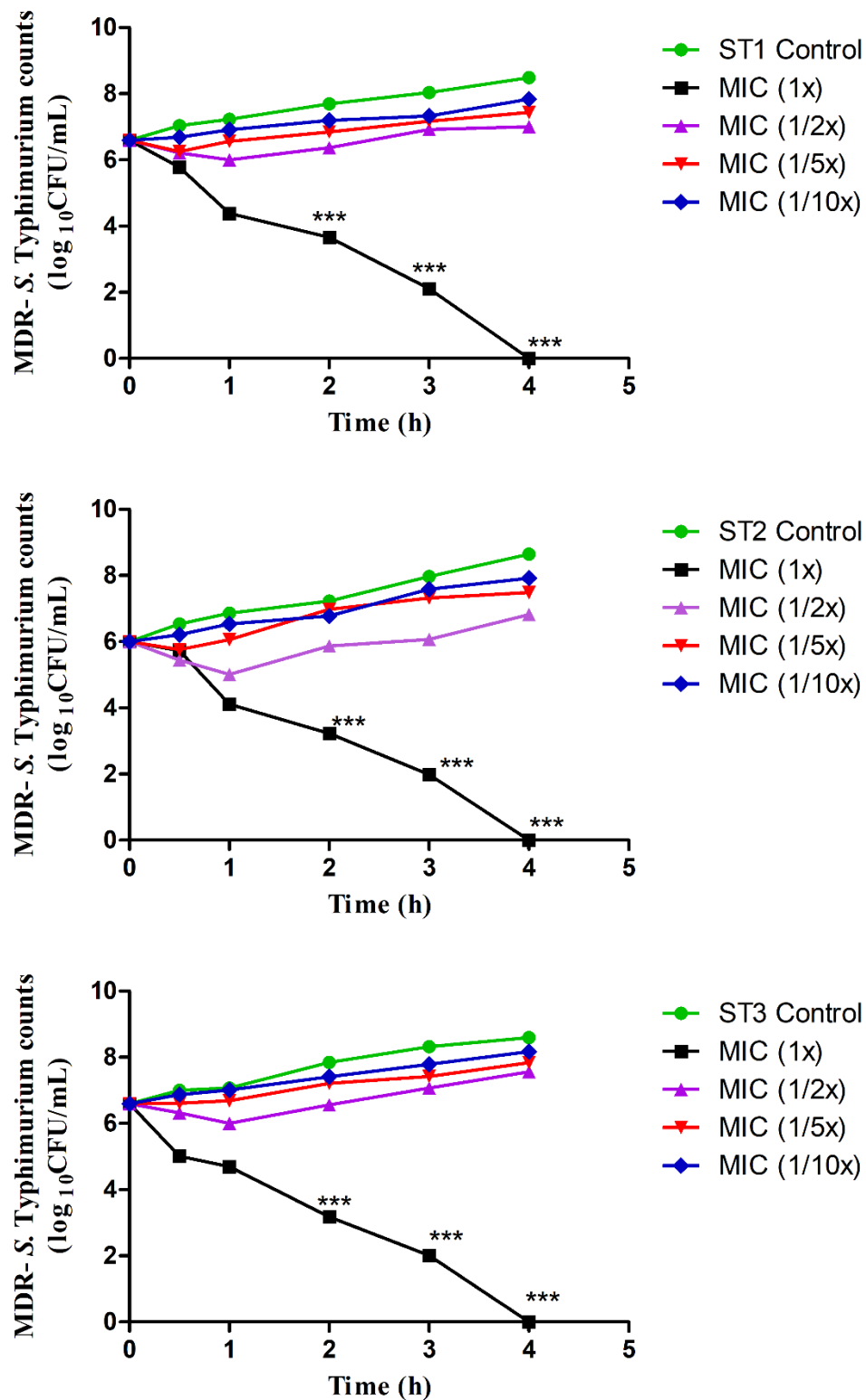


Fig. 14C. *In vitro* dose- and time-dependent photocatalytic killing kinetics of green synthesised ZnO NPs against MDR-S. Typhimurium strains

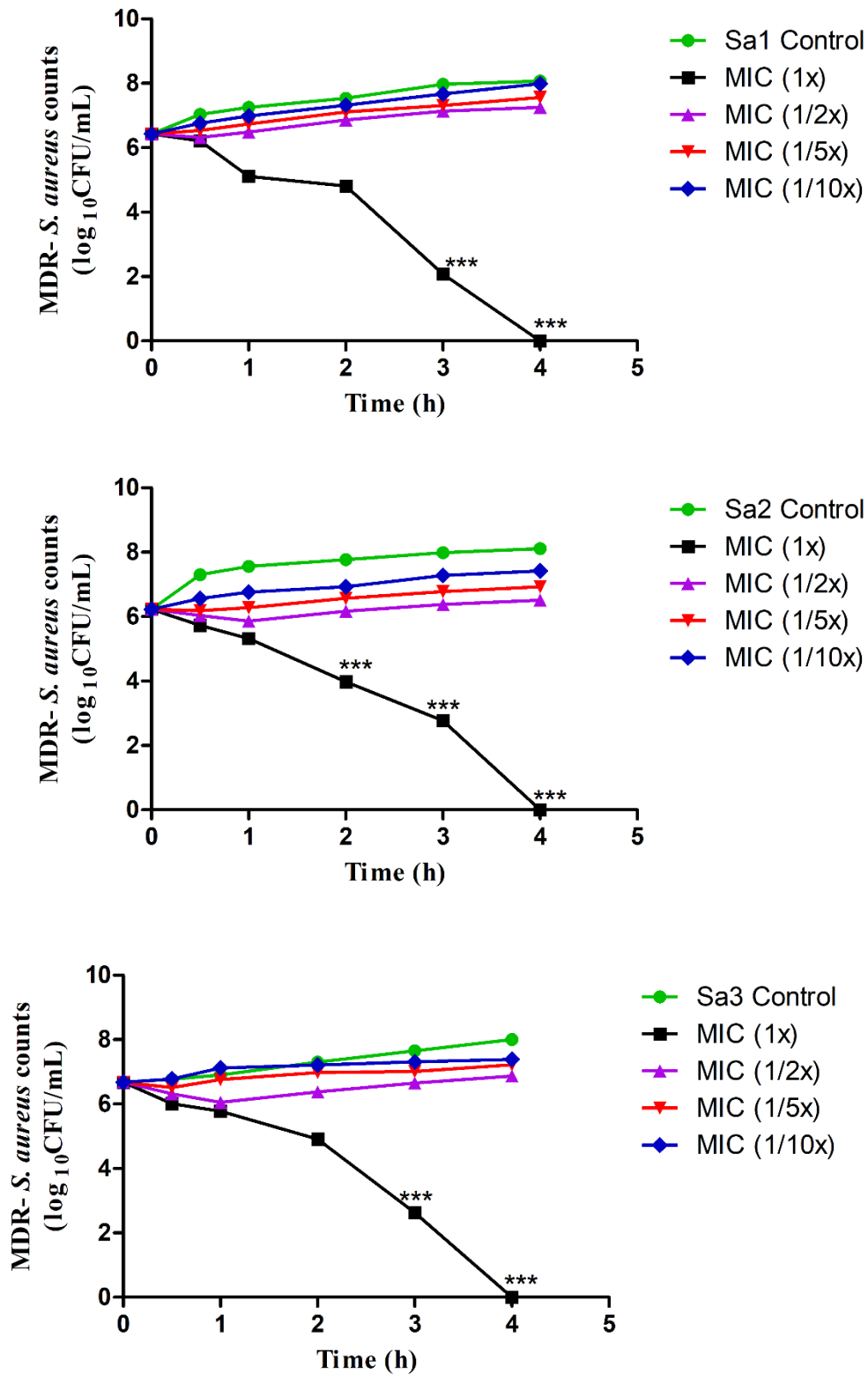


Fig. 14D. *In vitro* dose- and time-dependent photocatalytic killing kinetics of green synthesised ZnO NPs against MDR-MRSA strains

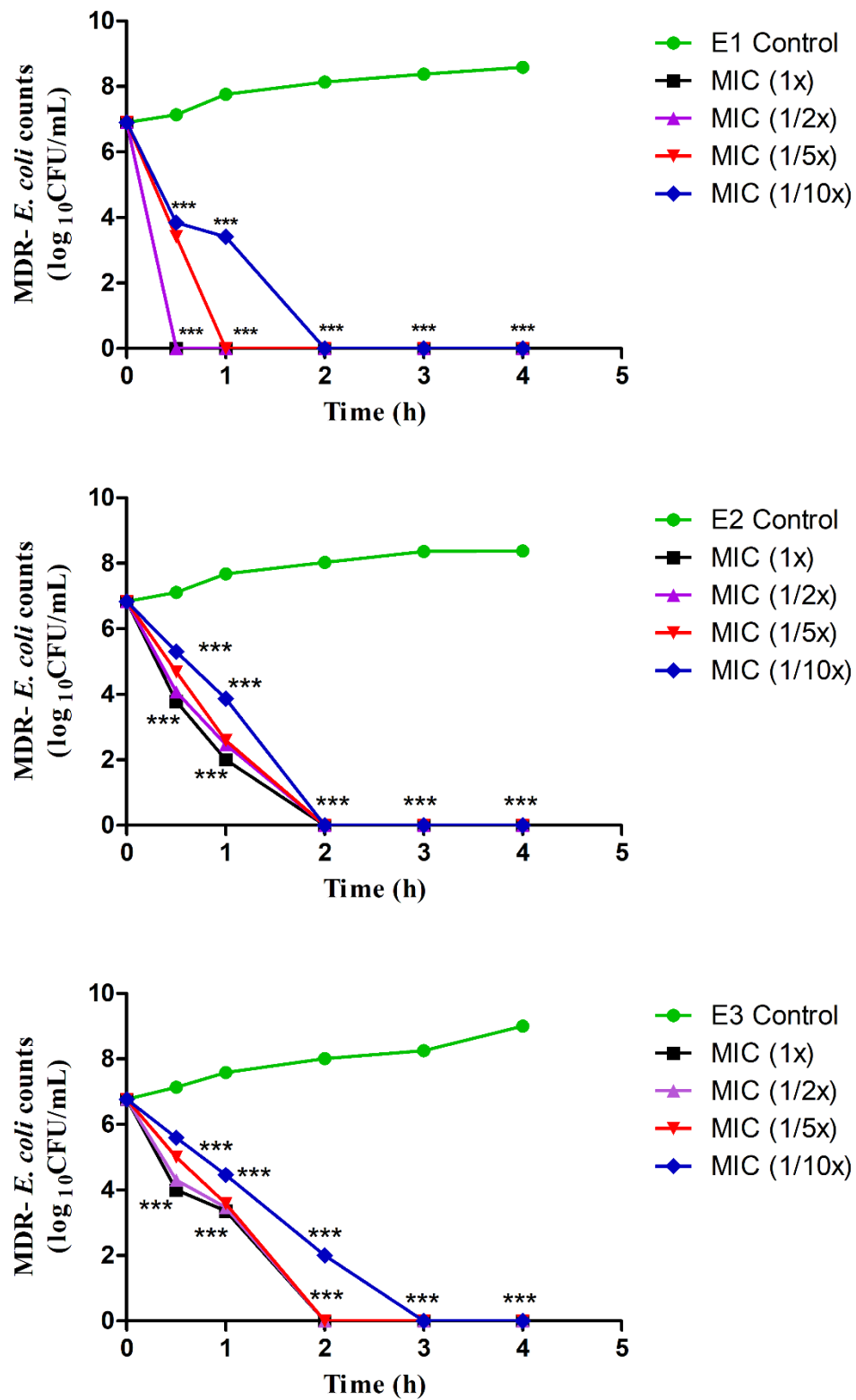


Fig. 15A. *In vitro* dose- and time-dependent photocatalytic killing kinetics of green synthesised Ag/ZnO NCs against MDR-EAEC strains

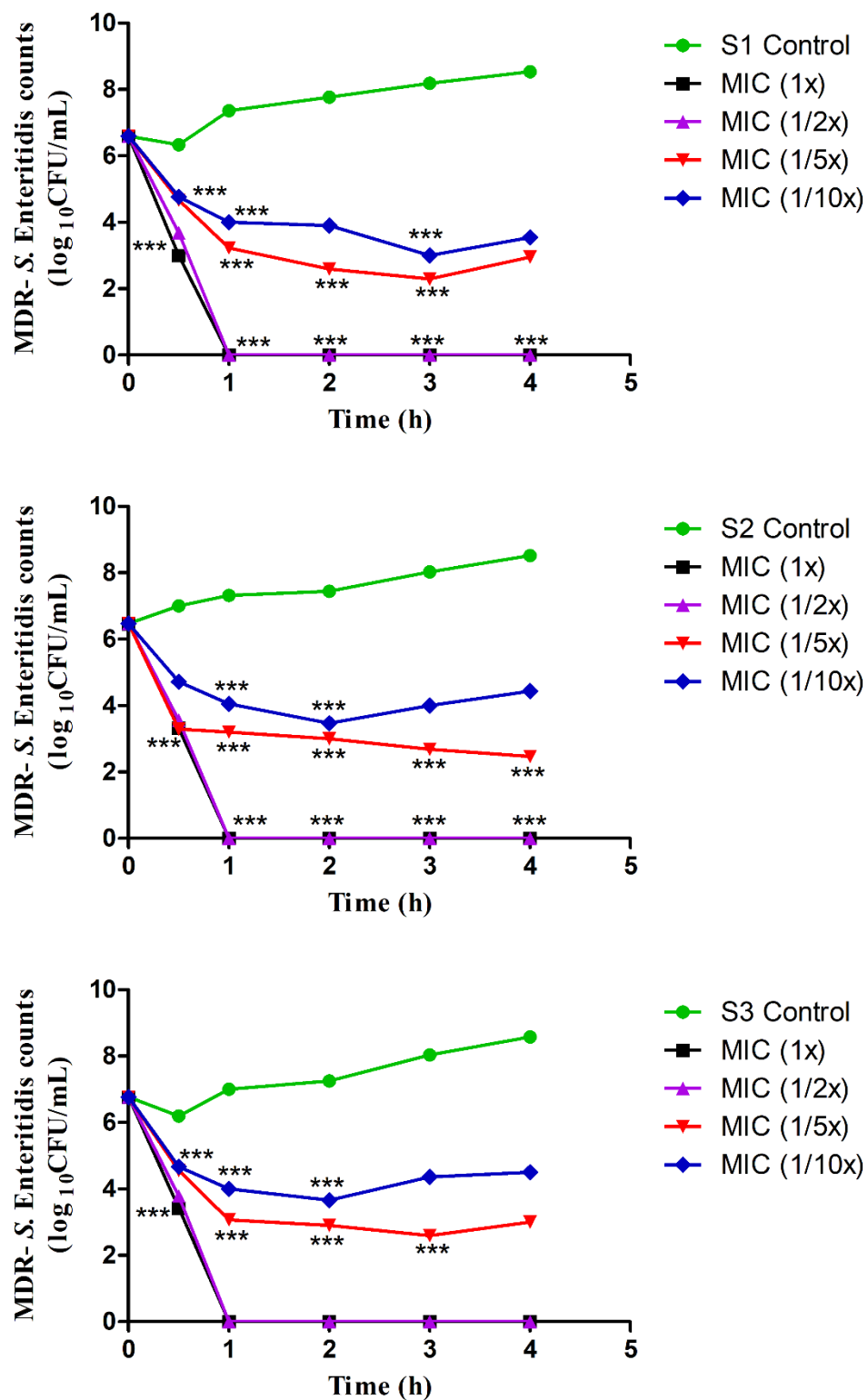


Fig. 15B. *In vitro* dose- and time-dependent photocatalytic killing kinetics of green synthesised Ag/ZnO NCs against MDR-*S. Enteritidis* strains

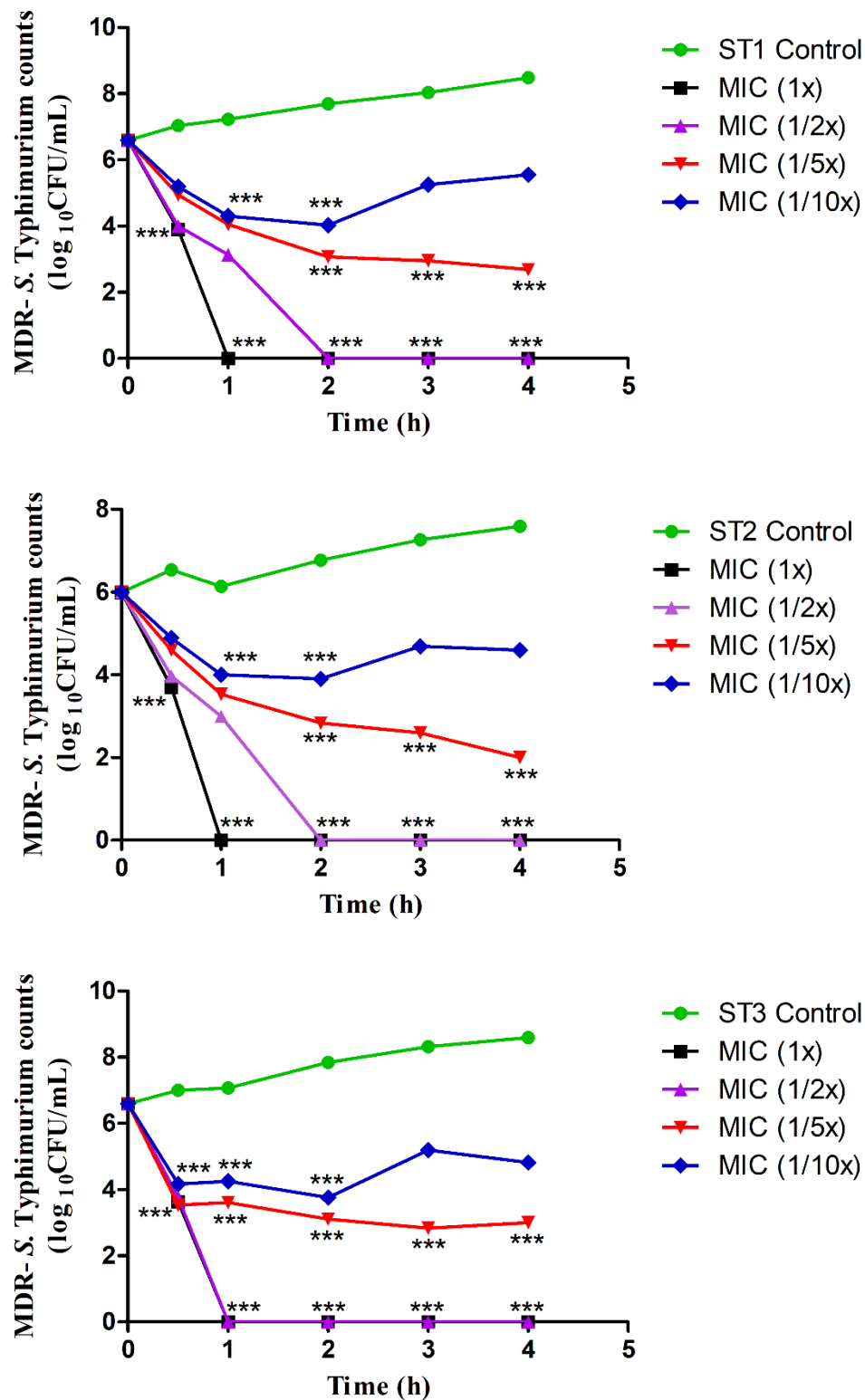


Fig. 15C. *In vitro* dose- and time-dependent photocatalytic killing kinetics of green synthesised Ag/ZnO NCs against MDR-*S. Typhimurium* strains

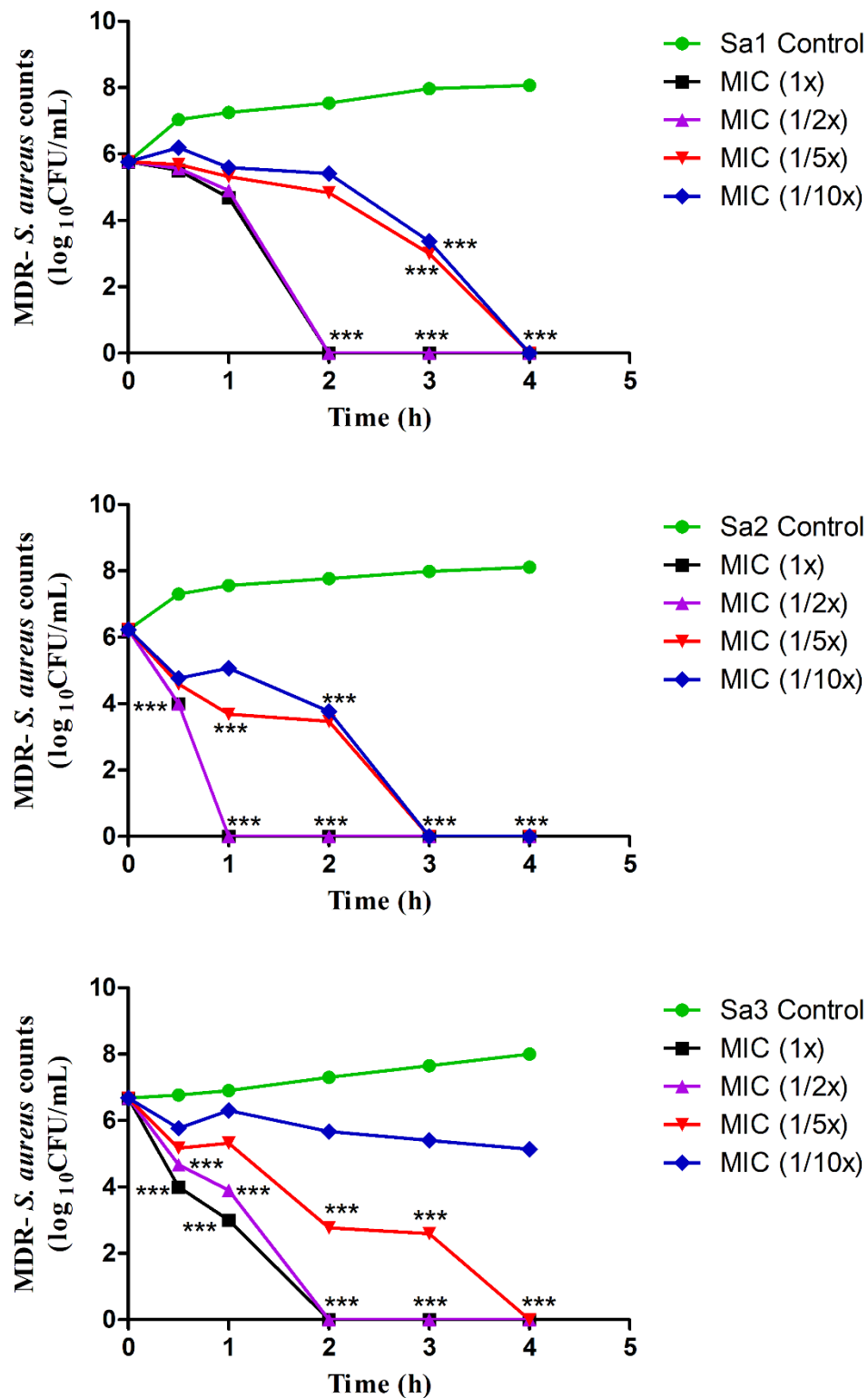


Fig. 15D. *In vitro* dose- and time-dependent photocatalytic killing kinetics of green synthesised Ag/ZnO NCs against MDR-MRSA strains

patterns of killing kinetics ranging from 60 min (Sa2 at 1X and 1/2X MIC) to 240 min (Sa1 at 1/5X and 1/10X MIC as well as Sa3 at 1/5X MIC) (Table 7; Fig. 15).

Table 7. *In vitro* dose- and time-dependent photocatalytic time-kill kinetics of MDR bacterial test strains treated with NPs under study on exposure to LED light

Test NPs	Concentration of NPs	Killing time (min)											
		MDR-EAEC			MDR-S. Enteritidis			MDR-S. Typhimurium			MDR-MRSA		
		E1	E2	E3	S1	S2	S3	ST 1	ST 2	ST 3	Sa 1	Sa 2	Sa 3
AgNPs	MIC (1X)	30	30	30	30	30	30	30	30	30	30	30	30
	MIC (1/2X)	30	30	30	30	30	30	30	30	30	30	30	30
	MIC (1/5X)	ND	ND	ND	ND	ND	ND	ND	ND	ND	30	30	180
	MIC (1/10X)	ND	ND	ND	ND	ND	ND	ND	ND	ND	120	120	ND
ZnO NPs	MIC (1X)	240	240	240	240	240	240	240	240	240	240	240	240
	MIC (1/2X)	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	MIC (1/5X)	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	MIC (1/10X)	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Ag/ZnO NCs	MIC (1X)	30	120	120	60	60	60	60	60	60	120	60	120
	MIC (1/2X)	30	120	120	60	60	60	120	120	60	120	60	120
	MIC (1/5X)	60	120	120	ND	ND	ND	ND	ND	ND	240	180	240
	MIC (1/10X)	120	120	180	ND	ND	ND	ND	ND	ND	240	180	ND

ND: Not detected

5. Discussion

5. DISCUSSION

The breakneck pace in the emergence of antimicrobial resistance (AMR) across the globe is a serious issue, not only for humans but also for animals. Moreover, the biofilm-forming ability of various bacterial species is an important contributing factor to the emergence of a resistance mechanism, as it decreases the effectiveness of the existing antibiotics and limits the penetration ability of antibiotics (Henostroza *et al.*, 2022). The environmental contamination caused by the drug-resistant bacteria along with antibiotics and their residues also poses a serious public health threat (Odoyo *et al.*, 2023). This public health issue has far-reaching consequences on the global as well as national health systems, thus reflecting the need to embrace the 'One Health' concept (Hu *et al.*, 2020).

Water pollution constitutes one of the major environmental public health threats that result in direct and indirect effects on the ecosystem (Lin *et al.*, 2022). With urbanisation, industrialisation, and a rise in agricultural output, novel water pollutants are on the rise. The monitoring as well as notification of emerging contaminants are sparse with regard to the water supply and wastewater (Morin-Criniet *et al.*, 2022). It has been reported that nearly 66 per cent of water-borne illnesses are caused by drug-resistant bacteria, while 26 per cent are associated with chemical pollutants (Gupta and Chaudhary, 2022). Although the current water purification system employs several techniques (physical, chemical, biological methods, or a combination), none of them were documented to be completely efficient (Crini and Lichtfouse, 2019).

Photocatalysis employing nanotechnological solutions has gained momentum in the recent past for treating wastewater (Aragaw, *et al.*, 2021; Shukla *et al.*, 2021; Ali *et al.*, 2023). Nanoparticles (NPs) have widely been employed as appropriate alternatives for wastewater treatment due to their high surface area-to-volume ratio and improved efficiency (Rajagopalachari *et al.*, 2022; Ahmad *et al.*, 2023). Likewise, NPs also possess high surface charge enabling them sensible contestants for photocatalytic studies (Hashemi *et al.*, 2022; Dhanalakshmi *et al.*, 2023). In general, metal and metal oxide NPs such as silver (Ag), zinc oxide (ZnO),

titanium-di-oxide (TiO₂), and copper oxides synthesized either biologically or hydrothermally are widely employed for wastewater treatment by photocatalytic applications (Elbadawy *et al.*, 2023; Lal *et al.*, 2023; Vasantharaj *et al.*, 2023). Green nanotechnology is an emerging field that provides safe nanomaterials with wide biomedical applications, low cost of production and environmental sustainability (Vijayaram *et al.*, 2023). The large surface area-to-volume ratio of NPs enables them an increased adsorbability and superior photocatalytic behaviour, due to which the NPs are widely employed for the removal of antibiotics (Noor *et al.*, 2022; Zhan *et al.*, 2023). Even though the potential of green synthesised AgNPs fabricated using several phytochemicals was estimated against the MDR strains of bacterial pathogens such as *Klebsiella pneumoniae* (Lakkim *et al.*, 2020) *Escherichia coli*, *Acinetobacter baumannii* (Said *et al.*, 2023), *S. aureus* (Jaffar *et al.*, 2023), *Pseudomonas aeruginosa* (Hassan Afandy *et al.*, 2023), their antibiofilm activity against biofilm-forming bacteria and their antioxidant activity remains naïve.

Hence, the present study was designed to evaluate the antibacterial as well as the antibiofilm potential of green synthesised AgNPs, ZnO NPs and Ag/ZnO NCs against MDR strains of enteroaggregative *E. coli* (EAEC), *Salmonella* Typhimurium, *S. Enteritidis* and methicillin-resistant *S. aureus* (MRSA). Further, the antioxidant, and photocatalytic potential of the selected nanomaterials for their dye degradation, antibiotic degradation as well as disinfection were evaluated.

5.1 GREEN SYNTHESIS OF NPs

5.1.1 Green Synthesis of AgNPs

The green synthesis of AgNPs was carried out using the cell-free supernatant of a potential probiotic strain- *Lactobacillus acidophilus* MTCC 10307 (Abishad *et al.*, 2022). The cell-free supernatant was used to reduce the silver nitrate solution (0.10 M) to AgNPs at 25°C under constant stirring for 60 min.

5.1.2 Green Synthesis of ZnO NPs

The green synthesis of ZnO NPs was carried out using the ethanolic extract of commercial piperine (Unni *et al.*, 2022). Piperine was used to reduce the zinc acetate dihydrate solution (0.10 M) to ZnO NPs at 60 °C under constant stirring for 120 min.

5.1.3 Green Synthesis of Ag/ZnO NCs

The green synthesis of Ag/ZnO NCs was carried out using the methanolic extract of *Curcuma longa* (Arya *et al.*, 2023). The methanolic extract reduced an aqueous mixture of silver nitrate (0.10 M) and zinc nitrate (0.10 M) solutions at the ratio of 2:3 to form Ag/ZnO NCs.

5.2 CHARACTERISATION OF NPs

The characterisation of the green synthesised NPs using UV-Vis spectroscopy was performed to revalidate their formation.

A characteristic absorbance peak at 430 nm (Fig. 1A) suggested the formation of AgNPs (Boldet *et al.*, 2022), while the characteristic peak noticed at 340 nm (Fig. 1B) indicated the formation of ZnO NPs (Daret *et al.*, 2022; Minhas *et al.*, 2023; Yousefi *et al.*, 2023). Moreover, two characteristic peaks observed at 340 nm as well as 430 nm (Fig. 1C) conferred the formation of Ag/ZnO NCs (Hosny *et al.*, 2022; Elumalai *et al.*, 2023).

5.3. *IN VITRO* ANTIMICROBIAL EFFICACY OF GREEN SYNTHESISED NPs

5.3.1 Determination of MIC and MBC of green synthesized NPs against MDR-EAEC, NTS and MRSA strains

The MIC and MBC values of AgNPs, ZnO NPs and Ag/ZnO NCs, as a measure of their antibacterial activity, were investigated against MDR strains of EAEC, NTS and MRSA by micro broth dilution technique (CLSI, 2019).

Irrespective of the tested MDR pathogens, the MIC values of biosynthesised AgNPs, ZnO NPs and Ag/ZnO NCs were found to be 7.8125, 62.50 and 31.25

µg/mL, respectively, while the MBC values obtained were 15.625, 125 and 62.50 µg/mL, respectively (Table 1). The comparatively lower MIC and MBC values observed in this study might be due to the oxidative stress as a result of the generation of reactive oxygen species (ROS) such as hydrogen peroxide (H₂O₂), singlet oxygen (¹O₂), superoxide anion (O₂⁻), hydroxyl radical (•OH), and hypochlorous acid (HOCl) that resulted in the bacterial cell death (Panchal *et al.*, 2020; Alavi, 2022; Mendes *et al.*, 2022). In addition, the antibacterial effect of the green synthesised NPs could also be attributed to the release of ions (Ag⁺, Ag²⁺, Zn⁺, Zn²⁺) produced by electrostatic interaction between the ions released and bacterial cell wall which might have resulted in the surface oxidation and complex porosity of bacterial cells (Xue *et al.*, 2021).

5.3.2. *In vitro* antibiofilm potential of NPs against MDR test isolates

The *in vitro* antibiofilm potential of the green synthesised AgNPs, ZnO NPs and Ag/ZnO NCs were assessed to estimate their reduction in the biofilm biomass at two different concentrations *i.e.*, MIC (1X) and MIC (2X), when co-incubated with the biofilm-forming isolates of MDR-EAEC, *S. Typhimurium*, *S. Enteritidis* and MRSA strains at 24 and 48 h of co-incubation.

This study exhibited a remarkable antibiofilm potential of AgNPs, ZnO NPs and Ag/ZnO NCs against the biofilm-forming MDR test strains. In particular, the reduction in the biofilm biomass was noticed to be higher in 48 h treated MDR-test strains as compared to the 24 h treated strains ($P < 0.001$). Moreover, AgNPs exhibited comparatively better antibiofilm properties (Fig. 2) than ZnONPs (Fig. 3) and Ag/ZnO NCs (Fig. 4). The antibiofilm activity exhibited by NPs could be achieved by an array of methods including the production of ROS, the destruction of extracellular polymeric substance (EPS), and the inhibition of quorum sensing (Daset *et al.*, 2021; Linet *et al.*, 2021). The fairly better antibiofilm activity of AgNPs could have either been due to the prevention of bacterial cells from adhering to surfaces or by damaging the intermolecular forces (Kalishwarala *et al.*, 2010).

An overall reduction in the biofilm biomass was observed to be higher in MDR-MRSA strains as compared to the MDR strains of EAEC and *Salmonella* spp. (Table 2). This difference in biomass reduction (Table 2) could be due to the changes in the cell wall of Gram-positive (MRSA) as compared to Gram-negative (EAEC and *Salmonella* spp.) (Sharma *et al.*, 2023).

5.4. *IN VITRO* ANTIOXIDANT ACTIVITY OF NPs

In order to evaluate the free radical scavenging abilities of the green synthesised AgNPs, ZnO NPs and Ag/ZnO NPs, the *in vitro* antioxidant activity was performed. The results obtained were compared with a standard antioxidant, ascorbic acid using DPPH (Fig. 5) and ABTS (Fig. 6) assays.

In this assay, AgNPs and Ag/ZnO NPs exhibited better antioxidant properties than ZnO NPs (Figs. 5 and 6). Moreover, this study exhibited a dose-dependent increase in the antioxidant capacity of the green synthesised NPs; however, the free radical scavenging ability of all the tested NPs was observed to be lower than the standard.

The intrinsic redox activity of metal-based NPs could be linked to their free radical trapping, as well as catalase- and superoxide dismutase-like activities (Valgimigli *et al.*, 2018). The green synthesised NPs (such as Ag and gold) act like enzymes and exhibit antioxidant activity that would be due to the free radical scavenging ability and reduction in the concentrations of ROS (Samrotet *et al.*, 2022). Moreover, the enhanced antioxidant properties of the green synthesised NPs over the physical or chemical methods could be related to the bioactive composition of the reducing agent which was used for their synthesis (Balkrishna *et al.*, 2021).

5.5. *IN VITRO* PHOTOCATALYTIC DYE DEGRADATION ABILITY OF NPs

5.5.1. *In vitro* photocatalytic potential of NPs for degradation of individual dye

The photocatalytic dye degradation assay was performed to estimate the ability of green synthesised NPs in degrading industrial dyes in the presence of a light source. In the present study, the degradation potential of AgNPs, ZnO NPs and Ag/ZnO NCs were evaluated using three cationic dyes *viz.*, methylene blue (MB), crystal violet (CV) and rhodamine B (RhB) on exposure to sunlight, LED light and UV light.

This study revealed significant dye degradation abilities of the green synthesised NPs under different light sources. Irrespective of the light sources, treatment with ZnO NPs exhibited a gradual increase in the photodegradation pattern for all the tested dyes throughout the study. However, AgNPs exhibited a drastic increase in the photodegradation pattern for MB and CV dyes, whereas degradation of RhB when treated with AgNPs was gradual throughout the entire duration. Moreover, Ag/ZnO NCs exhibited a momentous increase in the degradation of CV and MB irrespective of the light source. Nonetheless, the degradation of RhB when treated with Ag/ZnO NCs was gradual with LED and UV light. Furthermore, the degradation of RhB was negligible with AgNPs and ZnO NPs regardless of the light source.

The photocatalytic behaviour visualised by the change in colour of the dyes observed in this study upon photon absorption by NPs could mainly be due to the degradation of these toxic dyes (MB, CV and RhB) to smaller and non-toxic compounds like water, carbon-di-oxide, and organic acids (Saeed *et al.*, 2022). A decreased degradation of RhB observed in this assay could be attributed to their high solubility and stability in water (Mzimela *et al.*, 2022).

The proposed mechanism for dye degradation could be the generation of ROS such as superoxide anions, and hydroxyl radicals (Kannan *et al.*, 2022). Nonetheless, this increased dye degradation ability of NPs in the presence of different light sources could be attributed to the formation of electron-hole (e^-/h^+) pairs when photonic energy ($h\nu$) equal to or greater than the excitation energy (E_g) hits the surface of NPs. Later, the e^- from the filled valence band (VB) would be upgraded to an empty conduction band (CB) resulting in the formation of

e^-/h^+ pairs (Onget *et al.*, 2018). In addition, morphological characteristics, differences in energy gaps, and the light sources to which they have been exposed favours the effective charge separation determining the photocatalytic activity of NPs (Vasiljevic *et al.*, 2020).

5.5.2. Determination of photocatalytic dye degradation kinetics

The photocatalytic degradation kinetics of individual dyes were determined to assess the quantification of the individual dyes (MB, CV, and RhB). The concentrations of the aliquots taken at different time intervals were determined from the UV absorbance value by applying ‘Beer-Lambert’ law (Bhanot *et al.*, 2023).

The kinetics of degradation were estimated as zero order, first order, pseudo-first-order or second order. The reaction parameters envisaged a zero order kinetics for the degradation of RhB irrespective of the light source and NPs used except with Ag/ZnO NCs exposed to sunlight. However, Ag/ZnO NCs when exposed to sunlight, the RhB fitted to the first order kinetics, and with the UV light, RhB fitted to the second order kinetics. This could be due to the high stability of RhB in water, which made them fit zero-order kinetics, where the rate of degradation is not dependent on the concentration of the reactants (Table 3). The zero-order kinetics of RhB degradation using NPs were also reported in earlier studies (Li *et al.*, 2017; Fang *et al.*, 2020; Azeez *et al.*, 2022). Moreover, MB and CV followed primarily second-order kinetics. The differences in the rates of the reaction could be attributed to the differences in the amount of ROS released from the tested NPs upon photoexcitation with different light sources (Khan *et al.*, 2021; Kessler *et al.*, 2022).

In the photocatalytic dye degradation assay, Ag/ZnO NCs exhibited better photocatalytic potential; hence, further studies employed the use of Ag/ZnO NCs for conducting the selectivity as well as reusability assays.

5.5.3. Selectivity of dyes on photocatalytic dye degradation

The present study evaluated the selectivity of dyes on the *in vitro* photocatalytic dye degradation abilities of the green synthesised NPs. The

selectivity was then measured by mixing the dyes (MB, CV and RhB) and exposing the resultant mixture to sunlight.

The study exhibited a non-significant ($P > 0.05$) difference in the dye degradation pattern for Ag/ZnO NCs in degrading MB and CV either individually or in combination in the presence of sunlight, whereas a significant ($P < 0.01$) difference in the dye degradation pattern for Ag/ZnO NCs in degrading RhB individually and in combination in the presence of sunlight (Fig. 9). Further, the degradation of MB was estimated to be 97.82 per cent and 93.37 per cent when the dyes were used individually and in combination, respectively (Table 4). Similarly, degradation of 98.20 per cent and 95.79 per cent was obtained for CV (Table 4) and RhB exhibited degradation rates of 98.37 per cent and 79.52 per cent when treated individually and in combination, respectively (Table 4). This difference in the dye degradation of RhB individually or in combination over MB or CV could be due to the selectivity of the NPs for the dyes MB and CV. This selectivity of NPs to the dyes could conclusively be attributed to their differences in charge or structural morphology of the tested cationic dyes (Taghdiri, 2017; Prasad *et al.*, 2019).

5.5.4. Effect of reusability of NPs in photocatalytic dye degradation

This study analysed the reusability of Ag/ZnO NCs in a dye degradation experiment, wherein the used Ag/ZnO NCs were compared with fresh NCs for the degradation of a combination of MB, CV, and RhB.

This study exhibited diverse dye degradation patterns for degrading MB, CV and RhB by Ag/ZnO NCs when they were either freshly used or reused. For MB, 93.37 per cent degradation was obtained when treated with fresh Ag/ZnO NCs, while 84.25 per cent was obtained with the reused NCs (Fig. 10A). Similarly, for CV, a degradation of 95.79 and 90.59 per cent was obtained, respectively (Fig. 10B). Nonetheless, for RhB, a degradation of 79.52 and 61.50 per cent was obtained with fresh and reused NCs, respectively. It could be inferred that the tested NPs could be used as effective photocatalysts because of their high reusability and no significant differences in their performances (Nasriet *et al.*, 2021). The efficient reusability of

Ag/ZnO NCs in this study could be due to the high stability, inertness, photocatalytic mechanisms, easiness for separation and removal, controlled synthesis patterns and efficient charge separation (Du *et al.*, 2019; Ebrahimi *et al.*, 2020; Ravi *et al.*, 2021; Alanaziet *et al.*, 2023).

5.5. *IN VITRO* PHOTOCATALYTIC ANTIBIOTIC DEGRADATION ABILITY OF NPs

The *in vitro* photocatalytic antibiotic degradation assay was performed to estimate the ability of AgNPs, ZnO NPs and Ag/ZnO NCs to degrade the selected antibiotics (ampicillin, ciprofloxacin and tetracycline) on exposure to sunlight. The discarded antibiotics could act as a potential source of resistance development, which in turn could result in environmental pollution.

Irrespective of all the tested NPs, a highly significant ($P < 0.001$) degradation pattern was observed for tetracycline. Likewise, significant ($P < 0.05$) degradation of ampicillin as well as ciprofloxacin was observed on treatment with all the tested NPs on exposure to sunlight (Fig. 12). It was inferred that NPs-treated tetracycline resisted degradation on exposure to sunlight than ampicillin and ciprofloxacin.

It has been reported that adsorption and photocatalysis constitute the primary mechanisms for the degradation of antibiotics upon treatment with NPs. Besides, it could be inferred that the degradation of antibiotics could largely be attributed to photocatalytic degradation rather than adsorption (Dao *et al.*, 2018; Baaloudjet *et al.*, 2021). This phenomenon has been explained by the production of ROS such as $\cdot\text{OH}$, $\text{O}_2\cdot$, and holes (h^+) from the surface of NPs when irradiated with light sources, producing simple non-toxic molecules like CO_2 , water, and other smaller degradation products (Asadzadeh *et al.*, 2021). The elevation in the optical density readings obtained from UV-Vis spectroscopy (Fig. 11), as observed in this study, could likely be due to the increased production of the break-down products from the antibiotic over time and was directly proportional to each other (Lin and Cen, 2022).

5.6. *IN VITRO* DOSE- AND TIME-DEPENDENT PHOTOCATALYTIC KILLING KINETICS OF MDR- BACTERIAL STRAINS TREATED WITH NPs

The time-kill kinetic assay was estimated to evaluate the photokinetic disinfectant capabilities of AgNPs, ZnO NPs and Ag/ZnO NCs. In this assay, individual MDR strains of EAEC, *S. Typhimurium*, *S. Enteritidis* and MRSA were co-cultured with the four different concentrations (1X MIC, 1/2X MIC, 1/5X MIC and 1/10X MIC) of the test NPs along with their respective controls. The bacterial counts of the samples as well as controls that were exposed to LED light were estimated in log₁₀CFU/mL and expressed as mean $\pm$ standard errors at different time intervals (Table 7; Figs. 13-15).

In the present study, photocatalytic time-kill assay of MDR test strains treated with ZnO NPs resulted in the complete absence of bacterial growth in 240 min of exposure to LED light at the MIC doses. While AgNPs exposed to LED light resulted in the complete killing of MDR strains of EAEC and NTS isolates with the MIC and sub-MIC doses (1/2X MIC) at 30 min of co-incubation, the complete killing of MDR-MRSA strains was obtained with all the treated concentrations (including 1/10X MIC), thereby significantly reducing the concentration of NPs required for bacterial killing for MRSA than EAEC and NTS. Moreover, Ag/ZnO NCs resulted in the complete bacterial killing of MDR test isolates, even with sub-MIC levels at different time points.

The particle size, concentration, mode of distribution and time of incubation without modifying the mode of action determine the antibacterial potential of NPs (Zare *et al.*, 2019). In contrast to our findings with a non-photocatalytic assay, Varsha (2021) reported the time-dependent anti-bacterial activity of ZnO NPs against the tested MDR strains of *Salmonella* spp. with no discernible bacterial growth observed after 360 min of co-incubation at both their MIC and MBC doses. Moreover, the time-dependent anti-bacterial activity of AgNPs (Abishade *et al.*, 2022) and Ag/ZnO NCs (Arya, 2022) was observed to be 240 min both at MIC and MBC doses, indicating that they have a direct antibacterial effect before it enters the stationary phase of bacterial growth. The high surface area-to-volume ratio of

the tested NPs could be attributable to their antibacterial activity owing to the high ROS generation capacity (Chauhan *et al.*, 2020).

On exposure to the light source (LED light), pronounced antibacterial activity was produced by an even smaller concentration of NPs (1/10X MIC) which will subsequently lower the dose of NPs for exerting antibacterial activity. The enhanced antibacterial activity of the NPs observed in the present study could be due to the higher quantum of ROS generated, which actively participates in bacterial killing, upon photoexcitation from a light source.

Regardless of the treatment, MDR strains of MRSA and EAEC strains exhibited complete bacterial elimination at sub-MIC levels than the MDR test strains of *Salmonella* spp. The complete elimination of MDR-MRSA as well as EAEC strains could possibly be due to the extra-cellular nature of the bacterial pathogens when compared to the *Salmonella* strains which are capable of dwelling in distinct residential niches with varying rates of replication (Luk *et al.*, 2022).

In short, it was observed that the green synthesised AgNPs, ZnO NPs and Ag/ZnO NCs could be effectively applied in the fields of environmental remediation such as the photocatalytic degradation of industrial dyes and antibiotics along with photocatalytic disinfection of MDR pathogens. Further, the tested nanomaterials exhibited the antibiofilm property at their MIC and MBC doses against the biofilm-forming MDR test strains. Besides, the tested NPs exhibited potential antioxidant properties using DPPH and ABTS assays. Further, innovative strategies need to be developed for their future applications in slurry waste management and the incorporation of biodegradable plastics for their applications in food packaging.

6. Summary

6. SUMMARY

In recent times, wastewater treatment has become an important public health concern. As a result of rapid urbanisation, excessive discharge of effluents by industries directly into the water sources has been a threat for a long. Wastewater usually contains multi-drug resistant (MDR) bacteria which also possess fouling abilities. Moreover, the discharge of toxic chemicals, especially industrial dyes and antibiotic residues also poses direct as well as indirect hazards to the environment. Nanotechnology provides a single-point technological solution for the treatment of wastewater with its antioxidant, antibacterial, antifouling and photocatalytic properties. The present study has hence been envisaged to evaluate the antioxidant, and photocatalytic potential of green synthesised silver (Ag) nanoparticles (NPs), zinc oxide (ZnO) NPs and Ag/ZnO nanocomposites (NCs) in the dye removal and antibiotic degradation. Further, the anti-fouling as well as photocatalytic killing kinetics of these nanomaterials were assessed against MDR strains of enteroaggregative *E. coli* (EAEC), non-typhoidal *Salmonella* spp. (NTS), and methicillin-resistant *S. aureus* (MRSA).

Initially, the green synthesis of AgNPs, ZnO NPs, and Ag/ZnO NCs was carried out; the formation of synthesised nanomaterials was re-validated using UV-Vis spectroscopy to obtain characteristic peaks at 430 nm (AgNPs), 340 nm (ZnO NPs), and 340 and 430 nm (Ag/ZnO NCs). The antioxidant properties of AgNPs, ZnO NPs and Ag/ZnO NCs assessed using ABTS and DPPH assays revealed a dose-dependent increase in the antioxidant properties. Moreover, the IC₅₀ values determined for AgNPs and Ag/ZnO NCs were 60 and 80 µg/mL, respectively using ABTS assay, while 80 and 60 µg/mL, respectively using DPPH assay.

The antibacterial potential of the NPs was assessed against MDR strains of EAEC isolates (n=3), NTS isolates (*S. Enteritidis* and *S. Typhimurium*; n= 3 each) and MRSA isolates (n=3), maintained in the laboratory repository of the Department of Veterinary Public Health, College of Veterinary and Animal Sciences, Pookode. The antibacterial assay assessed by micro broth dilution technique exhibited the

MIC and MBC values of 7.80 to 15.625 $\mu\text{g/mL}$ (AgNPs), 62.50 to 125 $\mu\text{g/mL}$ (ZnO NPs), and 31.25 to 62.50 $\mu\text{g/mL}$ (Ag/ZnO NCs).

The antibiofilm potential of the tested NPs was evaluated both at 24 h and 48 h against the biofilm-forming MDR strains of EAEC, NTS and MRSA, keeping *E. coli* ATCC 25922 as a positive biofilm former and *E. coli* DH5 α as a non-biofilm producer. The results exhibited a significant ($P < 0.001$) antibiofilm potential of the tested AgNPs, ZnO NPs, and Ag/ZnO NCs at their MIC as well as MBC concentrations against the biofilm-forming MDR test strains (EAEC, NTS, and MRSA).

The photocatalytic potential of the green synthesised AgNPs, ZnO NPs and Ag/ZnO NCs were estimated as a measure of their degradation capacity of industrial dyes as well as antibiotics, and disinfection ability against the MDR strains of EAEC, NTS, and MRSA.

Initially, the photocatalytic dye degradation potential of the three NPs was tested against three cationic dyes- methylene blue (MB), crystal violet (CV), and rhodamine-B (RhB) on exposure to three different light sources (sunlight, LED light and UV light). The results showed that Ag/ZnO NCs could effectively degrade the dyes as compared to AgNPs and ZnO NPs regardless of tested dyes and light sources. However, the degradation of RhB was quite slower than MB and CV irrespective of different light sources and NPs. The sunlight as well as UV light exhibited better photodegradation capacity than LED light. Notwithstanding the light sources and NPs, RhB followed zero-order kinetics except in the cases treated using Ag/ZnO NCs; however, MB and CV followed primarily second-order kinetics. The selectivity of the tested dyes towards degradation was evaluated using Ag/ZnO NCs on exposure to sunlight. The results showed a non-significant ($P > 0.05$) degradation capacity of Ag/ZnO NCs for CV and RhB when used individually or in combination, while a significant ($P < 0.01$) degradation capacity was exhibited by MB on treating with Ag/ZnO NCs when used individually or in combination. Further, the reusability assay of the Ag/ZnO NCs revealed diverse dye degradation

patterns of reused as well as fresh Ag/ZnO NCs for the degradation of MB, CV, and RhB.

The photocatalytic degradation of ampicillin, ciprofloxacin and tetracycline when treated with AgNPs, ZnO NPs, and Ag/ZnO NCs was estimated on exposure to sunlight. The results revealed elevated absorbance peaks corresponding to the degradation products as time increases; further, the absorbance reaches a maximum degradation and remains unchanged thereafter. In this study, the results showed a significant antibiotic degradation potential of AgNPs, ZnO NPs and Ag/ZnO NCs against ampicillin, ciprofloxacin, and tetracycline when exposed to sunlight. The maximum degradation achieved was for ampicillin and ciprofloxacin when treated with Ag/ZnO NCs, while tetracycline was relatively resistant to degradation using AgNPs and Ag/ZnO NCs.

The photocatalytic time-kill kinetic assay of MDR-EAEC, NTS and MRSA strains was determined by treating the individual bacterial strains with different concentrations of NPs (1X MIC, 1/2X MIC, 1/5X MIC, and 1/10X MIC) on exposure to LED light. The results of photocatalytic time-kill kinetics depicted that the MIC dose of tested NPs required comparatively lower time to eliminate MDR-test strains of bacteria when compared to the non-photocatalytic killing kinetics with the same NPs under study. The study revealed bacterial killing by the NPs under study with even 1/10X MIC concentrations of the NPs. However, the time taken to effect killing was even higher than with 1X MIC concentrations. Further, innovative strategies need to be developed for their future applications in slurry waste management and the incorporation of biodegradable plastics for their applications in food packaging.

7. References

7. REFERENCES

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ANNEXURE

COMPOSITION OF REAGENTS

Buffered Peptone Water	g/L
Casein enzyme hydrosylate	10.00
Sodium Chloride	5.00
Disodium hydrogen phosphate	9.00
Monopotassium hydrogen phosphate	1.50
Final pH (at 25°C)	7.0±0.2

Mueller Hinton Agar	g/L
Beef infusion	300.00
Caesin acid hydrosylate	17.5
Starch	1.5
Agar	7.0
Final pH (at 25°C)	7.3±0.2

Nutrient Broth	g/L
Peptic digest of animal tissue	5.0
Sodium Chloride	5.0
Beef extract	1.5
Yeast extract	1.5
Final pH (at 25°C)	7.4±0.2

Normal Saline Solution	g/L
Sodium chloride	8.5g
Distilled water	1000ml

EMB agar	g/L
Peptone digest of animal tissue	10.00
Dipotassium phosphate	2.00
Lactose	5.00
Sucrose	5.00
Eosin-Y	0.40
Methylene blue	0.065
Agar	13.50

XLD Agar	g/L
Yeast extract	3.00
L-Lysine	5.00
Xylose	3.75
Lactose	7.50
Sucrose	7.50
Sodium deoxycholate	2.50
Ferric ammonium citrate	0.080
Sodium thiosulphate	6.8
Sodium chloride	5.00
Phenol red	0.080
Agar	15.00
Final pH (at 25°C)	7.4±0.2

**EVALUATION OF ANTIBIOFILM AND PHOTOCATALYTIC
POTENTIAL OF GREEN SYNTHESISED NANOPARTICLES
AGAINST MULTI-DRUG- RESISTANT BACTERIAL
PATHOGENS OF PUBLIC HEALTH IMPORTANCE**

**BIBIN MOHAN
(21-MVP-12)**

ABSTRACT

**Submitted in partial fulfilment of the requirement for the degree of
MASTER OF VETERINARY SCIENCE
(Veterinary Public Health)
2023**

**Faculty of Veterinary and Animal Sciences
Kerala Veterinary and Animal Sciences University**



**DEPARTMENT OF VETERINARY PUBLIC HEALTH
COLLEGE OF VETERINARY AND ANIMAL SCIENCES
POOKODE, WAYANAD – 673 576
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ABSTRACT

Water treatment is considered to be an important public health concern owing to the presence of drug-resistant bacterial pathogens as well as toxic industrial wastes. Nanotechnology possesses unique applications in the area of wastewater treatment. The present study has hence been envisaged to evaluate the antioxidant, and photocatalytic potential of green synthesised silver (Ag) nanoparticles (NPs), zinc oxide (ZnO) NPs and Ag/ZnO nanocomposites (NCs) in the dye removal and antibiotic degradation. Further, the anti-fouling as well as photocatalytic killing kinetics of these nanomaterials were assessed against multi-drug resistant (MDR) strains of enteroaggregative *E. coli* (EAEC), non-typhoidal *Salmonella* spp. (NTS), and methicillin-resistant *S. aureus* (MRSA). The synthesised NPs were re-validated using UV-Vis spectroscopy for their characteristic absorption spectra (AgNPs: 430 nm, ZnO NPs: 340 nm and Ag/ZnO NCs: 340 and 430 nm), while the antimicrobial efficacy was determined (AgNPs: 7.80-15.60 µg/ml, ZnO NPs: 62.50-125.0 µg/ml and Ag/ZnO NCs: 31.25-62.50 µg/ml) by micro-broth dilution technique.

The ABTS^{•+} and DPPH assay-based antioxidant analysis revealed a dose-dependent increase in the antioxidant potential of all three tested NPs, keeping ascorbic acid as the standard. The photocatalytic dye degradation potential of the three NPs was tested against three cationic dyes- methylene blue (MB), crystal violet (CV), and rhodamine-B (RhB) on exposure to three different light sources (sunlight, LED light and UV light). It was observed that the Ag/ZnO NCs could degrade the dyes more effectively than AgNPs and ZnO NPs regardless of tested dyes and light sources. The degradation of RhB was quite slower than MB and CV irrespective of different light sources and NPs. The sunlight as well as UV light exhibited better photodegradation capacity than LED light. Notwithstanding the light sources and NPs, RhB followed zero-order kinetics; however, MB and CV followed primarily second-order kinetics. The antibiotic degradation ability of the NPs was investigated by treating three different antibiotics (ampicillin, ciprofloxacin and tetracycline) on exposure to sunlight. Irrespective of the NPs, a

fairly good degradation ability was exhibited for ampicillin (71.22 per cent) and ciprofloxacin (84.83 per cent) which reached a maximum at 90 min post-exposure. However, ZnO NPs exhibited better antibiotic degradation potential (74.07 per cent) than AgNPs (12 per cent) and Ag/ZnO NCs (27 per cent) on degrading tetracycline, which reached a maximum at 60 min post-exposure.

In order to determine the antifouling ability of the AgNPs, ZnO NPs and Ag/ZnO NCs, biofilm-forming MDR test bacterial strains were co-incubated with the test NPs for 24 and 48 h. A significant inhibition ($P < 0.001$) in the biofilm formation was observed in all the treated groups both at 24 and 48 h. The reduction in biofilm biomass was found to be higher at 48 h when compared to 24 h, irrespective of the tested NPs and MDR strains. In addition, AgNPs could inhibit the biofilm-forming ability of the MDR strains when compared to Ag/ZnO NCs and ZnO NPs. All the tested NPs significantly ($P < 0.001$) inhibited the biofilm biomass of MDR-MRSA isolates as compared to EAEC and NTS isolates.

An *in vitro* time- and concentration-dependent photocatalytic killing kinetic assay was performed on treating the individual MDR bacterial test strains with the AgNPs, ZnO NPs and Ag/ZnO NCs on exposure to LED light. This study revealed a complete elimination of test strains at 1X MIC levels when treated with ZnO NPs at 240 min. Nonetheless, AgNPs exhibited a diverse photocatalytic killing kinetic pattern wherein MDR test strains when treated at 1X and 1/2X MIC levels revealed complete elimination at 30 min. While MDR-EAEC as well as NTS strains on co-incubation with sub-MIC levels (1/5X and 1/10X) could not exhibit complete bacterial elimination, MDR-MRSA test strains revealed complete elimination at 30 to 180 min and 120 min, respectively. On co-incubation with Ag/ZnO NCs (1X and 1/2X MIC), MDR-EAEC exhibited complete bacterial elimination at 30 to 120 min; however, bacterial counts could not be detected at 120 min for MDR-NTS and MRSA test strains. Moreover, at sub-MIC levels (1/5X and 1/10X), an increased clearance time (180-240 min) was reported for all the test strains exposed to LED light. In short, a reduction in the time as well as dose required for the bacterial

killing was observed irrespective of the NPs on exposure to LED light when compared to non-photocatalytic *in vitro* killing kinetic assay.

Overall, this study demonstrated the photocatalytic potential of green-synthesised AgNPs, ZnO NPs and Ag/ZnO NCs with antioxidant as well as antifouling properties.

KERALA VETERINARY AND ANIMAL SCIENCES UNIVERSITY
Faculty of Veterinary and Animal Sciences
PROGRAMME OF RESEARCH WORK FOR THESIS FOR MASTERS DEGREE

1. Title of thesis

Evaluation of antibiofilm and photocatalytic potential of green synthesised nanoparticles against multi-drug- resistant bacterial pathogens of public health importance

2. a) Title of the departmental / KVASU research project of which this forms a part

Exploiting encapsulated nanoparticle conjugated phytochemicals to combat antimicrobial resistance in poultry

b) Code No. if any, and order by which the departmental / KVASU research project is approved

No. KVASU/DAR/R1/35474/2019 dtd. 03/01/2020 of DAR, KVASU

3. a) Name of student

Bibin Mohan

b) Admission No.

21-MVP-12

c) Name of the discipline

Veterinary Public Health and Epidemiology

4. a) Name of Major Advisor

Dr. Jess Vergis

b) Address

Assistant Professor,
Department of Veterinary Public Health,
College of Veterinary and Animal Sciences,
Pookode, Wayanad- 673 576

5. Objectives of the study

1. Assessment of antibiofilm activity of green synthesised Silver (Ag), Zinc oxide (ZnO) and Ag-ZnO nanoparticles (NPs) against multi-drug-resistant (MDR) strains of non-typhoidal *Salmonella* spp. (NTS), enteroaggregative *Escherichia coli* (EAEC) and methicillin-resistant *Staphylococcus aureus* (MRSA)
2. Evaluation of the photocatalytic potential of green synthesised Ag NPs, ZnO NPs, and Ag-ZnO NPs

6. Practical/Scientific utility

Antimicrobial resistance (AMR) is emerging as a global public health threat and selection pressure is considered to be an important factor for the emergence. In order to palliate the public health menace of AMR, recent research has now been targeted towards alternate therapeutic strategies. In the recent past, NP-based therapeutics targeting MDR pathogens have gained considerable attention. The NPs exhibit potential antimicrobial, antibiofilm and antioxidant properties. These activities of NPs are primarily ascribed to the production of reactive oxygen species, cation release, biomolecule damage, energy depletion, and membrane interaction.

The global population is facing unprecedented environmental pollution due to antibiotics, industrial wastes, as well as wastes from various settings contaminated with microbes. The demand for mitigating pollution in developing countries is increasing day by day. The green synthesis could culminate in the development of safe, eco-friendly NPs and would have wide acceptance in nanotechnology. Moreover, the high surface-to-volume ratio of NPs controls the interaction with microbes and pollutants, especially during accelerated photoreaction. This photocatalytic property of NPs can be applied in various public health domains, including the removal of industrial pollutants and antibiotics, and also for wastewater treatment. Hence, the present study is envisaged to investigate the antibiofilm efficacy of green synthesised Ag NPs, ZnO NPs, and Ag-ZnO NPs against the MDR strains of NTS, EAEC, and MRSA and further evaluate the photocatalytic potential of green synthesised NPs.

7. Important publications on which the study is based

Paletta *et al.* (2020) reported the biofilm- forming ability of EAEC on abiotic surfaces and documented aggregate adhesion (AA) to human larynx epithelioma cancer (HEp-2) cell lines.

Panchal *et al.* (2020) performed green synthesis of ZnO NPs and Ag-ZnO NPs and were found to inhibit the growth of pathogenic *E. coli* and degraded methylene blue.

Shabaani *et al.* (2020) stated that the green synthesised ZnO NPs depicted potential bactericidal activity on *S. aureus* in a dose- dependent manner, and photodegraded methylene blue dye in an effective manner.

Abbas *et al.* (2021) documented that the green synthesised Ag-ZnO NPs exhibited photocatalytic degradation potential of methylene orange dye with a remarkable efficiency of 90 per cent.

The photocatalytic removal of ciprofloxacin was achieved through the green synthesised magnesium oxide doped with sulphur and nitrogen (Akbari *et al.*, 2021).

de Lacerda Coriolano *et al.* (2021) observed potential antibacterial and antibiofilm activity of green synthesised Ag NPs against antibiotic-sensitive and MDR *Pseudomonas aeruginosa*.

The ZnO NPs exhibited photocatalytic degradation of dyes (malachite green, methylene blue, and rhodamine-B) and removal of tetracycline (Li *et al.*, 2021).

The green synthesised ZnO NPs were found to inhibit the growth of pathogenic *E. coli*, *Salmonella* spp., and *S. aureus* by the generation of reactive oxygen species resulting in membrane damage and cellular material leakage, which ultimately led to cell death (Yusof *et al.*, 2021).

Abishad *et al.* (2022) stated that Ag NPs synthesised using *Lactobacillus acidophilus*, a potential probiotic strain, exhibited antibacterial activity against MDR-EAEC strains.

Unni *et al.* (2022) reported the green synthesis of ZnO NPs using the aqueous extract of *Piper longum* catkin and were found to exhibit antibacterial activity against MDR-NTS strains.

8. Outline of the technical programme

Three MDR isolates each of NTS (*S. Typhimurium* and *S. Enteritidis*), EAEC, and MRSA maintained in the laboratory repository of the Department of Veterinary Public Health, College of Veterinary and Animal Sciences, Pookode, will be used for the study. They will be revived and confirmed using polymerase chain reaction (PCR) assays (Nair *et al.*, 2015; Vijay *et al.*, 2015; González-Domínguez *et al.*, 2020) and antibiotic susceptibility testing (CLSI, 2019).

The characterised Ag NPs, ZnO NPs, and Ag-ZnO NPs in the laboratory repository will be freshly synthesised (Abishad *et al.*, 2022; Unni *et al.*, 2022) and employed for this study. The minimum inhibitory concentration (MIC) as well as minimum bactericidal concentration (MBC) of the green synthesised NPs will be re-validated against MDR strains of NTS, EAEC, and MRSA isolates (CLSI, 2019).

Initially, the *in vitro* time-dependent biofilm-forming ability of the MDR strains of NTS, EAEC as well as MRSA will be evaluated at 24 h and 48 h using a 96-well microtitre plate assay. Further, the antibiofilm potential of Ag NPs, ZnO NPs, and Ag-ZnO NPs will be assessed against the MDR strains of NTS, EAEC, and MRSA isolates (Vergis *et al.*, 2019).

The photocatalytic dye degradation activity of green synthesised Ag NPs, ZnO NPs, and Ag-ZnO NPs will be investigated using rhodamine-B, crystal violet, and methylene blue dyes (Rahman *et al.*, 2013).

In vitro photocatalytic time-kill kinetic assay of MDR strains of NTS, EAEC, and MRSA isolates treated with green synthesised Ag NPs, ZnO NPs, and Ag-ZnO NPs will be evaluated (Guan *et al.*, 2019).

The photocatalytic degradation efficiency of green synthesised Ag NPs, ZnO NPs, and Ag-ZnO NPs for the removal of antibiotics (ampicillin, ciprofloxacin, tetracycline) will be assessed (Baaloudj *et al.*, 2021).

In order to determine the *in vitro* antioxidant activity of green synthesised Ag NPs, ZnO NPs, and Ag-ZnO NPs, 2,2- diphenyl-1- picrylhydrazyl (DPPH) and 2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS•+)-based free radical scavenging assays (Dalmolin *et al.*, 2016; Zare *et al.*, 2019) will be performed.

The combined photocatalytic dye degradation, antibiotic removal, and time-kill kinetic assay against MDR strains of NTS, EAEC, and MRSA will all be further assessed for the three tested NPs.

The results obtained will be analysed using relevant statistical tools (SPSS version 24.0).

9. Main items of observations to be made

1. MIC and MBC values (mg/L) of green synthesised NPs
2. Biofilm-forming ability (absorbance) of MDR isolates
3. *In vitro* antibiofilm potential (per cent) of NPs against MDR isolates
4. *In vitro* photocatalytic dye degradation (per cent) of NPs
5. *In vitro* photocatalytic time-dependent log reduction in MDR isolates treated with NPs
6. *In vitro* photocatalytic antibiotic degradation efficiency (per cent) of NPs
7. *In vitro* DPPH and ABTS•+-based antioxidant activity (per cent) of NPs
8. *In vitro* combined photocatalytic dye degradation (per cent), antibiotic degradation efficiency (per cent), and time-dependent log reduction of all the NPs

10. Facilities

a) Existing:

Work will be conducted with the existing facilities available in the Departments of Veterinary Public Health, Veterinary Pharmacology and Toxicology, other departments and Central Instrumentation Facility of the College of Veterinary and Animal Sciences, Pookode.

b) Additional facilities required:

Chemicals and biologicals

11. Duration of study

Four semesters

12. Financial estimate

Chemicals and biologicals : Rs. 20000

Contingencies : Rs. 5000

Total : Rs. 25000

Sd/-

Signature of student

Project coordination group proposed

Animal Biotechnology- I

Sd/-

Signature of Major Advisor

Pookode

18.11.2022

Name, address and signature of other members of the Advisory Committee:

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14. Time frame of work

Semester I

1. Collection of literature
2. Preparation of research proposal
3. Revival and confirmation of MDR isolates

Semester II

1. Maintenance of MDR isolates
2. PCR-based re-validation of MDR isolates
3. Biofilm forming ability of MDR isolates
4. *In vitro* antibiofilm assays of synthesised NPs
5. *In vitro* antioxidant activity of synthesised NPs

Semester III

1. Maintenance of MDR isolates
2. *In vitro* photocatalytic dye degradation activity of synthesized NPs

3. *In vitro* photocatalytic time-kill kinetic assay of Ag, ZnO, Ag-ZnO NPs
4. *In vitro* photocatalytic antibiotic removal of Ag, ZnO, Ag-ZnO NPs
5. *In vitro* combined photocatalytic activity (dye degradation, time-kill kinetic assay and antibiotic removal) of Ag, ZnO, Ag-ZnO NPs

Semester IV

1. Analysis of data and interpretation of results
2. Preparation and submission of thesis

CERTIFICATE

Certified that the research project has been formulated observing the stipulations laid down under the Prevention of Cruelty to animals Act (Amendment, 1998).

Pookode
18.11.2022

Sd/-
Dr. Jess Vergis
Major Advisor

Curriculum vitae

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PGDAW	Indira Gandhi National Open University, New Delhi	School of Extension and Development Studies (SOEDS)	8.92	2023

Membership in professional bodies: Kerala State Veterinary Council (KSVC)