

ANALYSIS OF EFFICACY OF AG-CU NANOPARTICLE CONJUGATED THYMOQUINONE AGAINST *E. COLI* BIOFILM PROJECT WORK

Submitted in partial fulfillment of the requirements for the award of
Bachelor of Science Degree in Biotechnology.

By

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**SCHOOL OF BIO AND CHEMICAL ENGINEERING
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SATHYABAMA

**INSTITUTE OF SCIENCE AND TECHNOLOGY
(DEEMED TO BE UNIVERSITY)**

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BONAFIDE CERTIFICATE

This is to certify that this Project Report is the bonafide work of **PAVITHRA J (41770019)** who carried out the project entitled "**ANALYSIS OF EFFICACY OF AG-CU NANOPARTICLE CONJUGATED THYMOQUINONE AGAINST *E. coli* BIOFILM**" under our supervision from January 2024 to April 2024.

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ABSTRACT

This investigation evaluates the antibacterial potential of thymoquinone and Ag-Cu nanoparticles against *Escherichia coli* (*E. coli*) bacterial growth, both individually and in combination. Through swimming, swarming, biofilm assay and 96 well plate assays the study demonstrates that thymoquinone and Ag-Cu nanoparticles effectively inhibit *E. coli* growth, with Ag-Cu nanoparticles exhibiting stronger inhibitory effects compared to thymoquinone alone. Crystal violet staining in the biofilm assay illustrates significant reductions in bacterial growth in the presence of these agents. The study concludes that the conjugation of nanoparticles with thymoquinone enhances antimicrobial activity, highlighting the potential of these compounds as promising antibacterial agents. Further research is warranted to elucidate the mechanisms underlying their antibacterial activity and explore their application in combating bacterial infections.

Keywords: Antibacterial, Nanoparticles, Thymoquinone, Ag-Cu, *E.coli*, Biofilm

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LIST OF ABBREVIATION

MRSA	Methicillin-resistant <i>Staphylococcus aureus</i>
TQ	Thymoquinone
ROS	Reactive oxygen species
IC90	Inhibitory concentration 90%
PFTs	Pulmonary function tests
FEV1	Forced expiratory volume in one second
PEF	Peak expiratory flow
MMEF	Maximal Mid Expiratory Flow
FVC	forced vital capacity
MEF75	Maximum expiratory flow 75
MEF50	Maximum expiratory flow 50
MEF25	Maximum expiratory flow 25
PFT	Pulmonary functional test
SGew	Specific airway conductance
Tb –NPs	Biological Nano Thymoquinone
Tc- Nps	chemical Nano Thymoquinone
FESEM	Field Emission Scanning Electron
FTIR	Fourier-transform infrared spectroscopy
ATP	Adenosine triphosphate
QSI	Quorum Sensing Inhibition

THQ	Thymohydroquinone
MIC	Minimum Inhibitory Concentration
MBC	Minimum bactericidal concentration
NPO	Nitropyridine-N-oxide
Eos	Essential oils
QSI	Quorum sensing inhibition
PAE	Post-antibiotic effects
MHB	Mueller–Hinton Broth
ZnO-NPs	Zinc Oxide Nanoparticles
Ag	Silver
Cu	Copper
OD	Optical density

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CHAPTER- 1

INTRODUCTION

Worldwide, *Nigella sativa* (*N.sativa*) (Family Ranunculaceae) is a commonly used medicinal plant. It is widely used in many traditional medical systems, including Ayurveda, Siddha, Unani, and Tibb. Folklore has long used seeds and oil in a variety of culinary and medicinal systems. *N.sativa* seeds have been widely utilized to cure a variety of illnesses and conditions. It is regarded as one of the best types of therapeutic medicine in Islamic literature. It has been extensively utilized for many purposes such as liver tonic, diuretics, hunger stimulant, analgesics, antibacterial, digestion, anti-diarrhea, and skin problems. Numerous researchers have conducted extensive studies on *N.sativa*, exploring a wide range of its pharmacological actions, which may include hepato-protective, renal-protective, analgesic, antimicrobial, anti-inflammatory, spasmolytic, bronchodilator, antioxidant, anti-diabetic, and immunomodulation qualities. *N.sativa* is one of the best-ranked evidence-based herbal remedies because of its therapeutic miracles. It also reveals that thymoquinone, a significant bioactive component of the essential oil, is responsible for the majority of the plant's medicinal qualities (Ahmad *et al.*, 2013). However, the antibacterial potential of pure thymoquinone, one of its major ingredients and its mechanism of action is still intriguing.

A biofilm is a well-organized group of bacteria that are encased in a self-made polymer matrix made of DNA, protein, and polysaccharides. Because bacterial biofilms resist phagocytosis and other elements of the body's defense mechanism, they exhibit heightened resistance to antibiotics and disinfection chemicals, which contributes to chronic infections (Wingender *et al.*, 2001) (Keren *et al.*, 2004). Biofilm production, for instance, is responsible for the persistence of staphylococcal infections linked to external substances. Likewise, mucoid strains that develop biofilms are the source of persistent lung infections in people with cystic fibrosis (Equi *et al.*, 2002). Characteristically, gradients of nutrients and oxygen exist from the top to the bottom of biofilms and these gradients are associated with decreased bacterial metabolic activity and increased doubling times of the bacterial cell

these more or less dormant cells that are responsible for some of the tolerance to antibiotics (Hansen *et al.*, 2005). Biofilm growth is associated with an increased level of mutations as well as with quorum-sensing-regulated mechanisms (Tomlin *et al.*, 2005). Conventional resistance mechanisms such as chromosomal β -lactamase, upregulated efflux pumps and mutations in antibiotic target molecules in bacteria also contribute to the survival of biofilms. Biofilms can be prevented by early aggressive antibiotic prophylaxis or therapy and they can be treated by chronic suppressive therapy. A promising strategy may be the use of enzymes that can dissolve the biofilm matrix (e.g. DNase and alginate lyase) as well as quorum-sensing inhibitors that increase biofilm susceptibility to antibiotics (Hoiby *et al.*, 2010).

The escalating threat of antibiotic resistance and the persistence of biofilm-associated infections have spurred intense research into alternative therapeutic agents derived from natural sources. Among these, *Nigella sativa* (Goel and Mishra, 2018), commonly known as black seed, has attracted considerable attention for its rich medicinal properties and historical use in traditional medicine systems worldwide. Extracts from *N.sativa* seeds and its bioactive component, thymoquinone, have demonstrated a wide range of pharmacological actions, including antibacterial, antifungal, anti-inflammatory, and antioxidant activities. Notably, thymoquinone has emerged as a promising candidate due to its ability to inhibit biofilm formation and exert selective antibacterial activity through the generation of reactive oxygen species (ROS). Extensive research has elucidated the mechanisms underlying the antibacterial and antibiofilm properties of thymoquinone, providing valuable insights into its therapeutic potential against various bacterial pathogens, including *Escherichia coli*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa*. Moreover, recent studies have explored the synergistic effects of thymoquinone with conventional antibiotics (Goel and Mishra, 2018), highlighting its role in combating antibiotic-resistant bacteria. This review aims to provide a comprehensive overview of the literature on *N.sativa* and thymoquinone, encompassing their pharmacological activities, mechanisms of action, and potential applications in the treatment of bacterial infections and biofilm-associated diseases. By synthesizing and critically analyzing existing research findings, this review seeks to contribute to our

understanding of the therapeutic potential of *N.sativa* and thymoquinone (Goel and Mishra, 2018).

While *Nigella sativa* and its bioactive component, thymoquinone, have demonstrated promising antibacterial and antibiofilm properties, there remains a need for further investigation into their potential therapeutic applications. (Ahmad *et al.*, 2013) provided a comprehensive review of the therapeutic potential of *N.sativa*, highlighting its diverse pharmacological actions and historical significance in traditional medicine. However, the antibacterial potential of pure thymoquinone and its mechanism of action remain intriguing areas of study.

Biofilm-associated infections pose a significant challenge in healthcare settings due to their resistance to conventional antibiotics and disinfection methods (Keren *et al.*, 2004; Wingender *et al.*, 2001). The persistence of biofilm-related infections, such as those caused by *Staphylococcus aureus* and *Pseudomonas aeruginosa*, underscores the urgent need for alternative therapeutic agents (Equi *et al.*, 2002). Biofilm growth is associated with metabolic changes within bacterial cells and mechanisms such as quorum sensing, contributing to antibiotic resistance (Hansen *et al.*, 2005; Tomlin *et al.*, 2005).

Extracts from *N.sativa* seeds and thymoquinone have shown promising antibacterial, antifungal, anti-inflammatory, and antioxidant activities. Thymoquinone, in particular, has emerged as a potential candidate for inhibiting biofilm formation and exerting selective antibacterial activity through the generation of reactive oxygen species (ROS) (Goel and Mishra *et al.*, 2018).

Further studies have investigated the antimicrobial efficacy of *Nigella sativa* and its bioactive component, thymoquinone, against specific bacterial strains, including methicillin-resistant *Staphylococcus aureus* (MRSA). (Hariharan *et al.*, 2016) conducted *in vitro* profiling of TQ extracted from *N.sativa* against MRSA strains, revealing promising antibacterial activity with a minimum inhibitory concentration (MIC) range of 8–16 µg/ml and a prolonged post antibiotic effect (PAE). Importantly, TQ demonstrated bactericidal activity without inducing the selection of resistant mutants, highlighting its potential as a therapeutic agent against MRSA infections.

The challenges posed by bacterial biofilms, particularly in clinical settings, have prompted extensive research into alternative therapeutic strategies. Biofilm-associated infections, notoriously difficult to treat due to their increased tolerance to antibiotics, necessitate novel approaches for effective management (Hoiby *et al.*, 2010). Resistance mechanisms within biofilms, including decreased metabolic activity and up regulated efflux pumps, underscore the need for innovative interventions targeting biofilm disruption.

Different gene expression patterns distinguish the well-studied planktonic cells from the biofilm-forming cells. Recent genomic techniques like DNA microarray analysis have revealed the identities of several novel *Escherichia coli* genes involved in biofilm development. Nonetheless, a great number of other participants in this process may have gone undetected because of inadequate expression, regulatory mechanisms, or genetic backgrounds (Niba *et al.*, 2007). By using DNA microarray technology to assess the global gene expression profile of *Escherichia coli* growing in a sessile vs a planktonic state. In the adherent population, genes encoding adhesion-related proteins (type 1 fimbriae) and, specifically, autoaggregation (Antigen 43) were strongly expressed in a way that aligns with existing models of sessile community establishment (Schembri *et al.*, 2003).

In the context of combating bacterial infections, studies such as that by (Hosseinzadeh *et al.*, 2007) have evaluated the antibacterial activity of total crude extracts and essential oil of *Nigella sativa* seeds against gram-positive and gram-negative organisms in animal models. The results demonstrated dose-dependent antibacterial activities, further supporting the therapeutic potential of *N.sativa* in combating bacterial infections.

As the threat of antibiotic resistance continues to escalate, there is a pressing need for alternative therapeutic agents derived from natural sources. *Nigella sativa* and thymoquinone have emerged as promising candidates, exhibiting potent antibacterial activity against a wide range of bacterial strains, including multidrug-resistant pathogens like MRSA. Critically analyze existing research findings on the antimicrobial properties of *N.sativa* and thymoquinone, providing insights into their

mechanisms of action and potential applications in combating bacterial infections, including those associated with biofilm formation. By addressing the gaps in our understanding and exploring avenues for future research, this review seeks to contribute to the development of novel therapeutic strategies for combating antibiotic-resistant bacteria and biofilm-associated infections.

In addition to exploring the antimicrobial properties of natural compounds like thymoquinone (TQ) from *Nigella sativa*, research has extended to innovative approaches such as nanostructured coatings for medical implants and elucidating the anticancer mechanisms of antioxidants.

Zhao *et al.*, 2011 investigated the antibacterial potential of titanium implants coated with titania nanotubes (TiO₂-NTs) incorporated with silver nanoparticles (NT-Ag). Their findings revealed potent antibacterial activity against planktonic bacteria and prolonged prevention of bacterial adhesion for up to 30 days. This innovative coating holds promise for preventing implant-associated infections, addressing a significant concern in medical implantology.

Meanwhile, Zubair *et al.*, 2013 delved into the putative anticancer mechanism of antioxidants, focusing on the redox cycling of endogenous copper by thymoquinone. While TQ is recognized for its antioxidant properties at lower concentrations, recent studies have suggested its prooxidant effects at higher concentrations, particularly in the presence of transition metal ions like copper. Elevated copper levels are commonly observed in various malignancies, and TQ has been shown to induce oxidative DNA breakage in cellular DNA, mediated by reactive oxygen species (ROS). This prooxidant effect, attributed to the redox cycling of endogenous copper, provides a potential mechanism for the anticancer activity of plant-derived antioxidants like TQ.

These studies underscore the multifaceted nature of natural compounds and their applications in combating microbial infections and cancer. Nanostructured coatings offer innovative solutions for preventing implant-associated infections, while the elucidation of antioxidant mechanisms provides insights into their therapeutic potential in cancer treatment. By synthesizing and critically analyzing findings from

diverse research areas, this review aims to provide a comprehensive understanding of the therapeutic potential of natural compounds and innovative approaches in addressing contemporary healthcare challenges.

Many bacterial species form organized clusters called biofilms, which are the cause of most persistent and recurring illnesses. Infections linked to biofilms recur in 65–80% of cases. Antibiotic resistance is very strong in the bacteria connected to the biofilm. Due to the paucity of knowledge regarding biofilm development, spread, and resistance, a multidisciplinary approach is used to comprehend the science and create solutions for this issue (Venkatesan *et al.*, 2015). Novel therapeutic approaches that go beyond conventional approaches are required to combat bacterial infections that are resistant to antibiotics due to acquired resistance, biofilm formation, or both. With the ability to circumvent current processes linked to acquired drug resistance, nanomaterial-based therapeutics hold great promise in the fight against incurable bacterial diseases (Makabenta *et al.*, 2021).

CHAPTER-2

REVIEW OF LITERATURE

2.1 Thymoquinone inhibits biofilm formation and has selective antibacterial activity due to ROS generation.

Thymoquinone, a compound found in *Nigella sativa*, has garnered attention for its ability to inhibit biofilm formation and exhibit selective antibacterial activity, primarily attributed to its generation of reactive oxygen species (ROS). Studies have shown that thymoquinone effectively inhibits the growth of both Gram-positive and Gram-negative bacteria, with minimal inhibitory concentrations ranging between 1.56 and 100 µg/ml. Notably, scanning electron microscopy revealed significant alterations in bacterial cell morphology following treatment with thymoquinone, including dents, cell lysis, and reduction in cell size. Further validation of thymoquinone's bactericidal action was confirmed through live/dead imaging techniques, where treated bacteria exhibited a preferential uptake of ethidium bromide over acridine orange. Moreover, investigations using the HaCaT cell line demonstrated that thymoquinone exhibited an inhibitory concentration at 90% (IC90) value of 50 µg/ml, indicating its selectivity against bacterial cells compared to normal human cells. Thymoquinone's efficacy against biofilm formation was also demonstrated through crystal violet assays, colony-forming unit techniques, and scanning electron microscopy, showing promising antibiofilm activity against various bacterial strains, including *Bacillus subtilis*, *Staphylococcus aureus*, *Escherichia coli*, and *Pseudomonas aeruginosa*. To elucidate its mechanism of action, assays involving dichlorofluorescein fluorescence, DiSC3 and ROS were conducted. Interestingly, while bacterial cells treated with thymoquinone at MIC levels showed increased ROS production, no significant membrane damage was observed. This suggests that thymoquinone's antibacterial action may be primarily attributed to the generation of reactive oxygen species within bacterial cells, highlighting its potential as a therapeutic agent against bacterial infections and biofilm-associated diseases (Goel and Mishra, 2018).

2.2 A review on therapeutic potential of *Nigella sativa*: A miracle herb

Nigella sativa, commonly known as black seed, holds a prominent place in traditional medicine systems worldwide, including Ayurveda, Siddha, Unani, and Tibb. Both its seeds and oil have been extensively utilized for various culinary and medicinal purposes, with a rich history of use in treating a wide array of illnesses and conditions. Renowned in Islamic literature as a form of prophetic medicine, *N.sativa* has been recommended for regular use due to its myriad therapeutic benefits. Research on *N.sativa* has revealed its pharmacological actions, which include hepato-protective, renal-protective, analgesic, antimicrobial, anti-inflammatory, antioxidant, antidiabetic, and immunomodulatory properties, among others. Thymoquinone, identified as a significant bioactive component of *N.sativa*, has been implicated in many of these medicinal qualities, further substantiating its therapeutic potential (Ahmad *et al.*, 2013).

2.3 Fungicidal effect of thymoquinone involves generation of oxidative stress in *Candida glabrata*

Thymoquinone, a potent compound found in black seed essential oil, has demonstrated remarkable antifungal properties against various fungal species, including *Candida glabrata*. Despite limited understanding of its antifungal mode of action, recent studies have shed light on thymoquinone's fungicidal effects, particularly against *C. glabrata* planktonic cells and biofilms. Investigations have revealed that thymoquinone exerts its fungicidal action by acting as a pro-oxidant, inducing oxidative stress within fungal cells. This oxidative stress-mediated cell death was confirmed through measurements of reactive oxygen species production, decline in glutathione levels, and disruption of mitochondrial membrane potential. Importantly, thymoquinone-induced oxidative stress was identified as the primary cause of fungal cell death, underscoring its potential as a novel therapeutic agent against *Candida* infections (Almshawit and Macreadie, 2017).

2.4 Antiasthmatic effect of *Nigella sativa* in airways of asthmatic patients

On 15 asthmatic patients, the bronchodilatory effects of 50 and 100 mg/kg of boiled extract were compared with 6 mg/kg of theophylline. Pulmonary function tests (PFTs) were performed prior to and 30, 60, 90, 120, 150, and 180 minutes after the oral extract and theophylline were administered. These tests included forced expiratory volume in one second (FEV1), peak expiratory flow (PEF), maximal mid expiratory flow (MMEF), maximal expiratory flow at 75, 50, and 25% of the FVC (MEF75, MEF50, and MEF25, respectively), and specific airway conductance (sGaw). The findings demonstrated that, in the majority of time intervals, the extract significantly increased all assessed pulmonary function tests (PFTs) ($p < 0.05$ to $p < 0.001$). On the other hand, the increases in MEF75 and MEF25 as a result of its lower doses and the increases in FEV1, MMEF, and MEF50 as a result of both boiling extract doses were substantially less than those of theophylline ($p < 0.05$ to $p < 0.001$). After 150 minutes of treatment, the extract's bronchodilatory action began to wane in a manner comparable to that of theophylline, with an onset time of 30 minutes. At 30 minutes after delivery, the effect of both extract dosages was likewise considerably lower than that of salbutamol ($p < 0.001$ in all cases) (Boskabady *et al.*, 2010).

2.5 Experimental Trials for Reducing Biofilm-Producing *Escherichia Coli* Using *Nigella sativa* and Olive Oils' Nanoemulsions.

The creation of biofilms is thought to be an adaptive mechanism that promotes the development and proliferation of microbes. Furthermore investigated were the reported *E. coli* serotypes' capacities to form biofilm. The utilization of olive oil and *Nigella sativa* nanoemulsions to lessen the production of *E. Coli* biofilms was investigated in a reduction trial. The results showed that the prevalence rates of *E. coli* were 19.05%, 4.76%, 0%, 45.24%, and 30.96%, respectively, in the dairy plant floors, equipment, ice cream, and pasteurized milk that were analyzed. The current analysis found five different *E. Col* serotypes at varying rates: *E. Col*. O26:H11, *E. Col*. O55:H7, *E. Col*. O78:H-, *E. Col*. O111:H4, and *E. Col*. O127:H6. All of the

recovered *E. Coli* serotypes carried biofilm-coding genes, including fim H and *E. coli*-specific 16S rRNA, according to molecular identification (Aal *et al.*, 2021).

2.6 Removal of microbial pathogens and anticancer activity of synthesized nano-thymoquinone from *Nigella sativa* seeds

The objective of this study was to synthesize, describe, and compare the antibacterial and anticancer activities of nano thymoquinones that were generated chemically (Tc-NPs) and biologically (Tb-NPs). Wet milling was the basis for the chemical production of nano-thymoquinone (nTQ), whereas *N.sativa* seed extract was used in the biological procedure. The synthesized nTQ was subjected to examination using FESEM, FTIR, and UV. Agar well diffusion was used to assess the antibacterial activities of Tc-NPs and Tb-NPs against common antibiotic-resistant pathogens, including *E. coli*, *Pseudomonas aeruginosa*, *Enterococci faecalis*, and *Staphylococcus aureus*. The UV spectra investigation revealed that Tb-NPs and Tc-NPs both exhibited extremely strong absorption at 242 nm and 319 nm, respectively. Tb-NPs and Tc-NPs' FTIR spectra displayed distinctive peaks at five different region. Tc-NPs' homogeneously dispersed micro/nano fibers were revealed by the FESEM analysis, whereas Tb-NPs' agglomerates and spheres were scattered irregularly. With a lower dosage than Tc NPs, the cytotoxicity test demonstrated the possible anticancer action of Tb NPs. Additionally, by increasing the zone of inhibition, these nTQ particles demonstrated antibacterial action by eliminating the potential of bacterial and fungal infections. The acquired results indicated that because biosynthesized nTQ can accelerate cancer cell death and eliminate possible microbial infections, it has demonstrated greater activity. These results might favor the biosynthesis of nTQ over the chemosynthesis approach (Gnanasekaran *et al.*, 2021)

2.7 Antibacterial Activity of Thymoquinone Against *Shigella flexneri* and Its Effect on Biofilm Formation.

The study investigated the antibacterial activity of Thymoquinone (TQ) against *Shigella flexneri*, along with its potential mechanism and impact on biofilm formation. The findings revealed that TQ exhibited strong antibacterial properties against *S. flexneri*, with a minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of 0.2 mg/mL. Treatment with TQ significantly reduced the bacterial count below the detection limit within three hours in Luria-Bertani broth or 60 minutes in phosphate-buffered saline. Moreover, TQ demonstrated effectiveness against contaminated vegetable juice. Mechanistically, it induced alterations in intracellular protein, decreased adenosine triphosphate (ATP) concentration, increased intracellular reactive oxygen species (ROS), compromised cell membrane integrity, and caused morphological changes in bacterial cells. Furthermore, TQ effectively inhibited the formation of *S. flexneri* biofilm, leading to reduced biofilm and extracellular polysaccharide levels, as well as disruption of biofilm structure. These findings highlight the potential of TQ as a promising agent for controlling *S. flexneri* in fruit and vegetable processing industries, as well as other food sectors (Wang *et al.*, 2022).

2.8 Antibacterial and anti-biofilm activities of essential oils and their components including modes of action

Nowadays, there is a wealth of information available related to the biological activities of EOs and their components, which are the starting point for the development of many commercial products. The chemical characteristics and physiological properties of extracellular matrices, such as those of the extracellular polysaccharide secreted post-induction cell/surface, are dependent on the nature of the resident bacteria. Gram negative and Gram positive bacteria have revealed different auto inducers and their control could be used to regulate biofilm development. The determination of the enantiomeric composition in EOs is important

when using them as antibacterial compounds. Although monoterpene alcohols also have a hydroxyl group, they have a lower antibacterial activity than phenols. From the information obtained in the bibliography, the success of the antibacterial activity of the EOs would be based on its great molecular diversity. Hydrocarbons, ketones, aldehydes, alcohols and aromatic structures like phenols, going to a higher level of diversification for the stereochemistry of these molecules(Zygadlo *et al.*, 2017).

2.9 Inhibition of Biofilm Formation, Quorum Sensing and Infection in *Pseudomonas aeruginosa* by Natural Products-Inspired Organosulfur Compounds

The study conducted by (Cady *et al.*, 2012) investigated the efficacy of natural products-inspired organosulfur compounds in inhibiting biofilm formation, quorum sensing, and infection caused by *Pseudomonas aeruginosa*. Through a microplate-based screening assay, the effects of several S-substituted cysteine sulfoxides and their corresponding disulfide derivatives were evaluated. Notably, S-phenyl-L-cysteine sulfoxide and diphenyl disulfide demonstrated significant reductions in *P. aeruginosa* biofilm formation, comparable to the active concentration of 4-nitropyridine-N-oxide (NPO). Importantly, unlike NPO, these active compounds did not hinder planktonic cell growth but selectively targeted biofilm formation. In an infection model using *Drosophila*, both compounds exhibited significant reductions in *P. aeruginosa* recovery post-infection, without causing lethality to the host flies. Furthermore, the study explored the potential mechanism behind the observed biofilm inhibition, focusing on quorum sensing inhibition (QSI). Results indicated that S-phenyl-L-cysteine sulfoxide effectively inhibited quorum sensing in various reporter systems, whereas diphenyl disulfide showed limited QSI activity. Despite both compounds inhibiting biofilm formation, their differing QSI activities suggested potential variations in underlying pathways. The study hypothesized that biofilm inhibition by these compounds may occur through mechanisms other than QSI, warranting further investigation (Cady *et al.*, 2012).

2.10 Antibacterial activity of Thymoquinone, an active principle of *Nigella sativa* and its potency to prevent bacterial biofilm formation

Thymoquinone, a bioactive compound found in *Nigella sativa* seeds, has gained attention for its potential antibacterial properties. Investigating its efficacy, (Chaieb et al., 2011) evaluated the antibacterial activity of thymoquinone (TQ) against various pathogenic bacteria, focusing on its ability to prevent biofilm formation. Results revealed significant bactericidal activity of TQ against a range of bacteria, particularly Gram-positive cocci. Additionally, TQ demonstrated notable potency in inhibiting biofilm formation, with minimum biofilm inhibition concentrations observed for different strains. Moreover, the study highlighted the influence of TQ on cellular oxidative activity and its ability to prevent cell adhesion, suggesting its potential as a promising agent in combating biofilm-related infections. Further research is warranted to explore the full therapeutic potential of TQ in combating biofilm-associated infections (Chaieb *et al.*, 2011).

2.11 Bacterial biofilms: a common cause of persistent infections

Biofilm formation by bacteria represents a significant challenge in healthcare settings, contributing to the persistence and recurrence of infections. Through the aggregation of bacterial cells within a polymeric matrix, biofilms exhibit inherent resistance to antimicrobial agents, posing challenges for infection control. Recent advancements in understanding the genetic and molecular basis of bacterial community behavior offer promising avenues for developing targeted therapies to combat biofilm infections (Costerton *et al.*, 1999).

2.12 Plant Products as Antimicrobial Agents

The exploration of plant-derived compounds for their antimicrobial properties has garnered increasing interest in recent years. With a rich reservoir of secondary metabolites, plants offer a potential source of novel antimicrobial agents. Ethnopharmacologists, botanists, microbiologists, and natural-products chemists are

actively investigating phytochemicals for their effectiveness in treating infectious diseases. However, the use of botanical preparations necessitates careful consideration due to their unregulated nature and potential toxicity (Cowan, 1999)

2.13 Antibacterial Activity of Thymoquinone and Thymohydroquinone of *Nigella sativa* L. and Their Interaction with Some Antibiotics

Addressing the challenge of bacterial biofilm formation, described the synthesis and characterization of zinc oxide nanoparticles (ZnO-NPs) for their biocidal potential against *Pseudomonas aeruginosa*. Through in vitro assays, ZnO-NPs demonstrated significant inhibition of bacterial growth and biofilm formation, highlighting their potential as an antimicrobial surface coating material. Moreover, the study provided insights into the bactericidal mechanism of ZnO-NPs mediated by reactive oxygen species, underscoring their efficacy in controlling bacterial pathogens (Halawani, 2009).

2.14 In vitro profiling of antimethicillin-resistant *Staphylococcus aureus* activity of thymoquinone against selected type and clinical strains.

The antibacterial potential of thymoquinone (TQ) and thymohydroquinone (THQ), the main components of black seed essential oil from *Nigella sativa*, was investigated against a panel of bacteria including *Escherichia coli*, *Pseudomonas aeruginosa*, *Shigella flexneri*, *Salmonella Typhimurium*, *Salmonella Enteritidis*, and *Staphylococcus aureus*. Both TQ and THQ exhibited antibacterial activity against both gram-positive and gram-negative bacteria, irrespective of their susceptibility to conventional antibiotics. Notably, *S. aureus* displayed high susceptibility to TQ, with concentrations as low as three and 6 µg/ml being sufficient to inhibit and kill the bacteria, respectively. Conversely, THQ required concentrations 100 times higher to achieve similar effects. Gram-negative bacteria showed lower susceptibility, with minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values ranging between 200 and 1600 µg/ml for both TQ and THQ.

Furthermore, the study explored the synergy between TQ/THQ and antibiotics, revealing synergistic effects in *S. aureus* and variable effects in gram-negative bacteria, including synergism, antagonism, and indifference (Hariharan *et al.*, 2016).

2.15 Reactive Oxygen Species Mediated Bacterial Biofilm Inhibition via Zinc Oxide Nanoparticles and Their Statistical Determination

This study investigated the antimethicillin-resistant *Staphylococcus aureus* (MRSA) activity of thymoquinone (TQ) extracted from *Nigella sativa* Linn. Results indicated that TQ exhibited a minimum inhibitory concentration (MIC) range of 8–16 µg/ml and a MIC90 of 16 µg/ml against MRSA strains. Furthermore, TQ demonstrated bactericidal activity against MRSA, with a >3 log kill, and a prolonged postantibiotic effect (PAE) of 3.2 ± 0.2 hours. Importantly, TQ did not induce the selection of resistant mutants even upon exposure to high-density MRSA inoculum. Transmission electron microscopy revealed damage to the cell wall and membrane of TQ-treated MRSA strains, supporting its bactericidal mechanism. These findings suggest promising anti-MRSA activity of TQ, highlighting its potential as a therapeutic agent (Dwivedi *et al.*, 2014).

2.16 Antibiotic resistance of bacterial biofilms.

Bacterial biofilms, structured communities of bacteria encased in a polymer matrix, pose challenges in clinical settings due to their increased tolerance to antibiotics and disinfectants. Biofilm-associated infections, such as those caused by *Staphylococcus aureus* and *Pseudomonas aeruginosa*, are notoriously difficult to treat. Resistance mechanisms within biofilms include decreased metabolic activity, upregulated efflux pumps, mutations in antibiotic targets, and quorum-sensing-regulated mechanisms. Strategies to combat biofilms include early aggressive antibiotic therapy, chronic suppressive therapy, and the use of enzymes and quorum-sensing inhibitors to disrupt the biofilm matrix (Hoiby *et al.*, 2010).

This study evaluated the antibacterial activity of total crude extracts and essential oil (EO) of *Nigella sativa* L. seeds in mice infected intraperitoneally with *Staphylococcus aureus* or *Escherichia coli*. The methanol and chloroform seed extracts, along with the essential oil, exhibited dose-dependent antibacterial activities against both gram-positive and gram-negative organisms (Hosseinzadeh *et al.*, 2007).

2.17 Assessment of a microplate method for determining the post-antibiotic effect in *Staphylococcus aureus* and *Escherichia coli*

The post-antibiotic effect (PAE) stands as a pivotal parameter in understanding antibiotic efficacy, often serving as a predictor of pharmacodynamic activity. Traditionally, determining PAE has been laborious, involving the assessment of viable cell numbers. However, recent advancements propose spectrophotometric procedures as a promising alternative, offering notable speed advantages. In this study, they compared PAE values obtained using a rapid microplate method with those derived from the classical viable count procedure. PAEs for various antibiotics against *Staphylococcus aureus* and *Escherichia coli* were assessed following exposure to $5 \times \text{MIC}$ drug concentrations for 60 minutes in Mueller–Hinton Broth (MHB). The duration of PAE was monitored by tracking bacterial growth recovery in antibiotic-free MHB, measured either as colony forming units on Mueller–Hinton agar or as culture absorbance (at 600 nm) in a microplate reader. When analyzing PAEs for bacteriolytic agents, a poor correlation was observed between the two methods for both *S. aureus* ($R^2=0.096$) and *E. coli* ($R^2=0.5456$). However, for bacteriostatic agents and non-lytic bactericidal agents, a high correlation was found between the two methods for both *S. aureus* ($R^2=0.7529$) and *E. coli* ($R^2=0.7687$). These findings suggest that the spectrophotometric micro plate method could serve as a viable alternative to the classical method, particularly for antibiotics that do not induce bacterial cell lysis.

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2.18 Antibacterial nano-structured titania coating incorporated with silver nanoparticles

Titanium implants coated with titania nanotubes (TiO₂-NTs) incorporated with silver nanoparticles (NT-Ag) demonstrated potent antibacterial activity against planktonic bacteria and prevented bacterial adhesion for up to 30 days. The controllable NT-Ag structure holds promise for preventing implant-associated infections (Zhao *et al.*, 2011).

2.19 Redox cycling of endogenous copper by thymoquinone leads to ROS-mediated DNA breakage and consequent cell death: putative anticancer mechanism of antioxidants.

Plant-derived dietary antioxidants have garnered significant attention for their potential chemopreventive and cancer therapeutic effects in animal models. Thymoquinone (TQ), the major bioactive compound found in the volatile oil of *Nigella sativa*, possesses diverse pharmacological properties, including anti-inflammatory

cardiovascular, analgesic, anti-neoplastic, anticancer, and chemopreventive activities. While several mechanisms have been proposed to explain the anticancer activity of TQ, its precise mechanism of action remains elusive. TQ is recognized as an antioxidant at lower concentrations, and much of the research has focused on elucidating its antioxidant properties. However, recent studies have suggested that TQ may exhibit prooxidant effects at higher concentrations. It is well established that plant-derived antioxidants can transition to prooxidants, particularly in the presence of transition metal ions such as copper. Elevated levels of copper in tissues, cells, and serum are commonly observed in various malignancies. Copper is a crucial metal ion present in chromatin and closely associated with DNA bases, particularly guanine. Through experimentation using human peripheral lymphocytes and the comet assay, it has been demonstrated that TQ induces oxidative DNA breakage in cellular DNA. Importantly, this DNA damage can be mitigated by copper-chelating agents, such as neocuproine and bathocuproine, as well as scavengers of reactive oxygen species. Furthermore, TQ has been shown to target cellular copper in prostate cancer cell lines, leading to a prooxidant-mediated cell death. These findings suggest that the cytotoxic mechanism of TQ involves redox cycling of endogenous copper, resulting in reactive oxygen species (ROS)-mediated DNA breakage and subsequent cell death. This prooxidant effect provides a potential explanation for the anticancer activity of plant-derived antioxidants, such as TQ (Zubair *et al.*, 2013)

CHAPTER-3

AIM AND OBJECTIVES

3.1 AIM

To Analysis of efficacy of Ag-Cu Nanoparticle Conjugated Thymoquinone against *E. coli* Biofilms.

3.2 OBJECTIVES

- To analyze the effect on conjugate with binary Ag-Cu nano particles conjugated thymoquinone on the metabolism of *E. coli*
- To compare the efficacy of thymoquinone drug alone and drug with Nanoparticle conjugation in the inhibition of biofilm formation at varying concentration
- To validate the efficacy of Ag-Cu- Thymoquinone in the inhibition of *E. coli* biofilm formation using imaging techniques

CHAPTER- 4

MATERIALS AND METHODS

4.1 ISOLATION OF BACTERIA

4.1.1 Autoclave the test tubes

Autoclaving is a process of sterilization using high-pressure steam to kill microorganisms and their spores. This step ensures that the test tubes are free from any contaminants that could interfere with the bacterial culture.

4.1.2 Prepare required LB broth

LB broth, or Luria -Bertani broth, is a nutrient-rich medium that supports bacterial growth. It typically contains tryptone, yeast extract, and sodium chloride. Preparing the LB broth involves mixing these components in the correct proportions and sterilizing the mixture, usually by autoclaving.

4.1.3 For preparing culture pour 30l of Culture in 3ml of LB broth

This step involves inoculating the LB broth with the bacterial culture. A small volume of the bacterial culture (30µl) is added to a larger volume of LB broth (3ml) to create a diluted culture. This allows for the growth of individual bacterial colonies without overcrowding.

4.1.4 Keep in the Shaker for 24 hours at 37°C

The inoculated test tubes are placed in a shaker incubator set at 37°C, which is the optimal temperature for the growth of many bacteria. The shaker provides continuous agitation, ensuring that the bacteria are evenly distributed in the broth and have access to oxygen and nutrients.

4.2 DRUG PREPARATION



Fig:4.1: Drug preparation

4.2.1 Measure 5mg of the drug

Use a precise scale to measure out exactly 5 milligrams of the drug substance. Transfer the drug into an Eppendorf tube. Carefully place the measured 5mg of the drug into the Eppendorf tube. Be cautious to avoid spillage or contamination.

4.2.2 Add 5ml of ethanol

Using a calibrated pipette, add 5 milliliters of ethanol to the Eppendorf tube containing the drug. Ensure accurate measurement to maintain the desired concentration of the drug solution.

4.2.3 Shake well to mix

Cap the Eppendorf tube securely and shake it vigorously to ensure thorough mixing of the drug and ethanol. This step is crucial for achieving a homogeneous solution.

4.2.4 Prepare aliquots

After mixing, you can aliquot the drug solution into multiple Eppendorf tubes for storage or further experimentation. Use appropriate pipettes to transfer equal volumes of the solution into each tube.

4.2.5 Label the aliquoted tubes

It's important to label each aliquoted tube with relevant information such as the drug name, concentration, date of preparation, and any other pertinent details.

4.2.6 Store appropriately

Store the aliquoted tubes in suitable conditions, such as a refrigerator or freezer, depending on the stability requirements of the drug solution. Following these steps should result in the successful preparation and aliquoting of the drug solution in Eppendorf tubes. Make sure to adhere to proper laboratory practices and safety guidelines throughout the procedure.

4.3 SYNTHESIS OF THYMOQUINONE CONJUGATED NANOPARTICLES



Fig: 4.2: Preparation of conjugated drug

4.3.1 Prepare Ag-Cu solution

Measure out 100mg of Ag-Cu.

Add it to 2.5ml of distilled water in a suitable container.

4.3.2 Prepare thymoquinone solution

Measure out 10mg of thymoquinone.

Add it to 5ml of ethanol.

Check solubility:

If thymoquinone is soluble in ethanol, proceed to step 3.

If not soluble, add another 5ml of ethanol, and mix until fully dissolved.

4.3.3 Mixing thymoquinone with Ag-Cu

Using a vortex mixer, set up the Ag-Cu solution and start vortexing.

Dropwise, add the thymoquinone solution into the vortexing Ag-Cu solution.

Continue vortexing for 20 seconds to ensure thorough mixing.

4.3.4 Cooling the mixture

After vortexing, transfer the container with the mixed solution into an ice bag.

Leave the mixture in the ice bag for 5 minutes to allow it to cool down.

4.3.5 Repeat the process

After the cooling period, remove the container from the ice bag.

Repeat the vortexing process with the same parameters (20 seconds) and then return the mixture to the ice bag for another 5 minutes.

Repeat this vortexing and cooling process for a total of 4 hours, ensuring the solution remains well-mixed and cooled throughout.

4.3.6 Monitoring

Periodically check the mixture during the 4-hour process to ensure proper vortexing and cooling conditions are maintained.

4.4 SWIMMING ASSAY

4.4.1 Prepare LB agar

Follow the manufacturer's instructions to prepare LB agar medium.

Autoclave the LB agar to sterilize it.

Let the agar cool down to approximately 50-60°C before pouring into petri plates.

4.4.2 Prepare control plate

Take one petri plate and label it as "Control".

Pour the sterilized LB agar into the plate and allow it to solidify.

4.4.3 Prepare drug-treated plate

Mix the desired drug(s) into the LB agar medium while it's still in a liquid state. Ensure the drug(s) are evenly distributed.

Pour the drug-containing LB agar into another labeled petri plate and allow it to solidify.

4.4.4 Prepare nanoparticle and drug conjugation -Treated plate:

Mix the desired nanoparticles and drug conjugation into the LB agar medium while it's still in a liquid state. Ensure the nanoparticles are evenly dispersed.

Pour the nanoparticle and drug conjugation containing LB agar into another labeled petri plate and allow it to solidify.

4.4.5 Make holes in the plates

Once the agar in all plates has solidified, use a sterile cork borer or a pipette tip to make evenly spaced holes (wells) in each plate.

4.4.6 Inoculate with bacteria

Using a sterile pipette, add 5 microliters of the bacterial culture into each hole made in the plates.

Make sure to use different pipette tips for each plate to prevent cross-contamination.

4.4.7 Incubate plates

Seal the plates with parafilm or tape to prevent moisture loss.

Place the plates in an incubator set to 37°C.

Incubate the plates for 48 hours to allow bacterial growth.

4.4.8 Observation and recording

After 48 hours of incubation, remove the plates from the incubator.

Observe the plates for bacterial growth and any visible differences between the control, drug-treated, and nanoparticle-treated plates.

Note any variations in bacterial growth, colony morphology, or inhibition zones.

4.4.9 Data analysis

Analyze the results and draw conclusions regarding the effects of the drugs and nanoparticles on bacterial growth compared to the control.

Ensure proper aseptic technique and safety precautions are followed throughout the procedure to prevent contamination and ensure accurate results.

4.5 SWARMING ASSAY

4.5.1 Prepare LB agar with glucose or fructose

Prepare the LB agar according to standard protocol.

Add 0.5% glucose or fructose to the LB agar medium.

Mix well to ensure even distribution.

Autoclave the LB agar medium to sterilize it.

4.5.2 Prepare control plate

Take one petri plate and label it as "Control".

Pour the sterilized LB agar medium into the plate and allow it to solidify.

4.5.3 Prepare drug-treated plate

Mix the desired drug(s) into the LB agar medium while it's still in a liquid state. Ensure the drug(s) are evenly distributed.

Pour the drug-containing LB agar into another labeled petri plate and allow it to solidify.

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Note any variations in bacterial growth, colony morphology, or inhibition zones.

4.6 BIOFILM ASSAY

4.6.1 Prepare LB broth:

LB broth is a commonly used nutrient-rich medium for bacterial growth and is prepared according to standard laboratory protocols. Autoclaving is necessary to ensure sterility.

4.6.2 Prepare test tubes

Test tubes are used to contain the different experimental conditions: control, nanoparticle treatment, and drug treatment.

4.6.3 Control test tube

In the control test tube, 3ml of LB broth and 30µl bacterial culture are added. This serves as the baseline for biofilm formation without any additional treatments.

4.6.4 Nanoparticle and drug conjugation test tube

In the nanoparticle and drug conjugation test tube, 3ml of LB broth, 30µl bacterial culture, and 50µl nanoparticle and drug conjugation solution are added. Nanoparticles and drug conjugation may influence biofilm formation and are being tested for their effects.

4.6.5 Drug test tube

In the drug test tube, 3ml LB broth, 30µl bacterial culture, and 50µl of drug solution (specifically thymoquinone) are added. The drug's effect on biofilm formation is being investigated.

4.6.6 Incubation

All test tubes are incubated at 37°C for 48 hours. This allows the bacteria to form biofilms under the different experimental conditions.

4.6.7 Remove supernatant

After incubation, the supernatant (liquid above the bacterial biofilm) is carefully removed to prepare for further processing.

4.6.8 Air dry

The test tubes are allowed to air dry to remove excess moisture from the biofilms.

4.6.9 Crystal violet staining

Crystal violet dye is added to the test tubes to stain the biofilms. This allows for visualization and quantification of biofilm formation.

4.6.10 Wash

After staining, the test tubes are washed to remove excess dye and background staining.

4.6.11 Acetic acid solution

Acetic acid solution is added to solubilize the crystal violet dye bound to the biofilm. This step facilitates measurement of biofilm density.

4.6.12 OD measurement

Optical density (OD) of each test tube is measured at 595 nm using a spectrophotometer. The OD is directly proportional to the amount of biofilm formed, allowing for quantitative analysis of biofilm formation under different experimental conditions.

4.7 96 WELL PLATE

4.7.1 Prepare LB broth

LB broth is prepared according to standard laboratory protocols. It serves as the growth medium for bacterial cultures.

4.7.2 Take 96 well plate

A 96 well plate is chosen as the platform for conducting multiple experiments simultaneously.

4.7.3 Prepare blank medium

In the first line of the 96 well plate, 200 microliters of blank medium (LB broth without bacterial culture) is added to serve as a control.

4.7.4 Prepare bacterial culture wells

For each experimental condition, an Eppendorf tube is prepared. In the second line well, 200 microliters of LB broth and 10 microliters of bacterial culture are mixed in the Eppendorf tube. This mixture is then pipetted into the corresponding well in the 96 well plate. This serves as the control for bacterial growth.

4.7.5 Prepare nanoparticle and drug conjugation treatment wells

In the third line well, 200 microliters of LB broth, 10 microliters of bacterial culture, and 2 microliters of nanoparticle and drug conjugation solution are mixed in the Eppendorf tube. The mixture is then added to the corresponding well in the 96 well plate. This evaluates the effect of nanoparticles and drug conjugation on bacterial growth.

4.7.6 Prepare drug treatment wells

In the fourth line well, 200 microliters of LB broth, 10 microliters of bacterial culture, and 2 microliters of drug solution are mixed in the Eppendorf tube. The mixture is then added to the corresponding well in the 96 well plate. This assesses the impact of the drug on bacterial growth.

4.7.7 Incubation

The 96 well plate is placed in a static position for 48 hours to allow bacterial growth and biofilm formation.

4.7.8 Medium removal

After incubation, the medium from each well is removed.

4.7.9 Supernatant removal and washing

The supernatant (liquid above the biofilm) is removed, and the wells are washed twice with 200 microliters of water to remove any remaining debris or unattached bacteria.

4.7.10 Add ethanol

200 microliters of 30% ethanol is added to each well to fix for further analysis.

4.7.11 OD measurement

The optical density (OD) of each well is measured at 570 nm using a spectrophotometer. This measurement provides quantitative data on biofilm formation under different experimental conditions.

CHAPTER-5

RESULT

5.1 SWIMMING ASSAY

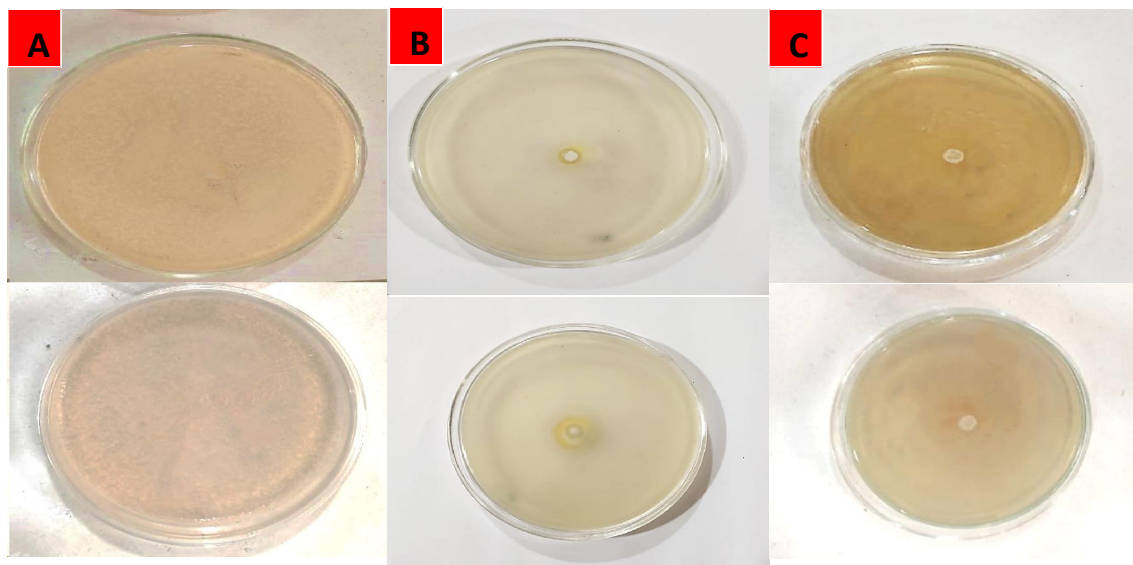


Fig: 5.1: Depicts three scenarios: A, B, and C of swimming assay

In A, the control plate exhibits normal LB Agar with a central application of 5 μ l E. coli bacterial sample. After an incubation period of 48 hours, significant E. coli growth is observed.

In B, LB agar supplemented with thymoquinone is presented. Following the addition of the same E. coli sample as in A, the subsequent bacterial growth is notably reduced compared to the control plate (A) after the same incubation period.

In C, the plate contains LB agar supplemented with Ag-Cu nanoparticles and drug conjugation. Despite the addition of an equivalent amount of bacterial sample,

the inhibitory effect observed surpasses both the control (A) and *thymoquinone*-treated (B) plates, signifying a lower inhibitory constant.

Table:5.1: Treatment of Drug and Nanoparticle by using Swimming Assay

Bactria isolates	Drug (cm)	Nanoparticle and drug conjugation (cm)
Growth in Diameter EV1	1.2	0.7
Growth in Diameter EV2	1.1	0.8
Growth in Diameter EV3	0.8	0.6
Growth in Diameter EV4	0.9	0.5
Growth in Diameter EV5	0.8	0.6
Growth in Diameter EV6	0.9	0.6
Growth in Diameter EV7	0.1	0.9
Growth in Diameter EV8	1.0	0.7
Growth in Diameter EV9	0.9	0.8
Growth in Diameter EV10	0.9	0.6
Growth in Diameter EV11	0.8	0.4
Growth in Diameter EV12	0.9	0.3
Growth in Diameter EV13	1.0	0.8
Growth in Diameter EV14	1.1	0.8
Growth in Diameter EV15	0.9	0.7
Growth in Diameter EV16	1.0	0.6
Growth in Diameter EV17	1.1	0.8
Growth in Diameter EV18	0.9	0.7
Growth in Diameter EV19	0.9	0.6
Growth in Diameter EV20	0.7	0.8
Growth in Diameter EV21	1.0	0.4

Growth in Diameter EV22	1.1	0.4
Growth in Diameter EV23	0.9	0.6
Growth in Diameter EV24	1.2	0.7
Growth in Diameter EV25	1.1	0.9
Growth in Diameter EV26	0.9	1.0
Growth in Diameter EV27	1.0	0.6
Growth in Diameter EV28	0.9	0.6
Growth in Diameter EV29	0.8	0.7
Growth in Diameter EV30	0.8	0.6

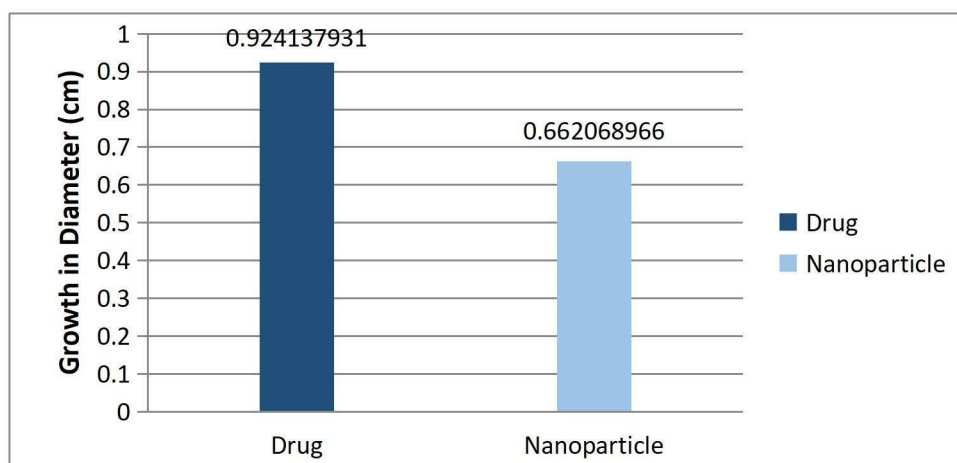
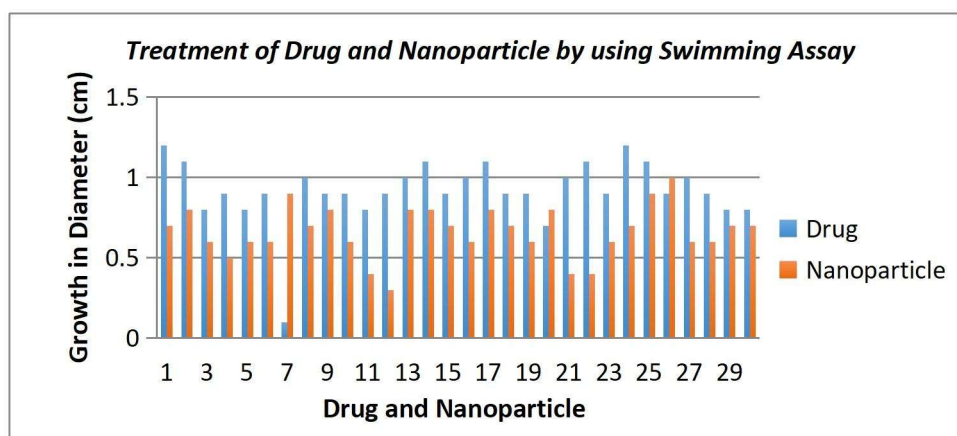


Fig:5.2: Graphical representation of swimming assay

Table :5.2 : At various concentration of Ag-Cu Nanoparticles and Drug conjugated

Bactria isolates	20 (cm)	30 (cm)	50 (cm)	80 (cm)	100 (cm)
Growth in Diameter EV1	0.9	0.8	0.6	0.6	0.6
Growth in Diameter EV2	0.7	0.6	0.5	0.4	0.4
Growth in Diameter EV3	0.8	0.7	0.6	0.6	0.6
Growth in Diameter EV4	1.1	0.9	0.6	0.6	0.6
Growth in Diameter EV5	1.0	0.8	0.7	0.6	0.6

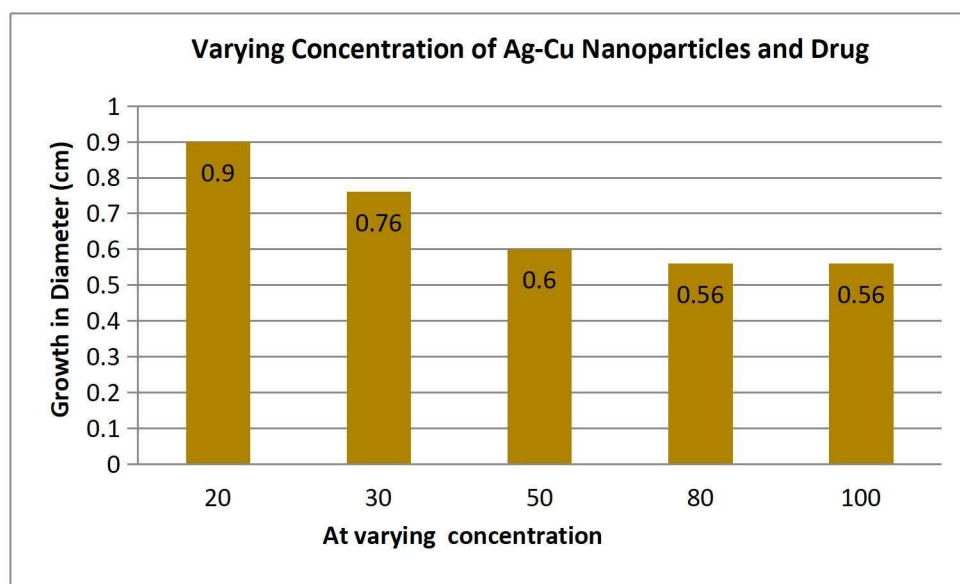
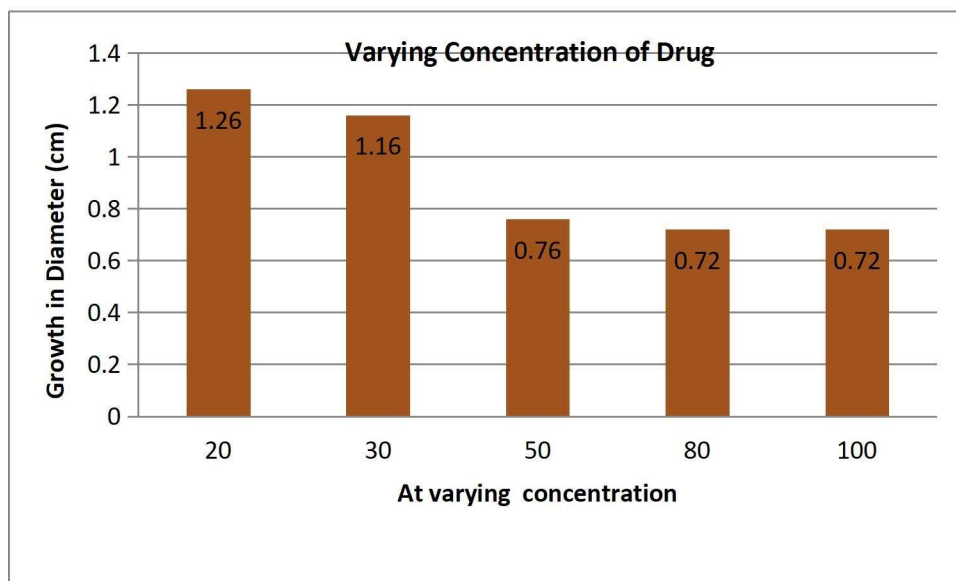


Fig:5.3 : Graphical representation of varying concentration of Ag-Cu Nanoparticles and Drug conjugated

Table 5.3 : At varying concentration of Drug

Bactria isolates	20 (cm)	30 (cm)	50 (cm)	80 (cm)	100 (cm)
Growth in Diameter EV1	1.1	1.0	0.8	0.8	0.8
Growth in Diameter EV2	1.6	1.3	0.6	0.6	0.6
Growth in Diameter EV3	1.4	1.8	1.0	0.9	0.9
Growth in Diameter EV4	1.2	0.9	0.7	0.7	0.7
Growth in Diameter EV5	1.0	0.8	0.7	0.6	0.6

Fig :5.4 : Graphical representation of Drug



5.2 SWARMING ASSAY

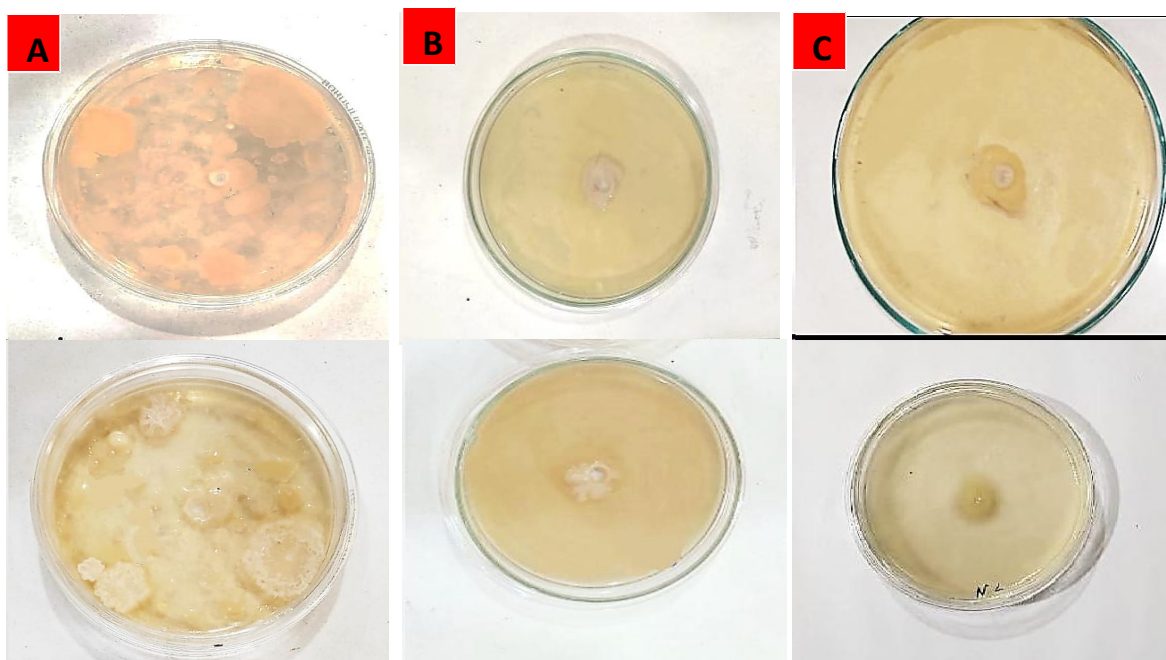


Fig:5.5: Illustrates three experimental conditions of swarming assay

In A, an LB agar plate supplemented with 0.5% glucose is depicted. Following solidification, *E. coli* bacterial samples were introduced into the central well. Subsequent incubation for 48 hours led to substantial bacterial growth.

In B, the same LB agar medium with 0.5% glucose was supplemented with the drug Thymoquinone. Despite the presence of this drug, the inhibitory effect observed was lower compared to the control (A), indicating some bacterial growth inhibition, but not as much as without the drug.

In C, the LB agar plate is modified with Ag-Cu nanoparticles and drug conjugation. Remarkably, the bacterial growth observed in this condition is significantly lower compared to both the control (A) and the Thymoquinone-treated plate (B), suggesting a potent inhibitory effect of the Ag-Cu nanoparticles.

Table: 5.4: Treatment of Drug and Nanoparticle by using Swarming Assay

Bactria isolates	Drug (cm)	Nanoparticle and drug conjugation (cm)
Growth in Diameter EV1	1.1	0.8
Growth in Diameter EV2	0.6	0.4
Growth in Diameter EV3	0.7	0.4
Growth in Diameter EV4	0.9	0.5
Growth in Diameter EV5	1.1	1.0
Growth in Diameter EV6	1.3	1.1
Growth in Diameter EV7	0.8	0.7
Growth in Diameter EV8	0.6	0.4
Growth in Diameter EV9	1.1	0.9
Growth in Diameter EV10	0.9	0.8
Growth in Diameter EV11	0.8	0.4
Growth in Diameter EV12	1.2	1.1
Growth in Diameter EV13	1.1	0.6
Growth in Diameter EV14	0.9	0.7
Growth in Diameter EV15	1.0	0.9
Growth in Diameter EV16	1.1	0.7
Growth in Diameter EV17	1.2	0.6
Growth in Diameter EV18	1.0	0.9
Growth in Diameter EV19	0.7	0.6
Growth in Diameter EV20	1.3	1.1
Growth in Diameter EV21	1.1	0.9
Growth in Diameter EV22	1.0	0.7
Growth in Diameter EV23	0.9	1.1
Growth in Diameter EV24	1.3	0.7
Growth in Diameter EV25	0.9	0.6
Growth in Diameter EV26	0.7	0.7

Growth in Diameter EV27	0.9	0.9
Growth in Diameter EV28	1.1	1.1
Growth in Diameter EV29	1.2	0.4
Growth in Diameter EV30	0.7	0.3

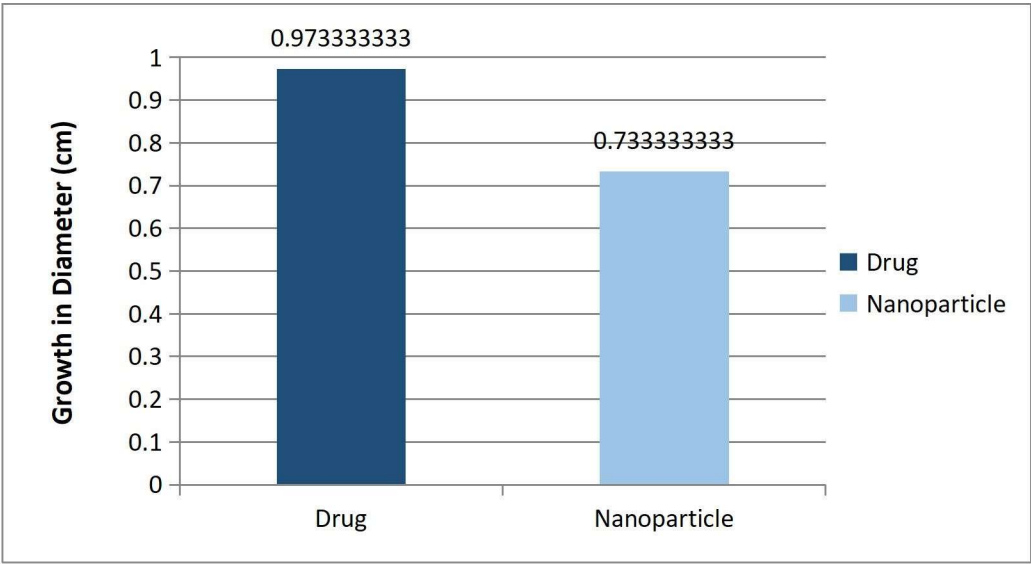
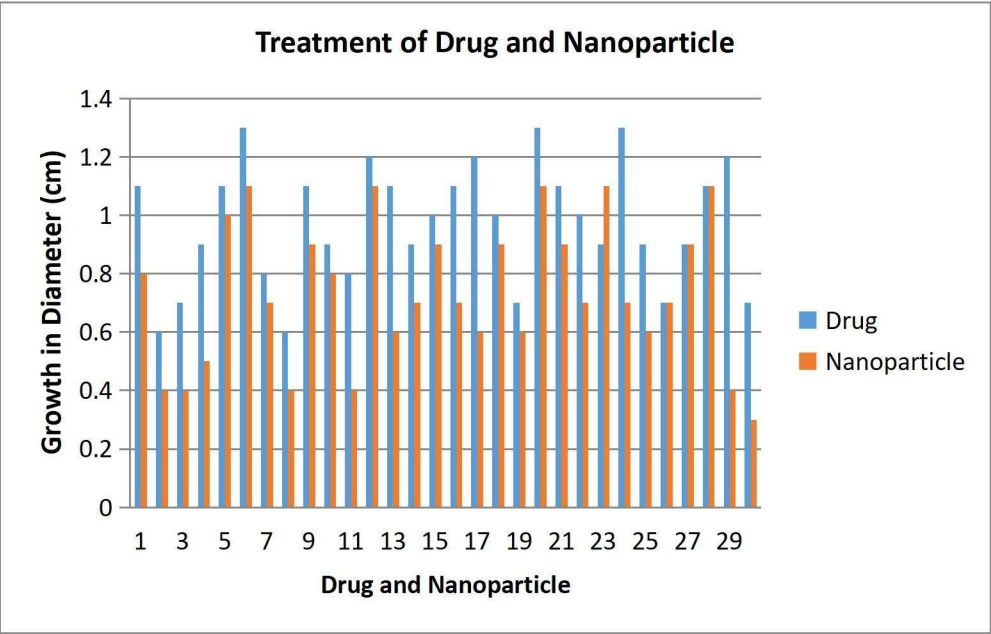


Fig: 5.6: Graphical representation of swarming assay

Table 5.5: At different concentration of Ag-Cu Nanoparticles and Drug conjugated of swarming assay

Bactria isolates	20 (cm)	30 (cm)	50 (cm)	80 (cm)	100 (cm)
Growth in Diameter EV1	0.9	0.6	0.7	0.9	0.7
Growth in Diameter EV2	0.9	0.6	0.5	0.5	0.5
Growth in Diameter EV3	1.0	0.7	0.4	0.4	0.4
Growth in Diameter EV4	1.0	0.8	0.7	0.6	0.6
Growth in Diameter EV5	0.9	0.8	0.6	0.6	0.6

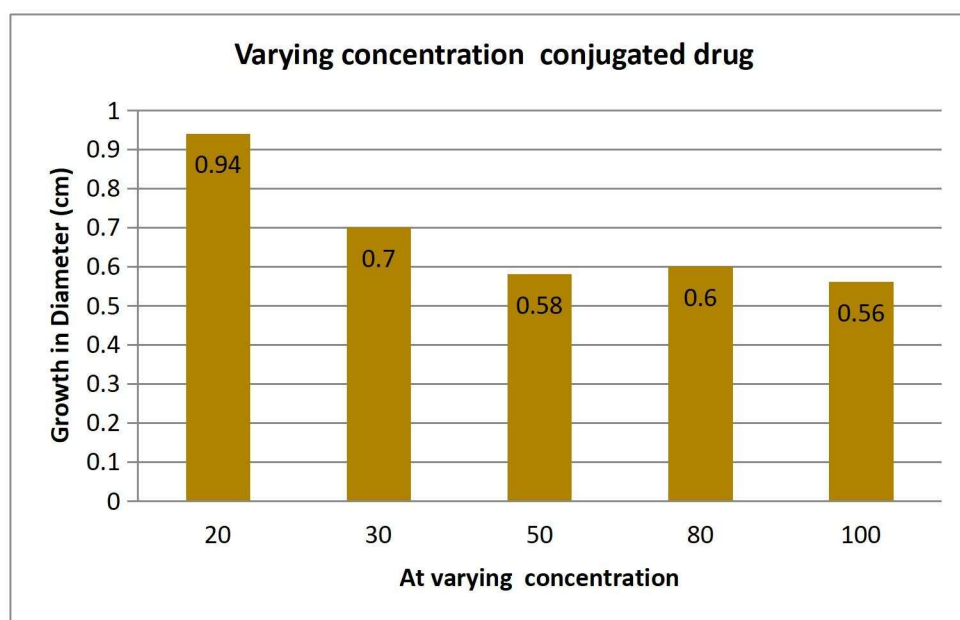


Fig: 5.7: Graphical representation of varying Ag-Cu Nanoparticles and Drug conjugated of swarming assay

Table 5.6 : At varying concentration of drug

Bactria isolates	20 (cm)	30 (cm)	50 (cm)	80 (cm)	100 (cm)
Growth in Diameter EV1	1.4	1.2	1.1	1.0	1.0
Growth in Diameter EV2	1	0.8	0.6	0.6	0.6
Growth in Diameter EV3	0.9	0.8	0.7	0.7	0.7
Growth in Diameter EV4	1.6	1.2	1.1	1.0	1.0
Growth in Diameter EV5	0.8	0.6	0.5	0.5	0.5

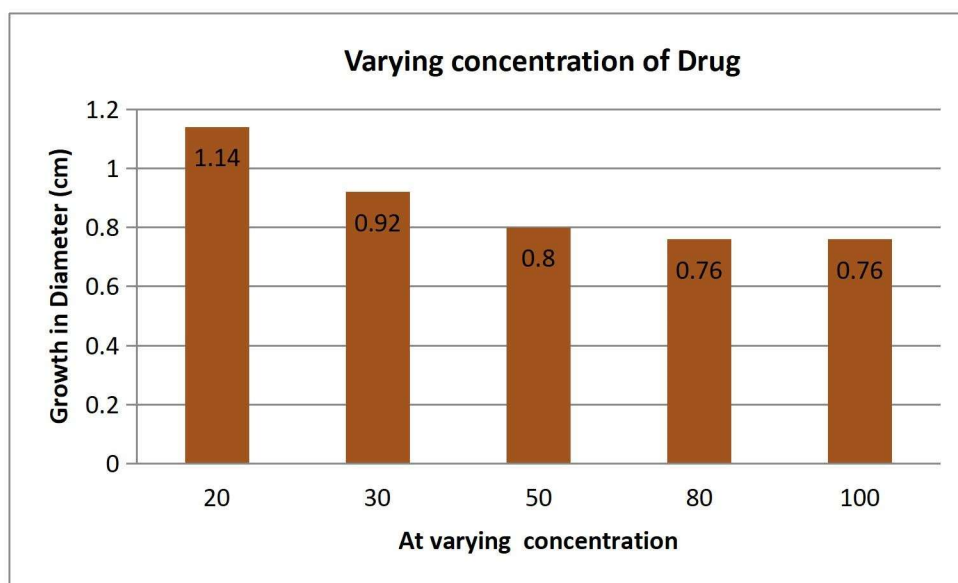


Fig:5.8 : Graphical representation of varying concentration of Drug of swarming assay

5.3 BIOFILM ASSAY



Fig:5.9 : Bioflim assay tubeswith crystal violet

The figure depicts the experimental protocol for evaluating the antibacterial efficacy of a drug and nanoparticles using crystal violet staining. LB broth is prepared and distributed into three labeled test tubes: Control, Drug, and Nanoparticle. In the Control tube, bacterial culture is added, while in the Nanoparticle and Drug tubes, bacterial culture is combined with respective solutions of nanoparticles and thymoquinone drug. After incubating for 48 hours at 37°C, supernatant is removed, and tubes are air-dried. Subsequently, crystal violet dye is added and incubated for 20 minutes, followed by washing with water thrice. Finally, 1 ml of acetic acid solution is added, and optical density is measured at 595 nm.

Table 5.7: Treatment of Control, Drug and Nanoparticle drug by using biofilm assay

Bactria isolates	Control (OD)	Drug (OD)	Nanoparticle and drug conjugation (OD)
Growth in Diameter EV1	0.89	0.77	0.63
Growth in Diameter EV2	0.92	0.89	0.74
Growth in Diameter EV3	0.97	0.83	0.81
Growth in Diameter EV4	0.91	0.84	0.79
Growth in Diameter EV5	0.97	0.89	0.84
Growth in Diameter EV6	0.87	0.74	0.71
Growth in Diameter EV7	0.89	0.84	0.81
Growth in Diameter EV8	0.91	0.79	0.74
Growth in Diameter EV9	0.92	0.83	0.82
Growth in Diameter EV10	0.93	0.89	0.84
Growth in Diameter EV11	0.97	0.83	0.77
Growth in Diameter EV12	0.99	0.93	0.82
Growth in Diameter EV13	0.97	0.94	0.89
Growth in Diameter EV14	0.91	0.84	0.72
Growth in Diameter EV15	0.97	0.84	0.67
Growth in Diameter EV16	0.91	0.79	0.74
Growth in Diameter EV17	0.93	0.82	0.76
Growth in Diameter EV18	0.89	0.62	0.52
Growth in Diameter EV19	0.91	0.74	0.56
Growth in Diameter EV20	0.93	0.79	0.76
Growth in Diameter EV21	0.92	0.84	0.81
Growth in Diameter EV22	0.92	0.71	0.63
Growth in Diameter EV23	0.87	0.84	0.78
Growth in Diameter EV24	0.81	0.79	0.63
Growth in Diameter EV25	0.92	0.89	0.87
Growth in Diameter EV26	0.96	0.94	0.91

Growth in Diameter EV27	0.82	0.79	0.37
Growth in Diameter EV28	0.92	0.91	0.70
Growth in Diameter EV29	0.78	0.74	0.55
Growth in Diameter EV30	0.89	0.82	0.65

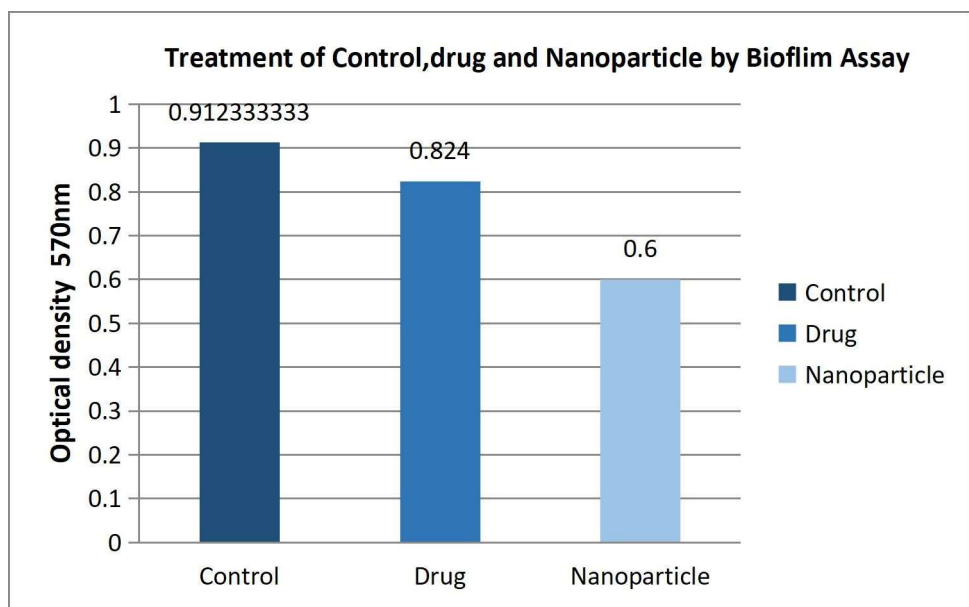
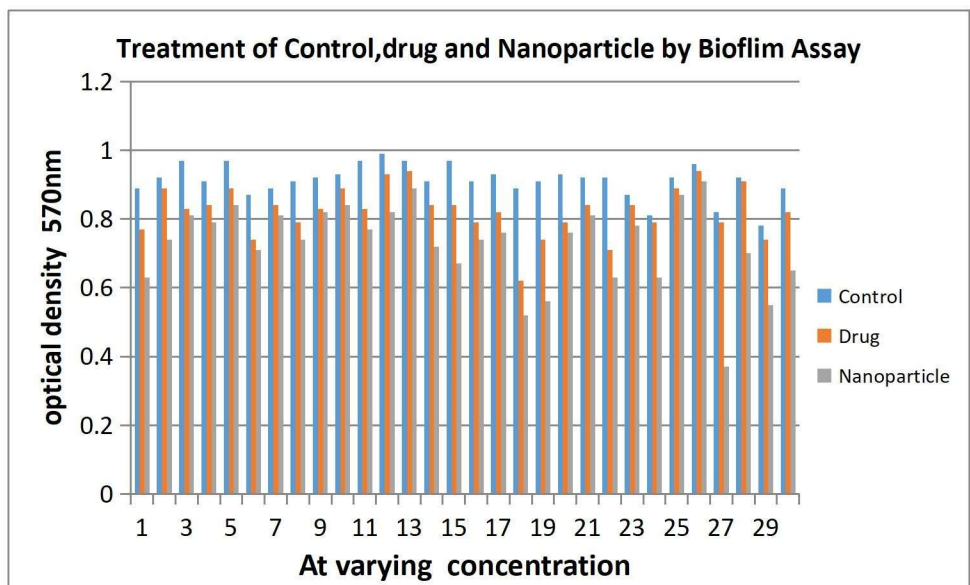


Fig.5.10: Graphical representation of Biofilm assay

Bactria isolates	10 (OD)	30 (OD)	50 (OD)	80 (OD)	100 (OD)
Growth in Calorimeter EV1	0.89	0.83	0.72	0.63	0.62
Growth in Calorimeter EV2	0.92	0.89	0.71	0.72	0.69
Growth in Calorimeter EV3	0.83	0.82	0.89	0.79	0.77
Growth in Calorimeter EV4	0.91	0.83	0.79	0.74	0.71
Growth in Calorimeter EV5	0.81	0.74	0.72	0.71	0.69

Table 5.8: At varying concentration of Ag-Cu Nanoparticles and Drug conjugated of Biofilm Assay

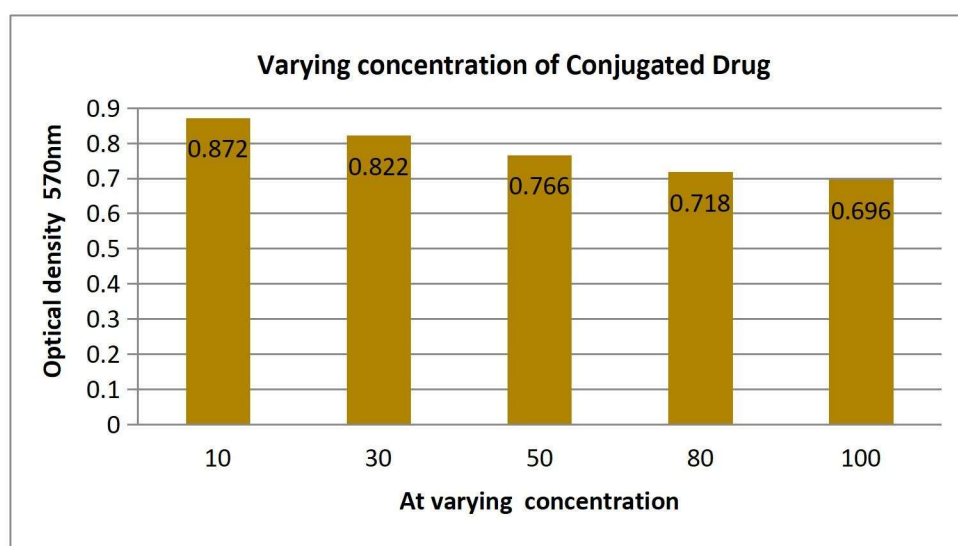
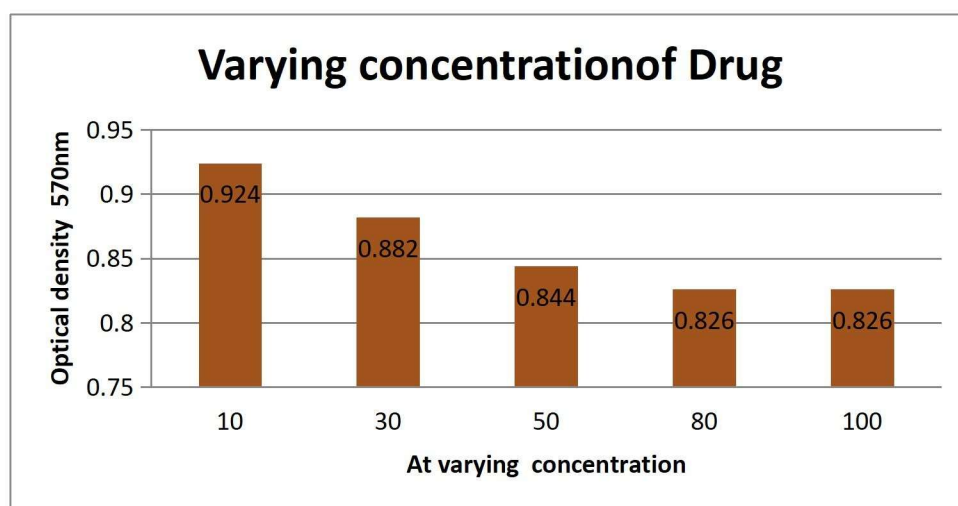


Fig:5.11: Graphical representation of at varying concentrationAg-Cu Nanoparticles and Drug conjugated of Biofilm Assay

Table 5.9: At varying concentration of Drug

Bactria isolates	10 (OD)	30 (OD)	50 (OD)	80 (OD)	100 (OD)
Growth in Calorimeter EV1	0.91	0.88	0.77	0.74	0.74
Growth in Calorimeter EV2	0.96	0.92	0.89	0.84	0.84
Growth in Calorimeter EV3	0.89	0.81	0.83	0.82	0.82
Growth in Calorimeter EV4	0.92	0.89	0.84	0.84	0.84
Growth in Calorimeter EV5	0.94	0.91	0.89	0.89	0.89



5.12: Graphical representation of at varying concentration of Drug

5.4. 96 WELL PLATE ASSAY



Fig: 5.13: 96 well plate

The figure depicts the experimental protocol for evaluating the antibacterial efficacy of a drug and nanoparticles using crystal violet staining. LB broth is prepared and distributed into four labeled wells: Blank, Control, Drug, and Nanoparticle. In Blank, LB broth was added. In the Control well, bacterial culture is added, while in the Nanoparticle and Drug well, bacterial culture is combined with respective solutions of nanoparticles and thymoquinone drug was added. After incubating for 48 hours at 37°C, supernatant is removed, and wells are air-dried. Subsequently, crystal violet dye is added and incubated for 20 minutes, followed by washing with water thrice. Finally, 1 ml of Ethanol is added, and optical density is measured at 570 nm.

Table 5.10: 96 well plate reading

Bactria isolates	Blank (OD)	Control (OD)	Drug (OD)	Nanoparticle and drug conjugation (OD)
Growth in OD at 570nm in EV1	1.816	3.719	3.599	2.527
Growth in OD at 570nm in EV2	1.590	3.634	3.612	2.239
Growth in OD at 570nm in EV3	1.889	3.559	2.826	2.523
Growth in OD at 570nm in EV4	1.721	3.646	3.599	3.112
Growth in OD at 570nm in EV5	1.753	3.340	3.224	2.157
Growth in OD at 570nm in EV6	1.926	3.512	2.286	2.119
Growth in OD at 570nm in EV7	1.882	3.619	3.603	3.512
Growth in OD at 570nm in EV8	1.953	3.476	2.000	1.976
Growth in OD at 570nm in EV9	1.872	3.826	2.579	2.227
Growth in OD at 570nm in EV10	1.996	3.494	3.119	2.987
Growth in OD at 570nm in EV11	1.863	3.559	3.527	3.439
Growth in OD at 570nm in EV12	1.739	3.474	3.117	2.989

Growth in OD at 570nm in EV13	1.836	3.649	3.236	2.191
Growth in OD at 570nm in EV14	1.769	3.494	3.291	3.117
Growth in OD at 570nm in EV15	1.939	3.569	2.579	2.123
Growth in OD at 570nm in EV16	1.797	3.943	2.527	2.437

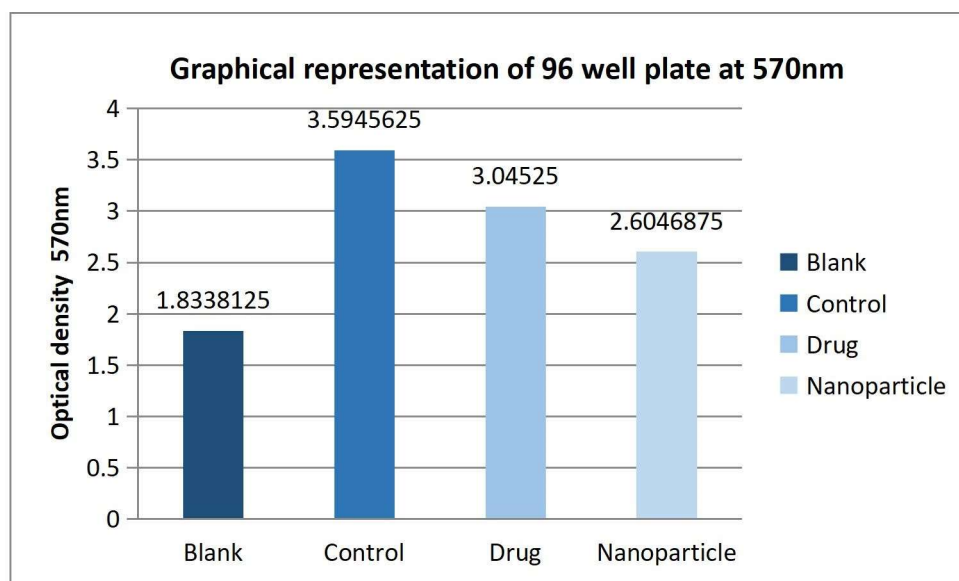


Fig:5.14: Graphical representation of 96 well plate reading

CHAPTER- 6

SUMMARY AND CONCLUTION

The investigation delved into the antibacterial potential of thymoquinone and Ag-Cu nanoparticles against *E. coli* bacterial growth, both individually and in combination. In the swimming assay (Figure 5.1), the control plate demonstrated robust *E. coli* growth, whereas the addition of thymoquinone or Ag-Cu nanoparticles notably reduced bacterial growth, with the latter exhibiting a stronger inhibitory effect than thymoquinone. Similarly, in the swarming assay (Figure 5.2), while the control plate exhibited substantial bacterial growth, the addition of thymoquinone or Ag-Cu nanoparticles led to varying degrees of inhibition, with Ag-Cu nanoparticles displaying the most potent inhibitory effect. Finally, the biofilm assay (Figure 5.3) illustrated the antibacterial efficacy of thymoquinone and Ag-Cu nanoparticles through crystal violet staining, showing significant reduction in bacterial growth in the presence of these agents compared to the control. Through the result of assay we can concluded that conjugated particle, that mean Nanoparticle with thymoquinone drug shows the efficient antimicrobial activity. These findings underscore the potential of thymoquinone and Ag-Cu nanoparticles as promising antibacterial agents, with Ag-Cu nanoparticles demonstrating superior efficacy against *E. coli* growth. Further research elucidating the mechanisms underlying their antibacterial activity and exploring their application in combating bacterial infections is warranted.

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