

PROFILING THE ANTIBACTERIAL ACTIVITY OF AG-CU NANOPARTICLE CONJUGATED THYMOQUINONE DRUG AGAINST MULTIDRUG RESISTANT CLINICAL ISOLATES

PROJECT WORK

Submitted in partial fulfilment of the requirements for the award of
Bachelor of Science degree in Microbiology

By

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SATHYABAMA

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

CATEGORY-1 UNIVERSITY BY UGC

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BONAFIDE CERTIFICATE

This is to certify that this Project Report is the bonafide work of **PRIYANKA.M (41300031)** and **ALFRED EMACULATE.A (41300056)** who carried out the project "PROFILING THE ANTIBACTERIAL ACTIVITY OF AG-CU NANOPARTICLE CONJUGATED THYMOQUINONE DRUG AGAINST MULTIDRUG RESISTANT CLINICAL ISOLATES" under our supervision from January 2024 to April 2024.

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We, **M.PRIYANKA (41300031)** and **A. ALFRED EMACULATE (41300056)** hereby declare that the Project Report entitled "**PROFILING THE ANTIBACTERIAL ACTIVITY OF AG-CU NANOPARTICLE CONJUGATED THYMOQUINONE AGAINST MULTIDRUG RESISTANT CLINICAL ISOLATES**" is done by us under the guidance of **Dr.INDUMATHI SM** at **Sathyabama Institute of Science and Technology, Chennai,Tamilnadu, India**, is submitted in partial fulfillment of requirements for the award of **Bachelor Of Science in Microbiology**.

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ABSTRACT

Various microorganisms can live on the inanimate surfaces. Numerous microbes can remain alive on surfaces for weeks or even months. Clinical bacterial isolates were collected from oral swabs (which were collected by our seniors and are ethically approved). These collected clinical isolates consist of *Escherichia coli* and *staphylococcus* around. As 56 samples for *Escherichia coli* and another 50 samples for *staphylococcus* isolates. Each one *Escherichia coli* and *staphylococcus* sample are plated using mannitol salt agar for *staphylococcus* and Eosin methylene blue agar for *Escherichia coli*, then were kept for 24 hours at 37 degree Celsius for confirmative test. The antibacterial activity method was used to screen the isolates for multidrug resistant bacteria. Ag-Cu nanoparticle conjugated thymoquinone drug is a novel therapeutic agent against multidrug-resistant bacteria. Nowadays Multidrug-resistant bacteria pose a significant threat to public health. Thus this study investigates the antibacterial activity of silver-copper (Ag-Cu) nanoparticle conjugated thymoquinone against multidrug-resistant clinical isolates.

Keywords: Thymoquinone drug, Ag-Cu nanoparticle conjugated thymoquinone drug, MDR resistant clinical isolates, Ag-Cu nanoparticles, Antibacterial activity.

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LIST OF ABBREVIATIONS

Ag-Cu	Silver copper
NPs	Nanoparticles
TQ	Thymoquinone
OD	Optical density
MDR	Multidrug resistant
LB	Luria Bertani
TEM	Transmission electron microscopy
GC-MS	Gas chromatography-mass spectrometry
EMB	Eosin methylene blue agar
MS agar	Mannitol salt agar
<i>E.coli</i>	<i>Escherichia coli</i>
<i>S.aureus</i>	<i>Staphylococcus aureus</i>
UV spec	Ultraviolet visible spectroscopy
Nm	Nano meter
DNA	Deoxyribonucleic acid
FESEM	Field emission scanning electron microscopy
HR-TEM	High resolution transmission electron microscopy
FDA	Food and drug administration
pH	Potential of hydrogen

CHAPTER 1

INTRODUCTION

1.1 MULTI DRUG RESISTANT IN BACTERIAL PATHOGENS

MDR microorganisms are widely recognised as one of the most important public health concerns of our day. According to the Infectious Diseases Society of America, antibiotic resistance is one of the biggest concerns to human health in the world. The major threat posed by the emergence of MDR microorganisms is rooted in several issues. First and foremost, patients with MDR bacteria usually have worse outcomes than people with organisms that are more sensitive. In this way, increasing rates of antibiotic resistance impact many aspects of modern medicine and threaten a number of medical advancements, such as the treatment of cancer, organ transplantation and surgery. These illnesses come with significant additional expenditures, Infections brought on by resistant organism as opposed to susceptible species are predicted to cost an additional 21 billion to 34 billion annually in the US. Third the usage of broad- spectrum antibiotics, both for empiric and definitive therapy, is strongly associated with the frequency of certain MDR bacteria. Vicious loop results from this increased use, which raises the prevalence of MDR even further. (David van Duin *et al.*, 2016) Antibiotic-resistant infections are becoming more commonplace globally these days, upsetting medical professionals. Deadly pathogenic multidrug resistant bacteria (MDR) are becoming more prevalent every day and represent a major threat to human health. These kinds of antibiotic-resistant bacterial strains were uncommon in the past and restricted to nosocomial infections, but they are now quite prevalent. The most common MDR bacteria include Gram-negative *E. coli* and *P. aeruginosa*, as well as Gram-positive *S. aureus*, *S. pneumoniae*, *E. faecium*, and *E. faecalis*.

1.2 PREVALANCE OF RESISTANCE TO FOURTH GENERATION ANTIBIOTICS IN ENTEROBACTERIACEAE

There are 53 genera in the Enterobacteriaceae family, and over 170 species have been identified so far. Of them, human bacterial illnesses have already been linked to 26 different bacterial genera. Gram-negative facultative anaerobic rods make up this family. The majority of species can grow at 37 °C,

although some do better at 25 to 30 °C. These microbes are widely dispersed throughout the natural world and can be found in plants, soil, water, and the gastrointestinal tracts of humans and other vertebrates. The majority of bacteria found in clinical samples are known to belong to the Enterobacteriaceae family, which is linked to a broad range of illnesses in both hospitals and the community. Urinary tract, bloodstream, and lower respiratory tract infections are among the many diseases that can occur in both the community and hospital settings. These infections are frequently caused by gram-negative bacteria, most especially by Enterobacteriaceae. Drug resistance genes can be spread from one bacterium to another through various mechanisms such as plasmids, bacteriophages, naked DNA or transposons. Infections due to these bacteria are mostly treated with beta-lactam antibiotics, often conveniently combined with aminoglycosides and, in some cases, with fluoroquinolones. A significant problem in intensive care units is constantly increasing resistance to these antibiotics. Resistance of Gram-negative bacteria to broad-spectrum penicillin and cephalosporin is currently so high that these important drug groups are very likely to lose their effectiveness. The increase in bacterial resistance is contributed to by selection pressure of antibiotics, the clonal spread of Multi resistant bacterial strains and recombination processes such as conjugation. Selection pressure refers to the environmental forces that favour the survival and proliferation of certain traits or characteristics within a population. In the context of multi drug resistant (MDR) bacteria, selection pressure specifically refers to the factors that drive the emergence and spread of antibiotic resistance. Because of a different approach to preventing the spread of resistance it is important to differentiate whether the higher prevalence of multi resistant bacteria is caused by increased prescription of antibiotics.

1.3 USE OF NANOPARTICLE IN TREATMENT OF BACTERIAL INFECTION

Nanoparticles have gained attention for their potential in treating bacterial infections due to their unique properties. They can deliver antibiotics directly to the site of infection, penetrate bacterial biofilms, and even enhance the effectiveness of existing antibiotics. Additionally, nanoparticles can be engineered to specifically target bacterial cells while sparing healthy cells, reducing side effects. Overall,

they offer promising avenues for combating antibiotic resistance and improving treatment outcomes in bacterial infections.

These are nanoparticles produced through metabolic processes, such as biosynthesis by microorganisms or enzymatic reactions. They can be derived from natural sources like bacteria, fungi, or plants. These nanoparticles often possess intrinsic antimicrobial properties due to the metabolic pathways involved in their synthesis. For example, silver nanoparticles synthesized by microbial reduction have shown potent antibacterial activity against a wide range of pathogens.

These are typically composed of carbon-based materials or organic polymers. They can be functionalized with antimicrobial agents or engineered to mimic antimicrobial peptides found in nature. Organic nanoparticles offer advantages such as biocompatibility, tuneable surface chemistry, and controlled release of therapeutic agents. For instance, chitosan nanoparticles, derived from the natural polymer chitin, have been explored for their antibacterial properties and ability to enhance drug delivery to bacterial infections.

Polymeric nanoparticles are composed of synthetic or natural polymers, which can be tailored to encapsulate antimicrobial drugs or directly interact with bacterial cells. They offer versatility in terms of size, shape, and surface properties, allowing for precise control over drug release kinetics and targeting mechanisms. Polymeric nanoparticles have been investigated for their ability to overcome bacterial resistance mechanisms and enhance drug efficacy. Examples include nanoparticles made from poly (lactic-co-glycolic acid), polyethylene glycol or polycaprolactone, which have shown promise in combating bacterial infections.

1.4 COMMERCIALY AVAILABLE NANOFORMULATION

A nanoformulation typically refers to a formulation of materials at the nanoscale, generally ranging from 1 to 100 nanometres in size. These formulations are designed to take advantage of the unique properties that arise at the nanoscale, such as increased surface area, quantum effects, and altered chemical reactivity.

Nano formulations are used across various industries, including pharmaceuticals, cosmetics, food and beverage, agriculture, and electronics, to improve product performance, efficacy, and safety. They often involve techniques such as nanoparticle synthesis, nanoencapsulation, and nanocoating. Nano-medicine companies often offer Nano formulations designed to enhance the delivery of drugs, such as liposomal formulations for cancer treatment or nanoparticle-based formulations for targeted drug delivery. Many skincare companies utilize Nano formulations in their products to improve skin penetration and efficacy of active ingredients. Some companies use nanoencapsulation techniques to improve the stability and bioavailability of nutrients or to create novel textures and Flavours in food products.

Nano formulations are being explored for agricultural applications, such as nanoencapsulated pesticides or fertilizers to improve efficacy and reduce environmental impact. Nanostructured materials are used in electronics manufacturing for applications such as high-performance batteries, nanoscale transistors, and advanced displays.

1.5 NEED FOR NEW TRATMENT OPTION FOR MDR PATHOGENS

There is an urgent need for new treatments targeting MDR pathogens for several reasons is rising Prevalence. MDR pathogens are becoming increasingly common globally due to factors such as overuse of antibiotics, inadequate infection control practices, and the widespread use of antibiotics in agriculture. This prevalence underscores the importance of developing new treatments to combat these resistant organisms.

Conventional antibiotics are losing their effectiveness against MDR pathogens, leaving healthcare providers with limited treatment options. In some cases, infections caused by MDR pathogens may be untreatable with currently available antibiotics, leading to higher morbidity and mortality rates. MDR pathogens pose a significant public health threat as they can spread rapidly within healthcare settings, communities, and even across borders. Outbreaks of MDR infections can be difficult to control and may result in substantial healthcare burdens and economic costs. Developing new treatments for MDR pathogens is

crucial for preserving the efficacy of existing antibiotics. By providing alternative treatment options, new therapies can help reduce the selective pressure driving the emergence and spread of antibiotic resistance. To address the need for new treatments in MDR pathogens, several approaches are being explored. Researchers are actively searching for new antibiotics with unique mechanisms of action that can effectively target MDR pathogens. This includes screening natural sources, such as soil bacteria and marine organisms, for compounds with antimicrobial properties, as well as developing synthetic antibiotics through rational drug design and high-throughput screening.

Combining existing antibiotics with adjuvants or other antimicrobial agents can enhance their effectiveness against MDR pathogens. Synergistic drug combinations can overcome resistance mechanisms and improve treatment outcomes while reducing the risk of further resistance development. Alternative treatment modalities, such as phage therapy, antimicrobial peptides, and immune-based therapies, offer promising avenues for combating MDR pathogens. These approaches harness the power of the immune system or other biological agents to target and eliminate resistant microorganisms. Tailoring treatment strategies to individual patients based on their genetic makeup, microbiome composition, and other factors can optimize therapeutic outcomes and minimize the risk of resistance development. Precision medicine approaches enable healthcare providers to personalize treatment regimens and select the most effective therapies for combating MDR infections.

Repurposing existing drugs for antimicrobial purposes is a cost-effective strategy for rapidly identifying potential treatments for MDR pathogens. By screening libraries of FDA approved drugs and investigational compounds, researchers can identify candidates with activity against resistant microorganisms and expedite their clinical development.

CHAPTER 2

LITERATURE SURVEY

2.1 ORAL PATHOGENS

Oral microbial populations have established themselves in the human mouth. The precise anatomical structure, external connectivity, and humid environment all play a role in the complexity and ecological site specificity of the microbiome that has colonized it. The oral microbiota is influenced by a complex web of endogenous and exogenous variables that both cause and sustain its dynamic balance. Oral and even systemic disorders can result from the dyspeptic state, which is characterized by a change in the microbial composition and a disruption in the micro ecological balance between the host and microorganisms (Xinzhi Liu, Yanmei Liu 2022).

The mouth provides various unique habitats for microbial colonization, including the tongue, cheek, lip, gingival sulcus, connected gingiva, teeth, and hard and soft palate. As a result, the resident microbiota does not live in a uniform environment. The development of remarkably diverse microbial communities is supported by these oral habitats, which together constitute a very varied biological system. Many bacteria thrive in the warm, moist environment of the mouth, which also provides nutrients derived from the host, including saliva proteins, glycoproteins, and gingival crevicular fluid. Being the only naturally non-shedding surfaces in the human body, teeth offer special chances for the production of large biofilms as well as a safe sanctuary for the persistence of microorganisms (Kilian. M, Chapple 2016).

Almost more than 1000 ml of saliva is produced daily by adult's ends up in the digestive system. Consequently, oral microbes are crucial for preserving the internal stability of the intestinal micro ecosystem since they are a significant source of intestinal microbes. Numerous intestinal inflammatory illnesses are impacted by the oral cavity's virulent strains, which spread to the intestine via the digestive system or blood. The prevalence of inflammatory bowel disease is rising continuously in developing and industrialized nations alike, with the highest frequency seen in China, where it stands at 3.44 per 100,000 people. The genesis

of chronic non- specific intestinal inflammatory illnesses is poorly known, it is thought to be influenced by both environmental and genetic factors (Peng X, Cheng L 2022).

2.2 COPPER (Cu) NANOPARTICLES SIGNIFICATION

It is well known that copper has a wide range of antibacterial and antifungal properties. Many scientists are looking for new antibacterial drugs due to the rise in infectious diseases and the development of antibiotics (M.J.Hajipour *et al.*, 2012). Although copper is one of the materials that is most frequently utilized in a variety of applications, copper nanoparticles need to be synthesized with specific care because of their high susceptibility to rust. Copper is far more air- sensitive than gold or silver, and its oxide phases are also more thermodynamically stable. As a result, the antibacterial capabilities of copper nanoparticles are inexorably diminished as an oxide layer form on their surface (S.Jeong *et al.*, 2008) Several organic copper complexes of Ni, Cu and Co have recently been studied for their potential to fight cancer and microorganisms. They presumably work by binding metal ions and amino acids, which may release some potential donors of free atoms and increase biological activity (S.Chen *et al.*, 2001). Because chelates are extremely durable compounds that can keep the surrounding metal ions from an organic molecule (chelating agent) in place, it is best to prevent precipitating them as insoluble hydroxides during storage(J.J Lucena *et al.*, 2004). In this study, we present the antibacterial activity of copper nanoparticles and copper chelated species that are an intermediary. We recently published about a soybean aqueous extract-mediated synthesis of very stable copper nanoparticle (M.J. Guajardo Pacheco *et al.*, 2010). The major goal was to determine the antibacterial qualities of several copper species, and including copper chelates and copper nanoparticles. Such a comparison analysis would highlight strain differences and ultimately improve the use of copper amino acids chelates for particular applications in which copper nanoparticles could not be used (Alba-Montero *et al.*, 2017).

2.3 SILVER (Ag) NANOPARTICLES SIGNIFICANCE

Silver nanoparticles (Ag NPs) have been promoted as a superior antibacterial agent capable of battling bacteria that cause infections both in vitro

and vivo. Gram -negative And Gram -positive bacteria, including multidrug drug resistant strains are all covered by Ag Nps antibacterial ability. Ag Nps have several, concurrent mode of action, when combined with antibiotic or other antibacterial agents such as chemical compounds they have a synergistic effect on pathogenic bacteria like *Escherichia coli* and *staphylococcus aureus*. Due to their properties, silver nanoparticles well suited for use in medical and healthcare products where they can effectively treat or prevent infections. This review attempts to identify variables impacting the antibacterial and cytotoxic effects of silver nanoparticles, as Well as to reveal the urgent need for more effective antibacterial effects (Xi-Feng Zhang *et al.*, 2016)

2.4 CHEMICAL SYNTHESIS OF AG-CU NPs

Metal nanoparticles have garnered a lot of interest lately because of their exceptional high activity against numerous kinds of fungus and bacteria. Different from the comparable metallic bulk materials, these metal nanoparticles showed unique optical, electrical, and mechanical capabilities as well as increased reactivity because of their high surface to volume ratio. Moreover, from a scientific and technological standpoint, bimetallic composite nanoparticles-which are made up of two distinct metal elements are more intriguing than monometallic nanoparticles. Comparing bimetallic nanoparticles to monometallic nanoparticles, their structure is determined by the distribution modes of the two elements and can be orientated in random alloy, alloy with an intermetallic compound, cluster-in cluster, and core-shell configurations, all of which lead to enhanced applications. The expensive cost of silver nanoparticles (Ag NPs) prevents their usage in commercial products, despite their benign nature, high electrical and thermal conductivity, and potent oxidizing capabilities. The common methods used to create monodisperse colloidal solutions of silver nanoparticles include chemical reduction, electrochemical, photochemical, sonochemical, and pulsed laser (Kim Seah Tan, Kuan Yew Cheong 2013).

Researchers also looked at copper nanoparticles (Cu NPs), which are less costly and have comparable characteristics. But because they oxidize easily, the synthesis of the Cu NPs presents a challenge. Thus, a great deal of research is needed to create straightforward shape-controlled synthesis techniques for Ag-Cu

bimetallic nanoparticles, or Ag-Cu NPs. The currently developed synthesis techniques for Ag and Cu NPs comprise thermal degradation, chemical reduction, and polyol, laser cutting, and exposure to an electron beam. Due to its affordability and ease of usage, chemical reduction is the most favoured technique among these. Chemical reduction allows for the optimization of the experimental parameters, hence enabling control over size and distribution. Silver citrate and copper acetate were utilized as metal precursors to create bimetallic nanoparticles. A volume 3:1 ratio water-isopropanol solution was utilized as the solvent, PVP was used as a stabilizer, and sodium borohydride was used as a reducing agent. Different forms of bimetallic nanoparticles have been created, depending on the synthesis technique. A double-step reduction has been used to create the core-shell nanoparticles and a single-step reduction of the Ag-Cu alloy. It is vital to note that synthesis variables such as temperature, pH, and concentrations can all have a substantial impact on the characteristics of the nanoparticles produced. Optimizing these characteristics is frequently required to generate nanoparticles with the desired size, shape, and properties for specific applications. When working with chemicals and nanoparticles, it is also important to exercise prudence (Marta Paszkiewicz *et al.*, 2016).

2.5 AG-CU NANOPARICLE ACTIVITY ON CLINICAL ISOLATES

Due to their exceptional electrical and thermal properties, which have led to a significant demand for them in the market, silver (Ag) and copper (Cu) nanoparticles have demonstrated remarkable potential in a range of applications. Microbes, including bacteria and fungi, can easily develop durable biofilms on a variety of surfaces. These biofilms are very resistant to antibiotics and induce nosocomial and postsurgical infections. The antibacterial and antiviral properties of Ag and Cu nanoparticles (NPs) are well recognized, and several mechanisms for their effects have been hypothesized, including released ions, reactive oxygen species, contact killing, the immunostimulatory effect, and others. Ag and Cu nanoparticles, as well as their derivatives, have varying antibacterial and cytotoxic properties. Size, shape, and surface treatment all have an impact on antibacterial activity. Antimicrobial Ag and Cu NPs are applied biomedically by coating them on substrates such as textiles, polymers, ceramics, and metals. Because Ag and Cu

are immiscible, synthesized Ag-Cu nanoalloys exhibit distinct microstructures that influence their antibacterial properties. Ag and Cu NPs work in concert when combined to provide significantly improved antibacterial activity. We have a chance to find a solution thanks to nanotechnology as the antibiotic resistance of bacteria to medications increases. Novel antimicrobial compounds have been proposed for metal nanoparticles, sometimes known as nanobiotics. They can lessen or completely stop the ongoing development of bacterial resistance. Almost all of the metals utilized to make these NPs are heavy metals, such Ag and Cu, for thousands of years, silver has been used as an antibacterial agent since Hippocrates recommended its application for the treatment of ulcers. Ag NPs have found widespread usage in antimicrobial applications as nanotechnology has advanced, particularly in the fight against nosocomial infections and microorganisms resistant to antibiotics. Regarding Cu, its antibacterials have been known to have antibacterial properties since four thousand years ago. Indeed, the first report of Cu as an antibacterial agent before that of Ag. Despite this, antibacterial investigations of Ag are more widespread than those of copper. What is notable is that, in comparison to the enormous number of antimicrobial investigations of Ag or Cu, the number of publications involved in the combination of antimicrobial Ag and Cu nanoparticles is only about 300 (X.Yahia 2021)

The antibacterial activity of Ag-Cu nanoparticles against clinical isolates of various pathogens. Their study highlighted the potential of Ag-Cu nanoparticles as effective antimicrobial agents, particularly in combating multidrug-resistant bacteria. (Singh *et al.*, 2016)

Antimicrobial properties of metal nanoparticles, including Ag-Cu nanoparticles. Their survey encompassed studies examining the efficacy of Ag-Cu nanoparticles against clinical isolates of bacteria and fungi, shedding light on their mechanism of action and potential applications in clinical settings (Martinez-Castanon *et al.*, 2018) .The synergistic antimicrobial effects of Ag-Cu nanoparticles in combination with conventional antibiotics against clinical isolates, including *E. coli* and *S. aureus*. Their research demonstrated enhanced antibacterial activity and reduced antibiotic resistance development, suggesting the potential use of Ag-Cu nanoparticles as adjuvants to conventional therapy (Kim *et al.*, 2020).

The biocompatibility and antibacterial activity of Ag-Cu nanoparticles in clinical isolates of wound infections. Their study assessed the cytotoxicity and antimicrobial efficacy of Ag-Cu nanoparticles in vitro and in vivo, providing insights into their safety and efficacy for clinical use (Sanchez-Lopez *et al.*, 2021). Antimicrobial activity of biocompatible silver-copper nanoparticle hybrids with different morphologies Investigated the antimicrobial activity of Ag-Cu nanoparticles against clinical isolates of various pathogens, including *E. coli* and *S. aureus* (Singh, R. *et al.*, 2016). Antimicrobial activity and mechanism of action of silver-copper nanoparticles against clinical isolates of pathogenic bacteria Explored the mechanism of action of Ag-Cu nanoparticles and their effectiveness against clinical isolates of bacteria, including *E. coli* and *S. aureus*. (Martinez-Castanon *et al.*, 2022). Silver-copper nanoparticles as potential therapeutics for bacterial infections: Current status and future prospects, Reviewed the current status and future prospects of Ag-Cu nanoparticles as therapeutics for bacterial infections, including their effectiveness against clinical isolates of *E. coli* and *S. aureus* (Garcia-Garcia *et al.*, 2024).

2.6 THYMOQUINONE DRUG

Nigella sativa has long been a favourite herb among academics and healers both. It is a plant that blooms annually. Its linear lanceolate leaves grow to a height of 20–30 cm (7.9–11.8 inches). The fragile flowers are typically yellow, white, pink, pale blue or pale purple, with five to ten petals. The plant's fruit is a big, inflated capsule made up of three to seven connected follicles, each of which contains many seeds. The black seeds are funnel-shaped, flattened, angular, and oblong, measuring 0.2 cm in length and 0.1 cm in width (Forouzanfar F, Bazzaz 2014). It has historically been used in a variety of ways to treat a wide range of illnesses. Black cumin, which comes from *Nigella sativa* seeds, is a popular spice and condiment. Several types of *Nigella sativa* have been used in traditional medicine to treat a wide range of illnesses, such as influenza, fever, dizziness, eczema, headaches, bronchitis, asthma, hypertension, and diabetes. *Nigella sativa* is frequently used by cancer patients as a dietary supplement in complementary and alternative medicine in addition to chemotherapy, according to recent research findings from Muslim nations. Due to their apparent non-toxicity to healthy cells and easy digestibility, natural chemicals with anti-cancer effects have

attracted increasing attention. Researchers studying cancer generally agree that TQ exhibits encouraging anti-cancer properties. Evidence for the chemo preventive or chemotherapeutic action was shown by numerous studies. Consequently, it might be helpful as a dietary supplement to boost the benefits of anti-cancer medications (Khader. M *et al.*, 2014).

Low concentrations, TQ has a considerable effect on harmful bacteria. Because of its potencies to inhibit biofilm formation and operate as an antibiotic, we may recommend adding it to the list of bioactive chemicals and conducting additional study on it, including in vivo compatibility tests in various biological models. Strong antioxidant activity is exhibited by thymoquinone, which aids in the body's defence against dangerous free radicals. This characteristic helps shield cells from harm and lower oxidative stress. Thymoquinone anti-inflammatory effects include its ability to block a number of inflammatory mediators and pathways, which suggests that it may be helpful in treating inflammatory diseases. Such as inflammatory bowel illness, asthma, and arthritis. Antimicrobial Activity of thymoquinone has the ability to fight against a variety of diseases, such as bacteria, viruses, and fungus. It might help strengthen the immune system and fight infections. Thymoquinone's potential as an anticancer agent is being supported by an increasing amount of data. Its capacity to stop tumour growth, cause apoptosis (programmed cell death) in cancer cells, and stop metastasis in a number of cancer types, including pancreatic, breast, prostate, and colon cancer, has all been investigated. Preclinical research has shown that thymoquinone has neuroprotective qualities. It might lessen brain inflammation, shield neurons from harm, and enhance cognitive performance. Conditions such as Parkinson's disease and Alzheimer's disease may benefit from these effects. Thymoquinone has demonstrated possible cardio protective effects through heart function improvement and reduction of oxidative stress, inflammation, and cholesterol. It could be beneficial for managing and preventing cardiovascular illnesses. The capacity to shield the liver from harm brought on by poisons, medications, and illnesses has been studied. It might promote liver health by reducing oxidative stress, fibrosis, and inflammation in the liver (Khader *et al.*, 2014).

2.7 ILLNESS BROUGHT ON BY *S. AUREUS* AND *E. COLI*

These two bacteria are major contributors to a wide range of infections that are acquired in hospitals and the community. One of the most prevalent nosocomial organisms that cause enterocolitis and urinary tract infections is *E. coli*. Another etiological infection agent that contributes significantly to morbidity and mortality is *S. aureus*. As per the findings of the Broad Institute (2010), *Escherichia coli* is the second most common cause of infection after *Staphylococcus aureus* 18.8%, accounting for 17.3% of hospital-associated clinical infections. Globally, it has been noted in recent years that certain strains of *Escherichia coli* and *Staphylococcus aureus* have become resistant to certain antibiotics. These have grown to be a significant worldwide health concern, boosting the demand for novel antibacterial substances. This review covered these two bacteria's taxonomy, morphology, biochemical traits, growth and habitat features, infections they caused, antibiotic resistance, and treatment options (Ghania Bachir, B Abouni 2015).

A large number of extracellular proteins that are harmful to the host are expressed by *S. aureus*. Pathogenesis is complex for most diseases produced by this bacterium. The germs must enter the host and adhere to host tissues or cells in order to start an infection. Many surface proteins on *S. aureus* facilitate attachment to host proteins including fibronectin and laminin, which are components of the extracellular matrix. Fibronectin is a component of blood clots and can be found on the surfaces of epithelial and endothelial cells. The bacteria are able to adhere to blood clots and injured tissue because they possess a fibrinogen/fibrin binding protein. This explains why *S. aureus* can cause infections following surgery as well as wound infections. Numerous other elements also aid *S. aureus* in eluding the host's defence mechanisms. For instance, the surface of numerous strains contains a polysaccharide that may assist them in fending off macrophage phagocytosis. Surface protein A of *Staphylococcus aureus* binds immunoglobulin G molecules via the Fc region. This interferes with phagocytosis and opsonisation (Mandal, Ananya. 2023)

Gram-negative, facultative anaerobic *Escherichia coli* bacteria are a natural component of the gastrointestinal tract. These microorganisms are primarily

located in the large intestine and are often linked to bacterial illnesses. The breakdown of the gut mucosal membrane that results in local tissue invasion and maybe distant tissue seeding through bacteraemia can be the cause of these illnesses. It is believed that bacterial migration proximally up the ureter causes colonization and possible infection of the bladder and more proximal tissues, which leads to urinary tract infections. Cholecystitis, bacteraemia, cholangitis, urinary tract infections, traveller's diarrhoea, pneumonia, and new born meningitis are among the common illnesses caused by *E. coli* (Ryan.P *et al.*, 2023).

2.8 PREVALENCE OF ANTIBIOTIC RESISTANCE

Antibiotic resistance is the ability of microorganisms to resist the effects of antimicrobial agents. This phenomenon happens when an antibiotic loses its ability to effectively stop bacterial growth. It leads to infections that are harder and more expensive to treat, increased illness and death rates. If the current trend of inappropriate and excessive use of antibiotics continues, it is anticipated that million fatalities would be caused by antibiotic resistance by the year 2050. Despite the fact that the discovery of antibiotics has saved countless lives over the past 80 years, the development of the clinical and agricultural sectors would be endangered by rising levels of bacterial resistance to antibiotics, which could result in life threatening situations even for bacterial infections that were once treatable. Antibiotic resistance would make low middle income countries poorer, mainly because it would result in more prolonged hospital admissions, more expensive medical care, and premature deaths, all of which would negatively impact the country's overall production rate. Antibiotic resistance cases have been steadily rising in recent years, which may enhance the horizontal spread of the resistant gene and has also been associated with cross- resistance to different antibiotic families. The review summaries the economic cost of antibiotics resistance globally, highlights recent cases of antibiotics of antibiotic resistance primarily caused by *Escherichia coli*, *Acinetobacter baumannii*, *klebsiella pneumonia*, *Staphylococcus aureus* and *Salmonella spp.*, and discusses the common mechanisms underlying antibiotic resistance as well as potential counter measures (Thiruchelvi pulingam *et al.*, 2022). The fourth-generation penicillin are modified version of natural penicillin that are semi- synthetic that have the advantage of a wider spectrum of activity, especially against gram – negative

bacteria like *pseudomonas*, *Enterobacter*, *proteus* and *klebsiella* species (Rammelkamp *et al.*,2006). Addressing this issue requires concerted efforts from healthcare, professionals, policymakers, researchers and the public to promote responsible antibiotic use.

CHAPTER 3

AIM AND OBJECTIVES

3.1 AIM

To study the antibacterial activity of Ag-Cu nanoparticle conjugated thymoquinone against pathogenic bacterial.

3.2 OBJECTIVES

- To study the effect of Ag-Cu conjugated thymoquinone on the growth of pathogenic bacteria *E.coli* and *S.aureus*
- To analyse the antibacterial activity of Ag-Cu, thymoquinone against clinical bacterial isolates
- To validate the Ag-cu, thymoquinone drug effecacy in 100 clinical isolates

CHAPTER 4

MATERIALS AND METHODS

4.1 MEDIA AND PREPARATION

Mannitol salt agar and Macconkey agar were used to isolate the bacterial species from the clinical swab sample. As for the bacterial culture Luria Bertani broth is used. For carrying out Agar Well Diffusion method to observe zone of inhibition and antimicrobial susceptibility Mueller Hinton agar/ Luria Bertani agar are used. All the media used for this research work were prepared according to the manufacturer's instructions, autoclaved for 15 minutes at 121°C and were aseptically poured into sterile petri dishes.

4.2 STERILIZATION METHOD

All the glass wares, micropipette tips, cotton swabs were properly washed and sterilized in an autoclave at 121°C for 15 mins. Glass rods and work bench were sterilized with 75% of alcohol by cotton before and after each procedure.

4.3 SAMPLE COLLECTION

Around 106 Clinical bacterial isolates were collected from 10 to 15 orally affected patients in swabs (which are collected by our seniors and are ethically approved). These isolates are used to check the efficacy difference between drug and conjugated nanoparticle drug. These 106 clinical isolates are mainly consisting of *E. coli* and *S. aureus* bacteria.

4.4 NANOPARTICLE (Ag-Cu) CHEMICAL METHOD

Synthesis of Ag-Cu, a 0.05M concentration of silver nitrate and copper acetate are mixed in a 50 ml de-ionized water under stirring. Then 0.5M of sodium borohydride and sodium citrate is added to the above solution. Then, the mixture was stirred for another 2 hours. After that, the precipitate was collected by centrifuge and washed with acetone and ethanol for several times. Finally, the obtained wet sample was dried in a vacuum oven at 80 degrees Celsius for 6h. After collecting the dried samples, they were grinded into powder formation using motor and pestle.

4.5 PREPARATION OF NANOFLUID FROM Ag-Cu NPs

Sonication of nanofluids is achieved by providing sound energy to agitate the nanoparticles in suspension. It is a process which breaks large particles into smaller fragments or better uniform sized particles in the base fluid. After that 50mg of Ag-Cu is then added to the 1ml of sterile water and sonicate for half an hour. To get the nanofluid from nanoparticles.

4.6 Ag-Cu CHARACTERISTIC TEM ANALYSIS

The nanoparticles are typically dispersed onto a TEM grid, often coated with a thin carbon film or other suitable substrate. This ensures the nanoparticles are well distributed and immobilized for imaging. The TEM instrument is configured for high-resolution imaging, typically using a beam of electrons transmitted through the sample. Parameters such as acceleration voltage, beam current, and aperture size are optimized for the specific sample and desired imaging conditions. Images of the nanoparticles are captured by directing the electron beam through the sample and onto a detector. Multiple images may be acquired at various magnifications and orientations to fully characterize the nanoparticles. The acquired images are then analysed to determine the size, shape, distribution, and other morphological characteristics of the silver-copper nanoparticles. Specialized software may be used for image processing and analysis, including particle size distribution analysis and measuring distances between nanoparticles.

4.7 PREPARATION OF THYMOQUINONE DRUG SOLUTION

The first step in the process is to accurately measure 5 mg of thymoquinone. This compound, derived from *Nigella sativa*, is the active ingredient in the solution. Next, 5 mg of ethanol is added to the thymoquinone. Ethanol serves as a solvent, facilitating the dissolution of thymoquinone. The choice of ethanol is significant as it's a polar solvent, which is compatible with many organic compounds, including thymoquinone. Once the thymoquinone and ethanol are combined, the mixture is shaken. This agitation helps to distribute the thymoquinone evenly throughout the ethanol, promoting dissolution. The solution is stored in a refrigerator at -4 degrees Celsius. This low temperature helps to preserve the stability of the thymoquinone, preventing degradation that could compromise its antibacterial efficacy.

4.8 PREPARATION OF Ag-Cu NPs CONJUGATED THYMOQUINONE DRUG

Initially, 100 mg of Ag-Cu nanoparticles are measured and added to 2.5 ml of sterile water in a container. Simultaneously, 10 mg of Thymoquinone is measured and added to 5 ml of ethanol. The solubility of Thymoquinone in ethanol is checked. If not, an additional 5 ml of ethanol is added to ensure complete dissolution of thymoquinone.

The Thymoquinone solution is then added drop wise to the Ag-Cu nanoparticle solution during vortexing in a vortex mixer. This step ensures the even distribution of thymoquinone on the surface of the Ag-Cu nanoparticles. The vortexing is carried out for 20 seconds, after which the solution is placed in an ice bag for 5 minutes. This rapid cooling helps to stabilize the newly formed Ag-Cu and Thymoquinone conjugates. This process of vortexing and cooling is repeated over a period of 4 hours.

4.9 Ag-Cu NPs CONJUGATED THYMOQUINONE DRUG FESEM ANALYSIS

The SEM is a microscope that uses electrons instead of light to form an image. FESEM is typically performed in a high vacuum because gas molecules tend to disturb the electron beam and then emits secondary and backscattered electrons used for imaging. FESEM images and the Energy Dispersive X-ray of these samples were obtained by placing a minimal amount of the powder sample on the sample holder using carbon tape. Crystallite size, morphology and elemental composition of these samples were determined by analysing FESEM images.

4.10 STUDY OF ANTIBIOTICS EFFECT AGAINST CINICAL ISOLATES

Effectiveness of antibiotics against clinical isolates is crucial for understanding drug resistance and developing new treatments. It involves testing various antibiotics against bacteria other pathogens isolated from clinical samples to determine which drugs are most effective in combating infections.

4.10.1 Piperacillin

Piperacillin is a broad-spectrum Beta-lactam antibiotic of the ureidopenicillin class. The chemical structure of piperacillin and other ureidopenicillin incorporates a polar side chain that enhances penetration into Gram-negative bacteria and

reduces susceptibility to cleavage by Gram-negative Beta lactamase enzymes. These properties confer activity against the important hospital pathogen *Pseudomonas aeruginosa*. Thus, piperacillin is sometimes referred to as an antipseudomonal penicillin.

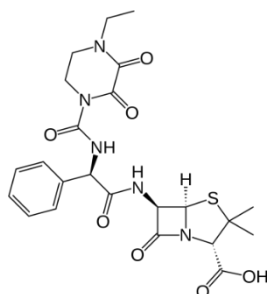


Fig 4.1: Piperacillin

4.10.2 Ertapenem

Ertapenem, sold under the brand name invanz, is a Carbapenems antibiotic medication used for the treatment of the infection of abdomen and lungs

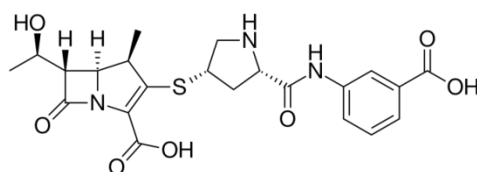


Fig 4.2: Ertapenem

4.10.3 Imipenem

Imipenem (trade name primaxin among others) is an intravenous beta-lactam antibiotic discovered by Merck scientists Burton Christensen, William Leanza and Kenneth Wildonger. In the mid 1970s. Carbapenems, are highly resistant to the beta-lactamase enzymes produced by multiple drug resistant gram-negative bacteria, thus play a key role in the treatment of infections not readily treated with other antibiotics. (Vardakas KZ *et al.*, 2012).

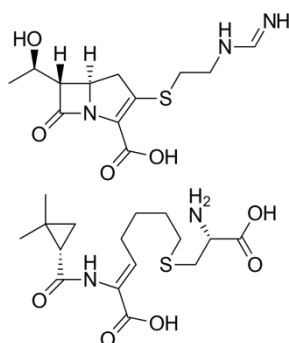


Fig 4.3: Imipenem

4.10.4 Meropenem

Meropenem, sold under the brand name Merrem among others, is an intervenors, Beta-lactam antibiotic used to treat variety of bacterial infections. Some of these include Meningitis, intra-abdominal infection, pneumonia, sepsis and anthrax.

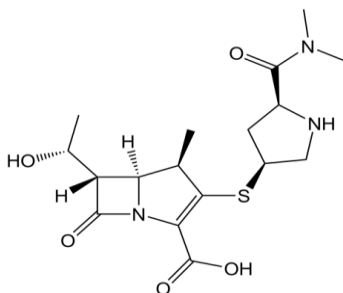


Fig 4.4: Meropenem

4.10.5 Methicillin

Methicillin, also known as methicillin, is a narrow spectrum beta-lactam antibiotic of the penicillin class.

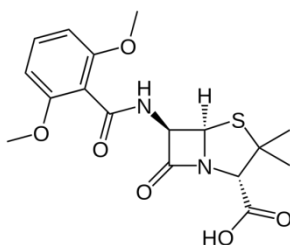


Fig 4.5: Methicillin

4.11 SPREAD PLATE TECHNIQUE

Spread plate techniques are a microbiological method of evenly distributing bacteria (*E. coli* and *Staphylococcus*) across the surface of a solid growth medium like LB agar. This method involves spreading a liquid sample containing the microorganisms onto the surface of the agar using a sterile spreading tool, such as a glass spreader. Bacterial colonies are then grown at 37°C for 24 hours in an incubator.

4.11.1 Well plate method

The hole with a diameter of 6 to 8 mm is punched aseptically with a sterile cork borer or a tip, and a volume (20–100 µL) of the antimicrobial agent or extract solution at desired concentration is introduced into the well. Then, agar plates are incubated under suitable conditions depending upon the test microorganism. The antimicrobial agent diffuses in the agar medium and inhibits the growth of the microbial. In well plate method multiple wells are used. Each well contains a different concentration of the antimicrobial agents (NP Ag-Cu & Drug & Conjugated drug), this creates a concentration of different range of inhibition. The size of these zones can give an indication of the microbial agents against the bacterial isolates. We are using three different agents - Nanoparticle Ag-Cu, Thymoquinone drug and Conjugated drug

4.11.2 Effect of Ag-Cu nanoparticle on growth of clinical isolates

The effect of nanoparticles on bacterial growth refers to the impact that tiny particles, typically ranging from 1 to 100 nanometres in size, have on the proliferation and viability of bacterial cells. Nanoparticles can exert different effects on bacterial growth depending on various factors such as their composition, size, shape, surface properties, concentration, and the characteristics of the bacterial species involved. Silver -copper, nanoparticles antimicrobial properties and can inhibit bacterial growth or even kill bacterial cells

Antibacterial activity of *S.aureus* and *E. coli*, Copper as monovalent and Ag-Cu nanoparticles were carried out using well diffusion method against pathogenic bacteria. These bacterial cultures were freshly cultivated for 24hrs in Luria Bertani broth. Each bacterial culture were spread on the LB agar plates. Four different concentrations of Ag-Cu nanoparticles were placed and incubated. After 24 hours

of incubation, the zone formation was recorded. The experiments were repeated for several times in different isolates.

4.11.3 Effect of thymoquinone drug on growth of clinical isolates

Thymoquinone is a compound found in black seed oil, extracted from the seeds of *Nigella sativa*, a plant native to Southwest Asia. Thymoquinone, a compound found in black seed oil, exhibits significant antibacterial activity against a range of bacterial strains including both Gram-positive and Gram-negative species. Its mechanism of action involves disrupting bacterial cell membranes, inhibiting enzymes, and interfering with DNA replication. Notably, thymoquinone targets specific bacteria such as *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, and *Helicobacter pylori*.

4.11.4 Effect of conjugated drug on growth of clinical isolates

Thymoquinone has known antibacterial properties, and silver and copper nanoparticles have also been studied for their antimicrobial effects. When combined, these compounds may act synergistically to inhibit bacterial growth through various mechanisms, such as disrupting bacterial cell membranes, interfering with essential enzymes, or generating reactive oxygen species that damage bacterial DNA. Conjugating drugs with nanoparticles can enhance their efficacy in inhibiting bacterial growth. Nanoparticles offer advantages such as increased surface area and the ability to deliver drugs directly to bacterial cells. When combined with drugs, nanoparticles can improve drug stability, solubility, and bioavailability. Additionally, nanoparticles can penetrate bacterial biofilms and overcome bacterial resistance mechanisms, making them effective against a wide range of bacterial species.

4.12 LIQUID ASSAY – ANTIBACTERIAL ACTIVITY TEST

Liquid assay/Broth micro- or macro-dilution is one of the most basic antimicrobial susceptibility testing methods. The procedure involves preparing of the antimicrobial agent (Ag-Cu NPs, thymoquinone drug and conjugated drug) in a liquid growth medium dispensed in tubes containing a minimum volume of 3 ml (macro dilution). Then, each tube is inoculated with a microbial inoculum prepared in the same medium after dilution of standardized microbial

suspension adjusted to 30mg/ml concentration. After well-mixing, the inoculated tubes are kept in shaking incubator at 37°C for 24 hours incubation. Next morning after collecting the test tubes efficacy was observed for the concentration of bacterial growth in spectrophotometry. This method followed by sufficient growth, the efficacy of the nanoparticle, drug and the conjugated drug can be compared by observing the concentration of bacterial growth.

4.12.1 Validation of effect of Ag-Cu NPs

Liquid growth medium was dispensed in test tubes containing a minimum volume of 3 ml. From 24 hours grown isolated Bacterial culture, 30mg/ml is taken and mixed in the inoculated tubes, and then 30mg/ml microbial agent Ag-Cu Nano fluid is added. After well-mixing, the inoculated test tubes are kept in shaking incubator at 37°C for 24 hours. After sufficient growth, the efficacy of the nanoparticle can be compared by observing the concentration of bacterial growth in spectrophotometry.

4.12.2 Validation of effect of thymoquinone drug

Liquid growth medium was dispensed in test tubes containing a minimum volume of 3 ml. From 24 hours grown isolated Bacterial culture, 30mg/ml is taken and mixed in the inoculated tubes, then 30mg/ml of microbial agent thymoquinone drug solution is added. After well-mixing, the inoculated test tubes are kept in shaking incubator at 37°C for 24 hours. After sufficient growth, the efficacy of the nanoparticle can be compared by observing the concentration of bacterial growth in spectrophotometry.

4.12.3 Validation of effect of conjugated drug

Liquid growth medium was dispensed in test tubes containing a minimum volume of 3 ml. From 24 hours grown isolated Bacterial culture 30mg/ml is taken and mixed in the inoculated tubes, then 30mg/ml of microbial agent conjugated drug solution is added. After well-mixing, the inoculated test tubes kept in shaking incubator at 37°C for 24 hours. After sufficient growth, the efficacy of the nanoparticle can be compared by observing the concentration of bacterial growth in spectrophotometry. These experiments were repeated for several times.

4.13 GROWTH CURVE

Growth curves are fundamental in studying the growth and replication patterns of various organisms, including bacteria. Typically, a bacterial growth curve involves four main phases- lag phase, exponential (log) phase, stationary phase, and death phase. Prepare 10 ml broth medium add 30mg/ml isolates culture sample then added antibacterial agent Ag-Cu NPs, Thymoquinone drug and conjugated drug for 10mg/ml mixed well then kept on shaking incubator at 37°C for one hour once.

CHAPTER 5

RESULTS AND DISCUSSION

5.1 BROTH PREPARATION

Nutrient media is prepared by following concentrations; For a 100 ml solution, 2.5g of LB broth, 2g of Agar is added with 100ml water. Once the LB broth and agar is combined, the mixture is typically autoclaved to sterilize the medium, as well as to completely dissolve the agar.



Fig: 5.1: LB Broth

5.2 SAMPLE PLATE TEST

106 Clinical bacterial isolates which are collected from 10 to 15 orally affected patients as swabs (which are collected by our seniors and were ethically approved).



Fig: 5.2: E. coli - EMB agar



Fig5.3: S. aureus - MS agar



Fig: 5.4: Clinical isolates for 100 samples

5.3 CHEMICAL SYNTHESIS OF Ag-Cu NPs:

To synthesis Ag-Cu, a 0.05M concentration of silver nitrate and copper acetate were mixed in 50 ml de-ionized water under stirring. Then 0.5M of sodium borohydride and sodium citrate were added to the above solution. Then, the mixture was stirred for another 2h. After that, the precipitate was collected by centrifuge and washed with acetone and ethanol for several times.

Finally, the obtained wet sample was dried in a vacuum oven at 80 degrees Celsius for 6h. After collect the dried sample grind the powder formation using mortar and pestle.



Fig: 5.5: Ag-Cu chemical synthesis

5.4 AG-CU NANOFLUID PREPARATION

Take 50mg of Ag-Cu then add 1ml of sterile water and sonicate for half an hour.



Fig: 5.6: Ag-Cu Nanofluid

5.5 AG-CU TEM ANALYSIS

Multiple images may be acquired at various magnifications and orientations to fully characterize the nanoparticles. The acquired images are then analysed to determine the size, shape, distribution, and other morphological characteristics of the silver-copper nanoparticles.

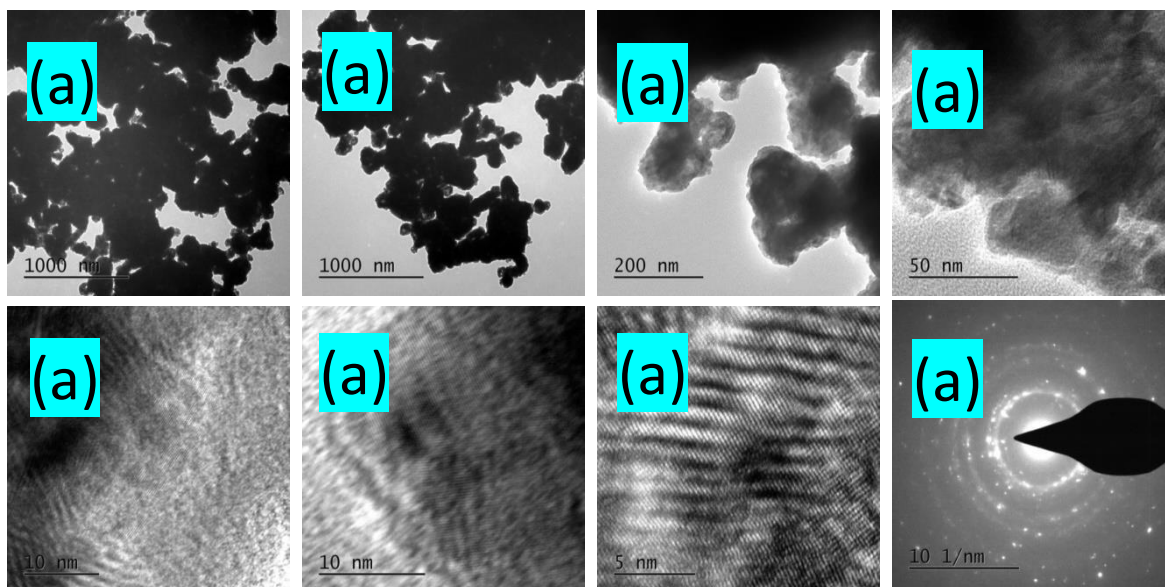


Fig: 5.7: Ag-Cu HR-TEM

5.6 THYMOQUINONE DRUG SOLUTION PREPARATION

Prepared drug solution is stored in a refrigerator at -4 degrees Celsius, after collecting the complete solution.

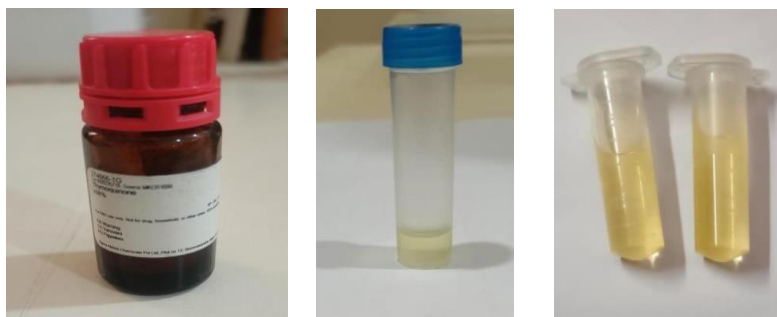


Fig: 5.8: Thymoquinone drug solution

5.7 AG-CU NPs CONJUGATED THYMOQUINONE DRUG PREPARATION

The conjugated solution is kept in rapid cooling, which helps to stabilize the newly formed Ag-Cu and Thymoquinone conjugates.

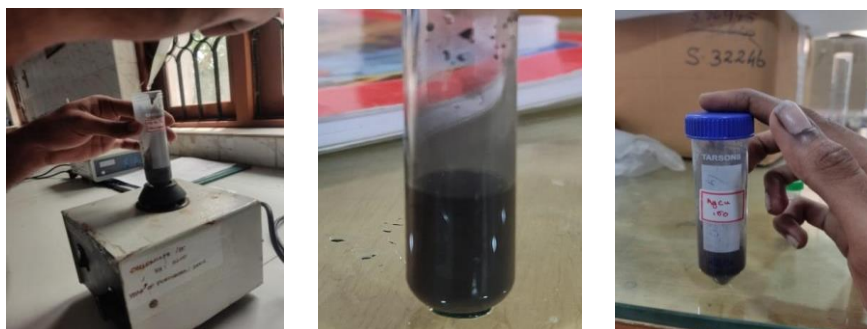


Fig: 5.9: Conjugated drug

5.8 Ag-Cu NPs CONJUGATED THYMOQUINONE DRUG FESEM ANALYSIS

FESEM images and the Energy Dispersive of these samples were obtained by placing a minimal amount of the powder sample on the sample holder using carbon tape. Crystallite size, morphology and elemental composition shown in fig: 5.10 .

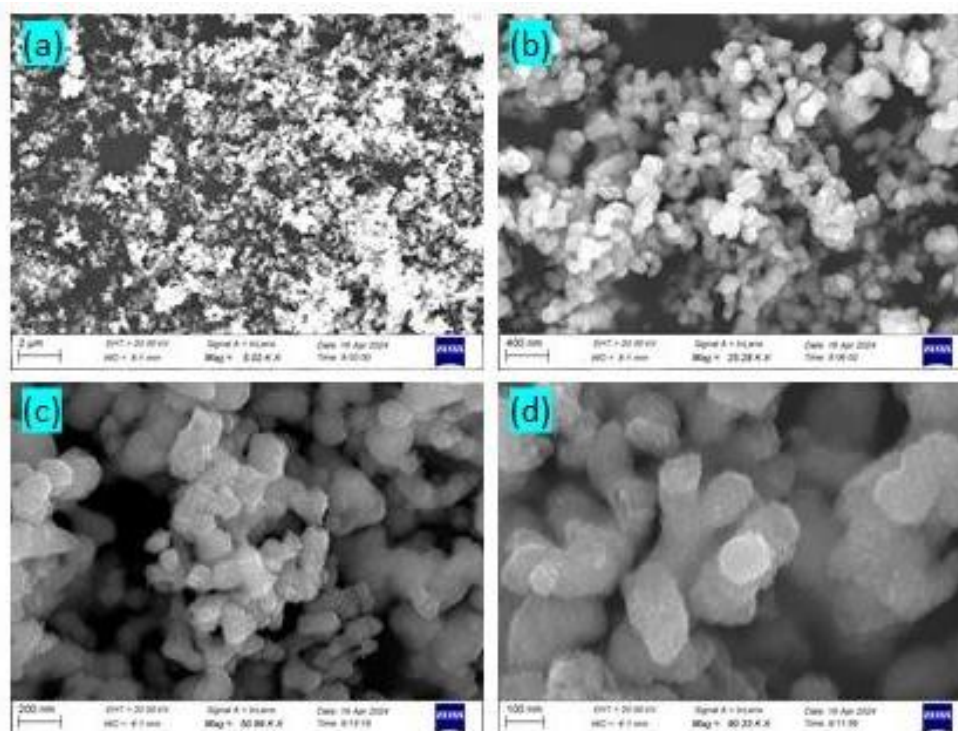


Fig: 5.10: Conjugated drug FESEM result

5.9: ANTIBIOTICS SUSCEPTIBILITY TEST FOR CINICAL ISOLATES

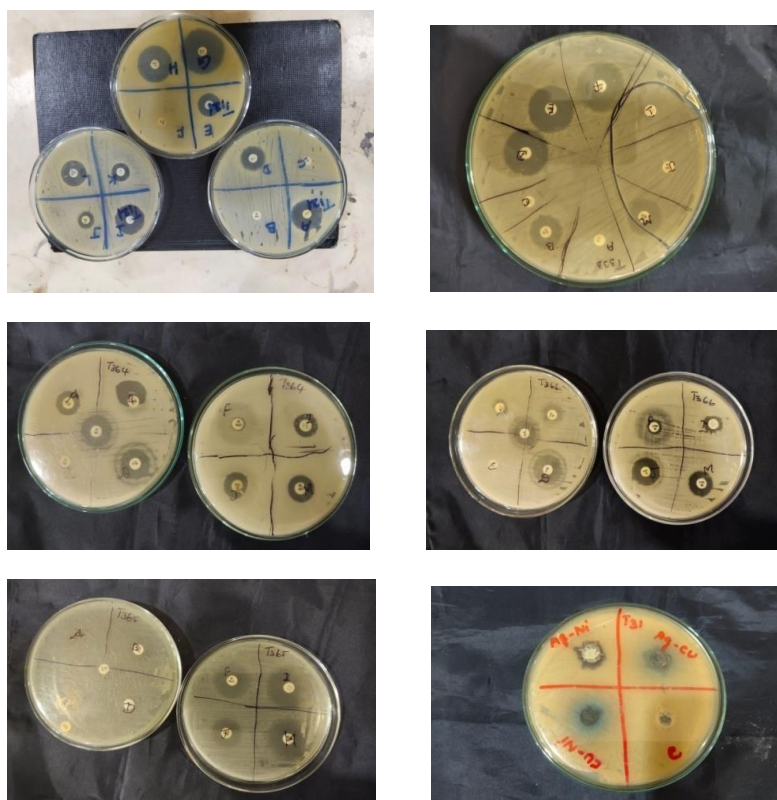


Fig 5.11: Antibiotics susceptibility test for clinical isolates

Different type of antibiotics (Piperacillin, Ertapenem, Imipenem, Meropenem, Methicillin) are used were observed for variable zone inhibition.

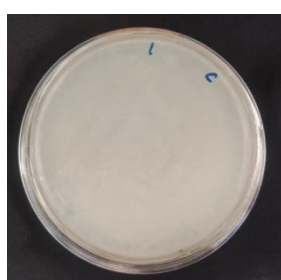
Table 5.1: Zone of inhibition (diameter-mm) against various antibiotics for clinical isolates

CULTURE NO	PIPRACILLIN	IMIPENEM	MERAPENEM	ERTAPENAM	METHICILLIN
CC1-01	5	9	30	0	13
CC1-02	7	20	30	10	10
CC1-03	0	21	30	12	18
CC1-04	3	19	13	12	11
CC1-05	0	14	12	6	12
CC1-06	4	18	17	5	15
CC2-51	4	17	15	6	9
CC2-52	0	8	12	0	8

CC2-53	0	9	15	0	8
CC2-54	0	8	11	0	14
CC2-55	0	11	13	0	12
CC2-56	0	9	14	0	11
CC2-57	0	9	15	0	12

5.10 SPREAD PLATE

Clinical isolates culture sample (*E. coli* and *Staphylococcus aureus*) completely spread evenly using L-Rod.



E. coli



S. aureus

Fig: 5.12: Control plate of clinical isolates

5.10.1 Well method

After preparing the agar plates, four holes will be punched for 6 to 8 mm. These four wells contain four different concentrations (10mg/ml, 30mg/ml, 50mg/ml, and 100mg/ml) of microbial agents Ag-Cu NPs, Thymoquinone drug and conjugated drug.

5.10.2 Effect of nanoparticles on growth of clinical isolates

Four distinct concentrations of nanoparticle inhibit the growth of bacteria in varying zone inhibition sizes, which are according to the measurements present in the well. Ag-Cu NPs less effect on zone of inhibition shown in fig:5.12.1



Fig: 5.12.1: Nanoparticles Ag-Cu effect on clinical isolates

1)10mg/ml 2)30mg/ml 3)50mg/ml 4)100mg/ml

Table 5.2: Zone of inhibition (diameter mm) for Ag-Cu against clinical isolates

CULTURE NO	10mg/ml	30mg/ml	50mg/ml	100mg/ml
CC101	12	13	28	40
CC102	11	17	26	39
CC103	10	16	25	36

As the concentration of the Ag-Cu NPs increases (from 10mg/ml to 100 mg/ml), the zone of inhibition appears to increase for both *E. coli* and *S. aureus*. This suggests that a higher concentration of the sample has a stronger inhibitory effect on the bacterial growth. The zone of inhibition for *E. coli* are generally larger than those for *S. aureus* for both sample and Ag-Cu NPs.

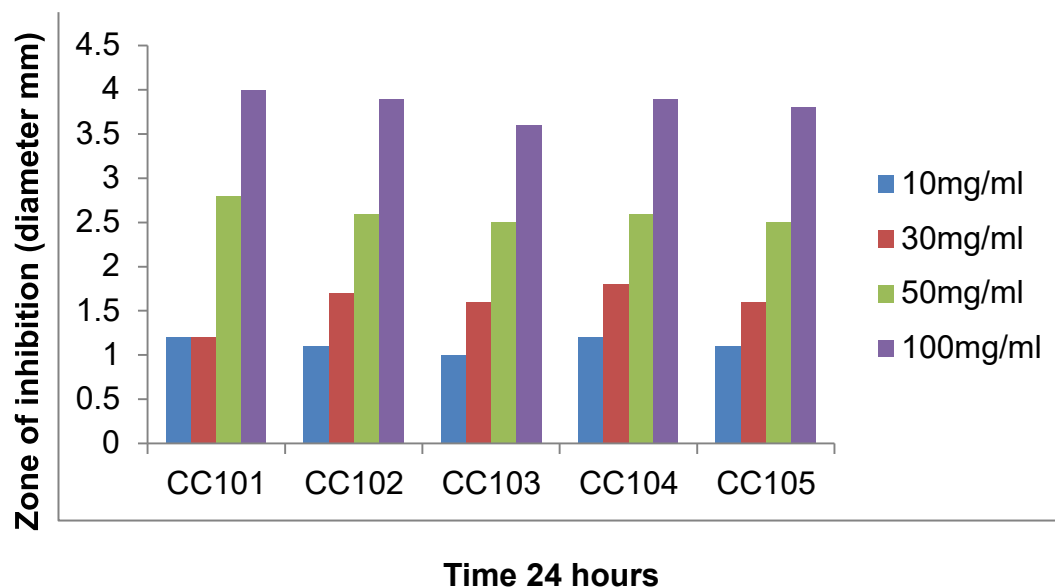


Fig: 5.12.2: Nanoparticle Ag-Cu antibacterial assay

5.10.3 Effect of thymoquinone drug on growth of clinical isolates

When the concentration of the drug increases (from 10mg/ml to 100 mg/ml), the zone of inhibition appears to increase for both *E. coli* and *S. aureus*. This suggests that a higher concentration of the sample has a stronger inhibitory effect on the bacterial growth. The zones of inhibition for *E. coli* are generally larger than those for *S. aureus* for both sample and thymoquinone drug. Different concentration-10mg/ml, 30mg/ml, 50mg/ml and 100mg/ml.

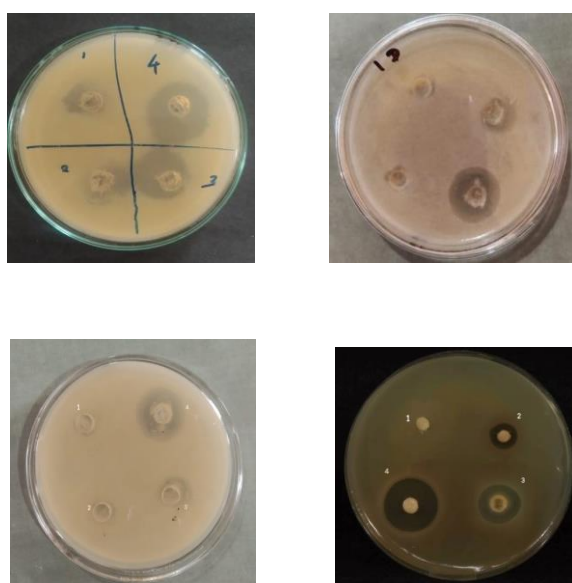


Figure 5.12.3: Thymoquinone drug effect on clinical isolates

Table 5.3: Zone of inhibition (diameter mm) for thymoquinone drug against clinical isolates

CULTURE NO	10mg/ml	30mg/ml	50mg/ml	100mg/ml
CC101	15	21	28	40
CC102	16	22	30	43
CC103	14	18	27	39

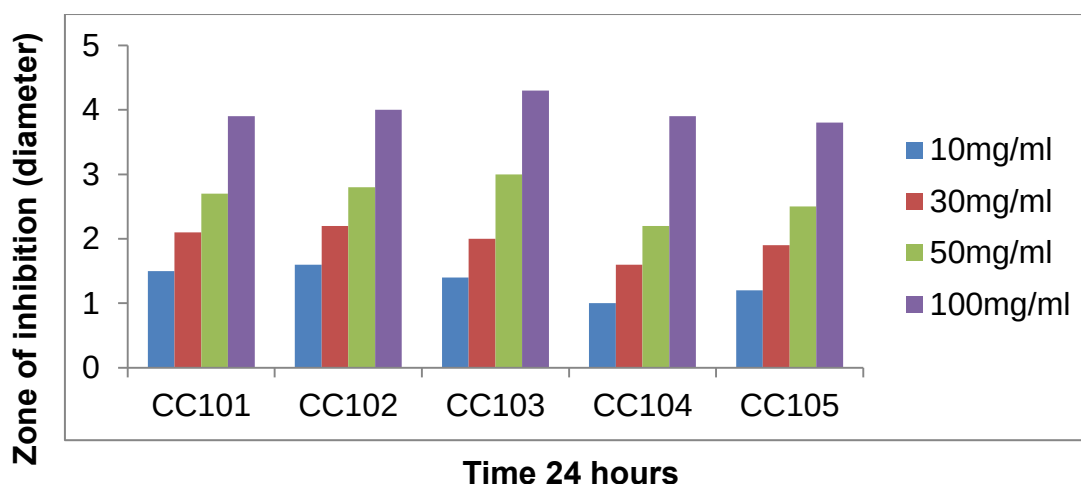
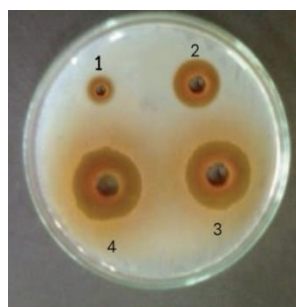
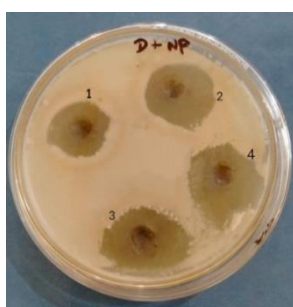


Fig: 5.12.4: Thymoquinone drug antibacterial assay

5.10.4 Effects of conjugated drug on growth of clinical isolates

As the concentration of the conjugated drug increases (from 10mg/ml to 100 mg/ml), the zone of inhibition appears to increase for both *E. coli* and *S. aureus*. This suggests that a higher concentration of the sample has a stronger inhibitory effect on the bacterial growth. The zones of inhibition for *E. coli* are generally larger than those for *S. aureus* for both sample and conjugated drug.



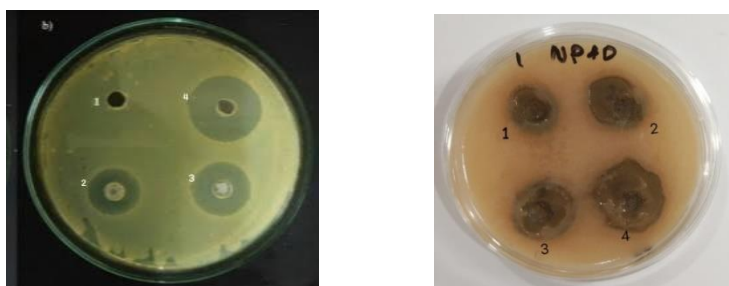


Figure 5.12.5: Conjugated drug effect on clinical isolates

1)10mg/ml 2)30mg/ml 3)50mg/ml 4)100mg/ml

Table 5.4: Zone of inhibition (diameter mm) conjugated drug against clinical isolates

CULTURE NO	10mg/ml	30mg/ml	50mg/ml	100mg/ml
CC101	18	22	29	58
CC102	16	20	25	54
CC103	19	25	32	69

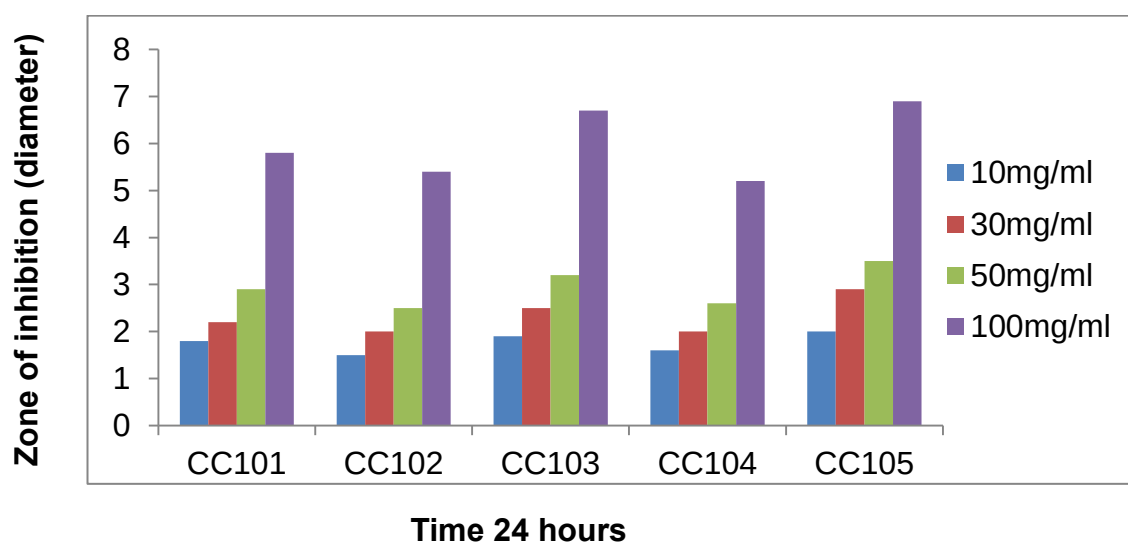


Fig: 5.12.6: Conjugated drug antibacterial assay

5.11 LIQUID ASSAY- ANTIBACTERIAL ACTIVITY

In the prepared broth medium, 30mg/ml of bacterial culture and 30 mg/ml of microbial agents (Ag-Cu NPs, Thymoquinone drug, and conjugated drug) are added, and mixed well. Place these tubes in a shaking incubator at 37°C to promote the development of bacteria. Following adequate growth, the concentration of bacterial growth in spectrometry can be used to compare the effectiveness of the drug, conjugated drug, and nanoparticle. The concentration of these can indicate how well a medication for nanoparticle, thymoquinone drug and conjugated drug work against the clinical isolates of bacteria.

5.11.1 Check the efficacy of Ag-Cu NPs liquid assay

In a 3ml of broth, 30mg/ml nanofluid solution is added and mixed well, and then it was kept in a shaking incubator at 37°C for overnight growth.



Fig: 5.13: Ag-Cu NPs effect on liquid assay

Table 5.5 Ag-Cu nanofluid added for different concentration reading (OD)

Culture No	control	10mg/ml	30mg/ml	50mg/ml	70mg/ml	100mg/ml	150mg/ml
CC101	1.25	1.10	0.89	0.69	0.57	0.49	0.34
CC201	1.28	1.07	0.84	0.65	0.54	0.47	0.31

5.11.2 Validation on effect of thymoquinone for 100 cultures

In a 3ml of broth, 30mg/ml thymoquinone drug solution is added and mixed, then it was kept in a shaking incubator at 37°C for overnight growth.

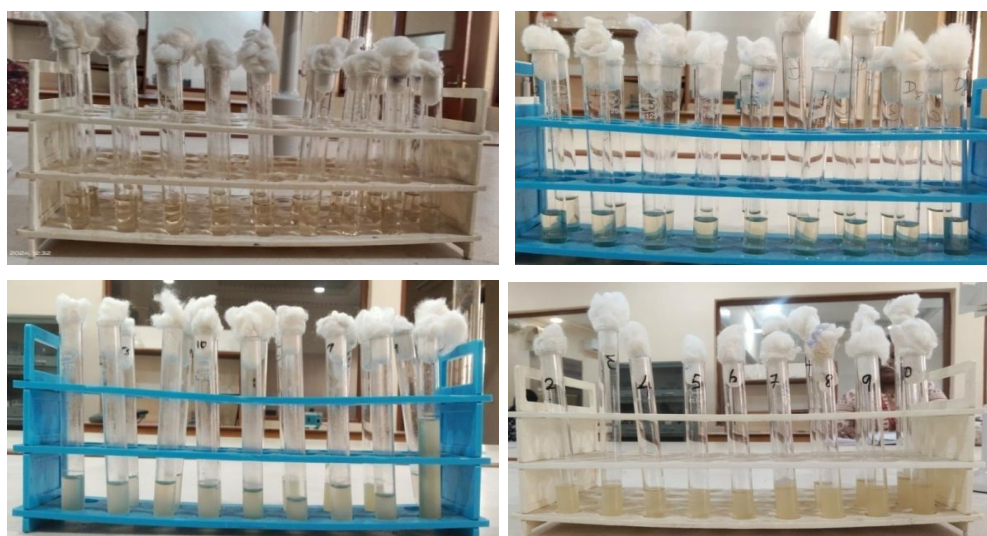


Fig: 5.13.1: Thymoquinone drug efficacy on liquid assay

The concentration of the sample decreases the efficacy for both *E. coli* and *S. aureus*.

Table 5.6: Thymoquinone drug against 100 clinical isolates reading (OD)

CULTURE NO	THYMOQUINONE DRUG
CC1-01	0.51 OD
CC1-02	0.55 OD
CC1-03	0.52 OD
CC1-04	0.49 OD
CC1-05	0.46 OD
CC1-06	0.54 OD
CC1-07	0.58 OD
CC1-08	0.58 OD
CC1-09	0.60 OD
CC1-10	0.57 OD
CC1-11	0.56 OD
CC1-12	0.60 OD
CC1-13	0.54 OD
CC1-14	0.43 OD
CC1-15	0.42 OD
CC1-16	0.43 OD
CC1-17	0.44 OD
CC1-18	0.50 OD
CC1-19	0.47 OD
CC1-20	0.43 OD
CC1-21	0.47 OD
CC1-22	0.59 OD
CC1-23	0.49 OD
CC1-24	0.47 OD
CC1-25	0.41 OD

CC1-26	0.52 OD
CC1-27	0.56 OD
CC1-28	0.53 OD
CC1-29	0.47 OD
CC1-30	0.58 OD
CC1-31	0.56 OD
CC1-32	0.47 OD
CC1-33	0.54 OD
CC1-34	0.56 OD
CC1-35	0.52 OD
CC1-36	0.46 OD
CC1-37	0.47 OD
CC1-38	0.48 OD
CC1-39	0.64 OD
CC1-40	0.51 OD
CC1-41	0.45 OD
CC1-42	0.57 OD
CC1-43	0.55 OD
CC1-44	0.53 OD
CC1-45	0.48 OD
CC1-46	0.47 OD
CC1-47	0.55 OD
CC1-48	0.41 OD
CC1-49	0.52 OD
CC1-50	0.49 OD
CC2-51	0.55 OD
CC2-52	0.46 OD
CC2-53	0.50 OD
CC2-54	0.47 OD

CC2-55	0.55 OD
CC2-56	0.54 OD
CC2-57	0.50 OD
CC2-58	0.47 OD
CC2-59	0.49 OD
CC2-60	0.52 OD
CC2-61	0.46 OD
CC2-62	0.45 OD
CC2-63	0.53 OD
CC2-64	0.54 OD
CC2-65	0.51 OD
CC2-66	0.50 OD
CC2-67	0.43 OD
CC2-68	0.49 OD
CC2-69	0.44 OD
CC2-70	0.51 OD
CC2-71	0.54 OD
CC2-72	0.46 OD
CC2-73	0.47 OD
CC2-74	0.50 OD
CC2-75	0.56 OD
CC2-76	0.49 OD
CC2-77	0.45 OD
CC2-78	0.53 OD
CC2-79	0.52 OD
CC2-80	0.50 OD
CC2-81	0.48 OD
CC2-82	0.44 OD
CC2-83	0.47 OD

CC2-84	0.53 OD
CC2-85	0.50 OD
CC2-86	0.48 OD
CC2-87	0.51 OD
CC2-88	0.50 OD
CC2-89	0.44 OD
CC2-90	0.46 OD
CC2-91	0.48 OD
CC2-92	0.51 OD
CC2-93	0.55 OD
CC2-94	0.52 OD
CC2-95	0.49 OD
CC2-96	0.46 OD
CC2-97	0.54 OD
CC2-98	0.58 OD
CC2-99	0.43 OD
CC2-100	0.51 OD
CC1-101	0.48 OD
CC1-102	0.47 OD
CC1-103	0.55 OD
CC1-104	0.41 OD
CC1-105	0.52 OD
CC1-106	0.49 OD

5.11.3 Validation on effect of conjugated drug for 100 cultures

In a 3ml of broth 30mg/ml conjugated drug solution is added and mixed well, and then it was kept in a shaking incubator at 37°C for overnight growth.



Fig: 5.13.2: Conjugated drug efficacy on liquid assay

The concentration of the sample decreases the efficacy for both *E. coli* and *S. aureus* shown in table: 5.7

Table 5.7: Conjugated drug against 100 clinical isolates reading (OD)

CULTURE NO	THYMOQUINONE DRUG	CONJUGATED (NP+DRUG)
CC1-01	0.51OD	0.25 OD
CC1-02	0.55 OD	0.29 OD
CC1-03	0.52 OD	0.21 OD
CC1-04	0.49 OD	0.19 OD
CC1-05	0.46 OD	0.14 OD
CC1-06	0.54 OD	0.23 OD
CC1-07	0.58 OD	0.19 OD
CC1-08	0.58 OD	0.21 OD
CC1-09	0.60 OD	0.29 OD
CC1-10	0.57 OD	0.22 OD
CC1-11	0.56 OD	0.21 OD
CC1-12	0.60 OD	0.29 OD
CC1-13	0.54 OD	0.18 OD

CC1-14	0.43 OD	0.11 OD
CC1-15	0.42 OD	0.27 OD
CC1-16	0.43 OD	0.15 OD
CC1-17	0.44 OD	0.19 OD
CC1-18	0.50 OD	0.24 OD
CC1-19	0.47 OD	0.07 OD
CC1-20	0.43 OD	0.08 OD
CC1-21	0.47 OD	0.09 OD
CC1-22	0.59 OD	0.21 OD
CC1-23	0.49 OD	0.19 OD
CC1-24	0.47 OD	0.13 OD
CC1-25	0.41 OD	0.16 OD
CC1-26	0.52 OD	0.29 OD
CC1-27	0.56 OD	0.37 OD
CC1-28	0.53 OD	0.31 OD
CC1-29	0.47 OD	0.20 OD
CC1-30	0.58 OD	0.26 OD
CC1-31	0.56 OD	0.24 OD
CC1-32	0.47 OD	0.18 OD
CC1-33	0.54 OD	0.29 OD
CC1-34	0.56 OD	0.23 OD
CC1-35	0.52 OD	0.38 OD
CC1-36	0.46 OD	0.12 OD
CC1-37	0.47 OD	0.19 OD
CC1-38	0.48 OD	0.07 OD
CC1-39	0.64 OD	0.35 OD
CC1-40	0.51 OD	0.16 OD

CC1-41	0.45 OD	0.23 OD
CC1-42	0.57 OD	0.28 OD
CC1-43	0.55 OD	0.23 OD
CC1-44	0.53 OD	0.19 OD
CC1-45	0.48 OD	0.11 OD
CC1-46	0.47 OD	0.09 OD
CC1-47	0.55 OD	0.28 OD
CC1-48	0.41 OD	0.12 OD
CC1-49	0.52 OD	0.25 OD
CC1-50	0.49 OD	0.19 OD
CC2-51	0.55 OD	0.24 OD
CC2-52	0.46 OD	0.10 OD
CC2-53	0.50 OD	0.19 OD
CC2-54	0.47 OD	0.12 OD
CC2-55	0.55 OD	0.22 OD
CC2-56	0.54 OD	0.20 OD
CC2-57	0.50 OD	0.18 OD
CC2-58	0.47 OD	0.14 OD
CC2-59	0.49 OD	0.16 OD
CC2-60	0.52 OD	0.21 OD
CC2-61	0.46 OD	0.13 OD
CC2-62	0.45 OD	0.11 OD
CC2-63	0.53 OD	0.18 OD
CC2-64	0.54 OD	0.21 OD
CC2-65	0.51 OD	0.19 OD
CC2-66	0.50 OD	0.17 OD

CC2-67	0.43 OD	0.09 OD
CC2-68	0.49 OD	0.12 OD
CC2-69	0.44 OD	0.08 OD
CC2-70	0.51 OD	0.23 OD
CC2-71	0.54 OD	0.26 OD
CC2-72	0.46 OD	0.14 OD
CC2-73	0.47 OD	0.14 OD
CC2-74	0.50 OD	0.19 OD
CC2-75	0.56 OD	0.24 OD
CC2-76	0.49 OD	0.17 OD
CC2-77	0.45 OD	0.15 OD
CC2-78	0.53 OD	0.21 OD
CC2-79	0.52 OD	0.20 OD
CC2-80	0.50 OD	0.18 OD
CC2-81	0.48 OD	0.10 OD
CC2-82	0.44 OD	0.07 OD
CC2-83	0.47 OD	0.17 OD
CC2-84	0.53 OD	0.23 OD
CC2-85	0.50 OD	0.20 OD
CC2-86	0.48 OD	0.15 OD
CC2-87	0.51 OD	0.17 OD
CC2-88	0.50 OD	0.18 OD
CC2-89	0.44 OD	0.11 OD
CC2-90	0.46 OD	0.13 OD
CC2-91	0.48 OD	0.15 OD
CC2-92	0.51 OD	0.25 OD

CC2-93	0.55 OD	0.29 OD
CC2-94	0.52 OD	0.21 OD
CC2-95	0.49 OD	0.19 OD
CC2-96	0.46 OD	0.14 OD
CC2-97	0.54 OD	0.23 OD
CC2-98	0.58 OD	0.19 OD
CC2-99	0.43 OD	0.09 OD
CC2-100	0.51 OD	0.16 OD

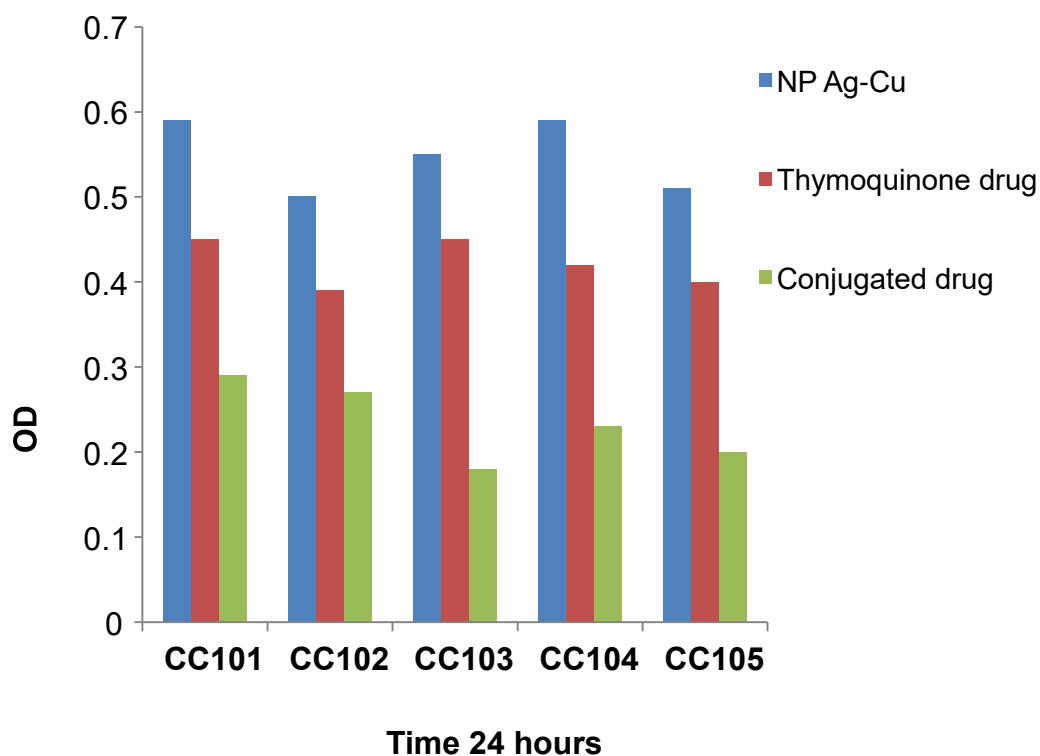


Fig: 5.13.3: Comparison of Ag-Cu NPs, Thymoquinone and conjugated drug

Conjugated drug highly effect compare to NPs and thymoquinone shown in fig:5.13.3

5.12 GROWTH CURVE FOR AG-CU NPs, THYMOQUINONE DRUG AND CONJUGATED DRUG AGAINST CLINICAL ISOLATES

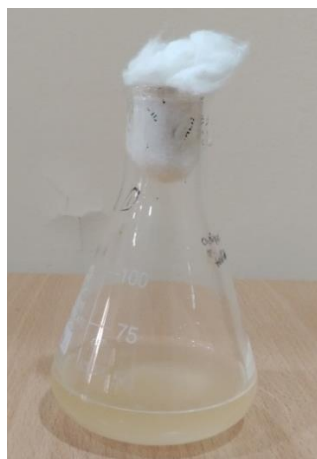


Fig: 5.14: Culture control growth medium

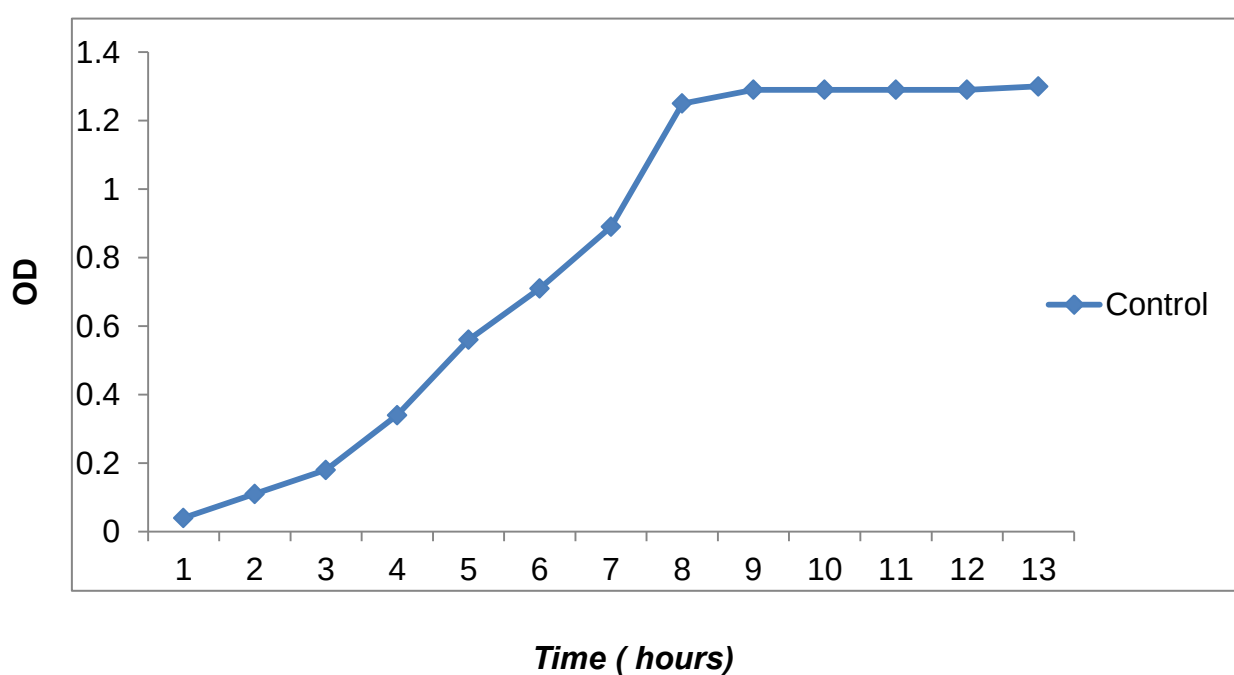


Fig: 5.14.1: Culture control growth curve

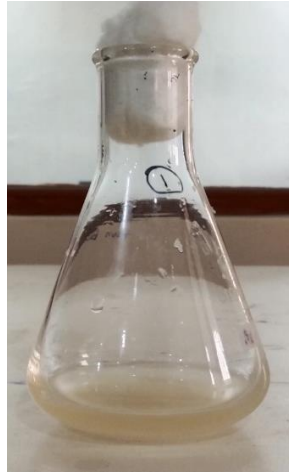


Fig: 5.14.2: Thymoquinone drug add culture growth medium



Fig: 5.14.3: Conjugated drug add culture growth medium

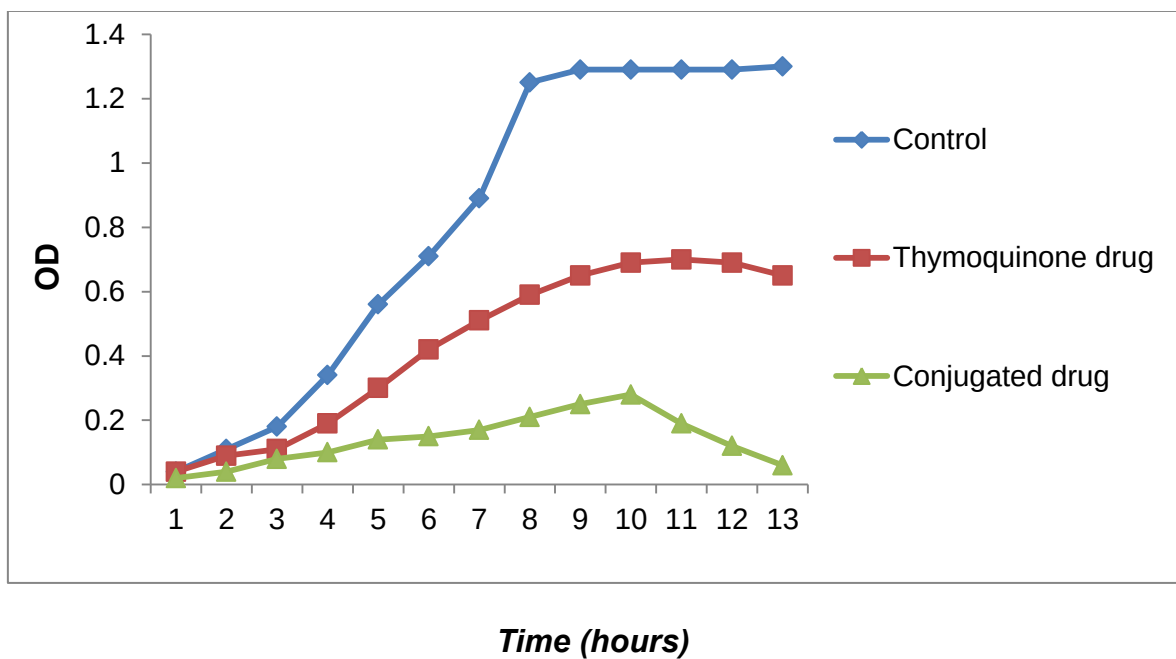


Fig: 5.14.4: Growth curve for S.aureus

Conjugated drug highly effect for clinical isolates for *S.aureus* and *E.coli* compare to NPs and thymoquinone shown in fig: 5.14.5

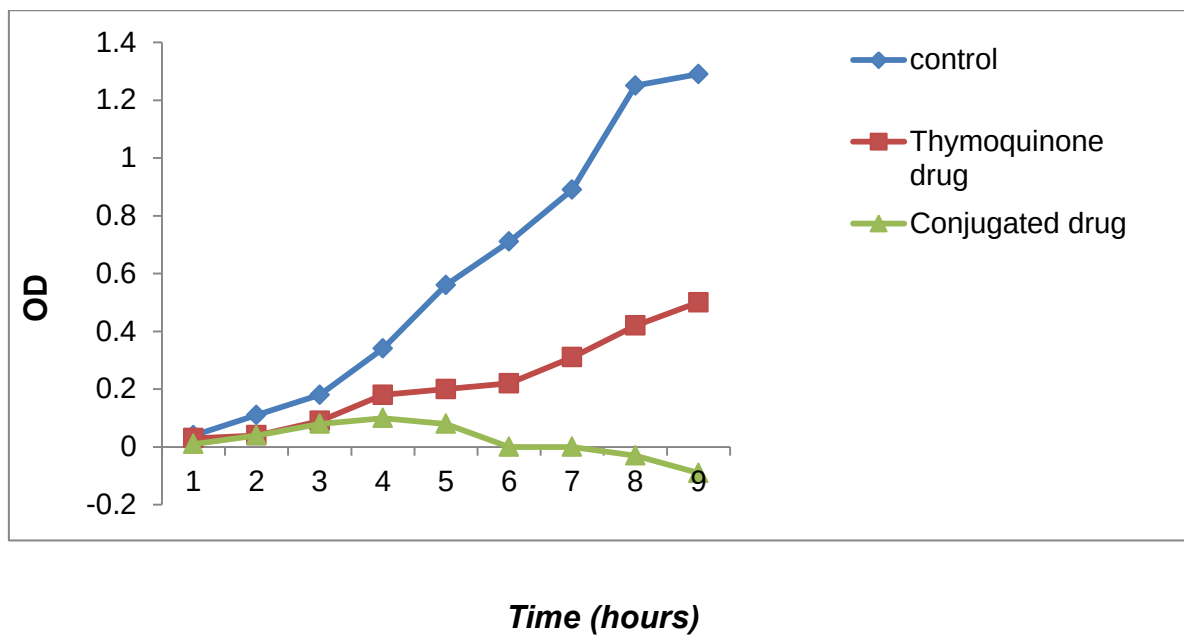


Fig: 5.14.5: Growth curve for *E.coli*

CHAPTER-6

SUMMARY AND CONCLUSION

Around 160 samples were collected and clinically isolated from 10 to 15 orally infected patients as swabs, in which 50 samples are *Staphylococcus* and 56 samples are *E. coli*, where it was collected by our seniors (ethically approved). The colony morphology of these isolates was golden yellow on MSA and pink coloured, circular, slightly raised and smooth colonies on macconkey agar. This was done as plating for identification and confirmation of the isolates. To check the antibiotic resistant pattern of bacterial isolates from different region of clinical isolates we used the commercial antibiotic disc such as Piperacillin, Ertapenem, Imipenem, Meropenem and Methicillin. This project is done to demonstrate the antibacterial effect of Ag-Cu NPs, Thymoquinone drug and Ag-Cu NPs conjugated thymoquinone drug against Multi drug Resistant Bacteria. As by the procedures observed above, less effect is observed when the bacterial isolates were treated with Ag-Cu NPs and thymoquinone drug. And effective results were observed when the bacterial isolates were treated with Ag-Cu NPs conjugated thymoquinone drug. Thus, our study using clinical bacterial isolates from swabs of orally affected patients proved that our Ag-Cu NPs conjugated thymoquinone drug is efficient in killing the bacterial cells. Ag-Cu nanoparticle conjugated thymoquinone against multidrug-resistant clinical isolates exhibits promising results. Our study confirmed the enhancement of thymoquinone activity when combined with NPs against these MDR bacteria. More than 200 clinical isolates are needed to be examined to check the efficacy and to collect a complete statistical data.

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