

ISOLATION AND IDENTIFICATION OF MULTI-DRUG RESISTANT BACTERIAL PATHOGENS FROM CLINICAL SAMPLES IN AND AROUND CHENNAI AND EFFICACY OF SELECTED HERBAL PLANT EXTRACTS

Submitted in partial fulfillment of the requirements for the award of

Bachelor of Science Degree in

Microbiology by

NANCY ROY M S (Reg.no 40300042)



DEPARTMENT OF BIOTECHNOLOGY

SCHOOL OF BIO AND CHEMICAL ENGINEERING

SATHYABAMA

INSTITUTE OF SCIENCE AND TECHNOLOGY

(DEEMED TO BE UNIVERSITY)

Accredited with Grade "A" by NAAC

JEPPIAAR NAGAR, RAJIV GANDHI SALAI, CHENNAI - 600119

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This is to certify that this Project Report is the bonafide work of

M S Nancy Roy (40300042) who carried out the project entitled
"ISOLATION AND IDENTIFICATION OF MULTI-DRUG RESISTANT BACTERIAL
PATHOGENS FROM CLINICAL SAMPLES IN AND AROUND CHENNAI AND
EFFICACY OF SELECTED HERBAL PLANT EXTRACTS" under our supervision
from September 2022 to March 2023.

INTERNAL GUIDE

(Dr. JAYASHREE)

HEAD OF THE DEPARTMENT

Dr. V. RAMESH KUMAR

EXTERNAL GUIDE

(Dr. D. SARAVANAN)

Submitted for viva voce examination held on 09/05/2023

Internal Examiner

Dr. Jayashree Nellore

External Examiner

Dr. Rakar

DECLARATION

I **M S Nancy Roy (40300042)** v hereby declare that the Project Report entitled **"Isolation and identification of multi-drug resistant bacterial pathogens from clinical samples in and around Chennai and efficacy of selected herbal plant extracts"** done by me under the guidance of **Dr. JAYASHREE (Internal)** and **Dr. D. Saravanan, Scientist, (External)** at **Centre for Laboratory Animal Technology and Research (CLATR), Sathyabama Institute of Science and Technology, Chennai** is submitted in partial fulfillment of requirements for the award of Bachelor of Science degree in Microbiology.

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SIGNATURE OF THE CANDIDATE

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ABSTRACT

Human health care is one of the most important topics for society. A microorganism that has the ability to cause disease is called pathogen. The purpose of the study is to find the microorganisms which are Multi-Drug Resistant. Clinical isolates were collected from hospitals in and around Chennai. The species level identification was then carried out by standard methods and by comparing their characteristics with those of known taxa. Susceptibility test were performed by Kirby – Bauer disc diffusion method. Since antimicrobial resistance patterns are continuously evolving and multi - drug resistant (MDR) organisms undergo progressive antimicrobial resistance, continuously updated data on antimicrobial susceptibility profiles will continue to be essential to ensure the provision of safe and effective empiric therapies. The preliminary screening of selected herbal plants, including *Syzygium cumini* and *Terminalia arjuna*, against Multi-Drug Resistant *Staphylococcus aureus* pathogens will be concluded in this in-vitro study. In further study, secondary screening will be carried out to check the MIC of the herbal plant extracts against MDR microorganisms.

KEYWORDS : Multi-Drug Resistant, *Staphylococcus aureus* , *Syzygium cumini*, *Terminalia arjuna*

LIST OF ABBREVIATIONS

❖ AST	- Antibiotic Susceptibility Test
❖ CLSI	- Clinical and Laboratory Standards Institute
❖ CRE	- Carbapenem Resistant Enterobacterales
❖ ESBL	- Extended spectrum Beta Lactamase
❖ ExPEC	- Extraintestinal Pathogenic <i>Escherichia Coli</i>
❖ HAI	- Hospital Acquired Infection
❖ IMViC	- Indole Methyl Red Voges Proskauer Citrate Utilization Test
❖ MDR	- Multi Drug Resistant
❖ MHA	- Mueller Hinton Agar
❖ MIC	- Minimum Inhibitory Concentration
❖ MRS	- Methicillin Resistant <i>Staphylococcus</i>
❖ MRSA	- Methicillin Resistant <i>Staphylococcus Aureus</i>
❖ MR-VP	- Methyl Red-Voges Proskauer
❖ PBP	- Penicillin-binding protein
❖ SCC	- Staphylococcal cassette chromosome
❖ UPEC	- Uropathogenic <i>Escherichia coli</i>
❖ UTI	- Urinary Tract Infection
❖ VRE	- Vancomycin Resistant Enterococcus
❖ VRSA	- Vancomycin Resistant <i>Staphylococcus Aureus</i>
❖ XDR	- Extensively Drug Resistant
❖ ZOI	- Zone Of Inhibition

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CHAPTER 1

INTRODUCTION

A pathogen is a disease causing organism, which shows various disease symptoms in the host. The severity of the disease symptoms is called as virulence. Pathogens can be categorized into 5 main groups: Bacteria, virus, protozoa, fungi and worms.

In our study, we mainly focus on the pathogenic bacteria. Though bacteria are natural commensals in the human body, there are still a hundreds of bacteria which cause infectious diseases in humans. Pathogenic bacteria also causes high infant mortality rates in developing countries. Even the free living commensal bacteria in the human body can turn into pathogens when threatened. People with weaker immune systems are more vulnerable to harmful microorganisms. Under some circumstances, such as penetration through a cut or wound, through sexual activity, or through weakened immune systems, some pathogenic bacteria can cause diseases.

Nowadays, the greatest challenges in the world health system are the rise of pathogenic microorganisms resistant to antibiotics. Antimicrobial resistance to at least one antimicrobial drug in three or more antimicrobial categories is known as Multiple Drug Resistance, Multidrug Resistance, or Multi Drug Resistance. Antimicrobial categories group antimicrobial substances according to the target organisms they are effective against. Infections caused by Multidrug-Resistant (MDR) represents serious problem for people health. At first, these MDR organisms were restricted to hospital environment, but now they can be found everywhere.

The increasing global development of multi-resistant and pan-resistant bacteria commonly referred to as "superbugs," which cause diseases that cannot be treated with current antimicrobial medications like antibiotics, is particularly concerning.

High doses of antibiotics administered to humans, farm animals, and even fish in aquaculture led to the selection of bacterial pathogens that were resistant to a range of medications. Bacteria may develop multidrug resistance through one of two ways. A bacterium may possess several resistance genes, each of which provides antibiotic resistance. Resistance genes are usually accumulated on tiny DNA

fragments called plasmids that can be exchanged across bacteria in a single event. The alternative hypothesis is that a single resistance mechanism results in resistance to multiple antibiotics called Cross-Resistance. (Nikaido, Hiroshi., 2009)

The most frequent type of resistance is acquired and transmitted horizontally through the conjugation of a plasmid. The increase in the prevalence of MDR indicates having very few antimicrobial agents to treat infections caused by these pathogens. One of the difficult issues for biomedical scientists has been overcoming bacterial drug resistance. Infections related to healthcare increase morbidity and mortality rates worldwide. Antimicrobial resistance restricts antibiotic therapy and makes it hard to treat conditions brought on by MDR microorganisms and this is heavily linked to the rise in mortality.

High rates of resistance against the major antibiotics used to treat common bacterial diseases, such as urinary tract infections, sepsis, sexually transmitted infections, and various types of diarrhea, have been documented globally, suggesting that we are running out of effective antibiotics.

Among the gram-positive species, *Staphylococcus aureus*, *Enterococcus faecium*, *Enterococcus faecalis*, and *Streptococcus pneumoniae* are the most frequent problem.

Staphylococcus aureus is responsible for a wide range of clinical illnesses. This bacterium frequently causes infections, both in conditions where they exist in the community and in hospitals. Therapy is still challenging due to the emergence of bacteria like MRSA that are multi-drug resistant. Normal healthy skin does not become infected by *S. aureus*; but, if the germs are permitted to reach internal tissues or the bloodstream, they may cause a number of potentially dangerous diseases.

Globally, MRSA is a significant cause of hospital-acquired infections (HAI). MRSA prevalence in India ranges from 20% to 80%. People with Methicillin-Resistant *Staphylococcus aureus* (MRSA) infections are 64% more likely to die than people with drug-sensitive infections. (World Health Organization Antimicrobial Resistance).The threat posed by MRSA had time for research to respond. As a

result, there are recently established medicines that are efficacious against vancomycin-resistant MRSA.

The evolution of gram-negative infections which are resistant to almost all of the known medications could pose a far more significant danger. Among the gram-negative strains, *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, and *Acinetobacter baumannii* have been most common (Vivas, Roberto, et al.2019)

In hospitalized patients, *E.coli* can lead to bloodstream infections, pneumonia, and urinary tract infections. One of the most frequent causes of HAIs is *E.coli*. Some *E.coli* strains are said to as multidrug-resistant because they have developed a high level of resistance to many, if not all drugs.

One of the major microorganisms responsible for urinary tract infections (UTIs) is Uropathogenic *Escherichia coli* (UPEC). Recent years have seen a sharp rise in the prevalence of UPEC and multidrug-resistant bacteria, which could make treatment more challenging. (Allocati, Nerino, et al.2013)

Pseudomonas aeruginosa isolates that are extensively drug-resistant (XDR) and multidrug-resistant (MDR) are frequently the cause of serious nosocomial infections and may affect the choice of antibiotic therapy. MDR/XDR-*P.aeruginosa* has traditionally been treated with conventional antipseudomonal Beta-lactam antibiotics as an initial course of treatment.(Kunz Coyne AJ.,et al,2022)

The cholera pathogen is able to adapt to unfavorable environmental conditions quickly and resist the negative effects of antimicrobial drugs thanks to its exceptional competency and distinctive genetic make-up. The MDR enteric pathogen *Vibrio cholerae*, which causes the acute watery diarrheal illness *cholera*, has gained notoriety in recent years. (Garbern, Stephanie C., et al.2021). *Salmonella* develops multidrug resistance as a result of efflux pumps that expel drugs from bacterial cells.

It is extremely concerning that gram negative bacteria have developed recombinant beta-lactamases that can degrade cephalosporins and carbapenams. Such

organism infections increase morbidity, mortality, length of hospital stay, and treatment costs. In more recent decades, "pan-resistant" strains of *Pseudomonas aeruginosa* and *Acinetobacter baumannii* have developed as a result of the majority of major pharmaceutical companies ceasing to develop new antibacterial drugs. As a result, there are hardly any medications that could be intended to treat these strains.

The most often prescribed medications for treating *A.baumannii* are carbapenems, which are Beta-lactams. These bacteria are challenging to eradicate due to the high amount of acquired and inherent carbapenem resistance mechanisms they have developed. The pharmaceutical sector lacks an answer to this issue. Finding an appropriate substitute for carbapenem, a routinely recommended medication for *Acinetobacter* infection, is therefore urgently needed.

Carbapenem-Resistant Enterobacterales (CRE) are considered the main causative agents of nosocomial infections. The two most prevalent bacterial infections are VRE and MRSA , and hospitals have also isolated these pathogens from water, animals, and animal products.(Vivas, Roberto, *et al.* 2019). MDR *P. aeruginosa*, Carbapenem-resistant *Enterobacteriaceae*, and *A.baumannii* have been mainly associated with clinical samples, but some strains have also been isolated from foods, animals, and water.

In *P.aeruginosa* infections, unsuitable treatment has been linked to a higher death rate, and delaying the initiation of adequate therapy may extend hospital stays and worsen infection persistence. Since there is a limited number of effective antibiotic treatments, MDR infections may lead to even worse clinical outcomes.

Resistance to fluoroquinolone antibiotics in *E.coli*, used in treating the UTI are widespread. For life-threatening infections brought on by *Enterobacteriaceae* that are resistant to carbapenem, colistin is the only treatment left (i.e. *E.coli*, *Klebsiella*, etc.). Moreover, colistin-resistant bacteria have been found in numerous nations and regions, where they are producing diseases for which there is now no efficient antibiotic treatment.

The number of people whose treatment is failing or who pass away from illnesses will rise in the absence of efficient methods for the prevention and sufficient

treatment of drug-resistant diseases, as well as enhanced access to current and novel antimicrobials with high levels of quality assurance.

According to statistics on infections brought on by MDR bacteria, bacterial resistance to antibiotics has multiplied significantly in recent years. Infections caused by multidrug-resistant organisms are challenging to treat as they do not respond to even the most potent medications. Even though, certain medicines can still help most people with MDRO management. The doctor will look for the specific type of MDRO that is causing the sickness. This can aid in making the optimal antibiotic choice. Therapy with the incorrect antibiotic can delay healing and make the infection more difficult to treat.

People diagnosed have larger doses of antibiotics and other medications, which could cause severe difficulties. A low HAI rate and high adherence to infection control procedures help the hospital's reputation. Multidrug-resistant (MDR) microorganisms have risen alarmingly in the last few decades, posing a severe threat to human health. Morbidity and mortality have increased along with the prevalence of infections caused by MDR bacteria, making the need for proper treatment of bacterial resistance an important and unmet problem.

If no new medication is created or discovered, it is predicted that there won't be any effective antibiotics available to treat illnesses by the year 2050. This necessitates seeking for alternate means of containing antibiotic-resistant infections, and numerous research organizations around the globe are already actively looking for fresh approaches. This goal is to identify the most prevalent bacteria that are resistant to antibiotics and outline alternate approaches that have been suggested to control them.(Vivas, Roberto, *et al.* 2019) Because of this, scientists have begun focusing on herbal remedies in an effort to find fresh inspiration for creating more effective medications to combat MDR microorganism strains.

The current rise in antibiotic resistance drives the quest for novel medications to tackle resistant microbes. Thus, due to the variety of plants' secondary metabolites and their capacity to exert antimicrobial activities, species that are frequently used as herbal medicines appear biologically active components separated from plant as a good alternative (Tchana ME, *et al.* 2014). Due to these issues, there is resurgence in interest in research into alternative antimicrobials and more focused treatment

methods, such as those employed before the development of antibiotics, as well as a return to conventional treatments and medications. While many of these alternative therapies can be applied topically, some of them can be used systemically. These new treatments could support antibiotic therapy and lessen the spread and emergence of antibiotic resistance in the future.

Natural remedies have been utilized in traditional medicine for thousands of years and predate the invention of antibiotics and other contemporary medications. Unbelievable claims have been made about the antibacterial effectiveness of several plants in the treatment of diseases. Although just 1% of flowering plants have been recognized by current scientists, local people are thought to have employed roughly 10% of them to treat various illnesses. Searches for plants that contain antimicrobial compounds are common since they are widely used as treatments for numerous infectious diseases (Khan, Rosina, *et al* 2009)

Many secondary metabolites found in plants, including tannins, alkaloids, and flavonoids, have been shown to exhibit antibacterial activities *in vitro*. Due to their accessibility, lack of adverse effects, and low toxicity, a variety of medicinal plants have been recommended in several phytotherapy guides for treating infectious disorders. Several herbal extracts have been reported to have antibacterial properties. Many plants have been discovered to treat skin infections, respiratory illnesses, gastrointestinal issues, and urinary tract infections (Khan R., *et al*, 2009). Significant antibiotic activity against MRSA has also been demonstrated by pleuromutilin and its derivatives. Protein synthesis inhibition may be the likely mechanism. By altering the permeability of the bacterial inner membrane, they stop such drug resistance. Epidithiodioxopiperazine, an indole-based natural chemical, has also shown notable bactericidal activity against MRSA.

In many research articles, it has shown that methanolic extracts of *Syzygium cumini* seed showed more efficacy against MDR bacterial pathogens. In this study, we used hexane, chloroform and methanolic extracts of *Terminalia arjuna* bark and *Syzygium cumini* seed against multi drug resistant *Staphylococcus aureus*.

In order to effectively treat staphylococcal infections and stop the spread of infections, precise and quick antibiotic resistance gene detection is crucial. Antibiotic

resistance genes are frequently determined using PCR-based molecular techniques.(Woodford N., *et al* ,2005)

According to the kind of SCCmec, MRSA strains have a mec gene on their bacterial chromosome that is a part of the wider Staphylococcal chromosomal cassette mec (SCCmec) area and confers resistance to many antibiotics. Penicillin-binding protein 2a (PBP-2a) is a protein that is encoded by the mec gene. PBP-2a is an important bacterial cell wall enzyme that catalyses the formation of the peptidoglycan in the bacterial cell wall. It is a penicillin-binding protein (PBP).

Since methicillin-resistant staphylococci (MRS) are resistant to the majority of antibiotics, including beta-lactams, aminoglycosides, and macrolides, it produces infections that are difficult to cure. The synthesis of beta-lactamase, which renders penicillin inactive by hydrolyzing its beta-lactam ring, is the most significant mechanism of penicillin resistance. The penicillin-binding protein 2a (PBP2a), which is encoded by mecA2, is connected to another process. The blaZ gene, which codes for BETA lactamase, is another one that contributes to staphylococci's penicillin resistance.(Zapun A., *et al* , 2008)

CHAPTER 2

REVIEW OF LITERATURE

- ❖ Multi-Drug Resistant bacteria are mostly clinical pathogens which are accompanied by morbidity and mortality. Several alternatives are being developed for effective treatment against Multi-Drug Resistant bacteria.(Morris, Sabrina, and Elizabeth Cerceo.,2020)
- ❖ According to the Infectious Diseases Society of America, antibiotic resistance "is one of the main threats to human health worldwide.". (David Van Duin, David L Paterson.,2016)
- ❖ Scientists around the world have suggested recent developments in combination medicines for the treatment of multi-drug resistant bacterial infections. The creation of adjuvants that either directly target resistance mechanisms, such as the suppression of indirectly target resistance or beta lactamase enzymes, by interfering with bacterial pathways of signaling are some of the efforts made in this direction. (Worthington, Roberta J., and Christian Melander.,2013)
- ❖ The first MRSA was isolated from clinical isolates in the year 1961. The most feared resistant types are; MRSA and VRSA. Methicillin resistance was achieved by interspecies transfer of *mec A* gene from and ancestral staphylococcal species to *Staphylococcus aureus* mediated by a unique staphylococcal mobile genetic element. Vancomycin resistance was achieved by horizontal transfer of a plasmid-born *van A* gene transposon from vancomycin resistant enterococcus to *Staphylococcus aureus* across the genus barrier. (Hiramatsu, K., *et al*, 2014)
- ❖ *S. aureus* has two key characteristics that affect its pathogenicity: its capacity to create a variety of toxins and its capacity to resist more than three kinds of antibiotics. Worldwide, the prevalence of multidrug-resistant bacteria has increased, and as a result, human commensal microbes now have some of these bacteria's genes.(Silva, Vanessa, *et al*.,2021)

- ❖ *P.aeruginosa* was defined as being MDR when the organism is resistant to all antibacterial agents studied except (carbapenems, amikacin and colistin). The most common site of isolation of MDR *P.aeruginosa* was a wound (39%) followed by respiratory tract (22%), urinary tract (18%) and blood(8.5%). Among the antipseudomonal agents that have been used carbapenems were the only class for which exposure didn't confer increased risk of isolation of MDR *P.aeruginosa*. (V Aloush , S Navon-Venezia., 2006)
- ❖ *K. pneumoniae* has been found to produce enzymes such Extended Spectrum -Lactamase (ESBLs) and Carbapenemase, which allow it to become resistant to antibiotics more quickly than most bacteria. Another characteristic of *K. pneumoniae* that is well known is its capacity to create biofilms, which are bacterial populations embedded in an extracellular matrix. Proteins, exopolysaccharides, DNA, and lipopeptides make up this matrix. The initial description of *K. pneumoniae* and the biofilm-forming phenomena was made in 1988. (Nirwati, Hera, *et al*,2019)
- ❖ MDR recently spread significantly, particularly in Gram-negative bacteria like *E.coli*, which is a "versatile" species that can include various pathotypes as well as being a normal component of the gut flora. As a result, commensal *E. coli* may have a unique role in the development and interaction of resistance characteristics. (Szmolka., 2013).
- ❖ Attempts to estimate the proportion of *E. coli* having extended-spectrum beta lactamases, Amp C beta-lactamases, and plasmid-mediated quinolone and fluoroquinolone resistance have had an impact on our understanding of the development and transmission of various antimicrobial resistance in ExPEC (Extraintestinal pathogenic *Escherichia coli*) (McKinnon.,2018)
- ❖ As a result of metagenomic research demonstrating the prevalence of antibiotic resistance genes in the environment, it has been hypothesized that there is a significant likelihood that these genes would transmit to bacteria that cause infections in humans. (Vivas, R., Barbosa, A.A.T *et al*.,2019)
- ❖ The vast majority of antibiotic resistances, however, are likely acquired resistances, the result of an indirect transfer of genes that resist antibiotics from other geographically and taxonomically remote bacteria. (Aminov, R.I. and Mackie, R.I., 2007)

- ❖ Many dynamic combinations of bioactive chemicals are found in many natural antimicrobials which appear as an alternative to fight against bacterial drug resistant. (Haque, R., Sumiya., *et al.*, 2017)
- ❖ The plants medicinal potential comes from a number of chemically active compounds that have a clear physiological effect on the human body. (Bag, A., Bhattacharyya, S.K., *et al.*, 2012)
- ❖ The plants medicinal potential comes from a number of chemically active compounds that have a clear physiological effect on the human body. Traditional medical practices using herbal medicines are of valuable sources for the development of novel antibiotics. From ancient times, rural residents of our nation have used the seed, fruit, leaf and bark of this plant as folk medicine to treat various illness and problems. (Bag, Anwesa, *et al.* 2012)
- ❖ The initial step towards building on eco-friendly therapy for many infectious diseases in our population is through the exploration of new biomolecules of plant origin by the laboratory testing of plants for antibacterial activity. There is a need to look for some alternative antimicrobial from other sources due to the rise in multi drug resistance of bacteria to the currently used antibiotics and the expensive process of synthesis of synthetic compounds. (Bag, Anwesa, *et al.* 2012)
- ❖ One of the possibilities would be medicinal herbs, which tend to be inexpensive, safe and effective against a variety of pathogens that are resistant to some antibiotics. It has not yet been sufficiently examined but the enormous chemical variety of plants holds the promise of becoming a source of chemicals that could change or modulate antibiotic resistance. (Bag, Anwesa, *et al.* 2012)
- ❖ The heritage of human cultures has a long recognized value of using plants to treat illness. Many infectious diseases have been cured using herbal plants by our ancestors. Plants with potential antibacterial action are being cultivated and yielding fruitful results. According to who 80% of people in developing countries depend on traditional health practices for their career needs. (Bag, Anwesa, *et al.* 2012)
- ❖ Antimicrobial action against a variety of bacteria and fungi that are resistant to antibiotics as demonstrated by essential oils from plants and the antibacterial capabilities of low bush blueberry. .(Haque, Razoanul, *et al.* 2017)

- ❖ Many dynamic combinations of bioactive chemicals are found in many natural antimicrobials which appears as an alternative to fight against bacterial resistance and also preserve probiotic species. Jambul (*Syzygium cumini*) contain several biological activities including anti-diabetic, anti-inflammatory, gastroprotective, and anti-bacterial, hepatoprotective, anti-diarrhoeal and anti-oxidant. (Haque, Razoanul, *et al.* 2017)
- ❖ Tannins, saporins, phenols, cardiac glycosides, terpenoids, phytosterols, steroids and amino acids were all present in ethanol extract of jamun stem, bark, seed, fruit pulp and leaf with the exception that anthroquinones were not present in seed extracts. Compared to the pulp and seed extracts , the leaf and bark extracts showed more potency according to the analysis.(Jagetia, Ganesh Chandra., 2017)
- ❖ Studies showed that *Syzygium cumini* seed ethanolic extract possesses strong antibacterial, anti-oxidant and cytotoxic properties. E.coli , bacillus subtilis , p aeruginosa and s aureus were all found to be showing inhibition in their growth by the ethanol extract of jamun seeds.(Jagetia, Ganesh Chandra., 2017)
- ❖ Studies on phytochemistry revealed the presence of polyphenols, tannins, flavonoids, terpenoids and other compounds that provides *S.cumini* seed to obtain therapeutic potential. Due to high antibacterial activity of this seed extract, it can be used to treat bacteria that are MDR and it can also be used as herbal remedy in place of antibiotics. The high concentration of polyphenolic components found in the seed extracts of *S.cumini* may be the basis for it possible anti-oxidants activity. (Jagetia, Ganesh Chandra., 2017)
- ❖ In the past few decades and also in some present investigations, *S.aureus* being the predominant organism of burn and wound infections has shown a rise in antibiotic resistance. With consideration to the resistant *S. aureus* strains the plants used in this research shown encouraging antibacterial activity. More research in this plant potential is necessary to create and alternative therapy for disease brought on by bacteria resistant to antibiotics. *Syzygium cumini* leaf extract showed variable antimicrobial activity against *S.aureus* isolates. (Jahan, Firdaus, *et al.*2011)
- ❖ *Terminalia arjuna* includes antimicrobial, antioxidant and anti-inflammatory as their pharmacological applications. *T.arjuna* bark contains a high level of flavonoids and tannins. 10% and 15% of aqueous and methanolic extracts of

T.arjuna have a high range of 8-11mm and 7-13mm zone of inhibition on *E.coli* dk1 and *S.aureus* mRS901 strain respectively.(Kumar, Sanjay, *et al.*2013)

- ❖ In particular, clinical samples of *S.aureus* have displayed an apparent inhibitory response to water and methanol extracts which was identified to be an MRSA by confirmed phenotypically.(Debnath, Sukalyani, *et al.* 2013)
- ❖ Acetone, ethanol and methanol extracts of the bark of *T.arjuna* were considered to be more effective in treating enteropathogenic growth.(Rath, S. and Padhy, R.N., 2015)
- ❖ The growths of the bacterial pathogens are inhibited more effectively by apical barks methanolic extract than by the middle or mature bark. Of all bacterial species, studies have prominently shown that *T.arjuna* extracts were more susceptible to *S.aureus*. The apical stem bark had a high concentration of secondary metabolites compared to the middle and mature bark. Flavonoids , tannins and alkaloids and other secondary metabolites serves as a protective mechanism against microorganisms attacking.(Patil, U.H. and Gaikwad, D.K., 2011)
- ❖ It was discovered that the polar extracts polyphenol content was high while the chloroform fractions polyphenol content was low. The high level of polyphenol in the polar extracts may therefore be favorably associated with *T arjuna* barks potent antibacterial activity. The methanol and water extracts of barks have shown a visible inhibition of growth of ATCC and the clinical cultures of gram positive and gram negative bacteria. Methanol extracts have showed a greater potential in the case of gram negative bacteria compared to the polar extract. Chloroform extracts was generally ineffective against all the samples and they have only shown very low response towards *Proteus vulgaris*. Presently studies have showed that the chloroform extracts are largely ineffective and only the water and methanol extracts could exhibit a considerable amount of antimicrobial activity , regardless of the particular pathogenic strains and the resistant profile against various drugs.(Debnath, Sukalyani., *et al*,2013)

CHAPTER 3

AIM

To study the prevalence of Multi-Drug Resistant bacterial pathogens from clinical samples in and around Chennai and to determine the efficacy of selected herbal plant extracts against the MDR *Staphylococcus aureus*.

SCOPE OF THE STUDY

- Collection of clinical cultures from the hospitals in and around Chennai.
- Isolation and identification of the cultures collected from clinical samples by standard methods.
- Analyzation of multi-drug resistant bacterial pathogens prevalence in and around Chennai using Kirby-Bauer disc diffusion test.
- The antimicrobial activity of herbal plant extracts against the multi-drug resistant *Staphylococcus aureus* bacteria was examined.

CHAPTER 4

MATERIALS AND METHODOLOGY

1. COLLECTION

Unknown cultures are collected from hospitals in and around Chennai with all safety measures.

2. ISOLATION AND IDENTIFICATION OF UNKNOWN CULTURES

- The primary step of identification process is to extract pure bacterial colonies. Only if the microbe is available in its purest form can examinations on the biochemical, antigenic, and other features of bacteria be conducted.
- One of the streaking techniques that are frequently used to get a pure culture is the quadrant streaking technique. Isolating a single colony or species from the mixed culture is helpful. In the quadrant streaking method, a small quantity of the inoculum to be isolated is deposited on the first quadrant of an agar plate and it is successively spread over the remaining three quadrants with the help of a sterile inoculation loop. The third and fourth quadrants are where distinct microbial colonies typically emerge. The isolated colonies are aseptically transferred into various nutrient agar slant tubes and cultured overnight at 37 degree Celsius in order to regrow the pure culture of the organism. It is then kept for further use.
- To learn about the development pattern and cultural traits of the targeted microorganisms, the isolated colony can be cultivated further in selective growth mediums.
- Then it is characterized by Gram staining (This differential staining technique is used to classify the bacteria into gram positive and negative) and Hanging Drop Motility test (To identify the motility of the microorganisms), oxidase (to identify the bacteria that produce an enzyme – Cytochrome C oxidase) and catalase (To determine the ability of the bacteria to degrade Hydrogen Peroxide by producing the enzyme catalase) and IMViC biochemical test.

1. **Cultural characteristics**

Media used;

(A) Solid media

➤ Nutrient agar

The most commonly used media in routine diagnostic laboratories. Consists of beef extract, peptone, sodium chloride and agar along with distilled water. It is the best media used for maintaining, cultivating, and isolating non-fastidious organisms. It is used in checking the purity of the culture. It is also used to study colony morphology of an organism.

➤ *Salmonella* and *Shigella* agar

A moderately selective, differential medium-consisting of beef extract, enzymatic digest of casein, enzymatic digest of animal tissue, lactose, bile salts, sodium citrate, sodium thiosulphate, ferric citrate, brilliant green, neutral red, agar. It is used for the isolation of *salmonella* and some *shigella* species from clinical and non-clinical specimens.

➤ Thiosulphate Citrate Bile Salt agar

A selective medium consisting of sucrose, dipeptone, sodium citrate, sodium thiosulphate, sodium chloride, yeast extract, ox bile, sodium cholate, ferric citrate, bromothymol blue, thymol blue, agar primarily used for the isolation of *V. cholerae* and other enteropathogenic vibrio species.

➤ Mannitol salt agar

A selective and differential medium consisting of pancreatic digest of casein, peptic digest of animal tissue, beef extract, sodium chloride, d-mannitol, phenol red, agar. It is utilized for determining *staphylococci* in dairy and food items as well as to selectively isolate and distinguish *staphylococcus aureus* from clinical samples.

➤ Mueller Hinton agar

Mueller Hinton agar is mostly used for assessing the susceptibility of microbes to various antibiotics. The Kirby-Bauer technique now uses it as the officially recognised medium, and the NCCLS specify its performance. For susceptibility testing, it demonstrates acceptable batch-to-batch repeatability. It allows the majority of non-fastidious infections to develop satisfactorily.

➤ MacConkey agar

The most common type of differential media consisting of peptone, lactose monohydrate, sodium chloride, bile salts, neutral red, crystal violet, and agar utilized mostly to separate gram-negative bacteria that digest lactose from those that don't, as well as to isolate gram-negative enteric bacteria.

➤ Blood agar

An enriched medium used in diagnostic laboratories. It is prepared by adding 5% sheep blood to the already prepared and sterilized nutrient agar. Often used to grow fastidious organisms. The main purpose is to study the hemolytic pattern of an organism.

➤ Eosin methylene blue agar

A differential medium consisting of peptic digest of animal tissue, dipotassium phosphate, lactose, sucrose, eosin-Y, methylene blue, agar, recommended for the isolation and differentiation of gram negative enteric bacteria from clinical and non-clinical specimens. It also helps in visual distinction of *E.coli* from other non-pathogenic lactose fermenting enteric gram negative rods.

➤ Cetrimide agar

A selective medium consisting of pancreatic digest of gelatin, potassium sulphate, magnesium chloride, cetyltrimethylammonium bromide, glycerine and agar. It is primarily used for the selective isolation and presumptive identification of *P.aeruginosa*

(B) Liquid media

➤ Nutrient broth

A nutrient agar without the agar-solidifying ingredient is called nutrient broth. In room temperature, it remains in liquid state.

Growth on selective and differential media:

The cultures are then subjected to identification using;

✓Differential media - Differential media are employed to differentiate certain closely related organisms or groups of organisms. (Blood agar and MacConkey agar) and

✓Selective media- Selective media allows only the growth of certain types of organisms, while inhibiting the growth of other organisms

- Eosin Methylene Blue agar – *E.coli*
- Mannitol Salt agar – *Staphylococcus* species
- *Salmonella Shigella* agar – *Salmonella* and *Shigella* species
- Cetrimide agar – *Pseudomonas* species
- Thiosulphate Citrate Bile salt Sucrose agar – *Vibrio* species

The colour and colony morphology (Pin pointed, circular, irregular, rough raised, swarming, opaque, transparent, translucent, mucoid colonies) of the cultures are observed.

4.2.2 Morphological characteristics

(A) Gram Staining

Gram staining is a differential staining technique used in the bacterial identification process to differentiate bacteria into Gram Positive and Gram Negative according to their cell wall composition. The ability of the bacterial cell wall to hold on to the crystal violet dye after solvent treatment is the fundamental principle of gram staining. Gram-positive bacteria contain more peptidoglycan, whereas gram-negative bacteria contain more lipids. All bacteria initially absorb the crystal violet

dye; however gram-negative bacteria's lipid coating is dissolved when a solvent is used. Gram negatives lose the main stain as the lipid layer dissolves. (Tripathi, N. and Sapra, A., 2020)

With a loopful of the sample, prepare the smear of suspension of the bacteria to be tested on the clean slide. Heat fix the bacteria to the slide after air drying. The primary stain, Crystal Violet, was applied, let it sit for 30 to 60 seconds, and then rinsed with water. For one minute, soak with gram's iodine (mordant) and then rinse with water. Next, wash with 95% alcohol or acetone (a decolorizer) for 10 to 20 seconds and then rinse it with water. Safranin (counterstain) added for approximately 1 minute, and then rinsed with water.

Blot dry, air dry, and observe under a microscope at 10x, 40x, and 100x magnifications. Gram-positive bacteria will stain blue/purple and Gram-negative bacteria will stain pink/red.

(B) Motility

Large hair-like appendages called "flagella" are present all over the body of bacteria. These are substantially responsible for the movement of those organisms. However, the capacity of the microbe to move in the growing media or by using the hanging drop technique which can be used to visualize the presence of bacteria in the medium or the activity of the flagella of bacteria (Jain, A., Jain, R., Jain, S. (2020). A unique kind of wet mount known as a "hanging drop preparation" is one in which a drop of medium comprising the organisms is suspended on a hollow slide. This method is frequently used in low light to study the movement of bacteria. This technique is used in detecting the motile bacteria present in the broth culture.

In this procedure, a drop of culture is applied to a coverslip that has petroleum jelly all around it. The coverslip and inoculum drop are then inverted over a depression slide's well. The petroleum jelly creates a seal to hold the drop to the coverslip and keep it from evaporating. This solution provides clear insights into microbial motility. Directional purposeful motility is a positive test.

4.2.3 Biochemical characteristics

(A) Oxidase Test

The oxidase test is a biochemical reaction that detects the presence of the enzyme cytochrome oxidase. Oxidase-positive organisms color the reagent blue or purple because they have cytochrome C as a component of their respiratory chain. Oxidase-negative organisms do not oxidize the reagent, retaining it colorless within the parameters of the test, and are therefore cytochrome c lacking.

There are several methods to perform the oxidase test. Some of the methods are; the filter paper test, direct plate method, swab method, impregnated oxidase test strip method and test tube method. In this study, we used dry filter paper disc method. In a petri plate, the oxidase disc is placed. A glass rod is used to pick up the test colony and apply it to the disc and observed for color change.

Appearance of a deep purple-blue/blue color within 5-10 seconds indicates oxidase positive test. No purple-blue color/No color change indicates negative oxidase test.

(B) Catalase Test

The catalase test makes it simple to find microorganisms that contain the catalase enzyme.

Hydrogen peroxide's bactericidal actions are counteracted by the catalase enzyme. Hydrogen peroxide (H_2O_2) is quickly broken down into water and oxygen by the enzyme catalase ($2H_2O_2 + \text{Catalase} \rightarrow 2H_2O + O_2$). The quick bubble creation is a sign that this reaction is occurring. (Reiner, K., 2010).

There are two methods in performing catalase test; tube method and slide method. In our study, we performed tube method. Fill a test tube with 2 ml of hydrogen peroxide solution. Take a few colonies of the 18 to 24 hours test organism and submerge them in the hydrogen peroxide solution using a clean glass rod or wooden stick. Keep monitoring out for immediate bubbling.

It indicates positive catalase test, if bubbles are produced. If no bubbles or very few bubbles produced, it indicates negative catalase test.

(C) IMViC

The acronym IMViC stands for indole, methyl red, Voges-Proskauer and citrate utilization.

➤ INDOLE

The indole test is a part of the IMViC protocols, a set of tests used to differentiate between members of the Enterobacteriaceae family. It evaluates an organism's capacity to break down the amino acid tryptophan and generate indole.

Inoculate the tryptone broth tube with a tiny quantity of a pure culture. Incubate for 24 to 48 hours at 35°C. Add few drops of Kovacs reagent straight to the tube to check for indole formation. The appearance of cherry-red ring on top of the medium within seconds after introducing the reagent is a sign of a positive indole test. The reagent layer will remain yellow or be slightly hazy if a culture is indole negative. (MacWilliams, M.P., 2012)

➤ Methyl Red

Methyl red test is used to determine the ability of microorganism to oxidize glucose with the production of acid end products. In this test, microorganisms are cultivated in a broth medium containing glucose. The color of the methyl red changes from yellow to red when introduced to the broth culture if the bacteria can use glucose and produce a stable acid. After an enteric Gram-negative rod has fully fermented glucose, methyl red is the indicator used to measure pH.

Using sterile technique, inoculate each experimental organism into its appropriate MR VP broth tubes with the help of inoculation loop. Incubate the test tubes at 37°C for 48-72 hours. Then add few drops of methyl red as an indicator and observe the color change. Positive test gives bright red in color and yellow color test indicates negative test.

➤ Voges-Proskauer

A biochemical test called Voges-Proskauer is used to assess an organism's capacity to make neutral or non-acidic end products.

Glucose → Pyruvic Acid → Acetoin.

Acetoin + added alpha-naphthol + added KOH = gives red color which indicates the positive reaction.

In test tubes, prepare MR VP broth and inoculate the appropriate bacterial culture aseptically. For 48 to 72 hours, incubate the test tubes at 37°C. Barrit's reagent A (alpha-naphthol) and Barrit's reagent B (40 percent KOH) should be added in a 3 is to 1 ratio. To aerate the tubes, take out the plug and shake them and then evaluate the outcomes. Pink–red color indicates positive test and copper color indicates negative test results.

➤ Citrate Utilization Test

Citrate utilization test is used to determine the ability of the microorganism to ferment citrate.

Citrate-permease, an enzyme produced by bacteria that may survive in this media, which is capable of converting citrate to pyruvate. Alkaline carbonates and bicarbonates are created when an organic acid like citrate is employed as a carbon and energy source, raising the medium's pH and causing the color change.

Using sterile technique, inoculate the test organism into the simmon citrate agar plate or slant. Incubate tubes for 24 – 48 hours at 37°C and observed for color change. If the organism ferment citrate there will be an appearance of intense Prussian blue and Negative test result is indicated by original green color.

4.3 ANTIBIOTIC SUSCEPTIBILITY TEST

Medical technologists use antimicrobial susceptibility testing (AST), a laboratory process, to determine which antimicrobial therapy is specifically beneficial for each patient. (Bayot ML, Bragg BN, 2022)

An antibiotic sensitivity test or antibiotic susceptibility test evaluates a bacteria response to antibiotics. This technique is used when some bacteria resist certain antibiotics.

Results of antibiotic sensitivity testing may enable a clinician to switch from empiric therapy, when an antibiotic is chosen based on clinical suspicion of the infection site of the patient to be treated and the most likely because of the causative bacteria.

One of the types of antimicrobial susceptibility test which is used widely in diagnostic and drug discovery laboratories is Kirby Bauer disc diffusion method.

The disc diffusion assay is used to screen biological material such as plant extracts for bactericidal activity in drug discovering laboratories, particularly bioprospecting facilities. In order to provisionally identify the antibacterial mechanism of action, the assay can be carried out during bioprospecting using coupled strains of bacteria.

There will be an area surrounding the disc where the bacteria have not developed sufficiently to be seen if an antibiotic prevents the bacteria from developing or kills the bacteria known as a zone of inhibition.

The antibiotics discs used in this study to check the Antibiotic Susceptibility of the collected cultures are Ceftriaxone, Gentamicin, Norfloxacin, Imipenem, Ciprofloxacin and Amikacin.

1. Preparation of Agar Plate and Inoculum

To compare the test organism to the 0.5 McFarland Turbidity standard, the test microorganism has to be grown in broth for an entire night. Mueller Hinton agar is used because it does not block sulphonamides and provides accuracy with the medium's composition and pH which should be between 7.2-7.4. It is poured onto Petri dishes; the agar should be 4mm in depth.

2. Inoculation and Incubation

- In aseptic technique, the culture is inoculated.
- In the MHA agar plate, the inoculum is lawn cultured by sterile cotton swab.
- Rotate the plate to completely swab its whole surface area to obtain uniform growth.

- For the medium to properly absorb the inoculum, the plates are allowed to dry for five minutes.
- In diagnostic labs the commercially available Discs are placed with proper space interval between them.
- The disc diffusion test is carried out slightly differently in drug development labs than it is in diagnostic labs. (Cushnie TP., *et al*, 2020)
- In this case, a test extract will be characterized rather than the bacterial strain. The test extracts are infused in the filter paper discs and placed on the inoculated agar plates.
- To prevent misplacement, lightly touch each disc with the flame sterilized forceps to make sure it is in contact well.
- Incubate it at 37 °C for 24 hours.

4.3.3 Interpretation

- Use a measuring scale to measure the zone of inhibition in the diameter of the disc after the incubation period.
- Compare the outcome against the CLSI standards before reporting it.
- The results are interpreted as Susceptible (S) or Resistant (R). This makes it feasible to choose the ideal antibiotic to treat a patient's ailment.

4. EFFICACY OF PLANT EXTRACTS

1. Selection of plant material

Terminalia arjuna (Neer Marutham Bark), *Syzygium cumini* (Jamun seeds) are selected for the evaluation of antimicrobial properties against the multi drug resistant *Staphylococcus aureus* strains. The selected plants are then shade dried and grinded into a powder.

2. Preparation of extracts

The powdered plant materials are weighed up to 50gms and it is transferred to separate conical flasks and labelled. 300 ml of hexane is measured and is added to the conical flasks. The plant-solvent mixture is mixed well and kept in rotary shaker for 2 days and At the end of extraction, each extract was passed through Whatman

filter paper No. 1. The hexane extract of each plant material is collected into separate beaker. The filtrate is then allowed to evaporate so that the final crude extract is collected. Again 300 ml of hexane is added to the filtered debris for secondary filtrate collection. The secondary filtrate is then collected after 2 days of occasional stirring and allowed to evaporate. Similarly, the chloroform and methanol extracts of each plant extracts is obtained. The collected crude extracts are then transferred into sterile Eppendorf tubes and then stored for screening of antimicrobial properties.

3. Microorganisms used

The 7 Multi drug resistant *Staphylococcus aureus* strains are used for testing of antimicrobial properties of the selected plant materials. Nutrient agar slants were used to cultivate the bacterial strains. The cultures were maintained by sub culturing periodically and preserved at 4 °C until further use.

4. Antimicrobial assay

The antimicrobial activity of the plant extracts was evaluated using paper disc Diffusion Method. The corresponding solvents are utilized in the preparation of a concentrated solution of those extracts. The discs are then infused with extracts of each plants using micropipette and also the test control for each of the solvents are also prepared. All the prepared discs are then fully dried before the application on bacterial lawn culture. Overnight cultures of multi drug resistant *Staphylococcus aureus* in nutrient broth are taken with 0.5Mcfarland turbidity. The inoculum was then lawn cultured in a Mueller Hinton Agar plate and the discs are placed with the help of forceps. Then it is incubated for 24-48 hours at 37 °C and the results are interpreted. By assessing the zone of inhibition against the test organism, antimicrobial activity was assessed.



JAMUN SEEDS



ARJUN TREE BARK



**PLANTS ARE POWDERED, WEIGHED
AND TRANSFERRED INTO CONICAL
FLASKS**



CHLOROFORM FILTRATE



HEXANE FILTRATE



METHANOL FILTRATE

Fig 4.1 Herbal plant extraction

CHAPTER 5

RESULTS AND DISCUSSION

5.1 COLLECTION



Fig 5.1.1- Collected cultures

(Total number of cultures collected = 50)

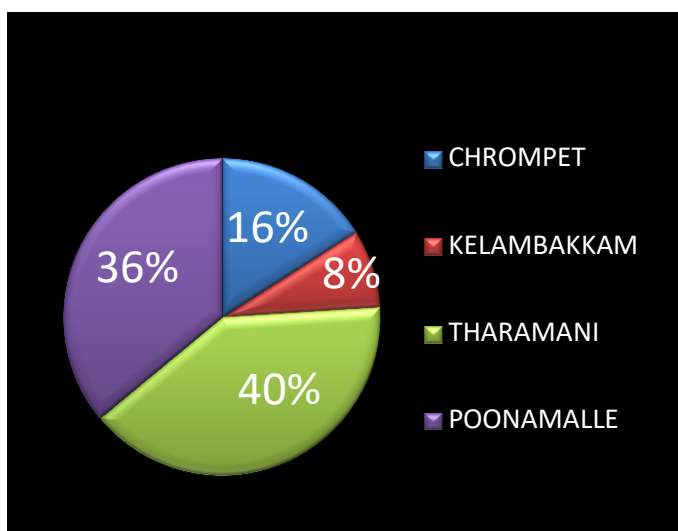


Fig 5.1.2 - Percentage of clinical cultures collected from hospitals in 4 different areas

Table 5.1.1 showing number of collected cultures according to gender

GENDER	NO. OF COLLECTED CULTURES
Male	18
Female	32
Total	50

(More than half of the clinical cultures are collected from females)

Table 5.1.2 showing number of collected cultures according to age group

AGE GROUP	NO. OF COLLECTED CULTURES
Adult	49
Child	01
Total	50

5.2 IDENTIFICATION OF MICROORGANISMS

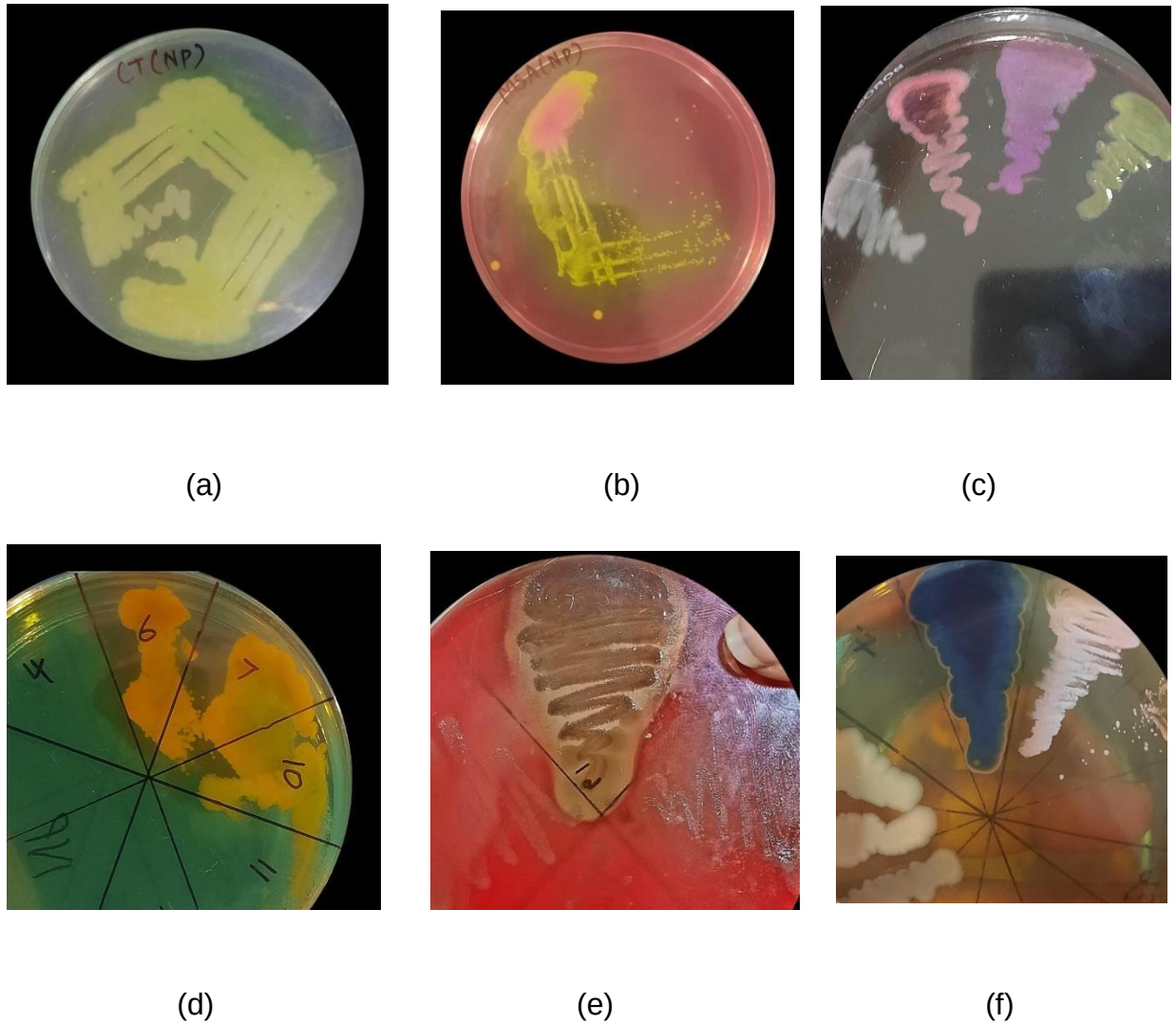


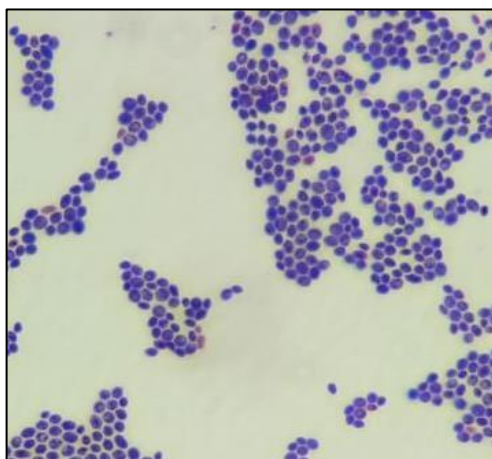
Fig 5.2.1 Growth on selective and differential media

(a) Yellow-green color growth of *Pseudomonas aeruginosa* in cetrinide agar
(b) Yellow colonies of *Staphylococcus aureus* growth in Mannitol Salt Agar
(c) Growth of enteric bacteria in EMB agar (d) Yellow colonies of *Vibrio cholerae* in TCBS agar (e) Hemolysis in Blood agar (f) Black colonies of *Salmonella* in SS agar

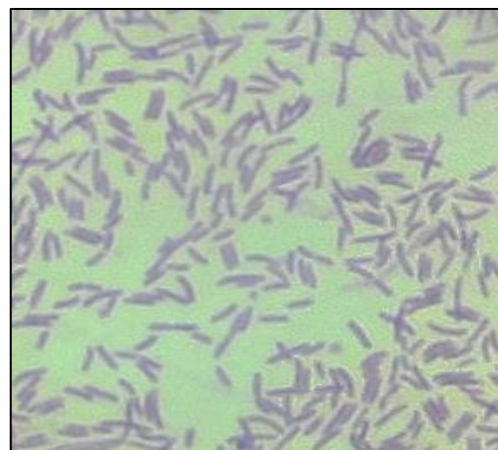
Table 5.2.1

Table showing gram positive and gram negative bacteria

GRAM STAINING	NO. OF MICROORGANISMS
Gram positive	19
Gram negative	31
Total	50



(a)



(b)

Fig 5.2.2- Gram Staining (a) Gram positive cocci in microscope, (b) Gram negative bacilli in microscope

Table 5.2.2 showing motility of the bacteria

MOTILITY	NO. OF ORGANISMS
Motile	27
Non motile	23
Total	50

Table 5.2.3 showing biochemical test results

ORGANISM	INDOLE	METHYL RED	VOGES PROSKAUER	CITRATE UTILIZATION	OXIDASE	CATALASE
<i>Staphylococcus aureus</i>	-	+	+	+	-	+
<i>Staphylococcus saprophyticus</i>	-	-	+	-	-	+
<i>Pseudomonas aeruginosa</i>	-	-	-	+	+	+
<i>Escherichia coli</i>	+	+	-	-	-	+
<i>Klebsiella pneumoniae</i>	-	-	+	+	-	+
<i>Proteus mirabilis</i>	-	+	-	+	-	+
<i>Salmonella typhi</i>	-	+	-	-	-	+
<i>Enterococcus faecalis</i>	-	-	+	-	-	-
<i>Vibrio cholera</i>	+	-	-	-	+	+

Table 5.2.4 showing the number of identified microorganism in the collected culture

NAME OF ORGANISMS	NO. OF ORGANISMS
<i>Staphylococcus aureus</i>	16
<i>Pseudomonas aeruginosa</i>	10
<i>Escherichia coli</i>	7
<i>Staphylococcus saprophyticus</i>	3
<i>Klebsiella pneumoniae</i>	3
<i>Proteus mirabilis</i>	1
<i>Salmonella typhi</i>	3
<i>Enterococcus faecalis</i>	1
<i>Vibrio cholerae</i>	6
TOTAL	50

Staphylococcus aureus is the most prevalent species isolated from the collected cultures (32% of all bacterial isolates) and this table also shows that *Enterococcus faecalis* and *Proteus mirabilis* are less prevalent and uncommon compared to the other microorganisms. *Pseudomonas aeruginosa* is the second most prevalent bacteria with a percentage of 20 of all bacterial isolates. Some previous studies show that *P.aeruginosa* accounts for about 20% of all HAI and *S.aureus* and *E.coli* were more prominent bacteria isolated from clinical specimens.

5.3 ANTIBIOTIC SUSCEPTIBILITY TEST

The identified organisms are then tested for their antibiotic susceptibility using the Kirby-Bauer disc diffusion technique. The organisms are tested using commercial antibiotic discs. In the below table,

- ✓ EC denotes *Escherichia coli*
- ✓ EF denotes *Enterococcus faecalis*
- ✓ KP denotes *Klebsiella pneumoniae*
- ✓ PM denotes *Proteus mirabilis*
- ✓ PA denotes *Pseudomonas aeruginosa*
- ✓ SA denotes *Staphylococcus aureus*
- ✓ ST denotes *Salmonella typhi*
- ✓ SS denotes *Staphylococcus saprophyticus*
- ✓ VC denotes *Vibrio cholerae*

Table 5.3.1 showing antibiotic susceptibility test results

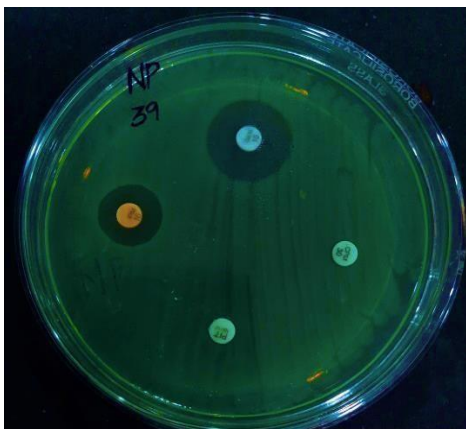
(R indicates Resistivity, S indicates Sensitivity)

ORGANISM	CEFTRIAXONE	AMIKACIN	IMIPENEM	NOR FLOXACIN	CIPRO FLOXACIN	GENTAMICIN
EC1	S	R	S	S	S	R
EC2	S	S	R	R	S	S
EC3	S	S	R	S	R	R
EC4	S	S	S	R	R	S

EC5	S	R	R	S	S	S
EC6	S	R	R	S	R	S
EC7	R	S	R	R	S	S
EF1	S	S	S	R	R	R
KP1	R	S	S	R	S	S
KP2	S	R	S	R	R	S
KP3	S	S	R	R	S	S
PM1	S	R	S	R	S	S
PA1	S	S	S	R	S	S
PA2	R	S	R	S	S	S
PA3	R	S	S	S	S	S
PA4	R	S	S	S	S	S
PA5	S	S	R	R	R	S
PA6	S	S	S	R	S	S
PA7	R	S	R	R	R	S
PA8	S	S	R	R	S	S
PA9	R	S	R	R	R	S
PA10	S	S	R	R	R	S

SA1	S	S	S	R	S	S
SA2	S	S	S	S	S	S
SA3	S	S	S	S	S	S
SA4	S	S	S	S	S	S
SA5	S	S	S	S	S	S
SA6	R	S	S	S	S	S
SA7	R	S	S	S	S	S
SA8	R	S	R	R	R	S
SA9	R	S	S	R	S	S
SA10	R	R	R	R	R	S
SA11	R	S	R	R	R	S
SA12	R	S	S	R	S	R
SA13	R	R	R	R	S	R
SA14	S	S	S	S	S	S
SA15	R	S	S	S	S	S
SA16	R	R	S	R	R	S
ST1	S	R	S	R	S	S
ST2	R	S	S	S	R	S

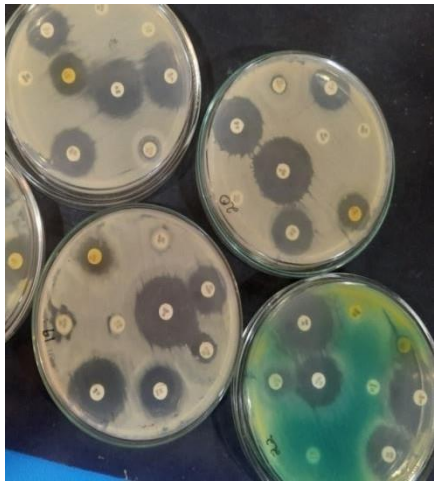
ST3	R	S	S	S	R	R
SS1	S	R	R	S	S	S
SS2	S	R	S	R	S	S
SS3	S	R	R	R	R	S
VC1	R	R	S	S	R	S
VC2	S	S	R	R	S	S
VC3	S	R	S	S	S	R
VC4	S	S	R	R	S	S
VC5	R	R	R	S	S	S
VC6	S	R	S	S	S	R



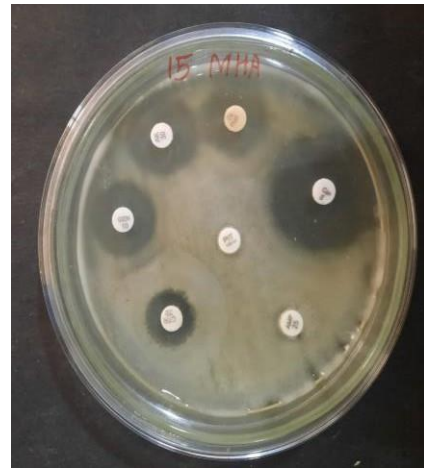
(a)



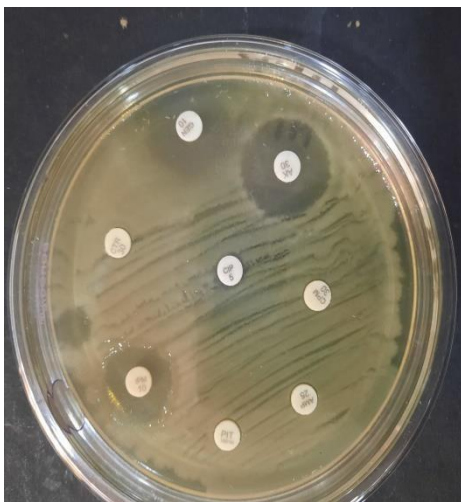
(b)



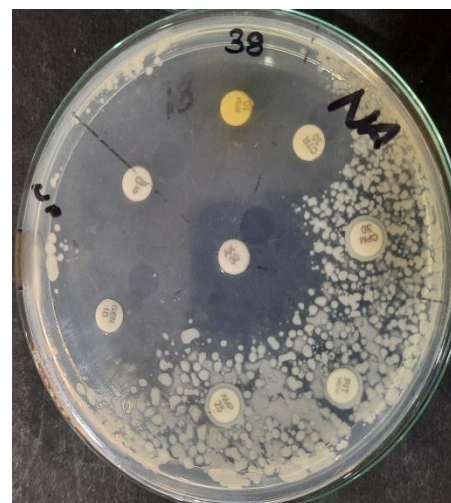
(c)



(d)



(e)



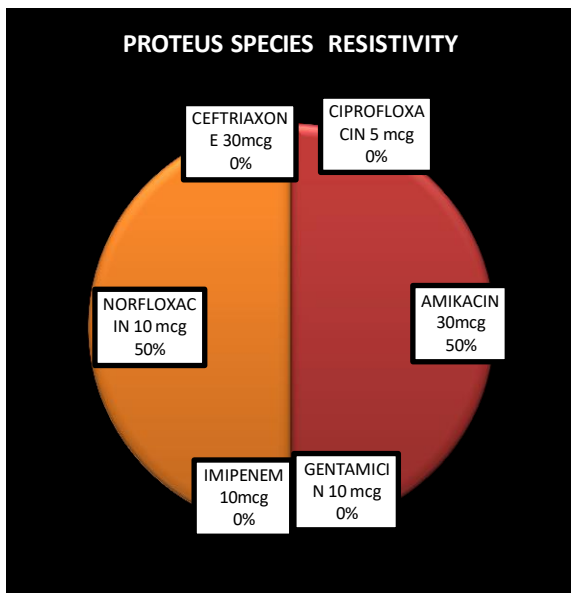
(f)

Fig 5.3.1(a) - (f) - Kirby-Bauer Antibiotic Susceptibility Test

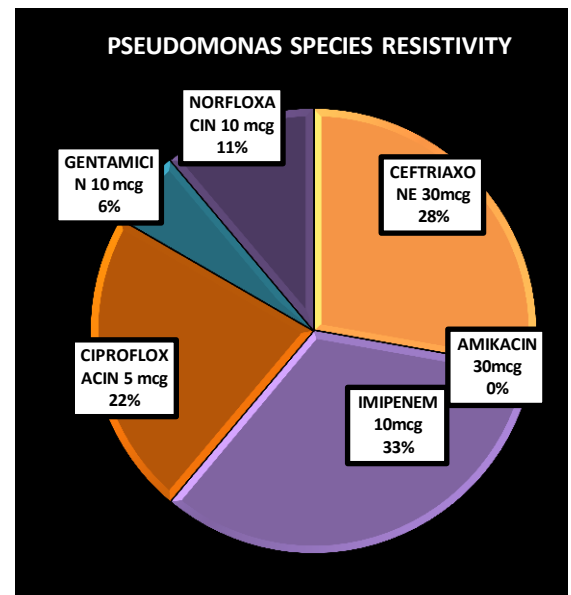
Table 5.3.2 showing the total count of MDR bacteria in the collected cultures

ORGANISMS	COUNT
<i>Staphylococcus aureus</i>	7
<i>Pseudomonas aeruginosa</i>	4
<i>Enterococcus faecalis</i>	1
<i>Vibrio cholera</i>	2
<i>Escherichia coli</i>	2
<i>Klebsiella pneumoniae</i>	1
<i>Proteus mirabilis</i>	0
<i>Staphylococcus saprophyticus</i>	0
<i>Salmonella typhi</i>	1
TOTAL	18

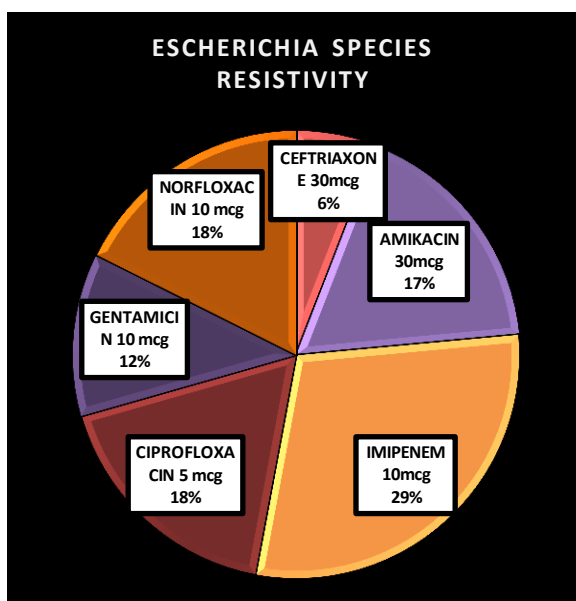
From the total 50 cultures collected and identified, about 36% of bacteria have shown Multi-Drug Resistance against the common antibiotics used. Microorganisms which have resistance over more than three antibiotics tested are taken as MDR. *Staphylococcus aureus* was the more prominent MDR compared to other pathogens isolated with high antimicrobial resistance rate of 26% against Norfloxacin and Ceftriaxone. And the second most prevalent MDR was *Pseudomonas aeruginosa* with Antimicrobial resistance rate of 33% and 28% against Imipenem and Ceftriaxone respectively. Amikacin is highly sensitive against all *P.aeruginosa* organisms. Fig 5.2 shows the resistivity of the isolated bacteria over some common antibiotics used.



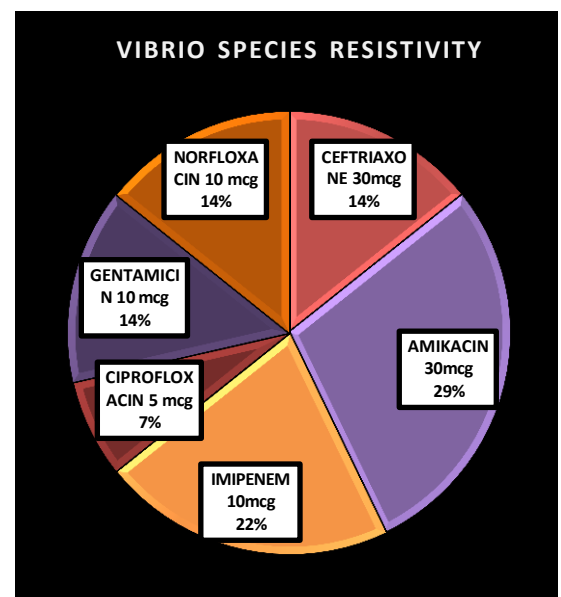
(a)



(b)

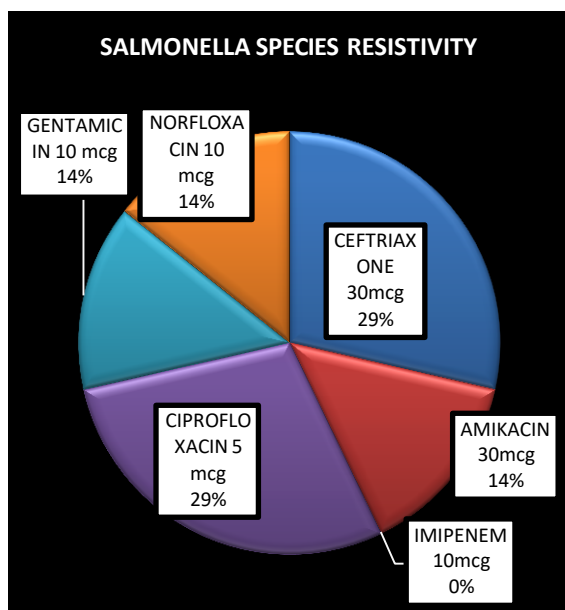


(c)

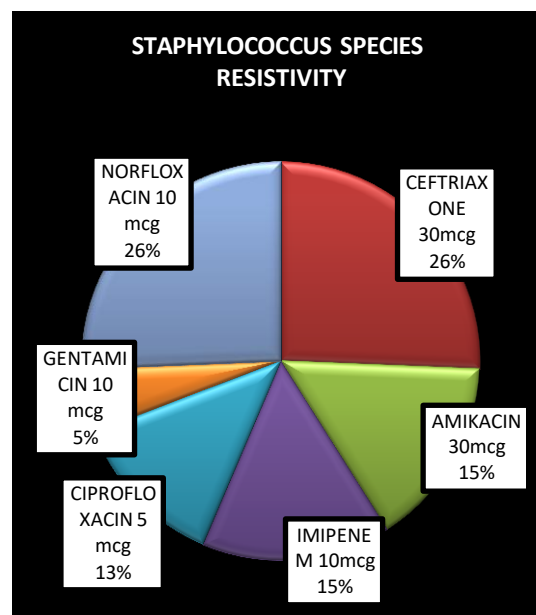


(d)

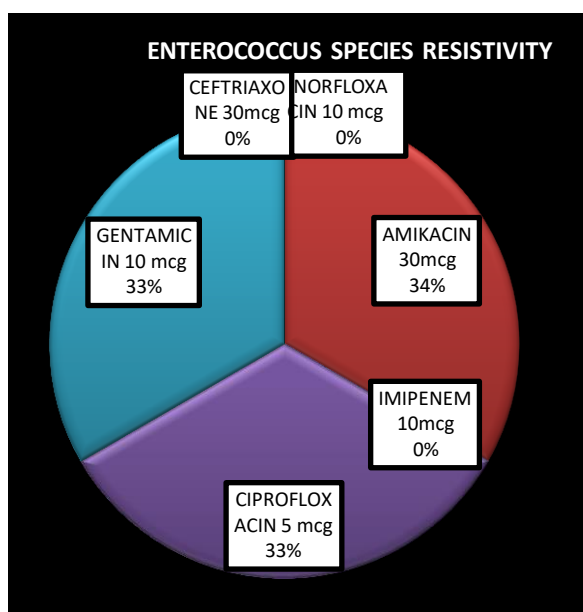
Fig 5.3.2- Resistivity chart (a) *Proteus mirabilis* resistivity (b) *Pseudomonas aeruginosa* resistivity (c) *Escherichia coli* resistivity (d) *Vibrio cholerae* resistivity



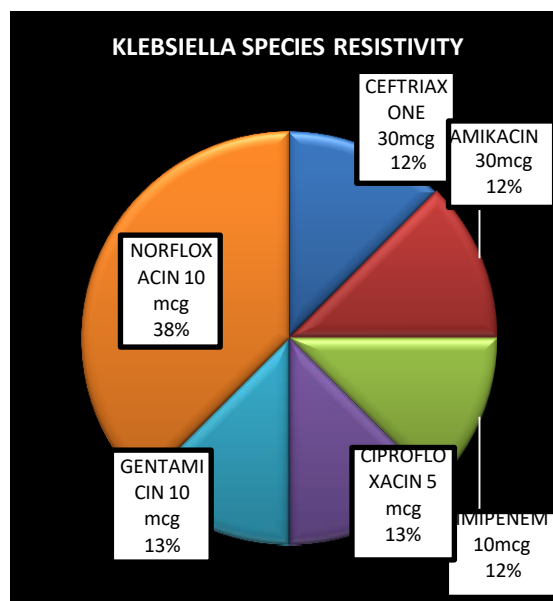
(e)



(f)



(g)



(h)

Fig 5.3.2- Resistivity chart (e) *Salmonella typhi* resistivity (f) *Staphylococcus* species resistivity (g) *Enterococcus faecalis* resistivity (h) *Klebsiella pneumonia* resistivity

Table 5.4 showing the efficacy of plant extracts against multi drug resistant staphylococcus aureus

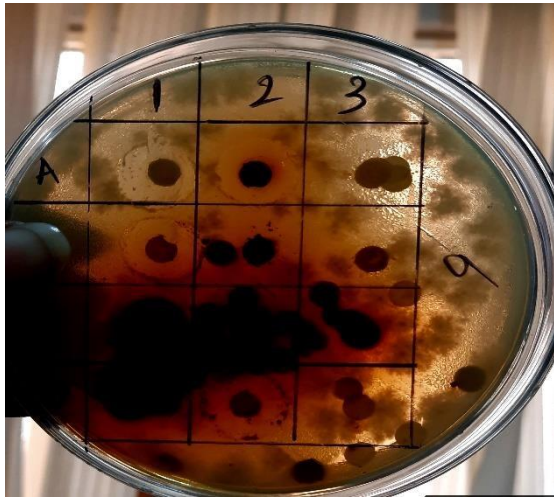
MICROORGANISM	<i>Terminalia arjuna</i>			<i>Syzygium cumini</i>		
	Hexane	Chloro Form	Methanol	Hexane	Chloro Form	Methanol
SA1	-	-	-	+	-	-
SA2	+	-	-	-	-	+
SA3	+	-	+	+	-	+
SA4	+	-	-	+	-	+
SA5	-	+	-	-	-	+
SA6	+	+	-	+	-	-
SA7	-	-	-	+	-	+

(SA denotes *Staphylococcus aureus*)

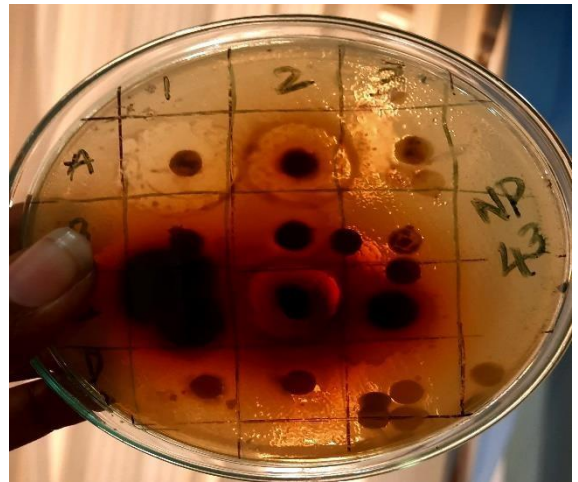
The antimicrobial effect of the selected plant extract against the *Staphylococcus aureus* are illustrated in table above. Herbal crude extracts of *Syzygium cumini* and *Terminalia arjuna* are prepared and tested against the identified MDR *Staphylococcus aureus*. Since it's just a preliminary test, only the presence of ZOI is noted and not the diameter of ZOI.

- ✓ + indicates sensitivity of herbal extracts against MDR *Staphylococcus aureus*.
- ✓ - indicates resistivity of herbal extracts against MDR *Staphylococcus aureus*.

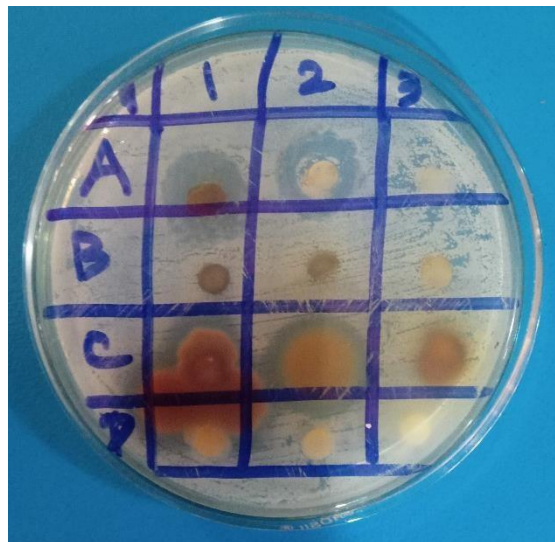
As indicated in the table 5.4, the chloroform extracts of *Syzygium cumini* seeds does not show any anti-microbial activity against the selected MDR *Staphylococcus aureus* organisms. The hexane extracts of *Terminalia arjuna* bark and methanol and hexane extracts of *Syzygium cumini* seed have shown strong inhibition zones against the MDR *Staphylococcus aureus*.



(a)



(b)



(c)

Fig 5.4- Paper disc diffusion of herbal plant extracts against MDR *S.aureus* (a) SA6 (b) SA4 (c) SA3

CHAPTER 6

SUMMARY AND CONCLUSION

In our in-vitro study, we found the prevalence of multi drug resistant bacteria in clinical samples in and around Chennai since the emergence of multi drug resistant pathogens increases day by day because of the overuse of antibiotics and resistant gene modifications. Firstly, we isolated the collected cultures and subjected it to identification by observing morphological and cultural characteristics and biochemical tests. Of all bacterial pathogens identified, *Staphylococcus aureus* is more prevalent than any other microorganisms. Then, we used the Kirby-Bauer disc diffusion technique to study the antimicrobial activity of the collected bacterial pathogens against common antibiotic discs such as ciprofloxacin, amikacin, imipenem, norfloxacin, gentamicin and ceftriaxone. The antibiotic resistance and prevalence of Multi- drug resistant bacterial pathogens highlights the necessity of extensive and up to date information on antibacterial susceptibility trends. We found that multi drug resistant *Staphylococcus aureus* is more in number than other bacterial pathogens. When considering the factor like sex and age group, MDR occurrence was predominant in female adults in our study. Recently WHO published a list of antibiotic resistant pathogens that pose the greatest threat to human health and the urge for developing new antibiotics and alternative treatments. We have checked the efficacy of herbals plants against the MDR bacteria and the preliminary testing of the selected herbal plant extracts have shown expected results against the test organisms. Hexane extracts of *Terminalia arjuna* (Arjun tree bark) and methanol and hexane extracts of *Syzygium cumini* (Jamun seeds) showed high efficacy against multi drug resistant *Staphylococcus aureus* in-vitro. Further, secondary screenings of the extracts are required to study more about the MIC against the MDR *Staphylococcus aureus*.

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