SCREENING OF HERBAL FRACTIONS FOR ANTIBIOTIC DRUG RESISTANCE REVERSAL

A

Thesis presented

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Degree of Master of Science in Biotechnology

BY

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Under The Guidance

of

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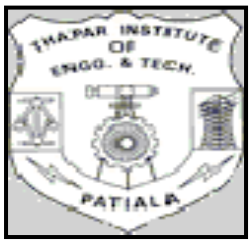

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CERTIFICATE

This is to certify that the thesis entitled, **"Screening of Herbal Fractions for Antibiotic Drug Resistance Reversal"** submitted by Gurdeep Singh in partial fulfilment of the requirement for the award of the degree of Master of Science in Biotechnology, to Thapar Institute of Engineering and Technology, Patiala, is an authentic record of his own work carried out by him during the period of six months from January 2005 to June 2005, under my supervision and guidance. This report has not been submitted for the award of any other degree or certificate in this or any other university or institute.

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EXECUTIVE SUMMARY

The increasing phenomenon of acquisition of resistance among microorganisms towards antimicrobial drugs is attributed due to their indiscriminate and improper use of current antimicrobial and the genetic plasticity of microorganisms converting them into drug resistant super infectious agents. These are difficult to treat and are responsible for variety of chronic infections.

The problem of microbial resistance is growing and the outlook for the use of antimicrobial drugs in the future is still uncertain. Therefore, actions must be taken to control the use of antibiotic, develop research to better understand the genetic mechanisms of resistance, and to continue studies to develop new drugs, either synthetic or natural. Natural products produced from plant extracts have great potential as antimicrobial compounds against drug resistant microorganisms.

Staphylococcus aureus is a commensal organism cited as major hospital acquired pathogen and strains of this species are becoming resistant to practically all classes of antibiotics. Most of the Methicillin-Resistant *Staphylococcus aureus* (MRSA) strains are rapidly becoming resistant to Ciprofloxacin (Fluoroquinolone). As the antimicrobial drug resistance in microorganisms increases rapidly, there is a continual need for a pipeline of new agents or NON-OTC (Over the counter) drug formulations to combat antimicrobial drug resistance in microorganisms.

Plants produce scores of phytochemicals, which have the innate ability to be used as antimicrobial drugs. However, there are some phytochemicals, which enhance the bioactivity of other compounds, referred to as biopotentiators or bioenhancers. These bioenhancers also work as synergists by reverting the antimicrobial drug resistance. The mechanisms of drug resistance reversal are being looked at for development of newer and effective drugs formulations of bioenhancers with resistant drug to overcome antimicrobial drug resistance.

In the present study, the Ciprofloxacin MIC determination was conducted in *Staphylococcus aureus*-NCTC-6571 strain by using Micro-broth dilution assay. *In vitro* development of resistance to Ciprofloxacin was investigated in the parent strain by exposing to subinhibitory MIC value of ciprofloxacin during a 50-days period. Subculturing led to resistance development, regardless to the initial potency of the Ciprofloxacin. The MIC of Ciprofloxacin for *Staphylococcus aureus* NCTC-6571 strain

increased from 4.9 mg/L to 625 mg/L. The selected mutant strain was subcultured in antibiotic free media for another 10-20 serial passage to get stable mutant.

The antimicrobial activity or MICs of different column chromato-graphically purified fractions of C*allistemon rigidus R.Br*. was conducted in *Staphylococcus aureus* NCTC-6571 and Mutant strain of *S. aureus* by Agar well diffusion assay and Broth dilution assay.

Finally, formulations were made by the synergistic combinations of resistant drug (Ciprofloxacin) and subinhibitory levels of different bioactive fractions of *Callistemon rigidus R.Br*. or fractions having no antimicrobial activity. The MICs of formulations were conducted in parent strain as well as in the mutant strain of *Staphylococcus aureus* by Micro-broth dilution method.

MIC of fraction fifth (F_5) against parent strain and Mutant strain were found 78 mg/L and 625 mg/L respectively. While other fractions had no antimicrobial activity against both the strains of *Staphylococcus aureus*. The Subinhibitory combination of fraction fifth (F_5) and resistant drug (Ciprofloxacin) showed antimicrobial activity against mutant strain of *Staphylococcus aureus* as well as parent strain. At the end of study, Death pattern of both the strains (*S.aureus* NCTC-6571 and Mutant strain of *S.aureus*) were conducted against Subinhibitory combination of fraction fifth (F_5) and resistant drug (Ciprofloxacin) by kill kinetics.

The present study indicates that Fraction (F_5) obtained from the leaf extracts of *Callistemon rigidus R.Br*, serves as potential lead Biopotentiator or Bioenhancer to revert Ciprofloxacin resistance in *Staphylococcus aureus* NCTC-6571. It further warrants studies to determine its FIC (Fractional Inhibitory Index) with Ciprofloxacin as well as its ability to enhance the antimicrobial potential of other Fluoroquinolone class of antibiotics like Ciprofloxacin, Pefloxacin, Oflaxacin and Norfloxacin and Gatifloxacin.

ABBREVIATIONS

AWD assay	**Agar Well Diffusion Assay**
Cf	**Ciprofloxacin**
CFU	**Colony Forming Unit**
DMSO	**Dimethyl Sulfoxide**
MBC	**Minimum Bactericidal Concentration**
MDR	**Multi Drug Resistant**
MRSA	**Methicillin Resistant *Staphylococcus aureus***
MHA	**Mueller-Hinton Agar**
MHB	**Mueller-Hinton Broth**
MIC	**Minimum Inhibitory Concentration**
MIZ	**Maximum Inhibitory Zone**
OD	**Optical Density**
SA	***Staphylococcus aureus* NCTC 6571**
SA (M)	***Staphylococcus aureus* (Mutant)**
TSA	**Trypticase Soya Agar**
TTC	**2, 3, 5-Triphenyl Tetrazolium Chloride**
UV	**Ultra Violet**
WHO	**World Health Organization**

INTRODUCTION

"Antimicrobial Resistance: Growing global public health threat"

The discovery of antibiotic Penicillin was the triumph over the disease causing bacteria, is one of the greatest success stories of modern medicine. Today after 50 years of the widespread use of antibiotics many of them do not pack the same punch once they did. This is due to antimicrobial drug resistance which is defined as "Survival of the fittest" microorganism in the presence of drugs.

The enhanced global flow of antimicrobial in terms of production and usage has been a precedent in development of antimicrobial drug resistance in human pathogens. These pathogens have common tendency to accumulate multiple resistance under antibiotic pressures and selection and at times are referred to as Multidrug resistant (MDR) pathogens/microorganisms.

The problem of antimicrobial drug reistance is not new, but has increased during the last decade, creating a serious threat to the treatment of infectious diseases (Conly, 2002). Frequently misuse and overuse of antimicrobials in many developing countries, had led to emergence of resistant pathogens which have been responsible for morbidity, mortality and cost of health care (Sharma *et al.* 2005). Some important human pathogens that have recently been reported to have acquired antibiotic resistance are; *Mycobacterium tuberculosis, Staphylococcus aureus, Neisseria gonorrhoeae, Haemophilus influenzae, and Pseudomonas aeruginosa* (WHO, 2002).

Staphylococcus aureus has been recognized as one of the most important gram positive bacterial pathogens contributing for infections and epidemic throughout the world. Resistance to Staphylococcus was reported in 1961, when first Methicillin (Penicillin) resistant *Staphylococcus aureus* (MRSA) was isolated in Europe, followed by Australia in 1966 and Unites States of America in 1968. MRSA continues to be a major cause of serious infection to man, both in hospitals and in the community. In the early 1980's MRSA reports consisted of isolated cases but later in 1982 epidemic MRSA strains (EMRSA) were described as multi-resistant strains with special capacity to colonize patients and staff and cause widespread outbreaks of infections (Pollivar *et al.*, 1982). These epidemic MRSA strains have subsequently spread to various parts of the world.

Resistance to antimicrobials is a natural biological phenomenon that can be amplified or accelerated by a variety of factors, including human practices. The use of

an antimicrobial for any infection, real or feared, in any dose and over any time period, forces microbes to either adapt or die, in a phenomenon known as “selective pressure”. The microbes, which adapt and survive, carry genes for resistance, which can be passed on when bacteria replicates. The resistance mechanisms in microorganisms are based on four strategies: i) Inactivation of the drug, ii) Prevention of the drug to reach its target, iii) Reduction of target’s susceptibility, or iv) Acquisition of new, insensitive target (Brigitte, 2002).

Resistance arises, either by mutation and selection for growth in presence of increasing concentrations of antibiotics, or by the acquisition of foreign resistance determinants. Resistance can spread either vertically, by dissemination of resistant clones, e.g. in case of MRSA or horizontally, by inter- and intra-species-specific gene transfer, such as by i) Transduction, ii) Conjugation, and iii) Transformation (Brigitte, 2002).

Staphylococcus aureus is a prevalent bacterium carried by humans that can cause a number of problems, from mild skin infections to serious diseases including food poisoning, wound infections, pneumonia, and toxic shock syndrome. The World Health Organization (WHO, 2002) recently reported that more than 95% of *S. aureus* worldwide is resistant to Penicillin, and 60% to its derivative Methicillin. Today in the U.S. more than 20% of all enterococcal infections, that is, infections caused by intestinal bacteria in the genus *Enterococcus*, are resistant to Vancomycin, once considered the antibiotic of last resort.

1.1 Factors that encourage the spread of antimicrobial resistance

The emergence and spread of antimicrobial resistance are complex problems driven by numerous interconnected factors, many of which are linked to the misuse of antimicrobials and thus amenable to change. In turn, antimicrobial use is influenced by interplay of the knowledge, expectations, and interactions of prescribers and patients, economic incentives, characteristics of a country's health system, and the regulatory environment (WHO, 2002).

Selective pressure exerted by widespread antimicrobial use is considered to be a major factor in the emergence of resistance. In some countries, antimicrobial drugs are available over the counter and are present in folk remedies; in others, their abuse in prophylactic and empiric therapy and the indiscriminate use of broad-spectrum antimicrobial drugs in the community have been major contributors. Resistance

factors can spread rapidly, not only locally but also, with greater movement of people around the world, globally. Microorganisms and their resistance factors may also be transferred from country to country in animals and commercially produced fruits and vegetables. (CCDR, 1997)

Hospitals are a critical component of the antimicrobial resistance problem worldwide. The combination of highly susceptible patients, intensive and prolonged antimicrobial use, and cross-infection have resulted in nosocomial infections with highly resistant bacterial pathogens. Resistant hospital-acquired infections are expensive to control and extremely difficult to eradicate (WHO, 2002).

The problem of microbial resistance is growing and the outlook for the use of antimicrobial drugs in the future is still uncertain. Therefore, action must be taken to reduce this problem, for example, to control the use of antibiotic, develop research to better understand the genetic mechanism of resistance, and to continue studies to develop new drugs, either synthetically or natural (Nascimento *et al.,* 2000).

1.2 Current antimicrobial drugs: Fluoroquinolones

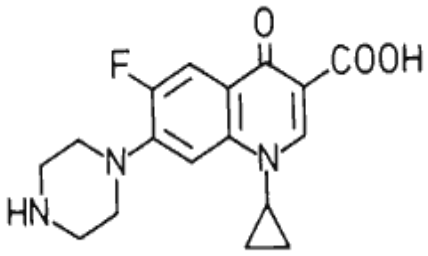
The fluoroquinolones are fluorinated derivatives of the quinolone, Nalidixic acid. Fluoroquinolone are highly active, broad spectrum antibiotics with many uses in both human and veterinary medicine (Piddock, 1998). Fluoroquinolone antimicrobial drugs were a major therapeutic advance of the 1980's because they have 100-fold greater activity than their parent compound, Nalidixic acid. Unlike Nalidixic acid, which is used only for urinary infections and occasionally Shigellosis, the fluoroquinolones have a broad range of therapeutic indications and are given as prophylaxis, e.g., for neutropenic patients (Livermore *et al*., 2002).

The first group, comprising the earliest quinolones such as Nalidixic acid and Cinoxacin, do not achieve systemic bactericidal levels; members of this group are useful for treating lower urinary tract infections. Members of the second group of fluoroquinolones, which includes Ciprofloxacin, Ofloxacin, and Levofloxacin, do achieve bactericidal levels in the blood. Unfortunately, a number of organisms, such as *S. aureus* and *P. aeruginosa*, quickly became resistant to these agents. These agents continue to be active against many gram-negative aerobes but have limited activity against gram-positive organisms. The third group of fluoroquinolones, which includes Gatifloxacin and Moxifloxacin, have improved activity against gram-positive cocci, including *S. pneumoniae* and some *Staphylococci*, but less activity against

gram-negative bacilli than Ciprofloxacin. The final group, which includes Trovafloxacin, has enhanced activity against gram-positive organisms. (American Pharmaceutical Association, 2001) Members of this group also have some activity against anaerobes, which is generally lacking in other fluoroquinolones. However, the use of Trovafloxacin has been restricted since 1999 due to reports of severe liver damage (FDA, 1999). Other more potent fluoroquinolones are currently under development that have activity against some organisms resistant to other agents in this class (Katzung, 2001).

Ciprofloxacin is 1-cyclopropyl-6-fluoro-1, 4-dihydro-4-oxo-7-(1 piperazinyl)-3-quinolinecarboxylic acid and has *in vitro* activity against a wide range of gram-positive and gram-negative microorganism. The bacterial action of Ciprofloxacin results from the inhibition of the enzymes topoisomerase II (DNA gyrase) and topoisomerase IV, which are required for bacterial DNA replication, transcription, repair and recombination. The mechanism of action of fluoroquinolones is different from that of Penicillins, Cephalosporins, Aminoglycosides, Macrolides and Tetracyclines; therefore, microorganisms resistant to these classes of drugs may be susceptible to Ciprofloxacin and other quinolones.

In vitro resistance to Ciprofloxacin develops slowly by multiple step mutations by serial passage technique (Kim *et al.*, 2003). Fluoroquinolone resistance increases stepwise when bacteria are sequentially challenged with increasing concentrations of drug (Zhao and Darlica, 2001).



Ciprofloxacin

The proportion of *E. coli* isolates reported as resistant to Ciprofloxacin rose slowly but steadily, from 0.8% in 1990 to 3.7% in 1999. For *Klebsiella species*, the resistance rate rose from 3.5% of reports in 1990 to 9.5% in 1996, before declining to 7.1% by 1999. *Enterobacter species* showed a similar pattern to *Klebsiellae*: the prevalence of resistance rose from 2.1% in 1990 to 10.5% in 1996, and then dipped to 7.9% in 1998 before rising to 10.9% in 1999 (Livermore *et al.,* 2002). According to the report of *Antimicrobial Management Program*, 39% of *Staphylococcus aureus* strains were found to be resistant to Ciprofloxacin from 1990 to 2000.

1.3 Antimicrobial agents from plants:

There are essentially two routes of drug discovery, firstly, synthesizing entirely new chemicals and evaluating them for a particular pharmaceutical use. Secondly, identifying the chemical of biological origin (natural product chemistry) and evaluate it for direct or indirect use as a template for the development of a new drug (Saxena and Kumar, 2002).

Plants have provided a source of inspiration for novel drug compounds, as plant derived medicines have made large contributions to human health and well-being (Iwu *et al.*, 1999). Their role is two fold in the development of new drugs by becoming

- Base for the development of a medicine, a natural blueprint for the development of new drugs, or;
- A phytomedicine to be used for the treatment of disease

Plant extracts have great potential as antimicrobial compounds against microorganisms. Thus, they can be used in the treatment of infectious diseases caused by resistant microbes. *Pinus densiflora* leaf extract have potential activity against the harmful gastric pathogens like *Clostridium perfringens*, *Staphylococcus aureus* and *Escherichia coli* without inhibiting the beneficial gastric flora like the *Lactobacillus* sp., *Bifidobacterium adolescentis*, *B. breve* etc. due to the presence of α-pinene (Ho-Joung Jeon *et al.,* 2001). The ethanolic extracts from Clove (*Syzygium aromaticum*), Lemonbalm (*Melissa officinalis*), have essential oils as the main active ingredient, showing antimicrobial activity against *Staphylococcus aureus* (Nascimento *et al.,* 2000).

Antimicrobial compounds from plants belong to different major groups like Phenolics, Quinones, Flavinoids, Tannins Terpenoids & Essential Oils, and Alkaloids etc. *Hypericum* extracts, polyphenolics from husk of *Cocos nucifera,* ethanol extract of the root of *Guatteria multivenia*, Allicin from Garlic (*Allium cepa*) have shown excellent activity against MRSA, *Candida albicans, Cryptococcus neoformans* and are the potential candidates to be developed into next generation of antimicrobials to combat multidrug resistance (MDR) in pathogenic

microorganisms (Ankri & Mirelman, 1999; Esquenazi *et al*, 2002; Gibbons *et al.,* 2002; Zhang *et al.*, 2002).

So far the clinicians are at a lurch position wherein they find themselves trapped in two opposite trends, the first one is prevalence and rapid increase in number of human pathogens and decrease in the contribution for research and development of antimicrobial drug is a cost intensive proposition demanding 100$ to 350 $ billion dollars. Moreover, there is no assurance that the product developed would not encounter resistance in due course of time. (Gold and Moellering, 1996) Therefore, the pace of development of the antimicrobial compounds of plant origin has been drastically reduced.

In the 1980's, Methicillin-Resistant *S. aureus* (MRSA) emerged and became endemic in many U.S. hospitals. Vancomycin was the only antimicrobial agent with uniform effectiveness against MRSA. In the 1990's, Vancomycin-Resistant *Enterococci* (VRE) emerged and also became endemic in many U.S. hospitals. In 1996, the first *S.aureus* strain with decreased susceptibility to Vancomycin (Glycopeptide intermediate-resistant *S. aureus* [GISA]) was reported in Japan. In 1997, the first GISA strains were reported in the United States. (CDC, 2002). The prevalence of nosocomial Vancomycin-Resistant *Enterococci* in the United States increased from 0.3% in 1989 to 7.9% in 1993 and 23% in 1999 (CDC, 1999).

1.4 Possibilities of reverting antimicrobial drug resistance

Two general strategies have been suggested to overcome antibiotic resistance: optimizing existing antibiotic therapies and designing new antibiotic therapies (American Pharmaceutical Association, 2001).

Firstly, the altered use of existing antibiotics in a number of ways: the duration may be decreased, the dose may be changed, or the use of particular agents can be limited or completely restricted (American Pharmaceutical Association, 2001). Secondly, the combination antimicrobial therapy has been shown to be effective in the treatment of Acquired Immunodeficiency Syndrome (AIDS) and Tuberculosis. The theoretical advantage to combination therapy is that the chance of mutant strains developing resistance to both (or all) antibiotics is very small. In fact, it is the product of chance that resistant mutants will develop to each antibiotic (American Pharmaceutical Association, 2001).

The synergistic effect from the association of antibiotic with the lead fractions obtained from plant extracts against resistant bacteria leads to new choice for the treatment of infectious diseases. These have been frequently referred to as resistance modifying agents or biopotentiators. These agents potentiate the activity of an antibiotic against a resistant strain. Synergistic effect of Anacardic Acid and Totarol with Methicillin inhibits MRSA. This effect enables the use of respective antibiotic when it is no longer effective by itself during therapeutic treatment (Nascimento *et al.,* 2000). Recently 5'- methoxyhydnocarpin (5' MHC) has been found to have a very strong synergistic effect and enhances the antimicrobial action of Berberine (Stermitz *et al.*, 2000 and Guz and Stermitz, 2000). A variety of resistance modifying agents like Epicatechin Gallate has been found to revert MRSA to Methicillin Susceptible Staphylococcus aureus (MSSA) (Gibbons, 2004).

The possible advantage of this approach is the same old drug can be reused as a combination and is less cost intensive approach as compared to new drug discovery altogether.

In the present study, we propose to develop drug formulations towards antimicrobial drug resistance reversal in ciprofloxacin resistant *Staphylococcus aureus* by using biopotentiators (bioenhancers) properties of different fractions of *Callistemon rigidus R.Br*.

REVIEW OF LITERATURE

2.1 Need of new Antimicrobials

Antibiotics were treated as miracle drugs when they first became available half a century ago. However, their popularity rapidly led to overuse. Over the last decade, it has become well known that antibiotics are losing their effectiveness as bacteria evolve resistance against them and new drugs only rarely reach the market (Cuevas, 2003). The problem of microbial resistance is growing and the outlook for the use of antimicrobial drugs in the future is still uncertain. Therefore, action must be taken to control the use of antibiotic, develop research to better understand the genetic mechanism of resistance, and to continue studies to develop new drugs, either synthetically or natural (Nascimento *et al.,* 2000).

Microorganisms with increasing rates of resistance to commonly used antimicrobials includes Methicillin-Resistant *Staphylococcus aureus* (MRSA), Vancomycin-Resistant *Enterococci* (VRE), *Shigella* and *Salmonella* species resistant to multiple antibiotics, Penicillin-Resistant *Streptococcus pneumoniae* (PRSP) (Conly, 2002).

Staphylococcus aureus is recognized as an important cause of community and nosocomial infections and the most common organism cultured from wound and soft-tissue infections (Smith and Jarvis, 1999). Superficial infections caused by *S. aureus* include folliculate, impetigo, and cellulitis. Severe infections such as endocarditis, osteomyelitis, and pneumonia are less common. However, *S. aureus* is the cause of 16% of all nosocomial bloodstream infections, which had a mortality rate of 82% before the development of antibiotics (Smith and Jarvis, 1999).

Currently, the overall mortality associated with *S. aureus* bacteremia ranges from 11% to 43% and has not declined in the past 15 years (Lowery *et al.,* 1998). Resistance of *S. aureus* to Methicillin, which indicates resistance to all ß-lactam antibiotics, was first described in 1961; soon after semisynthetic Penicillinase-Resistant Penicillin was introduced (Hryniewicz, 1999). In 1980's, Methicillin-Resistant *S.aureus* (MRSA) was found in many countries of Europe and in the United States, but only in sporadic outbreaks. It is believed that the emergence of MRSA was caused by overuse of antibiotics, coupled with inadequate infection control measures (Hryniewicz, 1999).

Staphylococcus aureus continues to be a major cause of community-acquired and health-care related infections in the United States and around the world (Lowy,

1998). Approximately 20% of community-acquired and nosocomial bacteremias in the United States are caused by *S. aureus* (Cockerill *et al,* 1997). The emergence of high levels of Penicillin resistance followed by the development and spread of strains resistant to the semisynthetic Penicillins (Methicillin, Nafcillin, and Oxacillin), Macrolides, Tetracyclines, and Aminoglycosides has made therapy of Staphylococcal disease a global challenge (Lowy, 1998, Struelens *et al.,* 1994 and Maranan *et al.,* 1997).

In the 1980's, because of widespread occurrence of Methicillin-Resistant *S. aureus* (MRSA), empiric therapy for Staphylococcal infections (particularly nosocomial sepsis) was changed to Vancomycin in many health-care institutions (Ena *et al.*, 1993). Vancomycin use in the United States also increased during this period because of the growing numbers of infections with *Clostridium difficile* and coagulase-negative *Staphylococci* in health-care facilities (Cunha, 1995, Ena *et al.*, 1993). Thus, the early 1990's saw a discernible increase in Vancomycin use. As a consequence, selective pressure was established that eventually led to the emergence of strains of *S. aureus* and other species of *Staphylococci* with decreased susceptibility to Vancomycin and other Glycopeptides.

The first case of GISA (Glycopeptide intermediate-susceptible *S. aureus)* was reported in Japan in 1996. Two additional cases were reported in the United States in 1999. It was believed that prolonged treatment with Vancomycin led to the emergence of a strain of *S. aureus* with intermediate resistance to Vancomycin (Waldvogel, 1999). In 1997, the first strain of *S. aureus* with reduced susceptibility to Vancomycin and Teicoplanin was reported from Japan (Hiramatsu *et al.*, 1997).

The widespread use of antimicrobial drugs for immunocompromised patients and in the intensive care units of modern hospitals clearly results in the selection of multidrug resistant organisms that causes serious nosocomial infections (Gold and Moellering, 1996). The prevalence of antimicrobial-resistant human pathogens is rapid increases, but the discovery and development of new antimicrobial drugs that are active against multidrug-resistant organisms have slowed dramatically (Gold and Moellering, 1996).

2.2 Resistance in gram-positive bacteria

The introduction and increasing use of antibiotics for antibacterial therapy has initiated a rapid development and expansion of antibiotic resistance in

microorganisms, particularly in human pathogens such as, *Staphylococcus aureus*. These pathogens have common tendency to accumulate multiple resistance under antibiotic pressure and selection (Brigitte, 2002). MRSA, that have acquired multiresistance to all classes of antibiotics, have become a serious nosocomial problem. In the industrialized world 60% hospital-acquired infections are caused by drug resistant microbes (Overcoming antimicrobial resistance, 2005).

The proportions of *Staphylococcus aureus* isolates resistant to Methicillin/Oxacillin (MRSA) increased from approximately 39% in 1999-2000. Nearly all strains of *Staphylococcus aureus* in the United States are resistant to Penicillin, and many are resistant to newer Methicillin-related drugs. In some countries up to 80% of nosocomial *Staphylococcus* infections are Methicillin resistant (MRSA) **(**WHO, 2000**)**. In 1991, 29% of *S. aureus* strains were Methicillin-resistant, and in 1996, there was a report of an *S. aureus* strain with intermediate Vancomycin resistance (VISA) (WHO, 2002).

Recently, the emergence of first MRSA with reduced Vancomycin susceptibility evoked the spectra of a totally resistant *S.aureus*. The rapid development of resistance is due to mutational events and/or gene transfer and acquisition of resistant determinants, allowing strains to survive antibiotic treatment (Brigitte, 2002). The problems with multiresistance expand also to PRSP that are partially or totally resistant to multiple antibiotics, and to Vancomycin resistant *Enterococcus* species, to commonly used antibiotics.

2.3 Strategies of resistance and dissemination of resistance determinants

Resistance mechanism in microorganisms are based on four strategies, *(i)* Inactivation of the drug, *(ii)* Prevention of the drug to reach the target, *(iii)* Reduction of the target's susceptibility, *(iv)* Acquisition of a new, insensitive target. Resistance arises either by mutation and selection for growth in presence of increasing concentrations of antibiotics, or by the acquisition of foreign resistance determinants (Brigitte, 2002).

Resistance can spread either vertically, by dissemination of resistant clones, as exemplified by the worldwide spread of epidemic Methicillin-Resistant *Staphylococcus aureus* (MRSA), or horizontally, by inter and intra-species-specific gene transfer, such as by 1) transduction, 2) conjugation; or 3) transformation (Brigitte, 2002). Transduction is mediated by bacteriophages and is generally of

narrow host range, restricted to particular species. A much broader host range, even crossing the gram barrier, is covered by conjugation, which requires direct cell-to-cell contact and the presence of conjugative elements. Finally the transformation is found in strains, which are naturally competent, as e.g. *Pneumococc*i, which have the ability to take up naked DNA from the environment. This allows them to remodel their DNA sequences by exchanging DNA cassettes leading to the formation of the so-called mosaic genes (Brigitte, 2002).

2.4 Fluoroquinolone Resistance mechanisms in gram-positive organisms

Fluoroquinolones are highly active, broad spectrum antibiotics with many uses in both human and veterinary medicine (Piddock, 1998). Fluoroquinolones were initially introduced for the treatment of Gram-negative bacterial infections in the 1980s. However, because of their Gram-positive bacterial spectrum, they have also been used to treat bacterial infections caused by *Pneumococci* and *Staphylococci* (Lowy, 2003). Genetic and biochemical experiments have shown that two enzymes are the targets of fluoroquinolones (Everett *et al.*, 1997). In Gram negative bacteria such as *Escherichia coli*, *Pseudomonas aeruginosa*, and *Neisseria gonorrhoeae*, the primary target is DNA gyrase and the secondary target DNA topoisomerase IV, but in Gram positive bacteria, such as *Staphylococcus aureus* and *S. pneumoniae*, the primary and secondary targets are reversed (Pan *et al.*, 1996).

Fluoroquinolones exert their bactericidal activity by interacting with two targets, the DNA gyrase and the DNA topoisomerase IV. They trap the enzyme reaction intermediate at a step in which the enzyme is bound to DNA and can be converted to a form in which both the strands of DNA are cleaved (Heisig, 2001). Both the enzymes are required for bacterial DNA replication, transcription, repair and recombination. The mechanism of action of fluoroquinolones is different from that of Penicillins, Cephalosporins, Aminoglycosides, Macrolides and Tetracyclines; therefore, microorganisms resistant to these classes of drugs may be susceptible to Ciprofloxacin (Second generation fluoroquinolone) and other quinolones (Teva Pharmaceuticals, 2004).

Fluoroquinolone resistance develops as a result of spontaneous chromosomal mutations in the target of the antibiotic, topoisomerase IV or DNA gyrase, or by the induction of a multidrug efflux pump (Lowy, 2003). The stepwise resistance to fluoroquinolones arises when bacteria are sequentially challenged with increasing

concentrations of drug (Zhao and Darlica, 2001). The confluence of high bacterial density, the likely preexistence of resistant subpopulations, and the sometimes limited quinolone concentrations achieved at sites of staphylococcal infections creates an environment that fosters selection of resistant mutants (Hooper, 2002). Fluoroquinolone resistant isolates usually contain one or more mutations in a small section of gyrA or parC; mutation in gyrB and parE is rare (Cohen, 1992).

New generations of quinolones may target both enzymes similarly and thus may contribute to the low frequency of mutant selection. Neither enzymatic mechanisms of quinolone inactivation exit in bacteria yet, not indication for transfer of clinically important quinolone resistance (Brigitte, 2002).

2.5 Plant Products as Antimicrobial Agents

The U.S. Pharmaceutical industry spent a record $4.1 billion on research and development in 1985, an increase of 11.6% from 1984 (Anonymous, 1986). In the same year, the American consumer purchased in excess of $8 billion in community pharmacies for prescriptions whose active constituents are still extracted from higher plants (Farnsworth and Soejarto, 1985). For the past 25 years, 25% of all prescriptions dispensed from community pharmacies in the United States contained active principles that are still extracted from higher plants (Farnsworth and Morris, 1976).

Approximately, 119 pure chemical substances extracted from higher plants are used in medicine throughout the world (Farnsworth *et al.,* 1985). The World Health Organization estimates that 80% of the people in developing countries of the world rely on traditional medicine (Anonymous, 1986) for their primary health care needs, and about 85% of traditional medicine involves the use of plant extracts. Therefore, about 3.5 to 4 billion people in the world rely on plants as sources of drugs (Farnsworth *et al.,* 1985).

Historically, plants have provided a good source of anti-infective agents; Emetine, Quinine, and Berberine remain highly effective instruments in the fight against microbial infections. Phytomedicine derived from plants have shown great promise in the treatment of intractable infectious diseases including opportunistic AIDS infections (Iwu *et al.,* 1999). Plants are rich in a wide variety of secondary

metabolites, such as tannins, terpenoids, alkaloids, and flavonoids, which have been found *in vitro* to have antimicrobial properties (Cowan, 1999).

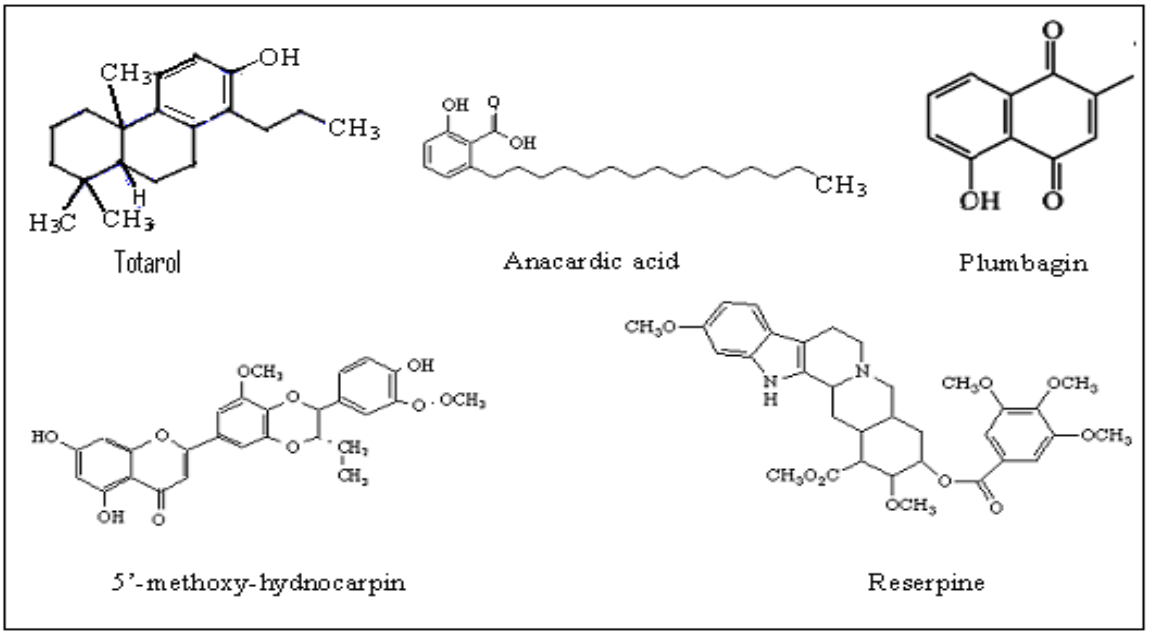


Fig. 1 Some Potential Antimicrobial Compounds Isolated From Plants

Diterpenes such as Totarol have been shown to potentiate Methicillin activity against MRSA *via* interference of Penicillin Binding Protein 2' expression (Nicolson *et al.,* 1999). The synergistic combination of hydrolysable tannins Tellimagrandin I with tetracycline reduces the MICs of Tetracycline against Methicillin-Resistant *Staphylococcus aureus* (MRSA) (Shiota *et al.*, 2000). The multidrug resistance inhibitor Reserpine caused a four fold reduction in the MIC of Tetracycline against a variety of multidrug resistant MRSA and MSSA (Gibbons *et al.*, 2000 and Schmitz *et al.*, 1998).

The polyketide derived Rhein and Plumbagin have striking activity against multidrug resistant *Staphylococcus aureus* and some gram negative bacteria (Tegos *et al.*, 2002). Synergistic effect of Anacardic acid and Totarol with Methicillin inhibits MRSA (Nascimento *et al.,* 2000).

Thus, the plants may have evolved compounds which evade multidrug resistance mechanisms and that plant antimicrobials might be developed into broad spectrum antibiotics in combination with inhibitors of multidrug resistance (Tegos *et al.*, 2002).

2.6 Development of a Fluoroquinolone resistant model

Fluoroquinolone resistance increases stepwise, when bacteria are sequentially challenged with increasing concentrations of drug starting from the subinhibitory levels (Zhao and Darlica, 2003). *In vitro* development of resistance to a novel fluoroquinolone, DW286, as well as to Ciprofloxacin, Gatifloxacin, Sparfloxacin and Trovafloxacin, was investigated in eight MRSA clinical isolates (Kim *et al.*, 2003).

The subculturing of *Staphylococci* and *Pneumococci* in subinhibitory concentrations of quinolones can lead to the *in vitro* selection of resistant mutants (Boos *et al*, 2001, Davies *et al.*,, 1999). The Serial passage method is widely used for the induction of *in vitro* resistance to fluoroquinolones in gram positive bacteria. First of all, MIC value of the respective fluoroquinolone was measured by Micro-broth dilution method with Mueller-Hinton broth (NCCLS, 1997). Then, the bacterial strains were subcultured in subinhibitory concentrations of fluoroquinolones during a 50 day period. When the MIC of any agent for any bacterial strain increased four fold the passage was stopped. The selected mutant strain was subcultured in antibiotic free MH Agar for another ten serial passage (Kim *et al.*, 2003).

2.7 Overcoming drug resistance

Prudent, conservative use of existing drugs is an important way to prolong their useful clinical life. However, the long-term solution to microbial drug resistance is to develop new antimicrobial drugs. Two strategies are used to find new agents: either analogs of existing compounds can be created, or entirely new classes of drugs can be developed.

The production of new analogs of existing antimicrobial compounds is generally straightforward and often productive; largely because of new compounds that are structural mimics of older ones have predictable site of action. In many cases, parameters such as solubility and affinity can be changed by altering chemical structure without altering the site of drug action. The new compounds actually be more potent than the parent compound, and, because resistance is based on structural recognition, the new compound may not be recognized by resistant factors. With this basic strategy in mind, new β-lactam antibiotics and new tetracycline related compounds are routinely synthesized and tested. (Brock, 1997).

New classes of antimicrobial compounds are much more difficult to identify and cost intensive. They must be isolated from the natural sources or synthetically designed to interact with specific microbial structures and then screened for efficacy and toxicity. To be effective, new antimicrobial compounds must work at novel sites in bacterial metabolism and biosynthesis, or be structurally dissimilar to existing compounds, thus avoiding existing resistance factors. To overcome the cost implication and time reverting drug resistance is a fruitful proposition to encounter antimicrobial drug resistance.

2.8 Bioassays

Bioassays play an important role while evaluating antimicrobial activity of plant fractions and antibiotics. Bioassays are divided into two categories based on their performance: **Prescreen assays** and **Screen assay**. Prescreen assay is applied to large number of initial samples to check whether the plant extracted fractions have desired bioactivity or not. While the screen assays are used to select a compound or material for the detailed study for its bioactivity. *In vitro* assessment of antimicrobial activity of any extract or material is done by two methods: **Agar well diffusion assay** and **Broth dilution method**.

2.8.1 Prescreen assay: Agar Well Diffusion Assay

The agar well diffusion assay is most commonly used prescreens to determine antimicrobial susceptibility (Cowan, 1999). Initial screening of potential antibacterial and antifungal compounds from plants may be performed with pure substances or crude extracts. Agar well diffusion assay (Reddish, 1929) is an advanced version of ditch plate assay initially designed by Alexander Flemming which uses wells prepared in agar to evaluate the antimicrobial qualities in antiseptic solutions *(*Fleming, 1924). Similarly, Heatley (1944) used agar diffusion assay as a means of monitoring the extraction and purification of penicillin. In this assay, the test material is placed in the agar well in the centre of agar plate and then seeded with test organism. If on incubation, a circular clear “zone of inhibition” surrounded the reservoir or agar well, that means the organism is sensitive to the test material.

Agar well assay is popular prescreen assay used by the clinical microbiologists and phytochemists to check the potential antimicrobial activity of plants and their use in traditional medicines for the treatment of infectious diseases (Navarro *et al.*, 1996).

2.8.2 Screening assay: Micro-Broth Dilution assay

Broth micro-dilution denotes the performance of broth dilution test in micro-dilution plate (microtitre plate) with a capacity of 300µl per well. This test is useful in the determination of Minimum Inhibitory Concentration (MIC) using dilution method. The lowest concentration that will inhibit the visible growth of the microorganism is referred is known as the MIC.This test is rapid in the assessment of antimicrobial activity of plant extracts or antibiotic compounds. The test also provides an opportunity to actually monitor the killing efficacy of the herbal extract by using a plate count method (Eloff, 1998 and Sparg, *et al.* 2002).

2.9 Callistemon rigidus R.Br.

Callistemon rigidus R.Br. is commonly known as "Bottlebrush" belongs to family Myrtaceae. It is a Stiff, upright shrub characterized by red flower. Spikes that are shaped like bottlebrushes (Fig. 2). Flowers are comprised of red, showy stamens each approximately 1 inch long. The flowers, with their showy stamens, encircle the stem and form 4-inch-long, cylindrical spikes that appear in the spring and summer. The persistent fruits are hard seed capsules that occur in tight clusters around the stem. The stiff, narrow leaves of this shrub are sharply pointed and fragrant when crushed. Bottle-brush grows from 5 to 15 feet tall. (Gilman, 1999).

Fig. 2 *Callistemon rigidus R. Br.*

Previous work has indicated that *Callistemon rigidus R.Br.* leaf extract has a potential antibiotic activity against human MDR pathogens (Anita Bansal, 2003). Further fractionation of the crude extracts lead to isolations of some fractions (Charu Gomber, 2004). These fractions have been evaluated for their efficacy as biopotentiators for reverting Ciprofloxacin resistance in *Staphylococcus aureus*.

AIM OF THE STUDY

The present work is oriented in screening the drug resistance reversing efficacy of lead fractions obtained from crude methanolic extract of leaves of *Callistemon rigidus R.Br*. The *Callistemon rigidus R.Br*. leaf extract has a potential antibiotic activity against human MDR pathogens (Anita Bansal, 2003). Further fractionation of the crude extracts lead to isolations of some fractions (Charu Gomber, 2004) that may act as biopotentiators or bioenhancers with resistant drug to overcome antimicrobial drug resistance in microbes.

The objectives of the present study were:

- Development of a Ciprofloxacin Resistant *Staphylococcus aureus* NCTC-6571 (surrogate/ teaching microorganisms) as a model for studying the phenomenon of antimicrobial drug resistance.
- Growth curve studies of *Staphylococcus aureus* NCTC-6571 and mutant *Staphylococcus aureus*.
- Screening of non-antimicrobial fractions of *Callistemon rigidus* leaf extract for their antimicrobial action and drug resistance reversal by *in vitro* methods.

MATERIALS AND METHODS

4.1 Test microorganism and growth conditions

Staphylococcus aureus-NCTC 6571 was used in the presence course of studies. It was converted into a resistant model (Mutant). Both the parent strain and mutant strain were activated in Mueller-Hinton Broth at 37°C for 18-24 hours @ 120 rpm prior to assays.

4.2 Maintenance of Microorganisms

The Staphylococcus aureus were maintained on Trypticase Soya Agar (TSA) slants and stored at 4°C. Activation of the culture was carried out by streaking culture from the slants on to a Muller-Hinton Agar (MHA) (HiMedia) plate and then incubating them overnight at 37°C. A single colony was then picked from this plate and transferred to Muller Hinton broth and incubated for 18-24 hours at 37°C prior to the test.

4.3 Antimicrobial agent

The antimicrobial agent used in the present study was **Ciprofloxacin**, a product of Cipla pharmaceutical Pvt. Ltd, India. The Ciprofloxacin used with an Expiry Date of June 2006 and potency of 500 mg / 704.125 mg of powder. Stored the drug in sealed containers in the dark at 4°C with a desiccant unless recommended by manufacturer. Prior to experiments these antibiotic powders/stock solutions of Ciprofloxacin should be brought to room temperature.

4.4 Turbidity standard for inoculum preparation (NCCLS, 1997)

To standardize the inoculum density for a susceptibility test, a Barium sulphate ($BaSO_4$) turbidity standard, equivalent to a 0.5 McFarland standard or its optical equivalent (e.g., latex particle suspension), should be used. A $BaSO_4$ 0.5 McFarland standard may be prepared as 0.5 ml aliquot of 0.048 mol/L $BaCl_2$ (1.175% w/v $BaCl_2$. $2H_2O$) is added to 99.5 ml of 0.18 mol/L H_2SO_4 (1% v/v) with constant stirring to maintain a suspension. The correct density of the turbidity standard should be verified by using a spectrophotometer with a 1-cm light path and matched cuvette to determine the absorbance. The absorbance at 625 nm should be 0.008 to 0.10 for the 0.5 McFarland standards. The Barium Sulfate suspension should be transferred in 4 to 6 ml aliquots into screw-cap tubes of the same size as those used in growing or diluting the bacterial inoculum. (NCCLS, 1997).

4.5 Preparation of stock solutions for MIC determination (Eucast, 2003)

For the preparation of stock solutions, potency of the drug powder has to be ascertained which can be done by using the formula:

$$\text{Weight of powder (mg)} = \frac{\text{volume of stock (mL) x concentration (mg/L)}}{\text{Potency of powder (mg/g)}}$$

A stock solution of 100mg/mL of ciprofloxacin was used in the present study (Table 1). The diluent used was sterile distilled water or 0.89% saline solution. This was then passed through a bacterial filter under aseptic conditions in sterile bottles/vials and stored at -20^{o}C or below until used.

4.6 *Staphylococcus aureus* growth kinetics

Growth kinetics studies were carried out to check the growth profile of the test microorganism. Such as, the duration of lag phase, log phase (active phase of growth), stationary phase as well as the death phase of the *Staphylococcus aureus*-NCTC-6571 (parent or surrogate strain). The growth kinetics of both strains (Parent and Mutant) were carried out by Microtitre plate reader.

Methodology

Muller-Hinton broth (50mL) was inoculated with an 18-24 hours (overnight grown culture) (500 µl) of *Staphylococcus aureus,* followed by incubation at 37^{o}C with aeration rate at 120 rpm. Growth was evaluated by measuring the viable colony count and optical density (O.D) of the culture at 578 nm. Prior to the measurement, the culture was vortexed for 1-2 minutes to gently break the *Staphylococcal* clumps and obtained consistent O.D reading through Spectrophotometer or Microtitre plate reader at 0h, 2h, 4h, 6h, 8h, 10h, 12h, 14h, 16h, 18 and 24h. The viable colony count was done by spot plating method by placing 10µl of serially diluted culture on one day old Mueller-Hinton agar plates and allowed to dry for 10 min under the laminar air hood. These plates were then sealed and incubated at 37°C for 24 hours and read after every 4 to 6 hours of incubation. The numbers of colonies on plates were counted.

Table 1: Preparation of dilutions of antimicrobials for use in micro-broth dilution tests (Eucast, 2003)

Antimicrobial Conc. (mg/mL) Stock solution	Volume of stock solution (µL)	Volume of saline (µL)	Antimicrobial conc. Obtained (mg/10µL)	Antimicrobial conc. Obtained (mg/mL)	Final conc. In m 200ul MHB (µg/ml)
100	10	0	1	100	5000
100	10	10	0.5	50	2500
100	10	30	0.25	25	1250
25	10	10	0.125	12.5	625
25	10	30	0.0625	6.25	312.5
25	10	70	0.03125	3.125	156.25
3.125	10	10	0.015625	1.5625	78.13
3.125	10	30	0.0078125	0.78125	39.00
3.125	10	70	0.0039063	0.39063	19.50
0.39063	10	10	0.0019532	0.19532	9.80
0.39063	10	30	0.0009766	0.09766	4.90
0.39063	10	70	0.0004883	0.04883	2.45
0.04883	10	10	0.0002442	0.02442	1.23
0.04883	10	30	0.0001221	0.01221	0.612
0.04883	10	70	0.0000611	0.00611	0.30
0.00611	10	10	0.0000305	0.00305	0.15
0.00611	10	30	0.0000152	0.00152	0.075
0.00611	10	70	0.0000075	0.00075	0.0375

4.7 *In vitro* development of ciprofloxacin resistance

Stepwise resistance to Fluoroquinolone arises when bacteria are sequentially challenged with increasing concentrations of drug. *In vitro* development of resistance to Ciprofloxacin was investigated by serial passage as carried out by Kim *et al.,* in 2003.

4.7.1 Serial passage

Staphylococcus aureus NCTC-6571 strain was grown in antibiotic-free Mueller-Hinton broth (HiMedia) at 37°C for 18 hours, and 1.5 x 10^8 cfu/mL of parent strain was inoculated into each of series of tubes, containing 4ml of an appropriate broth with antibiotic concentration consisting of doubling dilutions below and above the MIC of each agent for *Staphylococcus aureus*. After incubation at 37°C for 24 hours, an inoculum from the tube nearest the MIC, which had the same turbidity as the antibiotic-free control, was taken, diluted 1:100 with 0.89% saline and then transferred to new tube containing the appropriate broth with still higher concentration of antibiotic for three generations. Let it be stabilized on nutrient agar plate without antibiotic, and incubated the plate at 37°C for 24 hours. Took a single isolated colony from Nutrient agar plate and inoculated into fresh test tube, containing 4mL of Mueller-Hinton broth with still higher concentration of antibiotic. These passages were carried out, for 50 days, until an appreciably high MIC of Ciprofloxacin was not found. The selected mutant strain was subcultured in antibiotic-free Mueller-Hinton broth for another 10-20 serial passages and then again screened for its MIC value for Ciprofloxacin to check the stability of resistance.

4.8 Pre screen assay: Agar-well diffusion assay

Initial screening of potential antibacterial and antifungal compounds from plants may be performed with pure substances or crude extracts. Agar well assay is popular prescreen assay used by the clinical microbiologists and phytochemists to check the potential antimicrobial activity of plants and their use in traditional medicines for the treatment of infectious diseases (Navarro *et al.*, 1996).

4.8.1 Preparation of Mueller-Hinton Agar

Mueller-Hinton agar should be prepared from a HiMedia dehydrated base according to the manufacturer's instructions. Immediately after autoclaving, it was cooled and dispensed into glass or plastic, flat-bottomed petri dishes on a level, horizontal surface to give a uniform depth of approximately 4 ± 0.5 mm. This corresponds to 22.4 mL of medium for plates with a diameter of 100 mm. The agar medium was allowed to cool to room temperature and, unless the plate is used the same day, stored in a refrigerator (2 to 8°C). Plates should be used within seven days after preparation unless adequate precautions, such as wrapping in plastic, have been taken to minimize drying of the agar. A representative sample of each batch of plates should be examined for sterility by incubating at 37°C for 24 hours or longer.

4.8.2 Preparations of stock solution of Plant fractions *(Eucast, 2003)*

The provided column chromatographically purified fractions, isolated from *Callistemon rigidus R, Br.* were dissolved in the DMSO (Dimethyl sulfoxide) to give final stock concentration of 50 mg/mL. Two fold dilutions were made from this stock, according to **Table 1** (Eucast, 2003) and stored at –20°C.

Methodology

Poured 22.4 mL media into 100 mm sterile petriplates to give a mean depth of 4.00 mm ± 0.5 mm. Prepared 4 wells of 5 mm each using a sterile cork borer under aseptic conditions. Different volumes of stock solutions were then dispensed in a range of 5 µl - 30µl into the wells. One of the well is used as negative control (containing DMSO). After 10-15 minutes, when diffusion of extract has occurred, the wells were properly sealed with respective media and kept it for 15 min for solidification. Inocula of the target microorganisms were adjusted to 0.5 McFarland, against a card with a white background and contrasting black lines (to adjust turbidity of inoculum equal to 1.5 x 10^8 cfu/mL). Usually, within 15 minutes after adjusting the turbidity of the inoculum suspension, a sterile cotton swab was dipped into the adjusted suspension, rotated several times and pressed firmly on the inside wall of the tube above the fluid level. The dried surface of a Mueller-Hinton agar plate is inoculated by swabbing over the entire sterile agar surface. This procedure is repeated by swabbing two more times, rotating the plate approximately 60° each time to ensure an even distribution of inoculum. As a final step, the rim of the agar is swabbed.

Plates are then sealed with Para-film and incubated at 37°C for 18-24 hours. After 18 to 24 hours of incubation, each plate is examined. The diameters of the zones of complete inhibition were recorded in the test well as well as the control well.

4.9 Screening: Microplate -Broth Dilution assay (Eloff, 1998)

Broth micro-dilution denotes the performance of antimicrobial / antibacterial potential dilution at the least in 96 wells micro-dilution plate (microtitre plate) with a capacity of above 300µl per well. The MIC was determined using MH broth (HI Media) and using a color indicator, 2,3-5 Triphenyl tetrazolium chloride (TTC), which is reduced to formazan (pink to red color) by living cells. MIC concentration does not exhibit reduction of TTC into formazan.

Methodology

190µl of sterile Mueller-Hinton broth was dispensed in the wells of 96 well sterile microtitre plate and inoculated with 10µL of 0.5 McFarland adjusted overnight grown culture (18-24 hours old culture) of Staphylococcus aureus NCTC 6571, to each well. Plate was sealed with clean film or covered with microtitre plate lid. Incubated the plate at 37° C @120 rpm for 3-4 hours. After 3-4 hour incubation, added 10µL of different drug concentration in decreasing concentrations, as per (EUCAST, 2003) given in **Table 1**, in the respective wells of microtitre plate, marked properly according to different dilutions and sealed the plate with plate lid. Incubated the microtitre plate again at 37°C @120 rpm for 12 hours. After 12 hour incubation, added 10µL of 0.2% TTC (Triphenyl tetrazolium chloride) solution to each well of microtitre plate. Observed red colour formation, which showed the growth of organism. MIC is the minimum inhibitory concentration at which no visible growth of organism appears.

4.10 Plate count method to estimate the MIC

An aliquot of 100µl was withdrawn from test and control wells exhibiting the MIC value aseptically and diluted serially up to 10^{-6} concentration. 10 µl of the 10^{-6} concentration of the control and test wells were plated on one-day-old MH agar plate as a point inoculum and allowed to dry for 10 min under the laminar air hood. These were then sealed and incubated at 37°C for 24 hours (Eucast, 2003). These were then observed for colonies at regular intervals. These were counted to determine Colony forming units/mL.

4.11 Screening of Effective formulations by Broth dilution test

4.11.1 Preparations of Herbal formulations

The antimicrobial resistance reverting activity was accessed by designing combinations between Ciprofloxacin and non-antimicrobial fractions of *Callistemon rigidus R.Br*. The MIC of different fractions of *Callistemon rigidus* was first determined by using micro-broth dilution method.

In the formulations subinhibitory concentrations of Ciprofloxacin was kept constant throughout with decreasing concentration form subinhibitory concentrations of different fractions. These were tested against the parent and the mutant strains of *Staphylococcus aureus* NCTC 6571 by Broth Dilution Method (Eucast, 2003). TTC was used as a color indicator to judge the best combination. The best combination is described as one where there is no reduction of TTC to formazan.

Thereafter the concentration of the lead fraction was kept constant and the concentration of Ciprofloxacin was varied to arrive to an optimal combination.

4.11.2 Screening of formulations by Micro and Macro-Broth Dilution assays

In first set of experiment, 180μL of sterile MH broth was dispensed in the wells of 96 well microtitre plate. Then, inoculated with 10μL of 0.5 McFarland adjusted overnight grown culture (18-24 hours old culture), to each well of microtitre plate. Plate was sealed with clean film or covered with microtitre plate lid. Incubated the plate at 37° C @120 rpm. After 3-4 hour incubation, added 10μL of 6.25 mg/mL stock, to obtain a final Concentration 312.5 mg/L of Ciprofloxacin in 200μL MHB the respective wells of microtitre plate. Now, added 10μL of two fold dilution stock (Eucast, 2003) of F_5 fraction to obtain final concentration 312.5 mg/L, 156.25 mg/L, 78.125 mg/L, 39.06 mg/L, 19.6 mg/L, 9.8 mg/L, 4.9mg/L, 2.45 mg/L, 1.23mg/L in 200 μL MHB in respective wells of microtitre plate. The control wells contained either higher concentration of F_5 fraction and Ciprofloxacin with the resistant organism and resistant organism in the absence of both the agents. Covered the plate with plate lid. Incubated plate again at 37°C @120 rpm for 3-4 hours. After 12 hours incubation, added 10μL of 0.2% TTC (Triphenyl tetrazolium chloride) solution to each well of microtitre plate. Observed red colour formation, after 24 hours, which showed the growth of organism.

MIC is the minimum inhibitory concentration at which no visible growth of organism appears. Similarly, formulations of Ciprofloxacin with other fractions (F_6, F_7, and F_8) and were prepared and tested on Ciprofloxacin resistant as well as Ciprofloxacin sensitive *Staphylococcus aureus* by using micro-broth dilution assay. This test was also performed in test tubes by Macro-Broth Dilution method.

4.12 Death Kill Kinetics of *Staphylococcus aureus*

The best formulation which had highest killing efficiency was dispensed in the test tubes to make a final volume of 5 ml with Mueller-Hinton broth. The test was carried out in duplicate. Inoculated 50 µl of 0.5 McFarland adjusted overnight grown culture of mutant *S.aureus* to each of the test tubes containing MHB (control), MHB + Subinhibitory conc. of fraction F_5 (control 1), MHB + Subinhibitory conc. of Ciprofloxacin and MHB (control 2) + Best formulation (F_5 + Ciprofloxacin) (Test).The test tubes were then incubated at 37°C at 120 rpm in the shaker. Growth was evaluated by measuring the viable colony count and optical density (O.D) of the culture at 578 nm. Prior to the measurement, the culture was vortexed for 1-2 minutes to gently break the *staphylococcal* clumps and obtain consistent O.D reading at 0h, 6h, 12h, and 24h. The viable colony count was done by spot plating method by placing 10µl of serially diluted culture on one day old Mueller-Hinton agar plates and allowed to dry for 10 min under the laminar air hood. These plates were then sealed and incubated at 37°C for 24 hours and observed at regular intervals for appearance of Colony Forming Units on plates. The numbers of colonies on plates were counted. This experiment was repeated by taking the parent culture of *S.aureus* NCTC 6571.

RESULTS AND DISCUSSION

1. GROWTH CURVE STUDIES

Growth pattern of the test organism was studied on the basis of Optical Density (O. D) and Log_{10}cfu/mL. From our results based on both O.D. and Log_{10}cfu/mL, we concluded that the control organism (*Staphylococcus aureus* NCTC 6571) exhibited a lag phase of about 3½ to 4 hours. Then it entered into log phase (Active Phase of growth) that extended upto 13 hours of growth, after which the organism entered the stationary phase. Death phase was achieved after 18 hours of growth (Fig. 3).

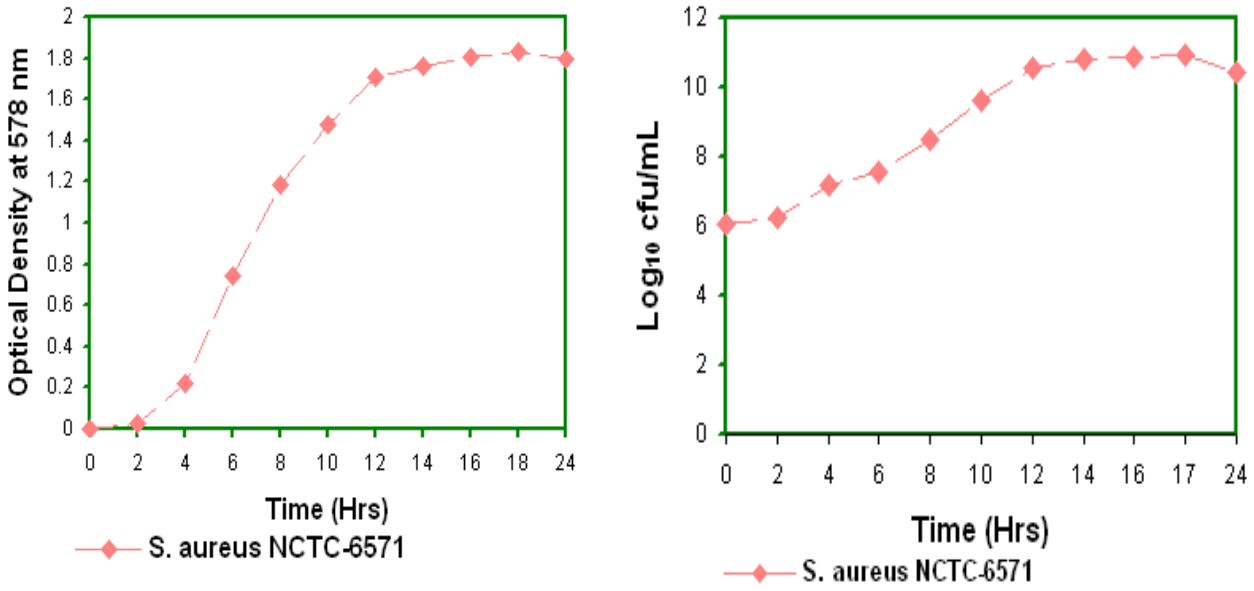


Fig. 3 Growth Curve Studies of *Staphylococcus aureus* NCTC 6571

2. Minimum Inhibitory Concentration of Ciprofloxacin against *Staphylococcus aureus* NCTC 6571

MIC was determined by microbroth dilution methodology according to Eucast guidelines. MIC was evaluated as the minimum inhibitory concentration at which no visible growth of microorganism appears. From our results, we concluded that MIC of Ciprofloxacin against control strain (*S. aureus* NCTC 6571) was found to be 4.9 µg/mL (Fig. 4).

3. Development of *In Vitro* Resistance (Kim *et al.*, 2003)

Stable *S. aureus* mutant was obtained after 50 days, sequentially challenged with increasing two fold concentration of Ciprofloxacin, according to **Table 1** (Eucast, 2003). The control strain passed in increasing double fold dilutions of Ciprofloxacin, developed High-Level-Ciprofloxacin-Resistance within 50 days exposure to

Ciprofloxacin. Reduced susceptibility towards Ciprofloxacin was detected after 5 to 7 serial passages. The experiment was terminated after 7 passages at which no significant increase in resistance towards the Ciprofloxacin was observed. The subculturing in Ciprofloxacin gave increased MIC, ranging from 4.9 µg/mL to 625 µg/mL.

4. Minimum Inhibitory Concentration of Ciprofloxacin against Mutant Strain of *Staphylococcus aureus* (Eucast, 2003)

MIC of the Stable *S. aureus* mutant, obtained after 50 days, sequentially challenged with increasing two fold concentration of Ciprofloxacin was determined by microbroth dilution methodology, according to Eucast guidelines. From our results, we concluded that MIC of Ciprofloxacin against mutant strain of *S.aureus* was found to be increased from 4.9 µg/mL to 625 µg/mL (Fig. 5).

5. In vitro stability of resistance (Kim *et al.*, 2003, Calvet et al., 1997)

After 23 days of exposure to Ciprofloxacin, Ciprofloxacin resistant *Staphylococcus aureus* was transferred in MH broth without Ciprofloxacin to assess the stability of resistance. The growth curve studies of mutant strain in the presence of resistant drug (312.5 µg/mL of Ciprofloxacin) were carried out at periodic intervals after 5 days, 10 days, and 20 days exposure to 312.5 µg/mL of Ciprofloxacin concentration along with stabilization in MH broth without drug for a 5 days period.

The Figure 6(a) shows the mutant was stable as it followed the same growth profile, in the presence or absence of resistant drug, as that of control strain(S.A NCTC 6571). While the growth of control strain was inhibited in the presence of 312.5 µg/mL of Ciprofloxacin and showed a 5 log reduction within two hours of exposure to 312.5 µg/mL concentration of Ciprofloxacin.

After 10 days exposure of resistant drug, the growth curve profile of mutant along with control strain was observed. The mutant strain was stable and grew in the presence or absence of resistant drug. The growth of control strain was inhibited in the presence of 312.5 µg/mL of Ciprofloxacin and showed 6 log reductions within two hours of exposure to 312.5 µg/mL concentration of Ciprofloxacin [Fig 6(b)].

After 20 days exposure to resistant drug, the mutant showed complete resistance against the Ciprofloxacin as it overwhelmed the growth of parent strain in the presence and absence of resistant drug (312.3 µg/mL of Ciprofloxacin) [Fig. 6(c)].

5000 µg/mL	2500 µg/mL	1250 µg/mL	625 µg/mL	312.5 µg/mL	156.25 µg/mL	78.125 µg/mL	39.06 µg/mL	19.6 µg/mL	9.8 µg/mL	4.9 µg/mL	2.45 µg/mL
1.23 µg/mL	0.612 µg/mL	0.30 µg/mL	0.15 µg/mL	0.075 µg/mL	0.0375 µg/mL	Control	Control				

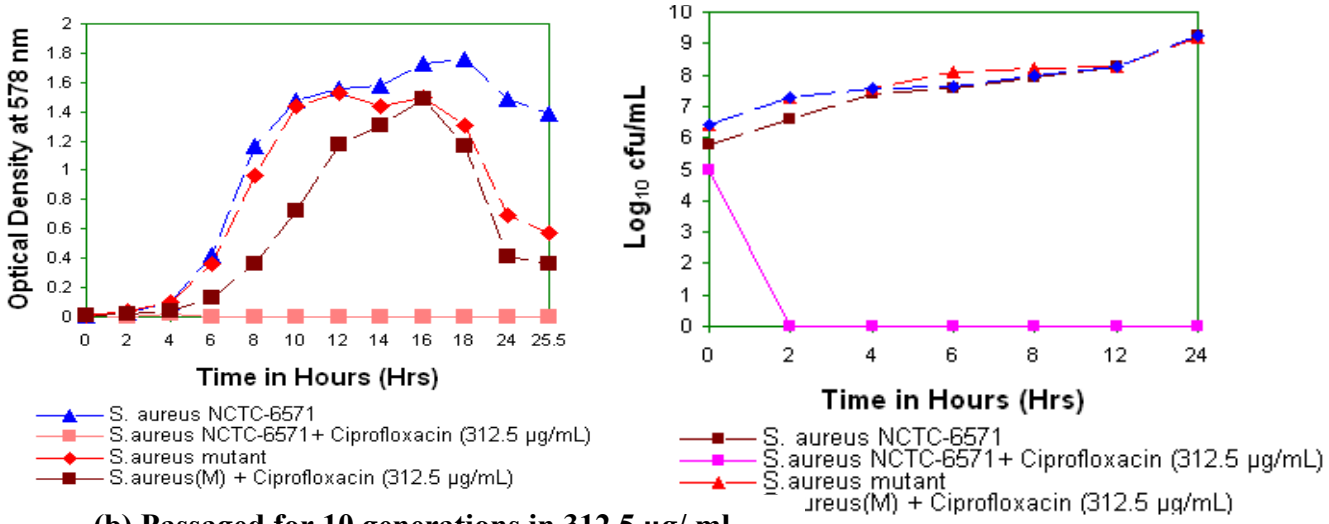
Fig 4: MIC Determination of Ciprofloxacin against *S.aureus* NCTC 6571

Drug + TTC	MHB + TTC		5000 µg/mL	2500 µg/mL	1250 µg/mL	625 µg/mL	312.5 µg/mL	156.25 µg/mL	78.125 µg/mL	39.06 µg/mL	19.6 µg/mL
9.8 µg/mL	4.9 µg/mL	2.45 µg/mL	1.23 µg/mL	0.612 µg/mL	0.30 µg/mL	0.15 µg/mL	0.075 µg/mL	Control			
Drug + TTC	MHB + TTC		5000 µg/mL	2500 µg/mL	1250 µg/mL	625 µg/mL	312.5 µg/mL	156.25 µg/mL	78.125 µg/mL	39.06 µg/mL	19.6 µg/mL
9.8 µg/mL	4.9 µg/mL	2.45 µg/mL	1.23 µg/mL	0.612 µg/mL	0.30 µg/mL	0.15 µg/mL	0.075 µg/mL	Control			

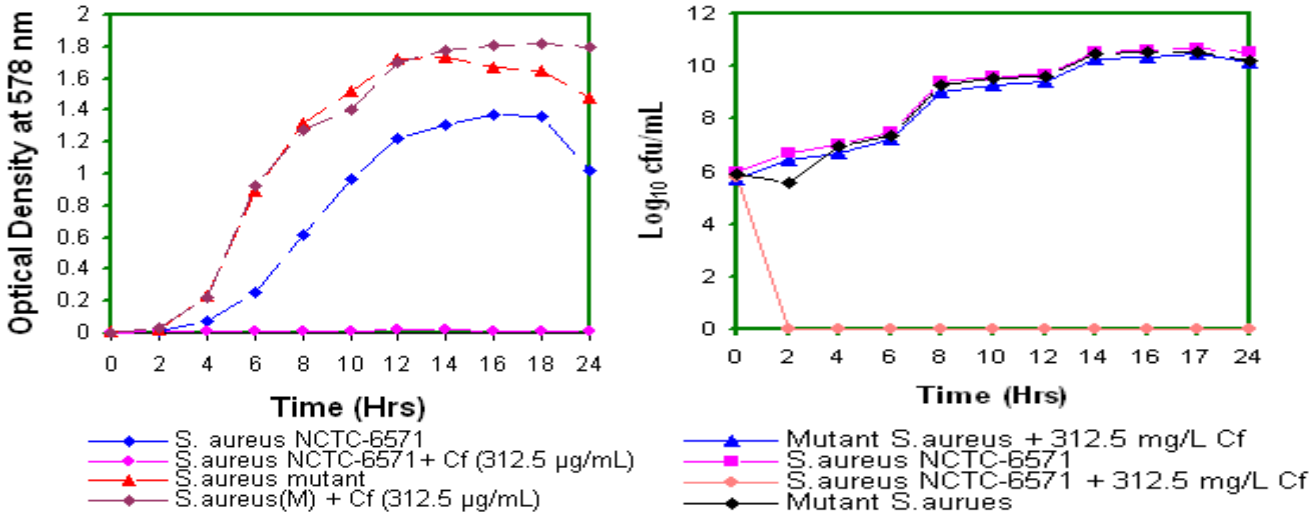
Fig 5: MIC Determination of Ciprofloxacin against Mutant *S.aureus* NCTC 6571

Fig.6: Growth Curve Studies of *S.aureus* NCTC 6571 and mutant *S. aureus* in the presence and absence of resistant drug.

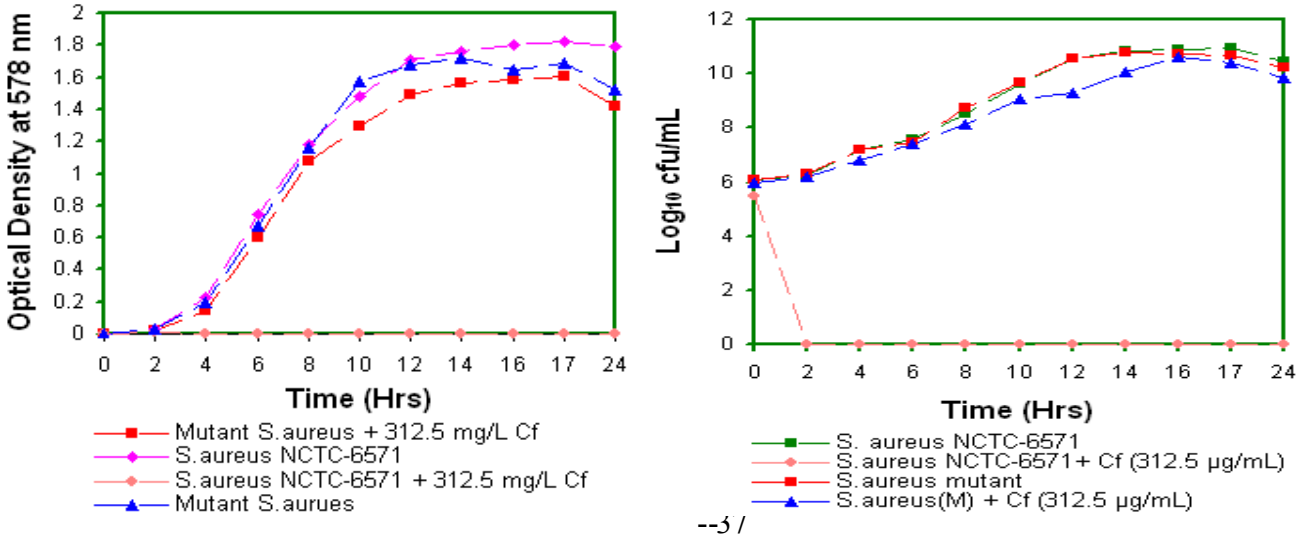
(a) Passaged for 5 generations in 312.5 µg/ ml



(b) Passaged for 10 generations in 312.5 µg/ ml



(c) Passaged for 20 generations in 312.5 µg/ ml



6. Agar Well Diffusion Assay

The stability of *in vitro* resistance was further confirmed by Agar Well Diffusion Assays, where in the growth *S. aureus* (control) and *S. aureus* (mutant) was evaluated at 1250 µg, 625 µg, and 312.5 µg of effective Ciprofloxacin in well. No zone of inhibition was observed for the mutant at 312.5 µg. However, a MIZ of 6.0 mm was observed at 625 µg, while 1250 µg of active compound produced a MIZ of 10 mm (Table 2, Fig. 9).

The parent strain of *S. aureus* gave an inhibitory zone of 76 mm, 83.33, and above 100 mm, at 312.5 µg, 625 µg, and 1250 µg of active compound (Ciprofloxacin) respectively.

Table 2. Agar Well Diffusion Assay

Sr. No.	Stock Concentration (µg/µL)	Volume Dispensed in Well (µL)	Effective Drug in Well (µg)	Zone of Inhibition (mm)	
				S. A. (NCTC 6571)	S. A (M)
1.	125	10	1250	100	10
2.	62.5	10	625	83.33	6
3.	31.25	10	312.5	76	0
4.	15.625	10	156.25	62	0
5.	7.8125	10	78.125	44	0
6.	3.906	10	39.06	36.5	0
7.	1.96	10	19.6	35	0
8.	0.98	10	9.8	30	0
9.	0.49	10	4.9	28.2	0

7. MIC Determination of Herbal Fractions

Minimum inhibitory concentrations of four methanolic fractions viz F_5, F_6, F_7, and F_8 of leaf extract of *Callistemon rigidus* was evaluated against *S. aureus* NCTC 6571, and *S. aureus* (mutant) by broth dilution assay. From the results, we concluded that of all

the four fractions, only fraction F_5 had an inhibitory activity against both *S. aureus* NCTC 6571 [Fig 11 (a)] and *S. aureus* (mutant) [Fig 11 (b)], at a concentration of 78 µg/mL and 625 µg/mL respectively. No antimicrobial activity was observed for the remaining three fractions (F_6, F_7, and F_8), up till a concentration of 2500 µg/mL (Fig. 11).

8. Combination Studies of Herbal Extracts and Ciprofloxacin (Nascimento *et al.*, 2000 , Muroi and Kubo, 1996 and Bisset *et al.*, 1994)

Based on the MIC values obtained by broth dilution assay for all the fractions and Ciprofloxacin, combination of herbal fractions with drug at sub MIC concentration were evaluated against *S. aureus* NCTC 6571, and *S. aureus* (mutant). Fraction (F_5) showed synergistic effect with resistant drug (312.5 µg/mL of Ciprofloxacin) at a concentration of 39.06 µg/mL. While fraction F_6, F_7, and F_8 did not exhibited synergistic effect with the resistant drug. From the results, we observed that the synergistic combination of fraction (F_5) at a concentration of 312.5, 156.25 and 78.125 µg/mL with resistant drug (312.5 µg/mL conc. of Ciprofloxacin) were inhibitory against mutant strain of *S.aureus*, which caused 8 log reduction within 12 hours of growth. The optimal synergistic combination of fraction (F_5) with resistant drug (at a concentration of 39.06 µg/mL and 312.5 µg/mL respectively) were also found to be effective against *S.aureus* (mutant) and caused eight logs reduction within 24 hours of growth (Fig 7, Fig. 12).

9. Agar Well Diffusion Assay

The synergistic effect of fraction F_5 with the resistant drug Ciprofloxacin was further confirmed by agar well diffusion studies. From the results, we observed that the control strain S. A (NCTC 6571) gave a MIZ of 46 mm against 312.5 µg of active compound (Ciprofloxacin) while it produced inhibitory zone of 54 mm against optimal combination (39.06 µg of Fraction F_5 + 312.5 µg of Ciprofloxacin). However, the mutant strain did not produce any zone against resistant drug (312.5 µg of Ciprofloxacin), but gave a MIZ of 33 mm against synergistic combination of 39.06 µg of Fraction F_5 and 312.5 µg of Ciprofloxacin. There was no zone of inhibition was found in case of fraction F_5 at a concentration of 39.06 µg against both the strains (Fig. 10)

10. Death Kill Kinetics

Based on the results of broth dilution assay using visible dye reduction method, optimal combination of fraction F_5 (39.06 µg/mL) with resistant drug (312.5 µg/mL) was evaluated by kill kinetics against *S. aureus* NCTC 6571, and *S. aureus* (mutant).

Fraction F_5 and ciprofloxacin alone did not inhibit the growth of Staphylococcus aureus (mutant). No bactericidal effect of fraction F_5 was observed against control *Staphylocoocus aureus*, however Cipofloxacin inhibited 99 % bacterial growth within 6 hours.

Combination of fraction F_5 and and ciprofloxacin at the above mentioned concentrations exhibited a synergistic effect against *Staphylococcus aureus* (mutant). However fraction F_5 alone at the concentration of 39.06 µg/mL did not inhibit the bacterial growth against both the strains of *S.aureus*. From our results of kill kinetics of *S. aureus* (mutant), an 8- log reduction in bacterial growth was achieved within 24 hours. No post antibiotic effect was observed.

The optimal Combination of fraction (F_5) with resistant drug (at a concentration of 39.06 µg/mL and 312.5 µg/mL respectively) showed 4 log reduction against control *S.aureus* within first 6 hours of growth (Fig. 8).

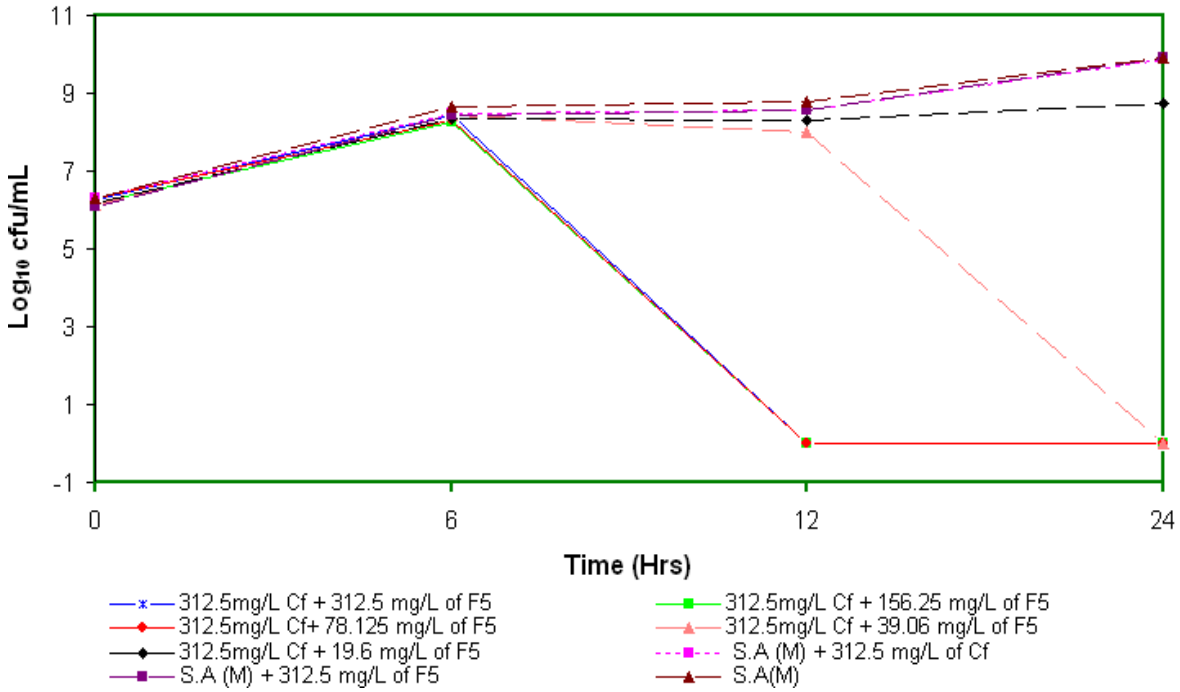


Fig. 7: Effect of Different Concentrations of Herbal Fraction (F_5) with Resistant Drug (312.5 µg/mL of Ciprofloxacin) on mutant strain of *S.aureus*.

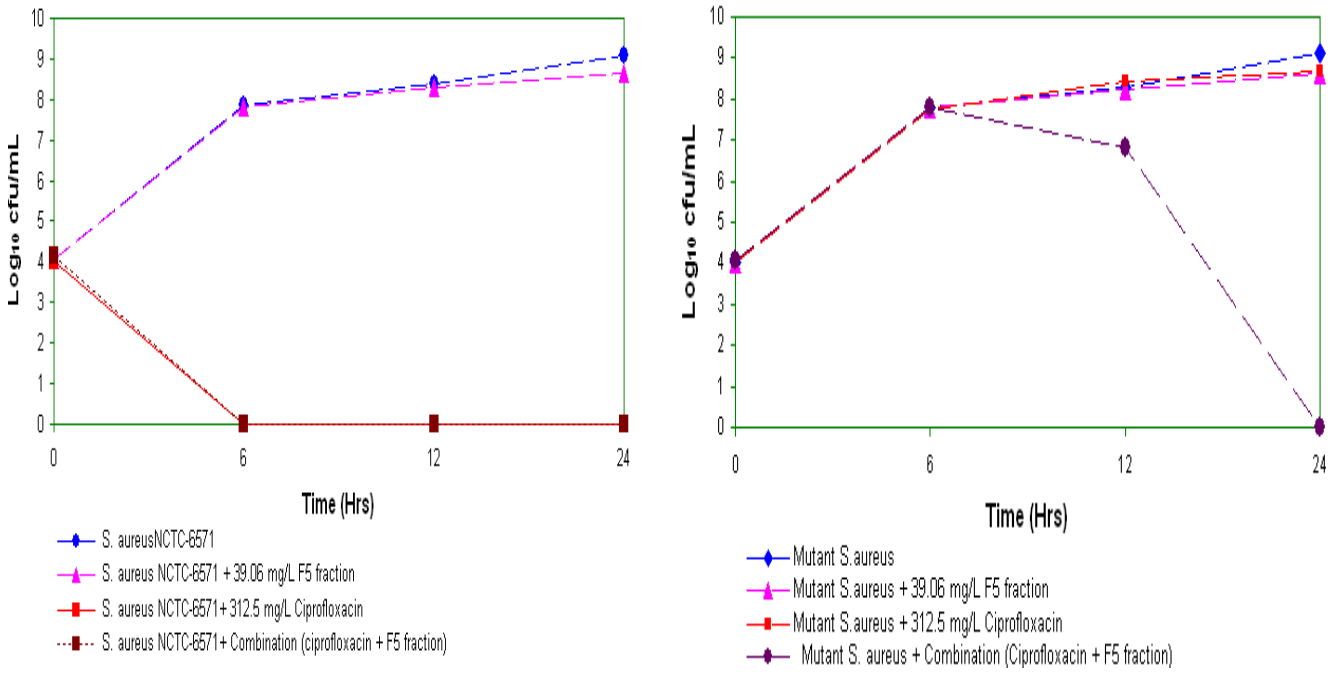


Fig. 8: Effect of Optimal Combination of Herbal Fraction (F_5) (39 µg/mL) with Resistant Drug (312.5 µg/mL of Ciprofloxacin) on Growth Profile of Mutant strain of *S.aureus*.

Fig. 9: Agar Well Diffusion Assay of Ciprofloxacin against Control and Mutant *Staphylococcus aureus.*

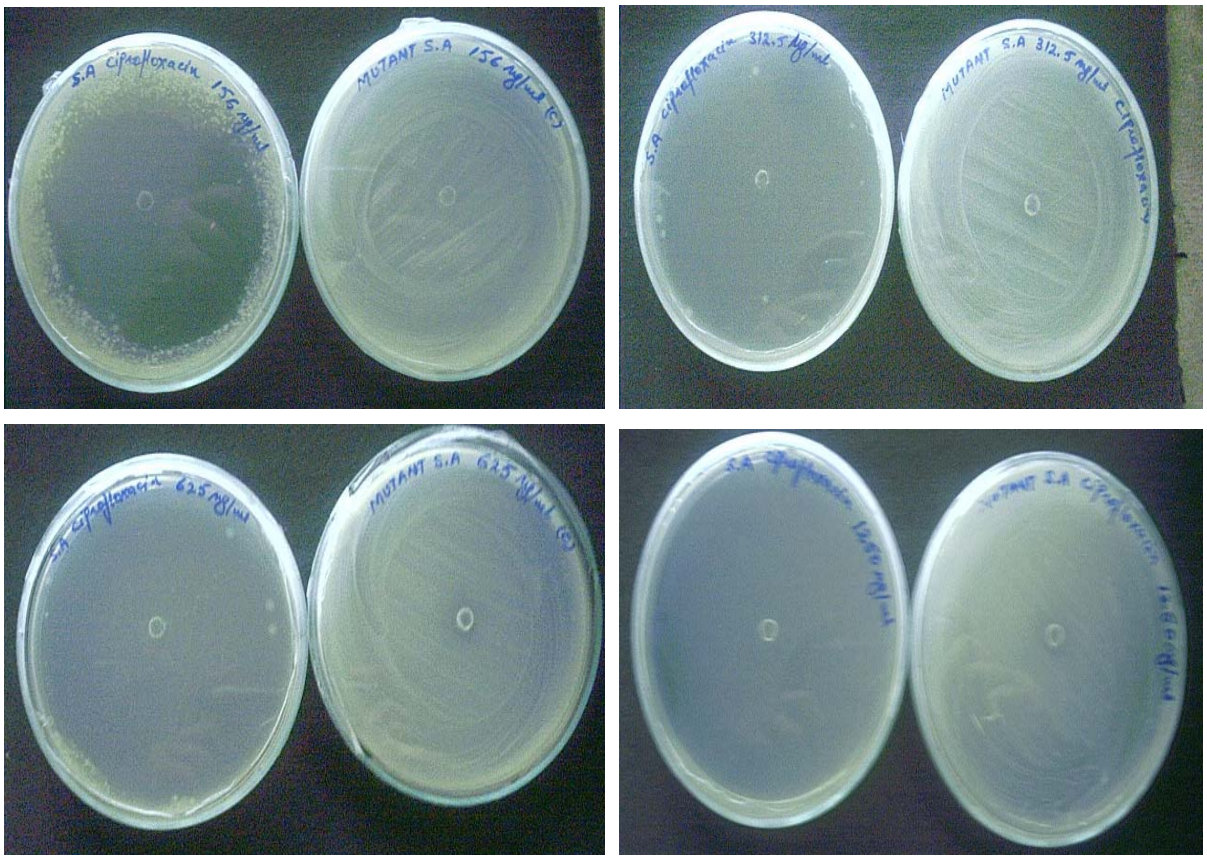


Fig. 10 : Agar Well Diffusion Assay of Optimal combination of Fraction 5 and Ciprofloxacin against Control and Mutant *Staphylococcus aureus.*

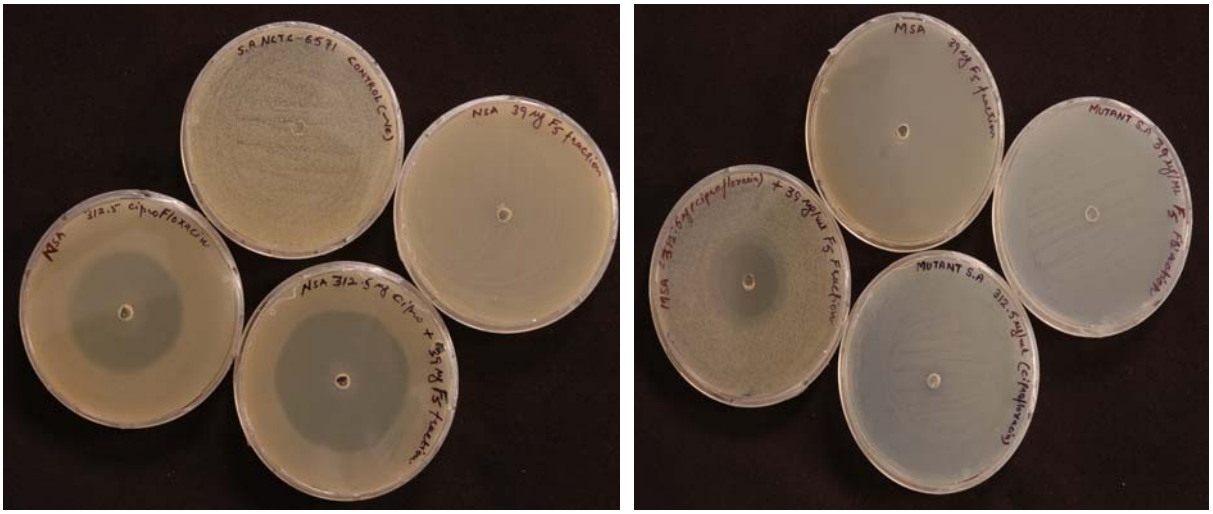
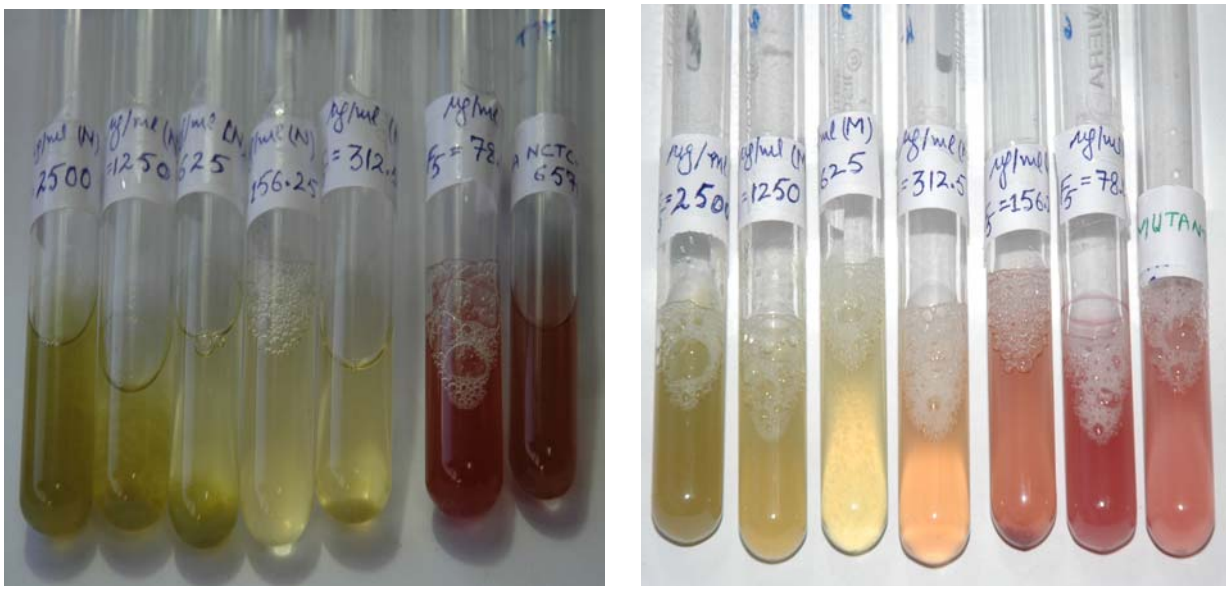


Fig. 11: MIC Determination of Fraction 5 against Control and Mutant *Staphylococcus aureus.*



11 (a) 11 (b)

Fig. 12: MIC Determination of Combination of Fraction 5 with Ciprofloxacin against Control and Mutant *Staphylococcus aureus.*

Conclusion

The use of plants to heal diseases including infectious ones, has been extensively applied by people. Data from literature as well as the present study results reveal great potential of plant extracts/fractions for therapeutic treatment and the importance of plant extracts/fractions, when associated with antibiotics to control multidrug resistant pathogenic bacteria, a major threat to human health.

In the present study, we concluded that fraction F_5, isolated from crude extract of leaves of *Callistemon rigidus R. Br.* was active against Ciprofloxacin resistant *Staphylococcus aureus* at a very low concentration (39.06 µg/ml) in combination with the resistant drug. Fraction F_5 thus, exhibits powerful *in vitro* activity against control as well as mutant strain of *S. aureus* and synergic interaction with resistant drug (Ciprofloxacin). These characteristics make fraction F_5 of *Callistemon rigidus R. Br.* potentially valuable for the future as a bioenhancer in antimicrobial drug resistance reversal therapy for *Staphylococcus aureus* infections.

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