Development of Herbal Mitigator Targeting Multi Drug Resistant Nosocomial Pathogen

To be submitted as Major Project in partial fulfilment of the requirement for the degree of

M. Tech. (Bio Medical)

Submitted by

RAGHAV NAGPAL
(2k14/BME/14)
Delhi Technological University, Delhi, India

Under the supervision of

Prof. B.D. Malhotra
Department of Bio-Technology
Delhi Technological University

Dr. Raman Chawla
Scientist 'D'
INMAS, DRDO

DECLARATION

I, **Raghav Nagpal**, hereby declare that the dissertation entitled **'Development of Herbal Mitigator Targeting Multi Drug Resistant Nosocomial Pathogen'** submitted is infulfilment of the requirement for the award of the degree of Master of Technology in Biomedical Engineering, Delhi Technological University. It is a record of original and independent research work done by me under the supervision and guidance of **Prof. Bansi. D. Malhotra**, Department of Biotechnology, Delhi Technological University, Delhi and **Dr. Raman Chawla**, Scientist 'D', INMAS , DRDO. The information and data enclosed in the dissertation is original and has not formed the basis of the award of any Degree/Diploma/Fellowship or other similar title to any candidate of the University/Institution.

RAGHAV NAGPAL

Roll No.: 2K14/BME/14

M. Tech. (Biomedical Engineering)

Department of Biotechnology

Delhi Technological University

Shahbad Daulatpur, Main Bawana Road,

Delhi 110042, INDIA

CERTIFICATE

This is to certify that the dissertation entitled**"Development of Herbal Mitigator Targeting Multi Drug Resistant Nosocomial Pathogen"** inthe fulfilment of the requirements for the reward of the degree of Master of Technology (Bio Medical Engineering),Delhi Technological University (Formerly Delhi College of Engineering, University of Delhi), is an authentic record of the candidate's own work carried out by him/herunder my guidance. The information and data enclosed in this thesis is original and has not been submitted elsewhere for honouring of any other degree.

Prof. B.D. Malhotra
Department of Bio-Technology
Delhi Technological University

Dr. Raman Chawla
Scientist 'D'
INMAS, DRDO

ACKNOWLEDGEMENT

I would like to pay my deepest respect to ***Dr. B D Malhotra****, Professsor, Delhi Technological University for being a constant inspirational idol. I am especially deeply indebted to* ***Dr. Raman Chawla****, Scientist 'D' INMAS (DRDO) for the exemplary guidance, monitoring, encouragement and the good working possibilities that he provided. The blessings given by him time to time shall carry me a long way in the journey of life on which I am about to embark.*

My deepest respect and heart full gratitude to Ms. ***Pallavi Thakur****, SRF, CBRN, She not only initiated my interest in the field of Biotechnology, but also led me through dark alleys and chasms to brighten the path. This project work would not have been completed without her pillared support and working with her has taught me great lessons not only related to the research field but also to be carried out in life.*

I greatly acknowledge Ms. ***Ankit Singh Chakotiya****, SRF, CBRN division, INMAS (DRDO), who supported me in every possible way to complete the project work and for providing me with valuable suggestions and all necessary facilities required to complete this project work.*

Lastly, this report would not have been possible if not for the patience and encouragement of my mother Mrs. ***Nisha Nagpal*** *and my deceased great grandmother Lt. Smt.* ***Khushabai Nagpal*** *for her blessings which she bestowed upon me.*

Raghav Nagpal

2K14/BME/14

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Symbols and Abbreviations

INMAS	*Institute of Nuclear Medicine and Allied Sciences*
µg	Microgram
ADR	Adverse drug reaction
AMR	Antimicrobial resistance
CBRN	Chemical, Biological, Radiological and Nuclear
CDC	Centre for Disease Control
EU	European Union
GI`	Gastrointestinal
Gm	Grams
ICU	Intensive Care Unit
LPS	Lipopolysaccharide
LRS	Lactated Ringer's solution
MBC	Minimum Bactericidal concentration
MDR	Multi- drug resistant
Mg	Milligram
MHA	Mueller Hinton agar
Ml	Milliliter
MRSA	Methicillin-resistant *Staphylococcus aureus*
NIH	National institute of health
OPW	Peptone water
OTC	Over the counter
QC	Quality Control
UTI	Urinary tract infection
WHO	World Health Organisation

DEVELOPMENT OF HERBAL MITIGATOR TARGETING MULTI DRUG RESISTANT NOSOCOMIAL PATHOGEN

Raghav Nagpal
Delhi Technological University, Delhi, India
raghavnagpal@ymail.com

ABSTRACT

Antibiotics have been used since several decades to treat patients from infectious diseases which altogether have resulted in reduced illness and death. The use of these antibiotics in patient care has been massivelyfavorable, when prescribed and taken correctly. However, these drugs have been used so widely and for a prolonged time, which the infectious organisms have adapted to them, making the drugs less effective. Several categories of drug resistant bacteria are now-a- days used in medical literature to characterize the different patterns of resistance spread found in healthcare-associated, antimicrobial-resistant bacteria. These patterns of resistance are acquired through mutation and horizontal gene transfer. Articulation of the drug resistance has led to such situations where, harmful bacteria once controlled through antibiotics are now virtually uncontrollable. As a result of emergence of multidrug resistant microbes, new alternatives are continually being searched, that could overcome the development of multi-drug resistance and hence aid in the suitable treatment of infectious diseases.

Therapy involving combinations of antimicrobials exhibiting multiple target sites are being used as an alternative by the medical practitioners to curb drug resistance. Parallel to these therapies, herbal plants have contributed immensely in managing microbial infections, as herbal medicaments contain various antimicrobial constituents like tannins, terpenoids, alkaloids and flavonoids . Hence, herbal plants have been considered as an innovative remedy for the

management of diseases which have been associated with antibiotic resistant microbes. Also a country like India, where folk medicinal literatures like Ayurveda, Unani have been followed since ages, has no dearth of medicinal plants which are a starting framework for remedial agents, being used for maintaining human health, supported by medical benefits and cultural believes.

CHAPTER 1

INTRODUCTION

The World Health Organization (WHO) states "the world is headed for a post-antibiotic era, in which common infections and minor injuries which have been treatable for decades can once again kill"[WHO, 2012].The infections "acquired in hospital by a patient who was admitted for a reason other than that infection [CDC, 1996] or an infection occurring in a patient in a hospital or other health care facility in whom the infection was not present or incubating at the time of admission including infections acquired in the hospital but appearing after discharge, and also occupational infections among staff of the facility [Bouvet, 1993]" are defined as Nosocomial infections by WHO. Nosocomial infections have become a troublesome situation in health care delivery across the world. Consistently millions of people have reported at least one form of nosocomial infection every year in the last decade. These often result in delayed recuperation of patients and even demise when not treated early. Different species of bacteria, fungi and viruses have been involved in the advancement of nosocomial infections among which, *E.coli* and *Klebsiella pneumoniae* ranked first and second as the most common cause of community and hospital acquired infections respectively. Urinary tract infection, pneumonia, tuberculosis, gastroenteritis, etc. are all included in Nosocomial infections [Syndor and Perl, 2011]. Management of nosocomial infections in immunocompromised patients has become challenging due to the growing menace of antibiotic resistance [Otter, Saber and Gary et al., 2011].

Conventionally antibiotics were defined as natural microbial products that can restrain the growth of other micro-organisms. However, synthetically produced chemotherapeutic

moieties have been likewise eluded under the same heading as antibiotics. Over the decades since their initial introduction antibiotics have not only ensured the successful handling of life-threatening infections but also have allowed for the use of interventions, such as surgery, for medical restorative conditions which were previously considered as unmanageable due to the high risks of infection. Overdependence on antibiotics as first line drugs and the irrational use of these modalities, has significantly contributed to the rise of global antimicrobial resistance (AMR) in microorganisms. In the recent times, virtually every ecosystem on earth has an occurrence of antibiotic resistant bacteria in large numbers. As more microbial species and strains are becoming resistant, antimicrobial resistance has risen as a serious threat to clinical medicine. The failure in maintenance of hospital hygiene due to antibiotic abuse create selective pressures on the bacterial strains and the mobile genetic elements which encode proteins endow bacteria with resistance mechanisms, Thus becoming the driving force behind AMR [Weinstein, 2001]. AMR also contributes to the degrading situation of health care management in developing countries where the disease burden is high and surrogating the old antibiotics with newer and more expensive ones enhances the cost constraints. This menace of growing infections has impelled scientists to seek for alternate therapeutic modalities which would help in combating drug-resistant pathogens causing nosocomial infections. To achieve prominent success in such endeavours, a new approach based on alternative or complementary medication is necessary which will bridge the gap between definitive allopathic care (modern medicine) and holistic cause management. This will constitute a branch of medicine wherein a plethora of natural remedies are used as provisions against the infections. The novel antimicrobial mechanism based on plant products will serve as a source of both traditional and modern medicines for the treatment of infectious diseases and hence will be vital for the health care systems of developing

countries like India.Relevant Pharmacological properties in promising herbal plants by the virtue of a diverse collection of phytoconstituents prove themselves as a candidate for novel drug generation.

One such method is classical bioprospection. The classical herbal bioprospection involves classification of herbal medicinal plants based on their ethnopharmacological significance testified in ancient literature as well in clinical folk literature of many countries. Though the process has been used for drug development in folk literature but it lacks scientific evident and proofs, is generally observation or experience based, is time consuming and tedious. The new techniques should have dynamic search modules with priority indexing, systemic categorization and also cross verification protocols. *In slico* bioprospection tool developed by research team of Division of CBRN defence, Institute of Nuclear Medicine and Allied Sciences, DRDO has been used to statistically jot down the clinically important plants from an extensive database of herbal plants relevant to the nosocomial pathogen under study [Nagpal *et al*, 2015]. Molecular Docking studies determine the binding interaction between a small molecular phytoligand and a virulent enzyme protein of *Klebsiella pneumoniae* which may result in activation or inhibition of the enzyme.The results obtained from *in silico* studies were then validated at *in vitro* level.

In this study Klebsiella pneumoniae, a key player in the spread of nosocomial infections is selected as a model organism for the bioprospection model proposed here, which is designed by the following sequential steps that involve, understanding the pathophysiology and drug resistance in the model organism, followed by extensive literature review to explore the utility of herbal drugs targeting *Klebsiella pneumoniae* infection leading to selection of plants and their correlation with the selected parameters. On the basis of scores obtained by different plants

against all parameters and optimization using the fuzzy set membership the promising plants with significant weightage score were selected. Also to validate the bioprospection model *in silico* docking simulations of most relevant bioactivity parameter against pre-selected phyto-ligands were carried using "Hex 8.0" as well as "iGemDOCK" software. *In silico* toxicity estimations using (Toxicity Estimation Software Tool) T.E.S.T. were also performed to screen out the false positives on the basis of LD50, Mutagenicity, Bioavailabilityand Developmental toxicity. Finally extraction and chemical and bioactivity based characterization/ standardization the aquo-alcoholic extract of the selected herbal is carried. Its antibacterial activityon *Klebsiella pneumoniae*was investigatedat *in vitro* level.

Specific Aims

1. *In silico* bioprospection studies for the selection of promising herbal candidates against *Klebsiella pneumoniae* mediated nosocomial infections.

2. Molecular docking studies for the validation of selected herbal candidates at primary level using Hex 8.0.

3. Secondary Molecular docking studies, using iGemDock and *in silico* Toxicity study of selected phyto-ligands using Toxicity Estimation Software tool.

4. To prepare aquo-alcoholic extracts of pre-selected herbal and screen extract for antibacterial activity using in vitro screening assays.

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CHAPTER 2
REVIEW OF LITERATURE

2.1 Nosocomial Infections

A prolonged stay in a hospital or health care providing settings with widespread unhygienic conditions can result in the acquisition of nosocomial infections. The infections typically appear after 48 or more hours of hospital admission or within 30 days after discharge [Archibald *et al.,* 2014].Microorganisms of variant specieshave been isolated in plenteous from different health care settings across the globe, indicatively directing of an upsurge of nosocomial pathogenesis [Markovic-Denic *et al,* 2009].Though,many of these microbial specieshave not been accounted for causing any intractable nosocomial outburst, yet because of their opportunistic nature the pathogens pretense a serious challengenot only to patients with immunocompromised conditions in the hospitals but also to the community. Nosocomial infections usually encountered include urinary tract infection, pneumonia, tuberculosis, gastroenteritis, legionnaire's disease and infections [Shears *et al.*, 2007].

Patients who encounter nosocomial infections are usually immuno compromised because of underlying chronic diseases, medical or surgical treatments and even due to age as aging reduces the immune strength to fight infections. Increasingly insistent medical and therapeutic interventions which include implantation of foreign materials, organ transplantations, etc. have not only created a risk factor but also given rise to a cohort of particularly vulnerable persons.As a result, the highest infection rates have been reported in intensive care unit (ICU) patients [Weinstein *et al.,* 2009].Scientific reports claim that nosocomial infection rates in adult and pediatric ICUs are approximately three times higher than elsewhere in the hospitals. The

infection sites and the pathogenesis concerned are more or less directly related to treatment in ICUs. In the vicinity of ICUs, patients with invasive vascular catheters and other medical or invasive monitoring devices are more prone to bloodstream infections caused by coagulase-negative *Staphylococci*. Also, studies have shown that cases of occult bacteremia in ICU patients are probably due to vascular access-related infections [Fridkin *et al.*, 2007].

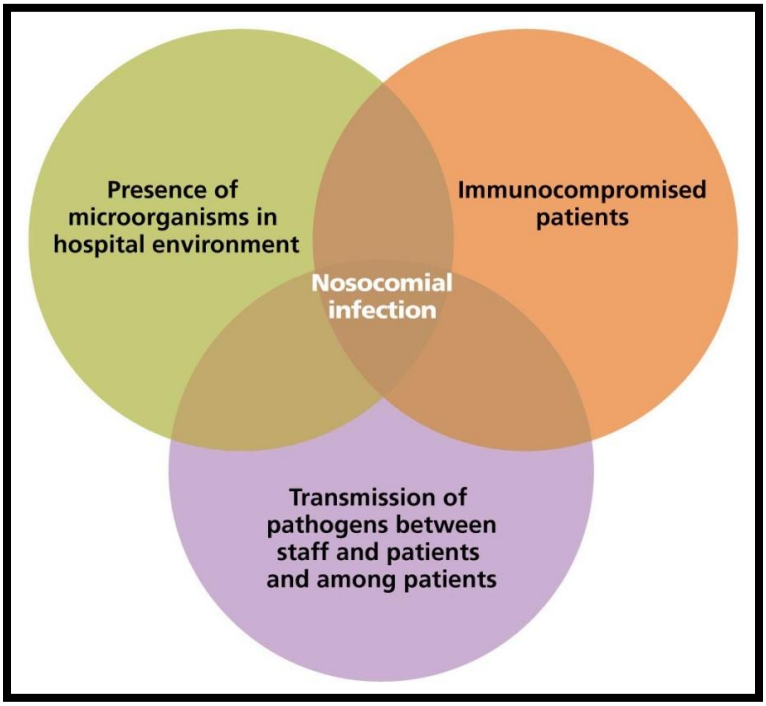

Figure 2.1 : Interplay of factors resulting in nosocomial infections

There are two main categories of nosocomial infections:

Endogenous Infections: The patient is infected by a germ that is already carrying (colonized patient), when undergoing a medical procedure such as the insertion of a catheter, treating an open wound or cut, an endoscopy, or surgery.

Exogenous Infections: are caused by germs found in the environment. These bacteria can be found virtually everywhere in a hospital; in beds, floors, walls, medical instruments, health care

workers clothing, ventilation systems, air conditioners, computer keyboards and cell phones etc. The hands of health care workers are the most common vehicle of infection transmission. One colonized infected patient can infect several others within a few days.

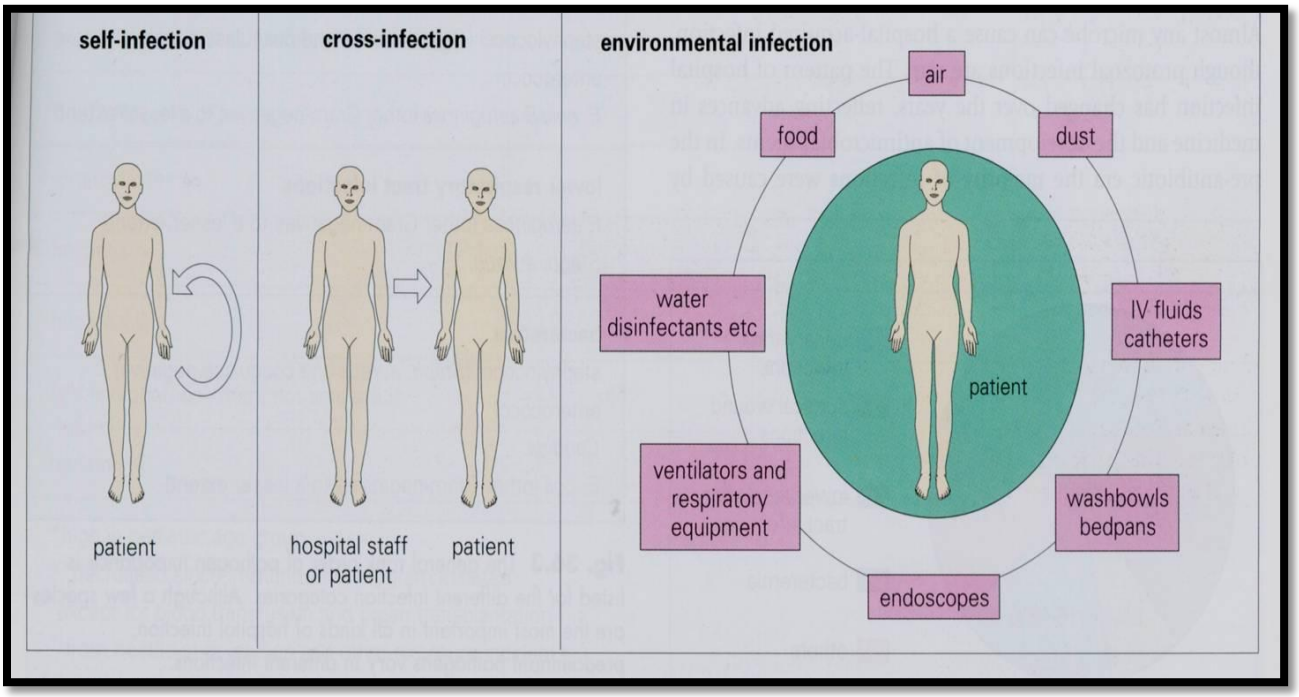

Figure 2.2: Endogenous and exogenous source of hospital acquired infection

2.1.1 Epidemiology of nosocomial infections

The data analysis report 2012 of National Nosocomial Infection Surveillance (NNIS) was a collaborative surveillance system report sponsored by the Centers for Disease Control (CDC) which proclaimed that gram negative bacilli were associated with majority of ICU infections along with almost75% of the total urinary tract infections, 65% of nosocomial pneumonia and 25% of bloodstream infections[CDC, 2012]. A study conducted in 12 intensive care units (in seven hospitals) of seven different Indian cities by the International Nosocomial Infection Control Consortium, observed 10,835 patients hospitalized for a total of 52,518 days for nosocomial infection. The report determined that patients acquired 476 different types of infections in the hospital, out of which about 46% were due to*Enterobacteriaceae*, 27%

Pseudomonas and 3% *Staphylococcus aureus* and 8% *Candida spp.respectively*[Yunus*et al., 2005*].

A survey done on 110,709 pedriatic ICU patients reported nosocomial infection in 6,290 patients. A six month study conducted in 2011 by United Health Commission in intensive care units of AIIMS, Delhi found that 11% patients out of a sample size of 1300 had contracted hospital acquired infections. It was found that 21% of the infections were caused by *Pseudomonas aeroginosa*, 23% *Staphylococcus aureus*, 16% *Klebsiella pneumoniae*, 15% *Acinetobacter baumanii* and 8% infection were *Escherichia coli* borne. An another parallel study conducted at Post Graduate Institute of Medical Education and Research (PGIMER) in Chandigarh revealed that up to 59 burn patients out of 71 i.e. 83% had some or the other kind of hospital acquired infections .In another study conducted in India on 422 patients, the prevalence of nosocomial infections was reported as 38.8% [Ganguly *et al*, 2012]. Another study on 629 patients in ICUin India, revealed that pneumonia (with a rate of 29.5%) was the most common infection reported and Gram negative bacterial species were detected as the most common pathogenic microbial agents [Orrett, 2012].

Not only in India but worldwide studies on nosocomial infections have indicated that more than 3.4 million people suffer from infectious complications acquired in hospital at a given point of time across the globe, and it may rise up to 400 percent in next 20 years [Tikhomirov et al., 2005]. Nosocomial infections occurred in 5-10% of all hospitalized patients in Europe and North America and more than 40% in parts of Asia, Latin America, and sub-Saharan Africa respectively [Lynch et al., 2007].

2.1.2 Causes of Nosocomial infections

There are three key factors involved in the spread of nosocomial infections. The first factor is many hospital personnel fail to follow basic infection control rules such as hand washing between patient contacts. In intensive care units, asepsis is often overlooked in the rush of crisis care [Weinstein *et al.,* 1991; Baeza and Ribeiro-Neto, 1999]. Secondly, long-term irrational use of antimicrobial in hospitals and other health care facilities has escalated the persistence of nosocomial infection. Thisabuse of antimicrobial drugs in ambulatory facilities and extended health care settings have also created a large reservoir of resistant strains in nursing homes [Tacconelli *et al.,* 2008].. Lastly, the long-term use of vascular or other device-related care in immunosuppressed patients has led to a higher prevalence of bloodstream infections and ventilator-associated pneumonia [Archibald *et al.,* 2004]. Unsafe medical care is the prime cause of spread of nosocomial infections, especially in underdeveloped and developing countries [WHO, 1999].

Nosocomial infections are most commonly caused by viral, bacterial and fungal pathogens. While the majority (nearly 60%) of nosocomial infection is caused by aerobic gram-negative bacilli, only 30% of nosocomial infections are caused by Gram-positive bacteria. Theanaerobes account for only 3% of total nosocomial infections. The remaining 7% of the nosocomial infections are caused by either fungi or viruses.

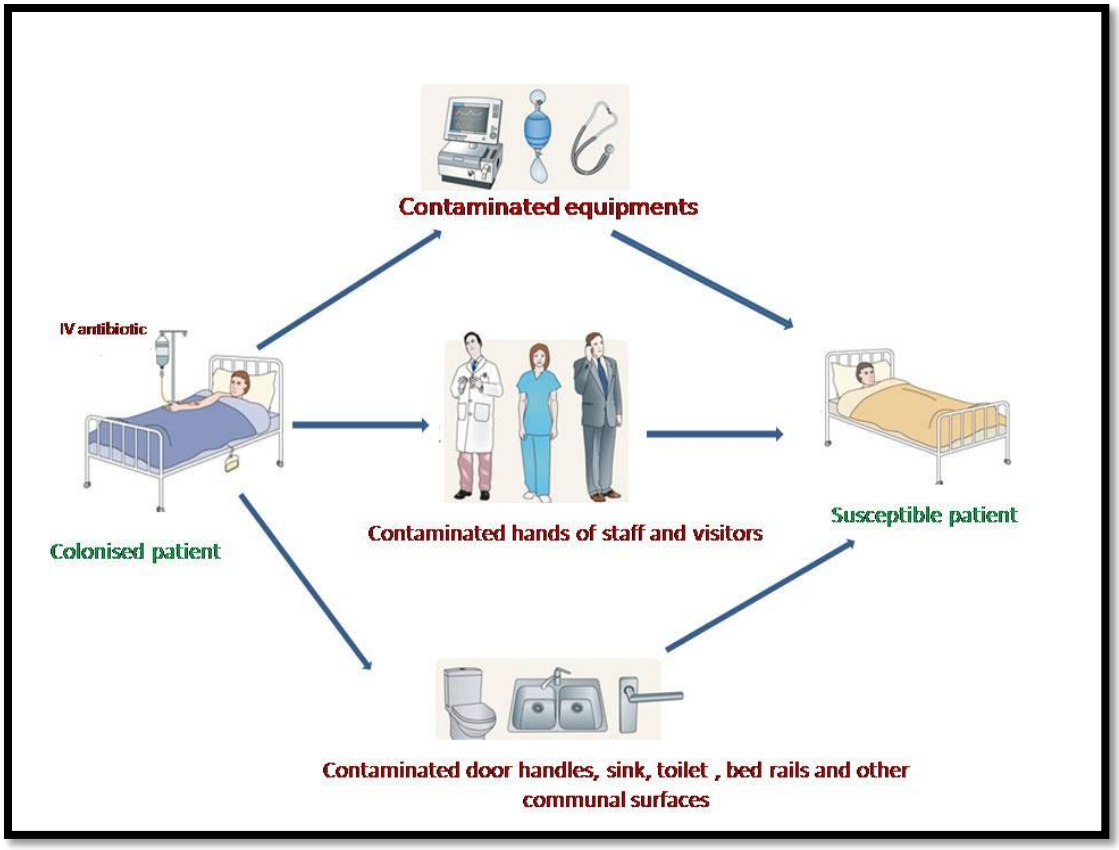

Figure 2.3: A) Factors associated with nosocomial bacterial pneumonia

In the period of 1970 till 2000 gram negative bacilli particularly *Pseudomonas aeruginosa* and *Enterobacteriaceae* were the chief causative agents of all the nosocomial infections [Robert *et al*., 2008]. A significant finding contributing the cause of nosocomial infections is that up to 60% of such infections are caused by drug-resistant microbes and in 35-40% of the infections the microorganism is resistant to the best drug commonly used to treat that infection. The excessive use of broad-spectrum antibiotics has led to the development of antibiotic resistance among the microorganisms [WHO, 2009]. Vancomycin-resistant *Enterococci* (VRE) and Methicillin-resistant *Staphylococcus aureus* (MRSA) are the major gram positive nosocomial microorganisms and *Pseudomanas aeroginosa*, *Klebsiella pneumoniae* and *Enterobacter* that harbor chromosomal or plasmid mediated beta-lactamase enzymes are major

gram-negative antimicrobial resistant pathogens of concern[Robert *et al.*, 2008]. *Klebsiella pneumoniae* accounts majorly (28%) for the morbidity and mortality in patients due to pneumonia and urinary tract infections followed by *Staphylococcus aureus* (17%) and *Hemophillus influenza* (13%). Nosocomial infection prolongs the hospital stays and hence increases the expense of the treatment. The worst part is that the nosocomial infections have affected those countries the most which have limited healthcare facility, limited resources and least capability to afford the expensive medication [WHO, 2009].

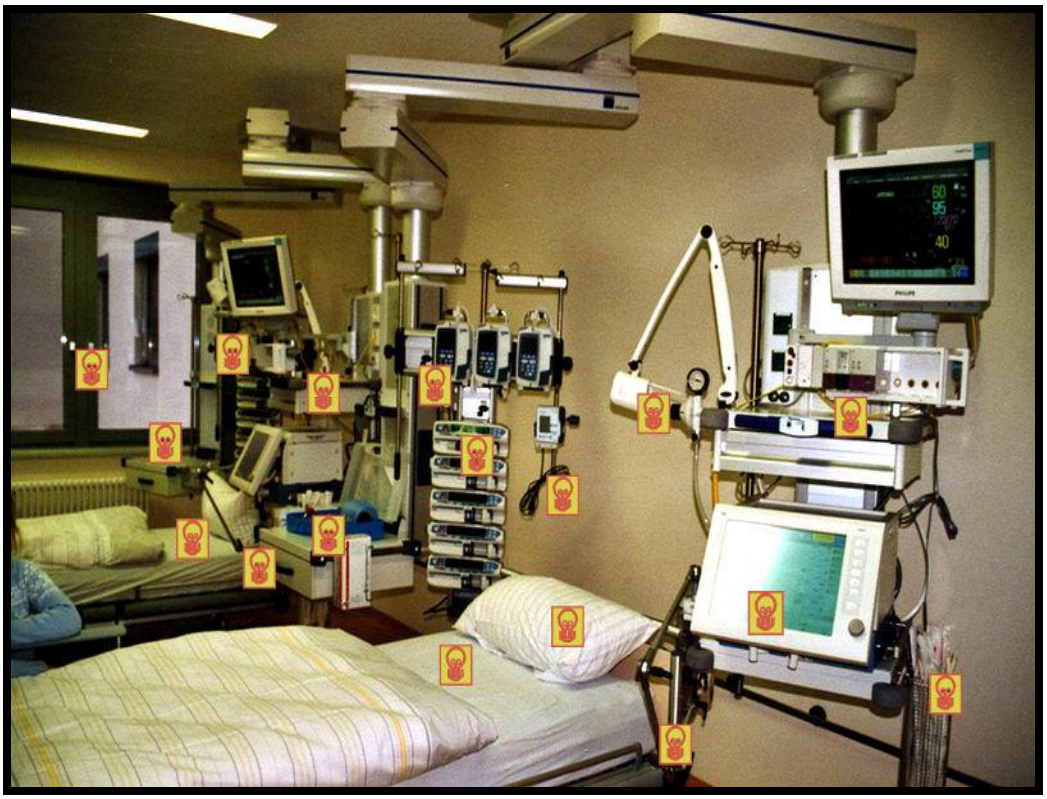

Figure 2.3: B) Sites of Nosocomial pathogens in Intensive Care Units

2.1.3 Types of nosocomial infections

Table 2.1: A glimpse of Nosocomial Infections – Type, Causative agent and Treatment associated

Common types of nosocomial infections	Commonly associated microorganisms	Treatment related factors contributing to Infection
Urinary tract infection	Gram-negative pathogens, *Escherichia coli*, *Proteus mirabilis*, *Klebsiella* spp and *P.aeruginosa*, *Enterobacter* and *Enterococci*	Use of catheter to measure output Disconnection of catheter from drainage tube Duration of catheterization; insertion of catheter late in hospital stay Retrograde flow of urine from drainage
Surgical site infections	*S.aureus*, coagulase-negative, *Staphylococci*,*Enterococcus* spp, *Enterobacter* spp., *P.aeruginosa*, *Escherichia coli*	Foreign material (including drains and sutures) Skin antisepsis Duration of operation Intra-operative contamination Duration of preoperative hospital stay Hypothermia during operation Duration of surgical scrub Antimicrobial prophylaxis Surgical technique Re-intubation
Ventilator-associated pneumonia	*P.aeruginosa*, *S.aureus*, *Haemophilus influenza*	Supine head position Aspiration of gastric contents Naso-gastric tube Use of paralytic agents Duration of mechanical ventilation
Intravascular device-related bloodstream infections	Coagulase-negative *Staphylococci*, *Entercoccus*, *S.aureus*, *Candida albicans*	Heavy colonization on skin at site of insertion Location in internal jugular or femoral vein Length of time in place Conatmination of catheter hub Type of infusate Location of insertion
Gastrointestinal tract infections	*Clostridium difficile*, Rotavirus	Antibiotic use, Naso-gastric intubation

† (Ref: Lore L. Alexander, CME Resource, 2007)

2.1.4 Prevention and control of nosocomial infections

Approximately one third of nosocomial infections can be prevented by adopting an appropriate level of preventive strategies/steps that are strictly needed to be pursued in an organized manner[Scheckler *et al.,* 2008]. Prevention protocol tends to vary according to the risk level of hospitals, clinics, and patients. Intensive care units and operating rooms carry a much higher risk that psychiatric units or a general practitioner's office. Effective surveillance and stringent preventative measures are required. Consequently, the following preventative measures should be standardized and implemented everywhere:

a. **Proper hygiene: washing one's hands is the basic preventative measure and reduces infection by at least 50%**.

Researchers have known for 150 years that medical personnel can save lives simply by washing their hands prior to treating a patient [Nayfield *et al.,* 2013].Isolating colonized and infected patients is essential. Ideally, each patient should be in a single room, but if space does not allow for this, only patients infected by the same bacteria should be kept together. Patients infected by different bacteria must be separated.Barrier measures such as wearing sterile clothing, latex gloves and masks reduces the spread of bacteria and protects immune-deficient and other high risk patients from contamination. This applies to all personnel, patients and visitors.

In hospitals,an aggressive infection control committee must be formed with a primary goal of reducing nosocomial infections through the identification and control of predisposing factors, education and training of hospital personnel. The committee should also bring up institutional policies and practices, for e.g.,the use of gloves and optimal hand washing must be facilitated and reinforced [Schwartz *et al.*, 2009; Alicia 1999]

b. **Restrict the use of antibiotics to prevent the development of antibiotic-resistant bacteria.**

Both inside and outside the hospital, clothing worn by health care workers constitutes a serious hazard. Hospital personnel should no longer be allowed to wear their hospital garments outside the establishment. Furthermore, surgical staff should not be permitted to wear their garb outside the operating room. When arriving for their shift, all hospitalpersonnel must remove their street clothing and put on the appropriate hospital garb. The British Medical Association recommended in July 2005 that all hospital clothing be cleaned and disinfected on a daily basis. Good hygiene and regular disinfection are standard preventative measures. Hospital rooms, beds, linen, doorknobs, toilets, and sinks, etc. must all be disinfected. Hospital maintenance workers require specific training in the techniques of disinfection. One simply does not clean a hospital room in the same manner as a hotel room.

c. Surveillance System

A surveillance system of nosocomial infections that occur outside the hospitals and other health care providing facilities must also be developed for the after the patients are discharged.

2.2 Antibiotics at a glance

According to the definition, the word "antibiotic" refers to substancesthatare produced by microorganisms and act against another microorganism. Thus the term antibiotics does not include synthetic substances (sulfonamides and quinolones), or semi-synthetic (methicillin and amoxicillin), or those which come from plants (quercetin and alkaloids) or animals (lysozyme). In contrast, the term "antimicrobial" includes all agents that act against all types of microorganisms – bacteria (antibacterial), viruses (antiviral), fungi (antifungal) and protozoa (antiprotozoal). This concludes to describing an 'antibiotic' asa low molecular substance

produced by a microorganism which inhibits or kills other microorganismsat a low concentration. Whereas, an antimicrobial is any substance of natural, semi-synthetic or synthetic origin that kills or inhibits the growth of microorganisms but causes little or no damage to the host. Thus, all antibiotics are antimicrobials, but not all antimicrobials are antibiotics. In a broader concept, antibiotics are medicaments that constrain or hold up the growth of bacteria. (CDC, 2013). Table 2 enumerates the commonly used antibiotics with their mechanism of action.

Table 2.2: Timeline of antibiotics

Antibiotic Class; Example	Year of Discovery	Year of Introduction	Year resistance observed	Mechanism of action	Mechanism of resistance	Activity or Target Species	Ref
Sulfadrugs; Protonsil	1932	1936	1942	Inhibition of dihydropteroate synthetase	Efflux, altered target	Gram positive bacteria	Lesch *et al*
β lactams; Penicillin	1928	1938	1945	Inhibition of cell wall biosynthesis	Hydrolysis, efflux, altered target	Broad spectrum activity	Toku E *et al*
Aminoglycosides Streptomycin	1943	1946	1946	Binding of 30S ribosomal subunit	Phosphorylation, acetylation, efflux, altered target	Broad spectrum activity	Leclercq *et al*
ChloramphenicolChoramphenicol	1946	1948	1950	Binding of 50S ribosomal subunit	Efflux, altered target	Broad spectrum activity	Falagas *et al*
Macrolides; Erythromycin	1948	1951	1955	Binding of 50S ribosomal subunit	Hydrolysis, glycosylation, phosphorylation	Broad spectrum activity	Hamilton *et al*
Tetracyclines; Chlortetracycline	1944	1952	1950	Binding of 30S ribosomal subunit	Monooxygenation, efflux, altered target	Broad spectrum activity	Jukes *et al*
Glycopeptides; Vancomycin	1953	1958	1960	Inhibition of cell wall biosynthesis	Reprogramming peptidoglycan biosynthesis	Gram positive bacteria	Small PM *et al*

Rifamycins; Rifampin	1957	1958	1962	Binding of RNA polymerase β subunit	ADP ribosylation, efflux, altered target	Gram positive bacteria	Sensi *et al*
Quinolones; Ciprofloxacin	1961	1968	1968	Inhibition of DNA synthesis	Acetylation, efflux, altered target	Broad spectrum activity	Ball P *et al*
Streptogramins; Streptogramin B	1963	1998	1964	Binding of 50S ribosomal subunit	C-O lyase, acetylation, efflux	Gram positive bacteria	Bouc heer *et al*
Oxazolidinones; Linezolid	1955	2000	2001`	Binding of 50S ribosomal subunit	Efflux, altered target	Gram positive bacteria	Swan ey *et al*
Lipopeptides; Daptomycin	1986	2003	1987	Depolarisation of cell membrane	Altered target	Gram positive bacteria	Wood worth *et al*
Diarylquinolines; Bedaquiline	1997	2012	2006	Inhibition of F_1F_0-ATPase	Altered target	Narrow spectrum activity (Mycobacterium tuberculosis)	Diaco n *et al*

Antimicrobials are classified in several ways, which include,

- Spectrum of activity
- Effect on bacteria
- Mode of action

2.2.1 Classification According To Spectrum of Activity

On the basis of the range of bacterial species susceptible to these modalities, antibacterials are classified as broad-spectrum, intermediate-spectrum, or narrow- spectrum.

a. **Broad spectrum antibacterials**: These show activity against both Gram-positive and Gram-negative microbes. Examples includes: tetracyclines, phenicols, fluoroquinolones, "third-generation" and "fourth-generation" cephalosporins.

b. **Narrow spectrum antibacterials**: These modalities have a limited activity and principally target a particular species of microorganism. For example, polymixins are usually only effective against Gram negative bacteria, whereasglycopeptides and bacitracin are only effective against Gram-positive bacteria.Likewise, Aminoglycosides and sulfonamides are only effective against aerobic organisms, while nitroimidazoles are generally effective agaisnt anaerobes.

2.2.2 Classification According toEffect on Bacteria

Different antibiotics exert their antimicrobial activity differently, an antibiotic can killthe microorganisms directly and be 'bactericidal', or it can be bacteriostatic by deterring the growth of microorganisms.

a. Bactericidal drugs are those which kill the target organisms. Due to the differences in the mechanisms by which antibiotics affect bacteria, the clinical usage of antibacterials may have different effects on bacterial agents, leading to an endpoint of either inactivation or actual death

of the bacteria. Examples of bactericidal drugs include cephalosporins, aminoglycosides, penicillins, and quinolones.

b. Bacteriostatic drugs delay or deter the bacterial growth and replication. Examples of bacteriostatic drugs are tetracyclines, sulfonamides, and macrolides.

c. Some antibiotics show both bacteriostatic and bactericidal properties. This depends on the dose, period of exposure and the state of the invading bacteria. For example, Drugs like aminoglycosides, fluoroquinolones, and metronidazole exert concentration-dependent killing characteristics. The rate of killing of these drugs increases with their concentration and hence the efficacy.

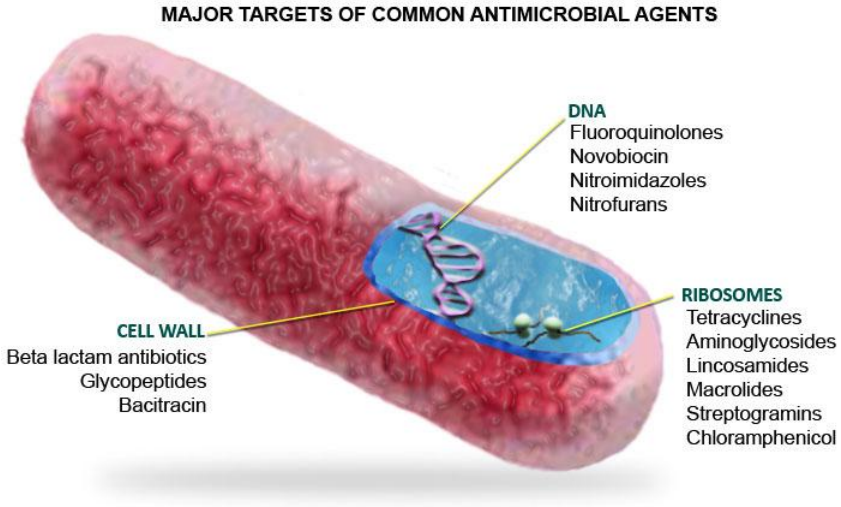

Fig. 2.4 – Targets of Antibiotics as shown in a given Bacteria cell

2.2.3 Classification According to Mode of Action

Due to the difference in their modes of action, variable nature of the structure and degree of affinity to their specific target sites, different classes of antibiotics have different modes of action. The various classes of antibiotics diversified on the basis of their mode of action are explained below,

a. **Inhibition of cell wall synthesis**: The presence of cell walls and its structure is critical for both the survival and life of bacterial species. A drug targeting cell walls can selectively inhibit the bacterial organisms. Penicllins, cephalosporins, bacitracin and vancomycin are some of the examples which kill the bacteria by cell wall synthesis inhibition.

b. **Inhibitors of cell membrane function**. Like cell wall, cell membranes are also important barriers that help in segregating and regulating intra- and extracellular movement of substances. Any disruption to this structure may result in leakage of important cell solutes essential for the cell's survival. The cell membrane is found in both eukaryotic and prokaryotic cells and hence the action of this class of antibiotic is often poorly selective and can often be toxic for systemic use in the mammalian host. Clinical usage of such drugs is only limited to topical use. Examples: polymixin B and colistin.

c. **Inhibitors of protein synthesis**. Protein synthesis is an essential process for the growth and survival of all bacterial cells. A number of antibacterial agents target bacterial this process of protein synthesis by binding to either of the 30S or 50S subunits of the intracellular ribosomes. This disrupts the normal cellular metabolism of the bacteria, and consequently leads to the death of the organism. The classes of antibiotics included in this class are: Aminoglycosides, macrolides, lincosamides, streptogramins, chloramphenicol, tetracyclines.

d. **Inhibitors of nucleic acid synthesis**. DNA and RNA are key to the replication process of all living forms, including bacteria. Some antibiotics bind to modules involved in the process of DNA or RNA synthesis.These causes intervention of the normal cellular processes which ultimately compromise the bacterial multiplication and survival. Examples include quinolones, metronidazole, and rifampin.

e. **Inhibitors of other metabolic processes**. Some antibiotics act on various cellular processes which are essential for the survival of the bacterial pathogens. This includes drugs like sulfonamides and trimethoprim which disrupt the folic acid pathway, a necessary step for the bacterial physiology to produce DNA synthesisprecursors. Sulfonamides bind to dihydropteroate synthase, trimethophrim inhibit dihydrofolate reductase and both of these enzymes are essential for the production of folic acid, anessential vitamin synthesized by bacteria for sustenance.

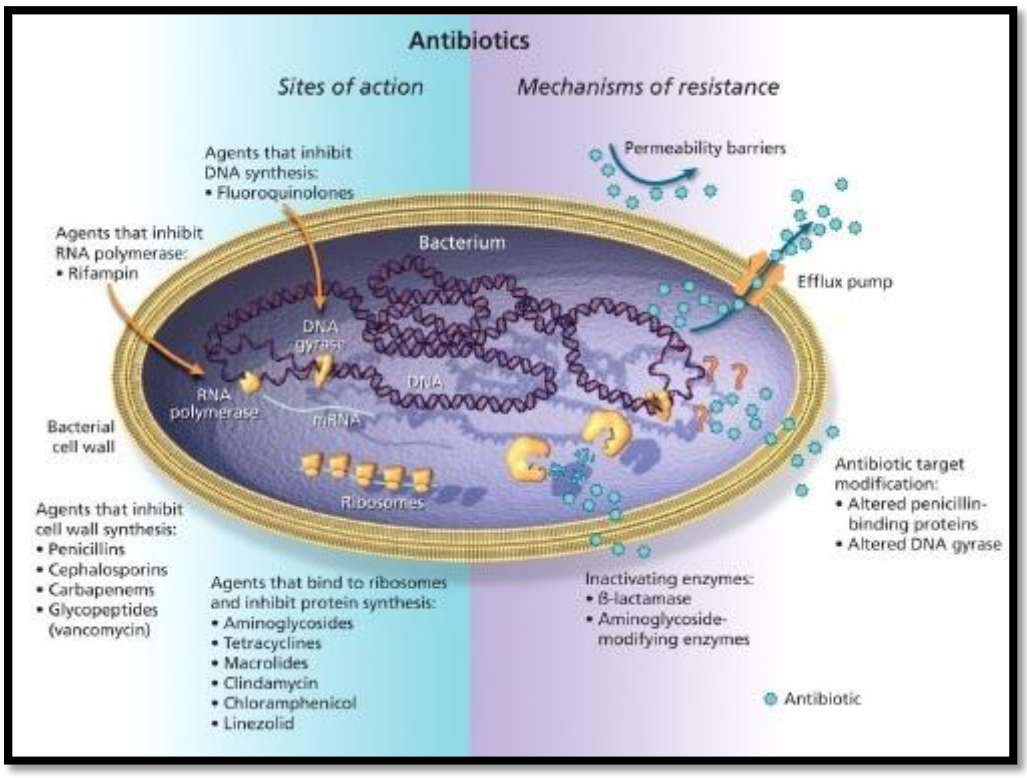

Figure 2.5: Resistance mechanism and sites of action[Mulvey and Salvor, 2009]

2.2.4Current scenario

There is a growing need for instigating the search for new antimicrobial agents. WHO's 2014 report on global surveillance of antimicrobial resistance reveals that antibiotic resistance is no longer a prediction for the future; it is happening right now, across the world, and is putting at risk the ability to treat common infections in the community and hospitals. Without urgent,

coordinated action, the world is heading towards a post-antibiotic era, in which common infections and minor injuries, which have been treatable for decades, can once again kill.

Drug resistance is spreading among pathogens, but the number of new antibiotics in the market is constantly decreasing. As a result, hospital-acquired bacterial infections now affect 1.7 million patients annually in responsible for 99,000 deaths every year. A scenario of increased drug resistance among pathogens versus reduced development of new drugs has arrived as shown in Fig 2.5.

2.3 Antibiotic Resistance

Over the years, the antimicrobial resistance has become a global problem. When an antibiotic fails in its ability to effectively control the bacterial growth and the microbes continue to proliferate even at therapeutic level of the drug, in such situation the bacteria becomes 'resistant' to that particular antibiotic and this phenomenon is termed as Antibiotic Resistance (Figure 2.6). It is one of the many adaptive traits that resilient bacterial subpopulations have started to possess, enabling them to out-compete and out-survive their microbial neighbors and overcome host strategies aimed against them. The phenomenon is as old as discovery of first antibacterial themselves. A constant selective pressure by numerous drugs resulted in organisms bearing surplus kinds of resistance mechanisms that led to multidrug resistancenovel,enzymatic mechanisms of drug modification,penicillin-binding proteins (PBPs), mutated drug targets, enhanced efflux pump expression, and altered membrane permeability. Few examples which have been exceedingly problematic are *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, *Escherichia coli* and *Klebsiella pneumoniae*bearing extended-spectrum β-lactamases (ESBL), vancomycin-resistant *enterococci* (VRE), methicillin-resistant *Staphylococcus aureus*(MRSA), vancomycin-resistant MRSA [CDC, 2103]

The problem of antimicrobial resistance has been persistent in developing countries, where the burdens of infection as well as cost constrictions are both high. Due to cost constrictions the replacement of older antibiotics with newer drugs is becoming a tedious task and the critical shortage of newer antibiotics is adding to the current situation [Larson E, 2007]. The major factor behind evolution of drug resistance is 'selection pressure'. This selection pressure has a multi-factorial involvement of irrational use of drugs at both human and animal level. Though drug resistance is a medical problem but the influential factors for its global spread are epidemiological, ecological, cultural, social, and even economic. WHO report 2014 lists latest resistance patterns in antibiotics which include the following,

- Treatment failure to the drug of last resort for gonorrhoea – third-generation cephalosporins – has been confirmed in several countries. Untreatable gonococcal infections result in increased rates of illness and complications, such as infertility, adverse pregnancy outcomes and neonatal blindness, and have the potential to reverse the gains made in the control of this sexually transmitted infection.
- Resistance to one of the most widely used antibacterial drugs for the oral treatment of urinary tract infections caused by *E. coli* – fluoroquinolones – is very widespread.
- Resistance to first-line drugs to treat infections caused by *Staphlylococcus aureus* – a common cause of severe infections acquired both in health-care facilities and in the community – is also widespread.

Resistance to the treatment of last resort for life-threatening infections caused by common intestinal bacteria – carbapenem antibiotics – has spread to all regions of the world.

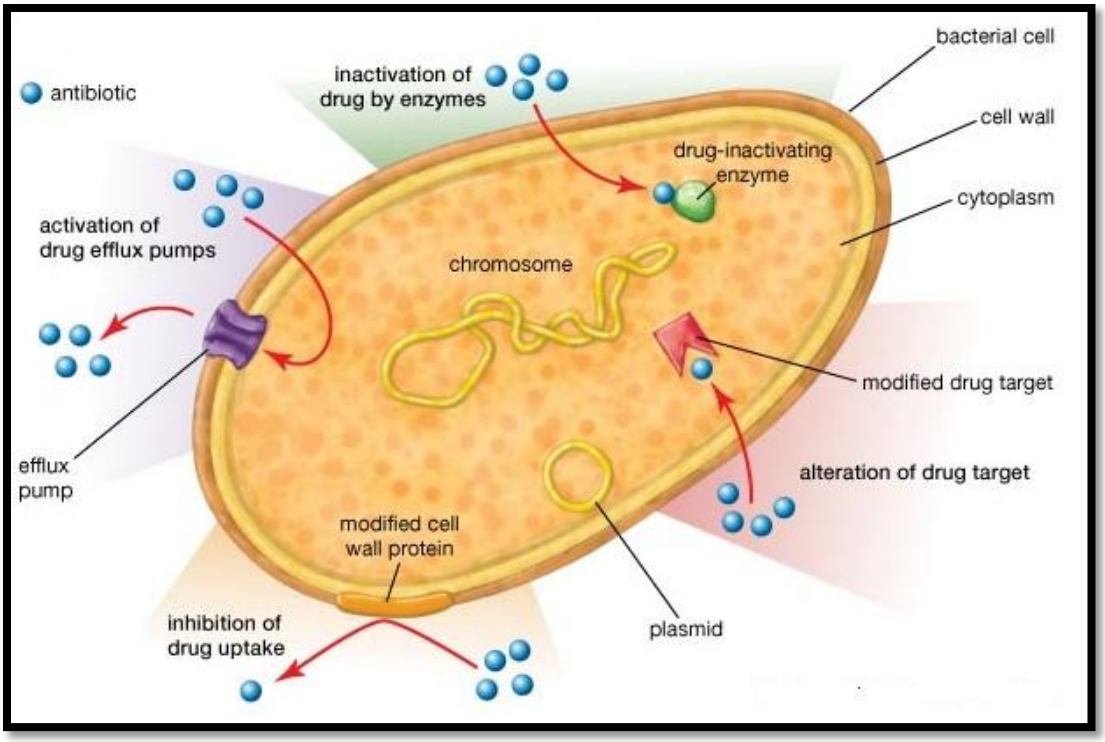

Figure 2.6: Antibiotic Resistance Mechanism

2.3.1 Mechanisms of Drug Resistance

Today, .all bacteria those were once susceptible to different antibiotics are resistant to at least one. Antibiotics were accounted as miracle drugs which cured many fatal infections. However, an over- dependence on these miracle agents soon developed and symptoms which could be handled by our body's immune system were treated with antimicrobials. After prolonged repeated use, bacteria was no longer killed or harmed by the drugs. Once resistant, the future generations of these strains were also resistant, capable of causing infections not cured by antibiotic drugs. The mechanisms of drug resistance are explained in detail.

a. Inhibition of Drug uptake : Preventing access

In this type of drug resistance mechanism the bacteria often modify the structure of their cell walls and do not permit the entrance of the drug.. In the bacterial anatomical structure, Porin channels are the corridors through which these antibiotics normally cross the bacterial outer

membrane(Ringler *et al*., 2006). Some bacteria protect themselves by prohibiting these antimicrobial compounds from entering past their cell walls. Restricting passage in this manner will constrain these antimicrobials from reaching their intentional targets, for aminoglycosides and beta lactams, are the ribosomes and the penicillin-binding proteins (PBPs), respectively. Many penicillin resistant Gram negative bacteria have an outer membrane of lipopolysacchrides and other modified materials covering the petidoglycan layer. These layers prevent penicillin is pass through to the site of peptidoglycan synthesis, which is the activity site of the antibiotic.

This strategy has been observed in:

- Pseudomonas aeruginosa against imipenem (a beta-lactam antibiotic)
- Enterobacter aerogenes and Klebsiella spp. against imipenem
- Vancomycin intermediate-resistant S. aureus (VISA) strains with thickened cell wall trapping vancomycin
- Many Gram-negative bacteria against aminoglycosides
- Many Gram-negative bacteria against quinolones

b. Drug Inactivation

Some bacteria resist antibiotic attack by incapacitating drugs though chemical alteration or through the addition of functional groups. Most favorable example of chemical modification is the action of enzymes called Label. Enzymes like β-lactamase work on penicillin antibiotics by hydrolysing the beta-lactam ring that provides them their function.

c. Alteration Of Antibiotic Target

In this mechanism the antibiotic target is altered to such a form where it is no longer susceptible to the drugs. Many antibiotics rely on the shape and stereochemistry of their specific binding targets and hence function. Thus, any change in the target site alters ability of the drug to

function by preventing the binding to the target. For example, chloramphenicol's activity is resisted by a modification of the rRNA in the large ribosomal subunit to which it binds.

- *Staphylococci* against methicillin and other beta-lactams (Changes oracquisition of different PBPs that do not sufficiently bind beta-lactams to inhibit cell wall synthesis.)
- *Enterococci* against vancomycin (alteration in cell wall precursor components to decrease binding of vancomycin)
- *Mycobacterium* spp. against streptomycin (modification of ribosomalproteins or of 16s rRNA)

d. Mutations

Impulsive mutations in bacterial chromosomes bring about drug resistance. The alterations in the primary sequence of bacterial DNA result in 3-D changes in the drug receptor sites. Thus, the drug is unable to bind at the site and hence cannot inhibit.

e. Expulsion via efflux pumps

Efficacy of antibiotics also depends on presence of the drug in sufficient concentration within bacterial cell. Various bacterial cells have membrane proteins which act as efflux pumps, thus extruding antibiotics out of the cell when it entrance. This results in low concentration of antibacterial drug which is inadequate to elicit effect. These pumps are selective to different antibiotics.

This strategy has been observed in:

- *Escherichia coli* and other Enterobacteriaceae against tetracyclines
- Enterobacteriaceae against chloramphenicol
- *Staphylococci* against macrolides and streptogramins
- *Staphylococcus aureus* and *Streptococcus pneumoniae* against fluoroquinolones
-

2.3.2 Current scenario

There is a growing need for instigating the search for new antimicrobial agents. WHO's 2014 report on global surveillance of antimicrobial resistance reveals that "antibiotic resistance is no longer a prediction for the future; it is happening right now, across the world, and is putting at risk the ability to treat common infections in the community and hospitals. Without urgent, coordinated action, the world is heading towards a post-antibiotic era, in which common infections and minor injuries, which have been treatable for decades, can once again kill."

Drug resistance is spreading among pathogens, but the number of new antibiotics in the market is constantly decreasing. As a result, hospital-acquired bacterial infections now affect 1.7 million patients annually in responsible for 99,000 deaths every year. A scenario of increased drug resistance among pathogens versus reduced development of new drugs has arrived as shown in Fig 2.7.

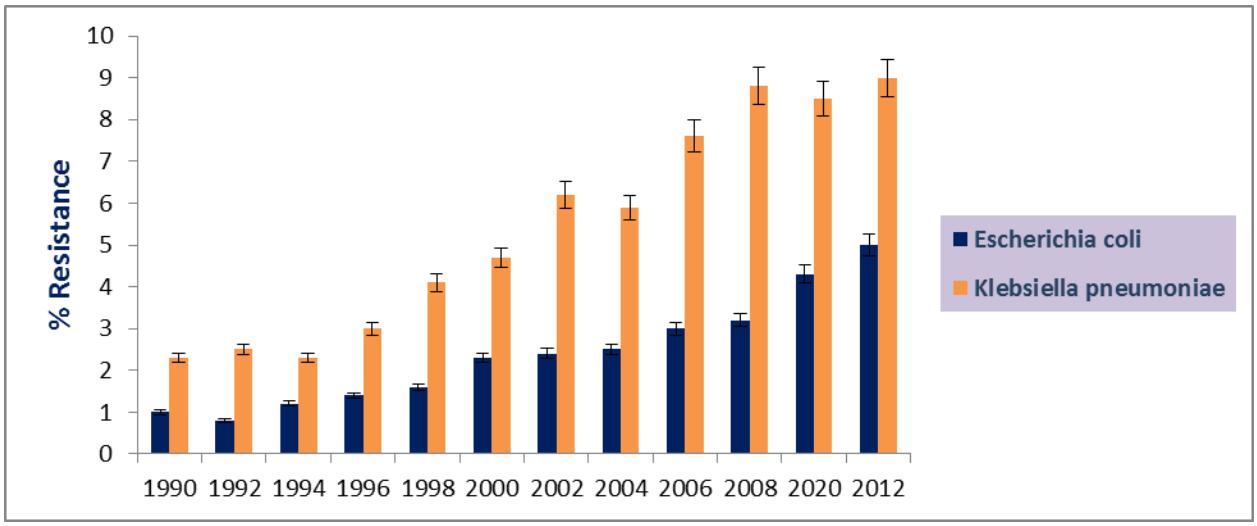

Fig 2.7: A) Increase in drug resistance among pathogens [Livemore *et al*., 2014]

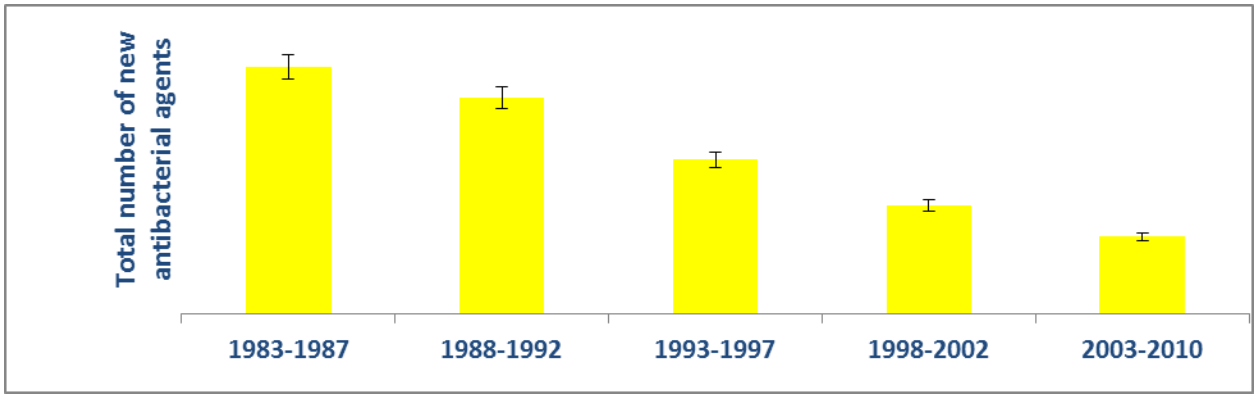

Figure 2.7: B) Decreased number of new drugs in the mark [Jefferey *et al*., 2014]

Antibiotic use has been increasing steadily in recent years. In a policy report released by the Infectious Disease Society of America (IDSA) on April 2013, IDSA expressed grave concern over the weak pipeline of antibiotics to combat the growing ability of bacteria, especially the Gram-negative bacilli (GNB), to develop resistance to antibiotics. Since 2009, only 2 new antibiotics were approved in United States, and the number of new antibiotics annually approved for marketing continues to decline. Some of these seven new antibiotics are listed in Table 2.3. Other New combination of antibiotics in the market which is used to treat drug resistant bacterial infections includes:

- Brilacidin (PMX-30063): Peptide defense protein mimetic (cell membrane disruption). In phase 2
- Ceftolozane/tazobactam (CXA-201;CXA-1/tazobactam):

  Antipseudomonal cephalosporin/β-lactamase inhibitor combination (cell wall synthesis inhibitor). In phase 3.
- Ceftazidime/avibactam (ceftazidime/NXL104): Antipseudomonal cephalosporin/β-lactamase inhibitor combination (cell wall synthesis inhibitor). In phase 3.
- Ceftaroline/avibactam (CPT-avibactam; ceftaroline/NXL104): Anti-MRSA cephalosporin/ β-lactamase inhibitor combination (cell wall synthesis inhibitor)
- Imipenem/MK-7655: Carbapenem/ β-lactamase inhibitor combination (cell wall synthesis inhibitor). In phase 2.

- Plazomicin (ACHN-490): Aminoglycoside (protein synthesis inhibitor). In phase 2.
- Eravacycline (TP-434): A synthetic tetracycline derivative / protein synthesis inhibitor targeting the ribosome being developed by Tetraphase. Phase 2 trials complete.

Table 2.3: List of Newer available antibiotics

Class of Compound	Phase of Development	Analogs	Mechanism of Action	Mechanism of Resistance	Drug Company
Oxazolidinones	FDA Approved 2000	Linezolid, Radezolid, Torezolid, RWJ-416457	Inhibits protein translation (initiation/elongation inhibition)	rRNA mutations	Pfizer, Rib-X, Trius Therapeutics, Johnson & Johnson
Glycopeptides	Phase III	Oritavancin, Dalbavancin, Telavancin	Inhibit peptidoglycan biosynthesis/ transglycosylation	Unidentified	Targanta/The Medicines Co, Pfizer, Theravance
Ketolides	Phase III	Cethromycin	Inhibits protein synthesis	rRNA dimethylation	Advanced Life Sciences
Glycylcyclines	FDA approved 2005	Tigecycline, PTK0796	Inhibits protein synthesis	Efflux pumps	Wyeth, Paratek Pharmaceuticals,
Carbapenems	FDA approved 2007	Doripenem, Raxupenem	Inhibits peptidoglycan biosynthesis	Carbapenemases, Efflux pumps, Porin mutations	Johnson & Johnson, Protez Pharmaceuticals
Streptogranins	Phase II	NXL103/XRP 2868	Inhibits protein translation	Unidentified	Novexel
Fluoroquinolones	Preclinical	JNJ-Q2, Finafloxacin	Inhibit type II topoisomerase	gyrA, parC mutations	Johnson & Johnson, MerLion Pharmaceuticals

2.4 *Klebsiella pneumonia:* Etiology and Characterstics of causative organism

*Klebsiella pneumoniae*belongs to *Enterobacteriaceae*family, whichis a heterogeneous family of Gram-negative, nonsporulating and facultative anaerobic rods.Like many *EnterobacteriaceaeK. pneumoniae*have type 1 pili (fimbriae), which enables bacterial adhesion toepithelial cells. It is a non-motile gram-negative rod-shaped bacterium which sustains both aerobically and anaerobically [Brisse*et al*., 2006].The skin, the naso- and oropharynx and intestinal tract of the patients are the main reservoir for the *Klebsiella pneumoniae* strains.On Mac Conkey Agar, it usually ferments lactose or produces pink colonies with surrounding areas of precipitated bile salts. It also grows characteristically with a green sheen on eosin methylene blue agar. *Klebsiella pneumoniae* strain will produce indole from tryptophan; it does not produce hydrogen sulfide, urease, and cannot use citrate as sole carbon source [Mahon *et al*., 2007]. Together with *E. coli* and *Pseudomonas aeruginosa*,it is consideredas oneof the three most common gram-negative pathogens, [Richard *et al.,* 2000]. Most clinical isolates have a polysaccharide capsule, regarded as a virulence determinant, which encapsulates the cells of the bacteria to prevent phagocytosis [Favre *et al*., 1999].*K. pneumoniae* is acommon member of the human intestinal flora, and it is said to be ubiquitously foundas a common opportunistic pathogen.It accounts for a significant proportion of healthcare-associated, or nosocomial infections that are frequently caused by gram negative E*nterobacteriaceae* members[Podschun*et al*., 2001; Brisse and Duijkeren2005].

2.4.1Pathogenicity and virulence factors of *Klebsiella pneumoniae*

The pathogenicity of *Klebsiella* spp. is associated with virulence factors like adhesins,siderophores, capsular antigens (O- and K-antigens), andlipopolysaccharides (endotoxins). The capsule protects the bacterium from phagocytosis as well as killing of

thebacteria by bactericidal and thus,is considered essential to the virulenceof the bacterial species [Poschdun and Ullmann, 1998]. A total of 77 capsular (K) serotypes have been described till now, and some of them have been reported to be the basis of severe infections in humans and animals. Lipopolysaccharide (LPS) is known to play a significant role in bacterial pathogenesis and is responsible for causing septic shock. It consists of 3 subunits: a hydrophobic lipid A domain (endotoxin), a non-repeating core of oligosaccharide and an outer structurally diverse polysaccharide (O-anitgen) [Caroff *et al*., 2001]. Fimbriae play an important role in the early stages of bacterial adhesion to host cells. The Fimbriae articulated by *K. pneumoniae*are divided into two major categories based on their adhesive interaction inhibitory response by D-mannose. The Type 1 fimbriae are D-mannose sensitive and easily bind to mannose containing trisaccharides on host epithelial cell glycoproteins. Type 2 fimbriae resist D-mannose inhibition and promote the bacterial binding to both endothelial and epithelial cells[Mayyan *et al.,* 1985].

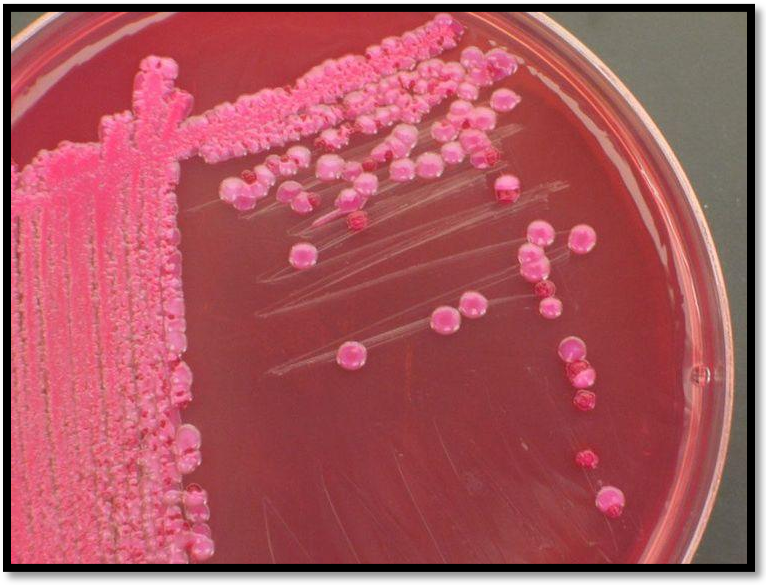

Figure 2.8: *K. pneumoniae* cultured on Mc Conkey agar

2.4.2 Mechanism of resistance in *Klebsiella pneumoniae*

Carbapenem-resistant Enterobacteriaceae (CRE) infection or carbapenemase-producing Enterobacteriaceae has become emerging as an important challenge in health-care settings. All Enterobacteriaceae are intrinsically resistant to penicillin G, glycopeptides, fusidic acid, macrolides, lincosamides, streptogramins, daptomycin and linezolid Klebsiella spp. with producing extended-spectrum beta-lactamases (ESBL) are resistant to many classes of antibiotics. Most frequent resistance pattern includes resistance against aminoglycosides, fluoroquinolones, tetracyclines, chloramphenicol and even sulfamethoxazole [Nathisuwan et al., 2001]. The inheritance of genes for chromosomal SHV-1-production has also made Klebsiella resistant to ampicillin, ticarcillin and piperacillin [Leclercq *et al*., 2016]. Efflux pumps extruding both multiple biocides and antibiotics have been described in Enterobacteriaceae and other Gram-negative bacteria [Levy, 2002]. .Clinical *K. pneumoniae* isolates have shown a reducedsusceptibility to the antiseptics like chlorhexidine, trigene and benzalkonium chloride.Additionally, heavymetals resistance to copper and silverwhich have been frequently used as antiseptics has been reportedin clinical MDR *K. pneumoniae* strains [Dolejska*et al*., 2012; Sandegren *et al*., 2012].

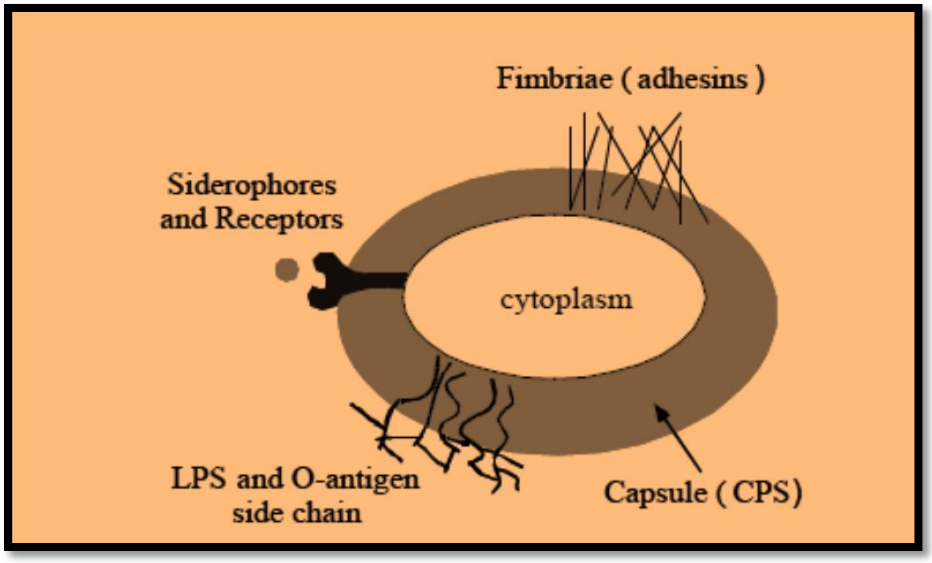

Figure 2.9: Klebsiella pneumoniae virulence factors[Podschun et al]

2.4.2.1 Mechanisms of β-lactam resistance in *Klebsiella pneumoniae*

The most common mechanism of β lactam resistance in Gram-negative bacteria is production of β-lactamase. β -lactamases enzymes inactivate β -lactam antibiotics by hydrolysing the amide bond of the β -lactam ring [Medeiros, 1984]. The first -lactamase resistance was observed against penicillinase in clinical isolates of *S. aureus, only* a few years after the introduction of penicillin. Ampicillin was the first penicillin to show activity against the Gram-negative bacteria. The first clinical *E. coli* isolate producing a plasmid-mediated broad-spectrum β -lactamase which was later termed as ampicillinase, was isolated in Athens from a patient called Temoneira in 1963This was named TEM-1 after this patient. Soon after, another plasmid-mediated ampicillinase, SHV-1, was detected among *K. pneumoniae*. Both TEM-1 and SHV-1 encoding genes, carried byconjugative transposons and plasmids, spread ubiquitously in Gramnegative bacteria [Philippon*et al*., 1989] and made *K. pneumoniae* an endemic in many hospitals [Medeiros, 1997].

2.4.3Diseases caused by *Klebsiella pneumoniae*

Enterobacteriaceae are important causes of UTIs, bloodstream infections, hospital and health-care-associated pneumonias, and various intra-abdominal infections. Pathogenic strains of *Klebsiella pneumoniae*are responsible for three types of infections in humans; urinary tracts infections, neonatal meningitis, and intestinal diseases [Brunder *et al.,* 1996]. Other common infections include pneumonia, wound infections, infections of intravascular and other invasive devices, biliary tract infections, peritonitis, and meningitis.

a) **Urinary tract infections(UTI)**

The urinary tract is the most common site of *Klebsiella pneumoniae*infection. It has been recognized as uropathogenic which causes 90 % of urinary tract infections in anatomically normal and unobstructed urinary tracts. A range of infections like uncomplicated urethritis/cystitis, symptomatic cystitis, pyelonephritis, acute prostatitis, prostatic abscess, and urosepsis have been associated with pathogenicity of the bacteria. The pathogenic strains have an adherence factor called 'P' fimbriae, which binds to the blood group antigen and mediates the attachment of *Klebsiella pneumoniae* to urinary epithelial cells. Thus, patients having intestinal carriage of the strain are at greater risk of developing UTI than the others. Elderly patients with structural abnormalities or obstruction such as prostatic hypertrophy, neurogenic bladders or in patients with urinary catheters,a series of infections involving complicated UTI and pyelonephritis caused by *Klebsiella pneumoniae*are observed [Madappa *et al.*, 2010].

b) **Pneumonia and respiratory infections**

K. pneumoniae associated pneumonia is stereotypic disease caused by aspiration of oropharyngeal microbes into the lower respiratory tract. A peculiar symptoms associated with hispneumonia is thick, bloody, mucoid sputum called "currant jelly" sputum. Other symptoms include, lung abscesses and pneumonia that can be difficult to distinguish from those caused by

other pathogens. *Klebsiella*also mimics pulmonary tuberculosis because it is present with hemoptysis and cavitating lesions. *Klebsiella pneumoniae* is a difficult infection to treat because of the organism's thick capsule Klebsiella infections are more likely to happen in immunosuppressed patients, such as patients with diabeties, alcoholics, patients who are intubated. *Klebsiella pneumonia* can spread through contact (for example, from patient to patient via the contaminated hands of healthcare personnel, or other persons) or, less commonly, by contamination in the environment [Prince *et al.,* 1997].

c) Intestinal disease

Diseases like pyogenic liver abscess are highly associated with intestinal colonization of virulent type *K. pneumoniae* strains. The pathogenesis of intra-abdominal diseases caused by *K. pneumoniae* includes haematogenous bacterial seeding from the gastrointestinal tract. In an animal study it was demonstrated that *K. pneumoniae* strains that have genetic regulatory complexes for translocation have the capability to surpass the intestinal barrier [Tu Y C *et al.,* 2009].

2.5 Alternatives to Antibiotics

Herbal medicaments have played a vital role in the health care systems of developing countries like India. Plant products serve as asource of both traditional and modern medication against infectious diseases. World Health Organization (WHO), in its recent reportshas advocatedtraditional treatments as safe therapies for ailments of microbial and non-microbial origins [WHO data]. Modern scientific reports have estimated that there are more than three hundred thousand species of medicinal plants which grow on the earth and it is described that in a single species of plant more than 5000 different chemical compounds are present. Apparently the secondary metabolites present in plants constitute an incredible source for exploring useful

anti-microbial drugs. [Kyo *et al.,* 2001]. This collection of phytoconstituents and their relevant pharmacological properties helps them to justify themselves as promising alternative candidate for novel drug generation. Also side effects associated with synthetics drugs have made herbal medicaments prospective alternate tools for treatment of various infections, thereby reviving our interest in folk systems of medicine such as Siddha, Ayurveda, and Unani.It has been studied that herbals adopt different strategies to serve as an efficacious therapeutic agent against multi-drug resistant microbial strains.

Most of the potent drugs used currently aredirectly or indirectly derivedfrom plant sources.In modern day Microbiological it has been demonstrated that higher plants exhibit a significant potency against various human bacterial and fungal pathogens [Mitscher *et al.*, 2007]. Using herbal extracts which demonstrating antimicrobial properties can be exceedingly significant in finding newer lead compounds, to counter ever-evolving human pathogenic diseases, like MDR tuberculosis, MDR pneumonia infections, antibiotic association diarrhea, MRSA (Methicillin Resistant *Staphylococcus aureus*) and also NDM-1 (New Delhi Metallo-beta-lactamase-1) *Escherichia coli* infections.

2.6 Herbal Informatics

'Herbalism' discussescustomary learning of medicaments to develop countermeasures employing scientifically evident attributes from natural flora. It is a validation based complementary study of medication which constitutes a branch of medicine wherein a plethora of natural remedies are developed and used for human necessities. The gap between modern medicine (allopathic care) and traditional medicine (holistic root cause management) is obligatory to be bridged using technological mediations. Additionally, the pace of emergence of extremely virulent microbes should be in cohesion with the drug discovery process of both folk

(herbal) and synthetic origin (antibiotics). “Herbal informatics” refers to multi-disciplinary field which integrates statistical, bioinformatics, chemi-informatics and herbal archiving tools.

In silico Bioprospection is a tool which works on the principals of herbal informatics. It is a statistical tool to identify herbal prospects based on binary; weightage matrix analysis followed by fuzzy set based prioritization indexing. It also comprises of validation protocols which take into account, extensive use of bio/chemi-informatics tools such as biological target identification, ligand designing, receptor optimization, molecular docking, toxicity prediction analysis, QSAR, Pharmacophore designing etc. These protocols exude potent leads out of identified prospects.

CHAPTER 3

MATERIALS AND MEHODOLOGY

3.1Herbal Bioprospection Model

The classical method of herbal bioprospection is identification of herbal plants with medicinal qualitiesdepending on the ethnopharmacological utility of the plants,which is not only testified in ancient literature,also in clinical literature of various countries. As it is an orthodox process, itis time consuming, monotonous, generally experience based. It also lacks scientifically evident and validated proofs. The evolution of new techniques which include dynamic search protocols along with priority indexing, systemic categorization and cross-verification could be referred to as an *in silico* bioprospection tool. The present study aims to simulate the above referred models utilizing *in silico* herbal bioprospection modeling, literature based parameter selection, priority indexing using random search model, scoring and decision matrix based analysis followed by optimization and validation.

3.1.1 Selection of microorganism

Microorganisms to be targeted using alternative system requires to follow some of the important characteristics i.e., a) lethal, sub-lethal, incapacitating or potentially dangerous Biothreat agent; b) either no treatment regime/vaccine available or limited availability; c) evolving virulent forms from past; d) possibly could be used as bio weapon which are lethal and/or panic creating agent.

3.1.2 Selection of bioactivity parameters using classical approach

The holistic mitigation requires multi-targeted approach. Based on the understanding of the mechanistic aspects of antibiotic resistant patterns of micro-organisms, as in present study,

nosocomial infections harbored *Klebsiella pneumoniae*, the various comparable targets attributing towards bactericidal activity of *Klebsiella pneumoniae* has been selected on the basis of extensive literature surge (Classical Bioprospection Approach). There are certain parameters which need to be assessed to analyze the bioactivity associated with a given herbal plant, with respect to its potential of treating dreadful infections allied with virulen multidrug resistant bacterial strains, like *Klebsiella pneumoniae*.

3.1.3 Evaluation of relevance factor using keywords hits scoring matrix approach

The analysis was conducted using PubMed as selected search engine. The random search model using combination keyword as Bioactivity Parameter + Antibacterial activity while advanced search model using the same combination keywords but in quotes, yielded 'N' hits. The first n=20 hits provided by the search engine, working on the principle of priority indexing, were based on the number of times a website is read/clicked. The first 20 hits are subjected to observational interpretation for assessing relevance using human interface. This sample set based analysis was used to evaluate the net weightage linked to each bioactivity parameter, using the following formula:

$$\%\,Relevance)avg = \frac{No.of\ relevant\ hits\ based\ on\ observational\ analysis}{n=20}\ X\ 100 \{\text{Eq. 3.1}\}$$

3.1.4 Selection of herbal plants using classical bioprospection approach

The classical bioprospection approach accounts for investigation of the following variables based on literature review to devise a logical conclusion, resultant in selection of plants. It includes a) Ethnopharmacological importance of plant; b) Relevance of Herb in traditional medicine; c) Availability factor or cultural acceptability in localized regions; d) Any vedic literature supporting its use; e) Investigations/ prior experience on potential of the herb; f)

Indirect indications, if any etc. The final conclusion to select a plant for in silico bioprospection is based on learning of the subject area conjugating with prior experiences/ investigations.

3.1.5 Binary coefficients matrix to evaluate the presence/absence of a parameter in selected plants

This methodology works on the principle of 0-1 binary code of absence/presence of a particular parameter in selected plants from previous step. The range of outcome of matrix lies within 1-6 for any plant. The cut off value selected for this matrix based analysis is closest value to the median of 1-6 range. Based on this, all the plants having more than 03 parameters, reported in PubMed search engine (n= first 20 hits) against 'Bioactivity Parameter + Selected Plant' random search model, were selected. It relates to the fact that only these plants which can support holistic approach should be screened for the next level analysis, in line with the rationale of present study.

3.1.6 Weightage matrix based analysis

This step includes evaluation of overall weightage of plants (Scores ≥3 in previous step) by multiplying their binary score with weightage obtained in Step No. 3.5. This is a primary step to screen the plants utilizable to subsequent analysis and removes fake positive results attributed towards investigator's biasness due to 'experience factor'. This step enhances the 'uncertainty factor' required for statistically valuable outcome. This step identifies potential plant leads based on *in silico* bioprospection approach subjected to fuzzy set membership analysis and optimization to validate the findings.

3.1.7 Fuzzy set membership analysis for decision matrix

In this approach, the given mathematical relationship was used to calculate the relevance of the variety/product;

$\mu_S = S\text{-}min(S)/[max(S)\text{-}min(S)]$ {Eq. 3.2}

Where: μ_S represents the desirability values of members of the fuzzy set S. Min(S) and max(S) are minimum and maximum values, respectively, in the fuzzy set S.

3.1.8 Optimization of decision matrix score

In this approach the numerical value of scores obtained were converted into a levelled score by using a scaled magnitude represented by a symbol "+".

3.2 Molecular Docking

To validate the bioprospection model *in silico* docking simulations of most relevant bioactivity parameter against pre-selected phyto-ligands were carried using Hex 8.0 and iGemDock softwares. *In silico* toxicity estimations using (Toxicity Estimation Software Tool) T.E.S.T were also performed to screen out the false positives on the basis of LD50, Bioaccumulation Factor, Developmental toxicity and Mutagenecity. Drug likeness of the phytoligands was calculated on the basis of their Lipinski Scores.

Softwares used: Argus Lab (4.0.1), Dog Site scorer (free online tool), Open Babel (2.4), Hex (8.0), iGemDock, Toxicity Estimation Software tool (T.E.S.T.), ACD Chemsketch (12.0).

3.2.1 Retrieval of 3D structure of Extended Spectrum Beta Lactamase Receptor

The experimental 3D tertiary structure of extended spectrum beta lactamase was retrieved from RCSB Protein Data Bank as "pdb" file (**doi:10.1016/j.jmb.2008.05.051**). Hydrogen atoms were introduced into the enzyme structure using Argus lab (4.0.1) to customize it as the receptor molecule for rigid docking. (www.arguslab.com).

3.2.2 Selection of Predominant Active Phyto Constituent from Plants and Preparation of Ligand Database

The predominant active phytoconstituentsof the selected plants were identified by extensive literature survey and using Dr. Duke's Phytochemicaland Ethnobotanical Databases. The selected phyto-ligands were drawn using ACD Chemsketch (12.0) and structures

werevalidated. The Structure of 7 standard chemotherapeutic inhibitors of SHV-1 beta lacatmase (Clavulanic Acid, Ampicillin, Aztreonam, Tazobactam, Tigecycline, Gentamicin, Polymyxin B) were also drawn and validated. Hydrogen atoms were introduced into the ligand structure using Argus Lab (4.0.1) to customize them for rigid docking (www.arguslab.com). The hydrogenated ligand molecules were then converted into 'pdb' format using Open Babel (2.4) interface as required for rigid docking simulations.

3.2.3 Active Site (Pocket) Analysis

DoG Site Scorer, a web based tool (dogsite.zbh.uni-hamburg.de/), was used to predict the possible binding sites in the 3D structure of extended spectrum beta lactamase enzyme. Predictions with DoG Site Scorer were based on the difference of Gaussian filter to detect potential pockets on the protein surface and thereby splitting them into various sub pockets. Subsequently, global properties, describing the size, shape and chemical features of the predicted pockets were calculated so as to estimate simple score for each pocket, based on a linear combination of three descriptors i.e., volume, hydrophobicity and enclosure. For each queried input structure, a druggability score in the range of 0 to 1 was obtained. Higher the druggability score, higher the physiological relevance of the pocket as potential target.

3.2.4 Primary Ligand Receptor Docking (Hex 8.0)

Receptor and Ligand files were imported in the Hex 8.0 software. Graphic settings and Docking parameters were customized as follows and rigid docking was performed. E values of the docking predicted the free energy of docking, which served as the basis for ranking phytoligands in increasing order of their docking abilities.

The parameters used for the docking process were:

a) Correlation type: Shape and electro only

b) FFT mode: 3D fast lite

c) Grid Dimension: 0.75

d) Receptor range: 180^0

e) Ligand range: 180^0

f) Twist range: 360^0

3.2.5 Secondary Ligand Receptor Docking Simulations

Receptors Files were loaded on to the Software interface in 'pdb' format and following parameters were adopted for all the docking simulations. Post docking analysis was carried out to obtain the "energy table**"** in which the fitness energy (E Value) with respect to each Amino Acid residue of the Receptor was and this served as the ranking parameter for the phytoligands.

Population Size: 200

Generations: 70

No. of Solutions: 2

Hydrophobic Preference: 1.0

Electrostatic Preference: 1.0

Scoring Function: GEMDOCK

3.2.6 Toxicity Prediction Analysis

Toxicity prediction analysis of predominant phytoconstituents was conducted using consensus clustering prediction methodology in rat model system (www.epa.gov/nrmrl/std/qsar/TEST). Oral Lethal Dose ($L.D_{50}$), Bioaccumulation factor, Developmental toxicity and mutagenicity of the ligand were used as the descriptors to filter the predominant phytoligands on the basis of being toxicants or non-toxicants respectively.

3.3 Preparation of Natural Plant Products

The dried plant material (~200g) was pulverized using pestle mortar till a moderately fine powder (coarse) was obtained and stored at room temperature till extraction. The powdered plant material was transferred to a Soxhlet apparatus and extracted with aquo-methanolic solvent system (30:70). The extract was prepared using hot continuous percolation method, for three consecutive cycles the respective filtrates were combined. The pooled filtrate was filtered through Whatman Paper No.1 and concentrated by the solvent evaporation in a rotary evaporator (EYELA SB-1200, Hyderabad, India) [at ~ 65 ± 2°C, 160-180 rpm and reduced pressure of 40 torr]. The residual solvent was removed using vacuum oven and yield was estimated. The extract was designated as RCTC-01 respectively. In the ensuing manuscript, extracts will be addressed with codes only.

3.3.1 Qualitative Chemical Fingerprinting

The qualitative estimates of various extracts with respect to Alkaloids (Wagner's test); Flavonoids (Sulphuric Acid test); Saponins (Froth test); Tannins (Ferric Chloride test); Steroids (Lieberman's test); Proteins (Ninhydrin test); Carbohydrates (Benedict's test); Glycosides (Keller-Killani test) and; Terpenoids (Salkowski test) was evaluated [Mariita *et al*., 2010; Usman *et al*., 2009]

3.3.2 Quantitative estimation of Total Phenolic Content

The total phenolic content in herbal extracts was estimated using the absorbance to concentration method [Singleton and Rossi,1965]. To an aliquot of 10µl of stock solution (1 mg/mL) of the extracts, 50µl of 10% Folin Ciocalteau reagent and 75µl of double distilled water were added. The mixture was kept for 5 min. at room temperature, and then 100µl of 20% sodium carbonate solution was added. The mixture was kept for 30 minutes and absorbance was recorded at 765 nm using UV visible spectrophotometer (Electronics Corporation of India Ltd,

Hyderabad, India). Based on the measured absorbance, the concentration of phenolic contents was calculated in terms of milligrams of Tannic acid per gram of dried extract.

3.3.3 Quantitative Estimation of Flavonoid Content

Aluminium chloride colorimetric method was used for the determination of total flavonoid contents in the extracts [Nasr *et al*]. 200 µl of double distilled water was added to 50µl of extracts and mixed with 15µl each of 5% Sodium nitrite and 10% Aluminium Chloride was added to the above mixture and incubated for 5min. 10µl of 1M Sodium Hydroxide solution was then added and the resulting solution was mixed with 10µl of distilled water and further incubated at 37°C for 10min. The absorbance was recorded at 510nm using a spectrophotometer (Electronics Corporation of India Ltd., Hyderabad, India). Based on the measured absorbance, the concentration of flavonoid contents was calculated in terms of milligrams of Quercetin per gram of dried extract.

3.4 Evaluation of Anti-oxidant activity

Reducing Power Estimation

The reducing power of herbal extracts was determined by the method of Oyaizu [Oyaizu, 1986].The aliquots (100µl) at different concentrations of the extracts were mixed with 250µl each of 0.2M phosphate buffer (pH 6.5) and 1% potassium ferricyanide and incubated at 50°C in a hot water bath for 20minutes. 250µl of 10% trichloroacetic acid was added to the mixture and centrifuged at 3000 × g for 10 min at room temperature [Sorvall (Kendro) Instruments, USA]. 250µl of resulting supernatant was taken and further diluted with 50µl double distilled water. The 100µl of 0.1% ferric chloride was added. The absorbance was recorded at 700nm using a spectrophotometer (Electronics Corporation Of India Ltd., Hyderabad, India). The different extracts were compared on the basis of their respective concentrations (mg/mL) corresponding to the unit absorbance expressed as ± S.D., using the formula:

$$Concentration\ (mg/mL) unit absorbance value$$

$$= C1/Abs.C1 + C2/Abs.C2 + C3/Abs.C3$$

{Eq. 3.3}

Where *C1*, *C2*,and *C3* are three randomly selected concentrations (mg/mL) from their linear response curve. Increased absorbance is indicative of increased reducing power.

3.5Radical Scavenging Activity

Super oxide ion Scavenging Potential

The superoxide ion quenching ability of the herbal extract was determined using nitroblue-tetrazolium reduction assay [Liu *et al*., 1997]. Aliquot (100 µl) of different concentrations of the herbal extracts was taken and mixed with 260 µl sodium pyrophosphate buffer (0.052 M, pH 8.3) and 10 µl phenazine methnosulfate (186 µM). 30 µl of Nitroblue-tetrazolium (300 µM) was added to the above solution and the final volume adjusted to 0.3 mL. The reaction was initiated by adding 20 µl of NADH (780 µM) and the solution was incubated at 37°C for 2 min. The reaction was terminated by adding glacial acetic acid to the resultant mixture, following 0.4 mL of butanol, which was added and mixed vigorously. The reaction mixture was allowed to stand for 10 min at room temperature; the absorbance was measured at 560 nm. The percentage inhibition of superoxide anion generation was calculated as follows:

$$\%\ \text{inhibition} = \frac{\text{Abs(contol)} - \text{Abs(sample)}}{\text{Abs(control)}} \times 100 \qquad \{\text{Eq. 3.4}\}$$

3.6Biochemical characterization of the given clinical isolate

a. **Fermentation profile fingerprinting of the clinical isolate(sugar based)**

The fermentation profiling of the clinical isolate was carried out using the Brown method [Brown *et al*., 2004]. When any bacterium utilizes carbohydrates for nutrients, acid and gas are

the end products. Some bacteria releases enzymes that enable it to use carbohydrates through fermentation and oxidation, but the gas may or may not be produced. Thus bacterial fermentation is distinguished by acid production which is observed by the means a **c**olor change in the test tube. Phenol Red acts as an indicator and normally is red colored in neutral or alkaline solution

If acidispresent(i.e. fermentation has taken place), phenol red will change its color from red to yellow.

b. Triple sugar- iron agar test

This test is basically designed to differentiate among the various genera of the *Enterobacteriaceae* family. In this family there are all gram negative bacilli which ferment glucose resulting in production of acid. This distinguishes them from other gram negative intestinal bacilli. This distinction among the various groups of intestinal bacteria is attributed to differences in carbohydrate fermentation patterns and hydrogen sulfide production by the organisms. Fermentation of carbohydrate is indicated by a visible color change of the pH indicator, phenol red. Whereasthe production of hydrogen sulphide is indicated by formation of a black precipitate atthe bottom of the tube [Versalovic *et al.*, 2011].

One loopful of the microbial culture was inoculated into the each sugar containing test tube,(to ensure proper inoculation loop was jabbed and enriched along the walls of the test tube).After inoculation, all the test tubes were subjected to incubation at 37 C overnight (24 hours).Also one single colony of the bacterium was picked and continuously streaked on to the TSI slant.After the appropriate streaking, the TSI slant was also incubated at 37C overnight.Observations were recorded the next day.

3.7Kirby- Bauer disk- diffusion test

This involves antibiotic susceptibility test to determine the susceptibility of bacterial species to various antibiotic and other synthetic/non synthetic agents. The method relies on the inhibition of bacterial growth measured under standard conditions.

The test organism is inoculate (bacterial lawn) on the culture medium, specifically the Mueller-Hinton agar. Following the inoculation filter paper discs impregnated with a specific concentration of a particular antibiotic are placed on the same cultureplate. The organism tries to growth under the selective pressure of the antibiotic and if the organism is susceptible to an antibiotic, no growth will be observed around the disc containing the antibiotic. Thus, a circular region called the "zone of inhibition" will be observed. Measurement of this zone of inhibition will determine the susceptibility of that particular organism to the antibiotic provided. The measurement is matched to the criteria set by the National Committee for Clinical Laboratory Studies (NCCLS). Based on the criteria, the organism can be classified as being Resistant (R), Intermediate (I) or Susceptible (S) [Ericsson*et al.*].

A colony of *K. pneumoniae* was picked from the culture and was added to 5ml of peptone water using an inoculation loop and was incubated for about 1 hour at RT so as to render the bacteria growth till the exponential phase. Followed by, streaking of lawn of bacteria from the incubated tube on a Mueller Hinton agar (MHA) plate using a sterile cotton swab. Selected antibiotic disks were gently placed on the MHA plate using a sterile needle in a way such that was a sufficient space for inspection of the growth of organism and concluding its susceptibility or resistance to the antibiotic. Incubate the plate overnight at 37°C. Following incubation cone sizes were measured using a ruler or caliper, including the diameter of the disk in the measurement (all measurements were done in millimetre).

3.8Modified Hodge Test

The cloverleaf technique, or modified Hodge test (MHT), is a phenotypic technique for detecting carbapenemase activity. It is based on the inactivation of a carbapenem by carbapenemase-producing strains that enable a carbapenem-susceptible indicator strain to extend growth towards A colony of *K. pneumoniae* was picked from the culture and was added to 5ml of peptone water using an inoculation loop and was incubated for about 1 hour at RT so as to render the bacteria growth till the exponential phase. Followed by a streaking of lawn of bacteria from the incubated tube on a Mueller Hinton agar (MHA) plate using a sterile cotton swab and allowed to dry for 3–5 minutes.A 10 µg meropenem susceptibility disk was placed in the center of the test area using a sterile needle. The test organism was streaked in a straight line from the edge of the disk to the edge of the plate (Up to four organisms can be tested on the same plate with one drug).The plate was incubated at 37°C for overnight.

3.9 DIATABS test: Carbapenemase/Metallo- Confirmative Identification Kit

The DIATABS are diagnostic tablets for detection and confirmation of Carbapenem and Metallo- β-Lactamase. These weredeveloped by Rosco and are used in qualitative procedures to identify in vitro antibacterial properties in microorganisms.

A colony of *K. pneumoniae* was picked from the culture and was added to 5ml of peptone water using an inoculation loop and was incubated for about 1 hour at RT so as to render the bacteria growth till the exponential phase. Followed by a streaking of lawn of bacteria from the incubated tube on a Mueller Hinton agar (MHA) plate using a sterile cotton swab and allowed to dry for 3–5 minutes. Diatabs discs were gently placed on the MHA using a sterile needle in such a way so that there was sufficient space for checking the growth of organism and

determining its susceptibility to the antibiotics. The plate was incubated overnight at 37°C and was observed for the growth pattern.

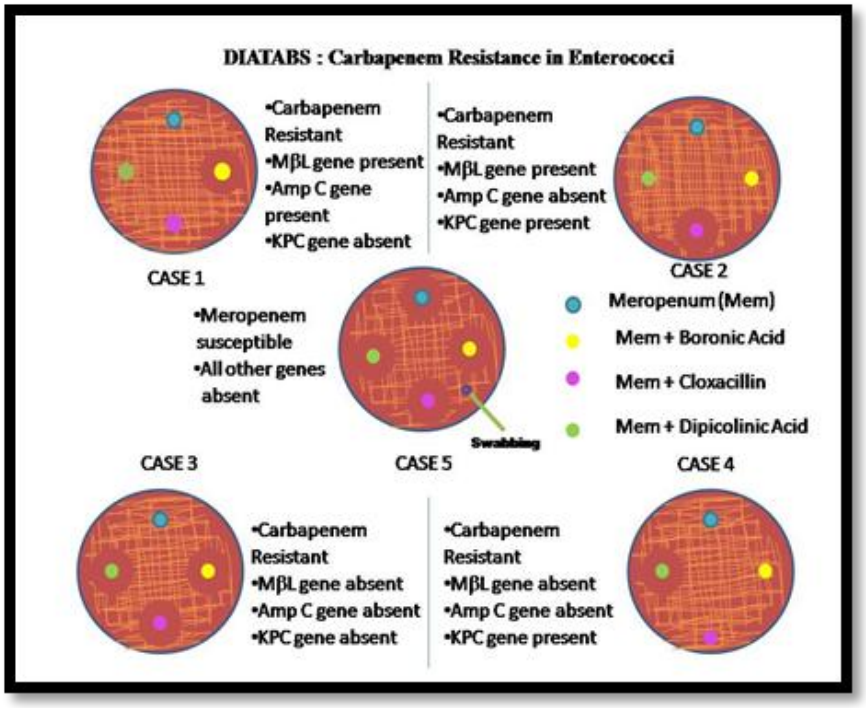

Figure 3.1: The interpretation of DIATABS test

3.10 Determination of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

Minimum inhibitory concentration (MIC) is the lowest concentration of an antimicrobial that will inhibit the visible growth of a microorganism after overnight incubation. The test broth along with positive control (Polymyxin B) solutions containing variable concentrations of extract, i.e. (6.25,12.5, 25, 50, 100, 200, 400, 800 and 1600 µg/mL) were inoculated with 50 µl of inoculum of the test organism (108 CFU/mL) followed by incubation at 37°C for 24 h. The MIC was corresponding to the lowest concentration shown turbidity in test tubes (matched with Mac Farland's solution). The colony count was also evaluated on MHAr plate (by seeding test

culture with different concentration of herbal extracts) after 24 h of incubation. Accordingly, MIC and MBC value of the five extracts were determined.

CHAPTER 4

RESULTS& DISCUSSION

4.1 Rationale for Selection of *Klebsiella pneumoniae*and bioactivity parameters using classical approach

Klebsiella pneumoniae was selected as one of the model organisms for the bioprospection studies on the basis of the rationales given in Table 4.1.The six testing parameters were selected for study based on mechanistic aspects of antibiotic resistance of *Klebsiella pneumoniae* harboring strains, including extended spectrum beta lactamase inhibition, MDR efflux pump Inhibition, adhesion inhibition, capsular polysaccharide inhibition, siderophore inhibition and symptomatic relief provision.

Table 4.1: Rationale for Selection of the Bioactivity parameters for Bioprospection Study

S.No	Parameter	Rationale for selection
1	Capsular Polysaccharide inhibition	a. A lipopolysaccharide membrane made of mannose sugar which prevents bacteria from phagocytosis by granulocytes and bactericidal serum factors. b. *Klebsiella pneumoniae* possesses K2 type of virulent capsular polysaccharide strain which prevents its recognition by macrophages. c. Several plants like *Rosemarinus officinalis, Glycyrrhiza glabra, Thymus vulgaris* etc inhibits capsular antigen of pathogenic *Klebsiella pneumoniae* rendering its cell wall permeable.
2	Extended Spectrum beta lacatamse inhibitor (ESBL)	a. Extended-spectrum beta-lactamases (ESBLs) are a rapidly evolving group of beta-lactamases which share the ability to hydrolyze third-generation cephalosporins and aztreonam yet are inhibited by clavulanic acid. b. ESBLs represent an impressive example of the ability of gram-negative bacteria like *Klebsiella pneumoniae* to develop new antibiotic resistance mechanisms in the face of the introduction of new antimicrobial agents.

		c. *Dortenia picta*, *Psidium guajava* etc have been reported to exhibit β-lactamase inhibition activity and can be used as antibiotic potentiation against resistant bacterial strains.
3	Efflux Pump inhibitor	a) Increased efflux decreases the intracellular concentration of the antimicrobial, thereby allowing bacterial survival for a greater length of time. b)Use of EPIs against AcrAB efflux pump of *Klebsiella pneumoniae* could facilitate the re-introduction of therapeutically ineffective antibiotics back into clinical use such as Ciprofloxacin and might even suppress the emergence of this MDR strain. c) Herbal EPIs like *Thymus vulgaris* serves as potential molecules to reverse antimicrobial resistance.
4	Adhesion inhibition	a) Microbes avoid antibiotic products by forming a lipopolysaccharide based biofilm which allows them to survive under hostile environmental conditions. b) Biofilm formation is one of the virulent factors of *Klebsiella pneumoniae* which promotes host colonization by increasing uroepithelial adhesion using Type 2 fimbriae. c) Plants like *Terminalia chebula* and *Mentha piperita* inhibits epithelial adhesion of *Klebsiella pneumoniae* thereby preventing caries formation.
5	Sidereophore inhibition	a) Bacteria have iron acquisition systems based on siderophores to grow successfully in host tissues. It obtains iron from host transport proteins by secreting iron-chelating compounds with extremely high Fe^{3+} affinity b) Aerobactin and Yersiniabactin have been shown to play a role in enhancing the virulence of *Klebsiella pneumoniae*.
6	Symptomatic relief	a) Curing symptoms associated with infections caused by pathogenic strains is an effective approach of treatment. b) Several plants especially those containing volatile essential oils like *Eucalyptus globules*, *Aloe barbadensis* exhibit property of relieving pain, fever and inflammation c) Klebsiella associated Urinary Tract infections, Gastroenteritis, Nosocomial Pneumonia, Bacteremia, Wound infection and Fever are treated using these phytochemical based modalities.

4.2 Evaluation of relevance factor using keywords hits scoring matrix approach

On the basis of the keyword hits scoring analysis, weightage was given to various parameters identified for screening of herbal plants with respect to antimicrobial activity, as exemplified in Table 2. Weightage was decided according to the percentage relevance obtained for each parameter, as elucidated in Figure 1. Highest relative percentage relevance was obtained for capsular polysaccharide inhibition (CPI) (i.e. 6), followed by other parameters like Extended Spectrum Beta Lactamase Inhibitor (ESBLI) (5.9), MDR Efflux Pump inhibition (EPI) (5.6), Adhesion Inhibition (AI) (4), Siderophore Inhibition (SI) (3.6), and Symptomatic Relief (SR) (3.2).

Table 4.2: Weightage assigned to the parameters based on Average Percentage Relevance

S.No	Bioactivity Parameter	Total no of Hits	Hits screened	Relevant Hits	% relevance	Probable relevance	Relative Weightage Assigned
1	*Capsular Polysaccharide inhibition*	*8*	*8*	*6*	*75%*	*6*	*6*
2	*Extended Spectrum beta lacatamse inhibitor*	*44*	*20*	*15*	*74.6%**	*33*	*5.9*
3	*Efflux Pump inhibitor*	*111*	*20*	*14*	*70%*	*77.7*	*5.6*
4	*Adhesion inhibition*	*210*	*20*	*10*	*50%*	*105*	*4.0*
5	*Sidereophore inhibition*	*37*	*20*	*9*	*45%*	*16.65*	*3.6*
6	*Symptomatic relief*	*27*	*20*	*8*	*40%*	*10.8*	*3.2*

4.3 Selection of herbal plants using classical Bioprospection approach

The classical bioprospection approach accounts for investigation of the following variables based on literature review to devise a logical conclusion, resultant in selection of plants. It includes a) Ethnopharmacological importance of plant; b) Relevance of Herb in traditional medicine; c) Availability factor or cultural acceptability in localized regions; d) Any vedic literature supporting its use; e) Investigations/ prior experience on potential of the herb; f) Indirect indications, if any etc. The final conclusion to select a plant for in silico bioprospection is based on learning of the subject area conjugating with prior experiences/ investigations.The rationale for selected plants is given in Table 4.3.

Table 4.3: Selected Herbal plants showing probable utility against *Klebsiella* infections

S.No	Herbal Plant	Common name	Predominant Phyto-constituents	Parts utilized	Availability	Relevance of Herb in Traditional Medicine	Vedic Literature supporting itsuse	Current Indications	Ref
1	*Allivum sativum*	*Garlic, Stinking Rose*	Allicin, ajoenes allyl disulfide, allypropyl disulfide	Bulbs, clove	Extensively grown in Batangas, Nueva Ecija, Southern Europe, cultivated in all parts of world	Arthritis, rheumatism, toothaches, digestive problems and gastrointestinal spasms	Mentioned in Ayurveda, Chinese traditional medicines	Antibacterial, antihelminthic, antimycotic, antiviral, antispasmodic, diaphoretic, expectorant, fibrinolytic, hypotensive, promoting leucocytosis, lipid lowering and platelet aggregation inhibition.	Watkins *et al*
2	*Artemesinin annua*	Sweet worm wood, sweet annie	Artemisinin, arteether, artemether, artemotil, artenimol, artesunate and dihydroartemisin	Dried aerial parts	Grown in China, Japan, Germany, Korea	Aqueous preparations of the dried herb were applied against fever, malaria, skin diseases, jaundice and haemorrhoids	Traditional Chinese medicine	Antiangiogenesis effects, Antimalarial effects, Antioxidant, Anticancer, Antimicrobial and Antiangiogenesis effects	Heide *et al*
3	*Brassica nigra*	Black mustard	Phytoalexins (sinalexin, sinalbins A and B), sterols and steryl esters (primarily sitosterol and campesterol), and flavonoids (eg, apigenin, chalcone).	Dried seeds	Native to the southern Mediterranean region of Europe and possibly South Asia	Food flavoring, for forage, as an emetic, and diuretic, as well as a topical treatment for inflammatory conditions such as arthritis and rheumatism.	Ayurveda	Antibacterial, Hypergylcemic and cardiovascular effetcs	Velisek *et al*

4	*Bridella micrantha*	Coast gold leaf	Benzene, 1.3-bis 2-Pinen-4-one, 1,8 Cineole, camphor, α-Pinene, borneol	Stem bark	Native to primarily tropical, northeast, western, west-central, and Southern Africa	Used locally in folk medicine, variously as an anti-abortifacient, an antidote, a laxative or purgative; and to treat diverse conditions of the central nervous system (headache), eye (infections, conjunctivitis), the gastrointestinal system (abdominal pain, constipation, gastritis), respiratory system (common cold	Mentioned in Ayurveda	Antiamebic, antianemic, antibacterial, anticonvulsant, antidiabetic, antidiarrheal, antihelminthic, anti-inflammatory, antimalarial, antinociceptive, antiviral, and hypoglycemic effects	Ngueyem *et al*
5	*Camella sinesis*	Tea	Caffeine, Theobromine, Theophylline, Purine derivatives like xanthine, methylxanthine, and adenine, tanning agents (tannin, polyphenols, gallic acid, and catechin derivatives)	Shrub	Originally from the triangle of countries of South China, Assam (northeastern India) and Cambodia. - Planted in almost all tropical and subtropical regions of the world.	Promote blood circulation, promote excretion of alcohol and other harmful substances, invigorate the skin, promotes digestion, combat tiredness and depression, among many others. Strong infusions were used as external applications for skin ailments, eruptions, abrasions and athlete's foot. - Decoction of leaves used as stimulant and to relieve fatigue. - Used to soothe headaches, aid digestion.	Recorded as early in the 6th century as a Chinese herbal medicine, recommended particularly for people who slept too long. It was used to promote	Anti-oxidant, anti-diabetic, hypolipidemic, antibacterial, hepatoprotective	Sharangi *et al*
6	*Curcuma domestica*	Long turmeric	Curcumin (diferuloylmethane) and various volatile oils, including tumerone,	Rhizome, leaves	Widely distributed in the Philippines Native of India	Decoction of rhizome, as tea, used for fevers, dysentery, abdominal pain, flatulence, abdominal spasm, arthritis. · In the Philippines,	Ayurveda, Malays in China	Antioxidant, antiinflammatory, cholesterol-lowering, antibacterial, antifungal, antiviral, immunomodulatory, hepatoprotective, and anticarcinogenic activity.	Singh *et al*

			atlantone, and zingiberone.		Now pantropic	rhizomes with coconut oil used as stomachic and vulnerary. · Internally, juice of fresh rhizome used as anthelmintic. · Used for menstrual irregularities, contusions and associated painful swelling. · Antiseptic for wounds: Crush rhizome and apply to wounds. Externally, rhizomes are applied to insect bites, ringworm, bleeding			
7	*Glycyrrhiza glabra*	Licorice	Glycyrrhizin (a triterpenoid saponin), glycyrrhizinic acid, glabin A and B, glycyrrhetol, glabrolie, isoglabrolide, isoflavones coumarins, triterpene sterols	Roots, leaves, and rhizomes	Cultivated. everywhere, Native of southeast Europe and southwest Asia	In China, it is an ingredient in many remedies and used for spasmodic cough. In ancient Greece, China and Egypt, used for gastritis and UGI tract ailments	Mentioned in Ayurveda, Synerveda	Antibacterial, anti-hepatotoxic, estrogenic, antifungal, antihemorrhoidal, antihyperglycemic, antimalarial, antioxidant, antiulcer	Gupta *et al*
8	*Mentha piperita*	Peppermint	Menthol, menthone and menthyl esters, particularly menthyl acetate, limonene, pulegone, caryophyllene and pinene	Fresh leaves	India , China, Europe, America, Australia	Nausea, vomiting, abdominal pain, indigestion, irritable bowel, and bloating	Ayurveda, Unani	Antitussive, anti-spasmodic , anti-emetic, radiprotective, antimicrobial effects	Schmidt *et al*

9	*Ocimum sanctum*	Holy Basil, Sulasi	Methyl homo anisic acid, plus cineol and linalool, Eugenol (1-hydroxy-2-methoxy-4-allybenzene)	Rhizomes, leaves	Found throughout the Philippines	Traditionally used for cough, bronchitis, asthma, malaria, dysentery, stress situations, worm infestations, superficial fungal infections, and as diuretic.	Ayurveda, Greek , Roman and Siddha	Antifertility, anticancer, antidiabetic, antifungal, antimicrobial, galactagogue, hepatoprotective, cardioprotective, antiemetic, antispasmodic, analgesic actions.	Joshi *et al*
10	*Piper nigrum*	Black pepper	Piperine, alkamides, piptigrine, wisanine, dipiperamide and dipiperamide	Fruits and roots	Indian sub-continent Vietnam, Burma ,Indonesia	Aid digestion, improve appetite, treat coughs, colds, breathing. Possess Antibacterial activity and reported use in antiprotozoalmedicine	Remedies in Ayurveda, Siddha and Unani medicine and also Chinese	Possesses alterative, tonic, appetizer & carminative activities, dyspepsia, flatulence & respiratory tract infection.	Namara *et al*
11	*Pongamea pinnata*	Karanj, Honge	Glabrin kanugin, gamatay, , glabrosaponin, kaempferol, kanjone, kanugin, karangin, neoglabrin, pinnatin, pongamol, pongapin, quercitin	Fruits and sprout	Coastal regions of India, Australia, Florida, Hawaii, India, Malaysia, Philippines	used in folk remedies for abdominal tumors in India, oil is used as a liniment for rheumatism	Mentioned in Ayurveda	Anti-inflammatory Activity, Anti plasmodial, Anti diarroheal, antiulcer and anti-oxidant property	Chopade *et al*
12	*Psidium guava*	Guava	β- caryophyllene, ß-sitosterol, maslinic acid, pinene	Leav, bark, fruit, roots	Introduced from tropical America. Thoroughly naturalized Pantropic in distribution	In India, water decoction of leaves used for treatment of jaundice, ulcers, rheumatism, wound cleaning and constipation	In Ayurveda it is considered as tridosha nashaka	Antidiarrheal, antiseptic, antispasmodic, antioxidant hepatoprotective, anti-allergy, antimicrobial, antigenotoxic, antiplasmodial, cardioactive, anticough, antidiabetic, antiinflammatory, antinociceptive	Martha *et al*
13	*Punica granatum*	Pomegranate	Ellagic acid ellagitannins (including punicalagins),		Native of southwestern Asia. - Has been introduced	In India, rind of fruit used for diarrhea, In Cuban traditional medicine, used for treatment of respiratory diseases.	Reported in Ayurveda and Chinese literatures	Anticarcinogenic, anti-inflammatory, antimicrobial properties, with beneficial effects in various disease processes such as Alzheimer's,	Jurenka *et al*

			punicic acid, flavonoids, anthocyanidins, anthocyanins, estrogenic flavonols and flavones.		in all tropical countries	- In traditional Thai medicine, used for diarrhea. - Juice of fresh fruit used for dyspepsia and as a cooling and thirst-quenching drink for fevers. - The Chinese and Annamites use the rind of the fruit and root bark as vermifuge.		osteoarthritis, neonatal brain injury, male infertility	
14	*Rosemarinus officinalis*	Rosemary	d-pinene, cineol, borneol, camphene and camphor.	Leave	Introduced from Europe. Commonly sold in markets. Cultivated in gardens for medicinal purposes.	Cough, Diuretic, Gas pains, Rheumatism, Conjunctivitis	Mentioned in the Greek folk medicinal system	Antioxidant, radio protective, Anti-inflammatory, Antibacterial, Ant hypotensive, Antispasmodic	Sereitia *et al*
15	*Sesame spp*		Sesamin, sesamolin, stigmasterol, ß-sitosterol, and stigmasterol-3-O-ß-D-glucoside.	Seeds	Native of tropical Asia	Oil of seed used for treatment of ulcers and suppurating wounds, antirheumatic, Alopecia (baldness) due to prolonged illness, pulmonary tuberculosis	Mentioned in Ayurveda, Unani and Siddha	Oil considered demulcent, emollient, diuretic, emmenagogue, lactagogue and laxative, Antioxidant / Analgesic, neuroprotective, Insecticidal	Sanskar *et al*
16	*Syzgium aromaticum*	Clove	Eugenol, acetyl eugenol, beta-caryophyllene and vanillin,crategolic acid	Flower buds	Harvested primarily in Indonesia, India,Madagascar, Zanzibar, Pakistan, Sri Lanka	Essential oilis used as an anodyne (painkiller) for dental emergencies, as a carminative, to increase hydrochloric acid in the stomach and to improve peristalsis	Indian Ayurvedic medicine, Chinese medicine, and western herbalism	Analgesic, aromatic Antibiotic, antiseptic Anthelmintic, mosquito repellent, anti-rheumatic and carminative agent	Alqareer *et al*

17	*Terminalia chebula*	Hardad, Myrobalan	Chebulinic acid, chebulagic acid, chebulic acid, ellagic acid and gallic acid	Fruit	Native to southern A sia from In dia and Ne pal east to southweste rn China (Yun nan), and south to Sri Lanka, Mal aysia and V ietnam.	Decoction of fruit used for thrush and as gargle for mucous membrane inflammations of the mouth, obstinate diarrhea. - In India, used for digestive disorders, irregular fevers, flatulence. - Used for renal calculi, dysuria, and urinary retentionUsed for fever, cough, and asthma.	Extensively used in Ayurveda, Unani, and Homeopathic medicine.	Antioxidant, antimicrobial, antidiabetic, hepatoprotective, anti-inflammatory, antimutagenic, antiproliferative, radioprotective, cardioprotective, anti-inflammatory, antimutagenic, radioprotective, anticaries, wound healing activity.	Saleem *et al*
18	*Thymus vulgaris*	Green thyme, Garden thyme	Thymol and cavacrol , thujone, pinene, camphene, pinene, cymene, terpinene, , caryophyllene	Sub shrub	It is native in the Mediterrani an Europe region	Used earlier for Cold, coughs, asthma, laryngitis, sinusitis, catarrh, whooping cough, sore throats and tonsillitis	Ayurveda ,Greek and ancient Egyptian medicinal folklore	Antiseptic, antibacterial, antispasmodic, astringent, disinfectant	Sasaki *et al*

4.4 Binary coefficients matrix to evaluate the presence/absence of a parameter in selected plants

Out of 44 identified herbals, 18 herbals exhibited a binary score of either 3 or more than 3, significantly ($p<0.05$) higher than that of the counterparts, e.g. *Punica granatum, Glycyrrhiza glabra, Thymus vulgaris, Rosemarinus officnalis, Ocimum sanctum, Terminalia chebula,*

Mentha piperita, Cucuma Domestica, Brassica nigra, Allivum sativum, Syzgium aromaticum, Psidium guajava, Sesame spp, Bridella micrantha and Pongamea pinnata, Artemisinin annua, Camellia sinesis. The weightage binary score matrix of the 44 identified herbals is shown in table 4.4. Weightage binary score is calculated by multiplying the binary score of each bioactivity parameter corresponding to the plant with the character weightage of the bioactivity parameter.

4.5 Weightage matrix based analysis

18 herbal plants (Fig 4.1), which contained either 3 or more than 3 characteristic and hence illustrated a better score as compared to other herbs, e.g. *Punica granatum,* Glycyrrhiza glabra, *Thymus vulgaris, Rosemarinus officnalis, Ocimum sanctum, Terminalia chebula, Mentha piperita, Cucuma Domestica, Brassica nigra, Allivum sativum, Syzgium aromaticum, Psidium guajava, Sesame spp, Bridella micrantha and Pongamea pinnata, Artemisinin annua,* and *Camellia sinesis.* This step identifies potential plant leads based on *in silico* bioprospection approach. The selected plants were subjected to fuzzy set membership analysis and optimization to validate the findings.

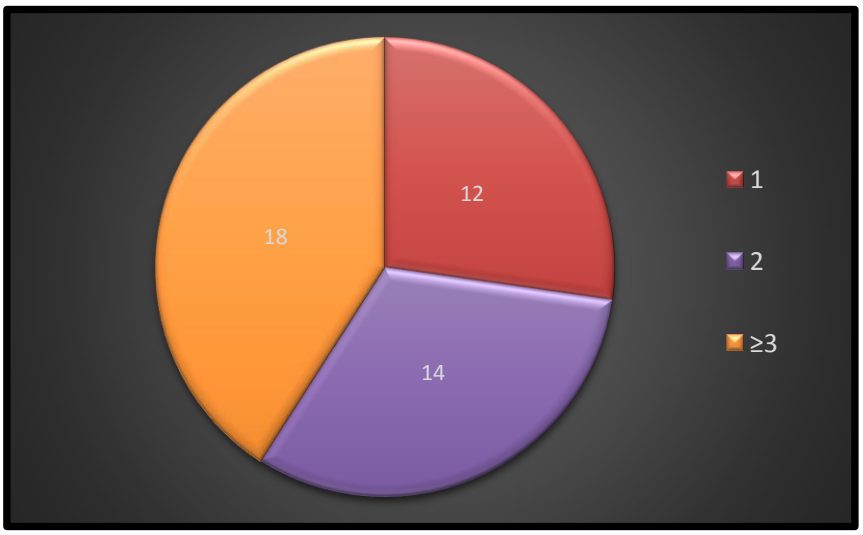

Figure4.1: Selection of plants on the basis of Binary Matrix Scores

Table 4.4: Weightage Matrix Scores for herbal plants screened on the basis of binary matrix score

S.No	***Character Weightage***	***Weightage 6***	***Weightage 5.9***	***Weightage 5.6***	***Weightage 4***	***Weightage 3.6***	***Weightage 3.2***	
	Herbal Plant	Capsular Polysaccharide inhibition	Extended Spectrum beta lacatamse inhibitor	Efflux Pump inhibitor	Adhesion inhibition	Sidereophore inhibition	Symptomatic relief	Total
1	*Rosemarinus offcinalis*	+	+	+	+	-	+	*24.7*
2	*Curcuma domestica*	+	+	-	+	+	+	*22.7*
3	*Punica granatum*	+	+	+	-	-	+	*20.7*
4	*Psidium guajava*	+	+	-	+	-	+	*19.1*
5	*Glycyrrhiza glabra*	+	-	+	+	-	+	*18.8*
6	*Thymus vulgaris*	+	-	+	+	-	+	*18.8*
7	*Ocimum sanctum*	+	-	-	+	+	+	*16.8*
8	*Terminalia chebula*	-	+	+	+	-	-	*15.5*
9	*Camellia sinesis*	-	+	+	+	-	-	*15.5*
10	*Bridella micrantha*	+	+	-	-	-	+	*15.1*
11	*Piper nigum*	+	-	+	-	-	+	*14.8*
12	*Brassica nigra*	+	-	-	+	-	+	*13.2*
13	*Allivum sativum*	+	-	-	+	-	+	*13.2*

14	*Mentha piperita*	-	+	-	+	-	+	*13.1*
15	*Syzgium aromaticum*	-	+	-	+	-	+	*13.1*
16	*Pongamea pinnata*	-	+	-	+	-	+	*13.1*
17	*Sesame spp.*	-	-	+	+	-	+	*12.8*
18	*Artemesinin annua*	-	-	+	-	+	+	*12.4*
19	*Berberis aristata*	-	-	+	+	-	-	*9..6*
20	*Citrus paradisi*	-	-	+	+	-	-	*9.6*
21	*Nelumbo nucifera*	+	-	-	-	-	+	*9.2*
22	*Picorhiza kurroa*	+	-	-	-	-	+	*9.2*
23	*Pimpinella anisum-*	-	+	-	-	-	+	*9.1*
24	*Holoptelea integrifolia*	-	+	-	-	-	+	*9.1*
25	*Jatropha elliptica*	_	-	+	-	-	+	*8.8*
26	*Daucus carota*	-	-	+	-	-	+	*8.8*
27	*Commifora molmol*	-	-	+	-	-	+	*8.8*
28	*Calendula officinalis*	-	-	-	+	-	+	*7.2*
29	*Eucalyptus globulus*	-	-	-	+	-	+	*7.2*
30	*Mellisa officinalis*	-	-	-	+	-	+	*7.2*
31	*Azadirachta indica*	-	-	-	+	-	+	*7.2*

32	*Andrographis peniculata*	-	-	-	+	-	+	*7.2*
33	*Dortenia picta*	-	+	-	-	-	-	*5.9*
34	*Prospis julifora*	-	-	+	-	-	-	*5.6*
35	*Centella asiatica*	-	-	+	-	-	-	*5.6*
36	*Coffea arabica*	-	-	-	+	-	-	*4*
37	*Ricinus communis*	-	-	-	+	-	-	*4*
38	*Carica papaya*	-	-	-	-	-	+	*3.2*
39	*Sinapis alba*	-	-	-	-	-	+	*3.2*
40	*Aloe barbadensis*	-	-	-	-	-	+	*3.2*
41	*Dasmodium gangeticum*	-	-	-	-	-	+	*3.2*
42	*Bergenia crassifolia*	-	-	-	-	-	+	*3.2*
43	*Apocynum cannabinum*	-	-	-	-	-	+	*3.2*
44	*Plumbago zeylanica*	-	-	-	-	-	+	*3.2*

4.6 Fuzzy set membership analysis for decision matrix

On the basis of Decision matrix, 6 plants were found to show high percentage relevance to be chosen as potent therapeutic herbal plants against drug resistant bacteria, as shown in Table 4.5. Amongst these, Rosmarinus offcinalis held the topmost position with 100% relevance, followed by *Glycyrrhiza glabra, Thymus vulgaris, Psidium guajava, Curcuma domestica, Punica granatum, Rosemarinus offcinalis.*

Table 4.5: Fuzzy Set Membership Analysis for herbal plants, Screened on the basis of Weightage Matrix scores

S.No	*Plants*	*μ_S**	*Optimized score*
1	*Rosmarinus offcinalis*	*1*	*+++++ (5)*
2	*Punica granatum*	*0.81*	*+++++ (5)*
3	*Curcuma domestica*	*0.9*	*+++++ (5)*
4	*Psidium guajava*	*0.73*	*++++ (4)*
5	*Thymus vulgaris*	*0.72*	*++++ (4)*
6	*Glycyrrhiza glabra*	*0.72*	*++++ (4)*

4.7 Active Site Analysis of the target virulence protein

Active Site Analysis of SHV β-lactamse, using DoG Site Scorer revealed that ten pockets (P0 to P9) of the extended spectrum protein as shown in table 4.6. Pockets P0, P1 and P2 were found to be energetically favourable on the basis of their relevant drug-ability score. Out of these pockets, P0 was found to be more druggable attributed to its descriptors (Figure 4.2), i.e., larger surface area, greater depth, less solvent-exposed surface, spontaneity of binding and higher hydrophobic.

4.8 Selection of Predominant Active Phyto Constituent from Plants and Preparation of Ligand Database

The predominant active phytoconstituentsof the selected plants were identified by extensive literature survey and using Dr. Duke's Phytochemicaland Ethnobotanical Databases. Theselected phyto-ligands were drawn using ACD Chemsketch (12.0) and structures were validated. The Structure of 7 standard chemotherapeutic inhibitors of SHV-1 beta lacatmase (Clavulanic Acid, Ampicillin, Aztreonam, Tazobactam, Tigecycline, Gentamicin, Polymyxin B) were also drawn and validated

Table 4.6: Active Site Analysis of SHV β-lactamse

Pocket No.	Volume (A^{o3})	Surface Area (A^{o2})	Depth (A^{o})	Drug Score
P0	697.66	846.18	22.88	0.41
P1	658.24	686.16	17.32	0.32
P2	428.42	717.87	15.29	0.26
P3	212.98	374.14	13.78	0.05
P4	196.03	277.25	10.76	0.00
P5	192.26	359.54	11.24	0.01
P6	160.64	238.04	7.82	0.03
P7	146.43	276.41	9.22	0.00
P8	142.02	267.13	12.66	0.00
P9	129.28	371.98	7.02	0.00

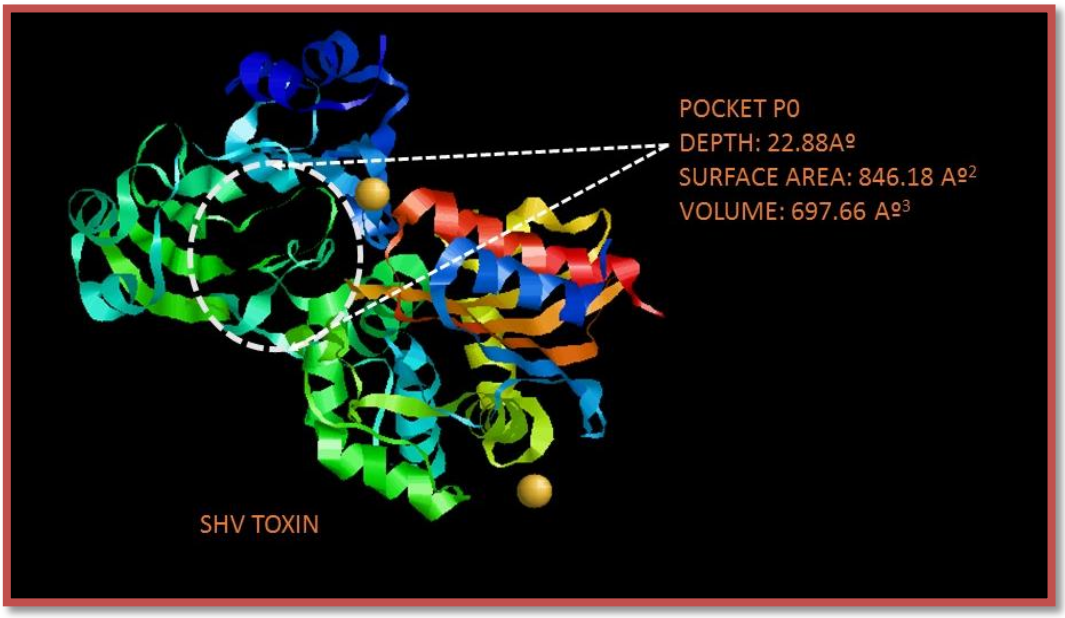

Figure 4.2: Active Site Analysis of SHV toxin Depicting Pocket P0

4.9 Primary Docking Simulations Using Hex 8.0

Energy of docking (E values) were calculated using Hex 8.0 and revealed that on the basis of the Energy interactions between the toxin and phyto-ligand, 37 ligands out of 95 had better spontaneity of binding interaction with the receptor than Tazobactam which is considered a novel first line antibiotic in treatment of *K. pneumoniae*. 24 ligands showed better interaction than Gentamicin.Also three phytoligands (dipiperamide, puncalagins and glycyrrhizin) showed better binding affinity than Polymyxin B which is consdered last line antibiotic against most of the gram negative infections (Appendix A). The phytoligands of the different plants selected are shown in Table 4.7. The primary docking simulation of SHV 1 β lactamase with Glycyrrhizin is shown in Fig. 4.3 (A). Also, figure 4.4 (A) shows the increasing order of E Value obtained using Hex 8.0.

4. 10 Secondary Docking Simulations using iGemDock

Energy interaction of the ligands with toxin revealed that, 10 phytoligands (pongapin, wisanine, chebulic acid, stigmasterol, maslicnic acid,insoglaboride, chebulagic acid,thujone, punicalagins, chebulinic acid)had better interaction in terms E value than Polymyxin

B(Appendix B). the results of the secondary docking simulations were more or less consensus with the primary docking, the docking scores of phytoligands are enlisted in Table 4.7. The range of binding energy was between -56.6 (piperedine) to -267.3 (chebulic acid). The secondary docking simulation of SHV 1 β lactamase with chebulinic acid is shown in Fig. 4.3(B).

Table 4.7: Phytoconstituents of selected plants with their Primary and Secondary Docking E Value

PLANTS/ Chemotherapeutics	Phyto compounds	Commmon Names	Primary Docking Using HEX 8.0 E value (Kcal/mol)	Scondary Docking Using iGemDock E value (Kcal/mol)
Antibiotics	Clavulanic Acid		-190.52	-82.3529
	Ampicillin		-193.7	-92.3674
	Aztreonam		-219.23	-102.508
	Tazobactam		-226.66	-97.3601
	Tigecycline		-243.62	-133.122
	Gentamicin		-271.19	-107.687
	Polymyxin B		-369.8	-142.24
Allium sativum	Ajoene	Garlic	-321.97	-90.6
	Allyl Propyl phosphate		-185.57	-62.5
	Allicin		-214.09	-62.7
	Allyl Disulfate		-188.94	-56.7
Artemesinin annua	β Artheeter	Sweet worm wood,	-229.76	-141
	Artemether		-273.36	-113
	Artenimol		-238.15	-126.5
	Artesunate		-338.83	-117.064
	Arthemisinin		-206.22	-134
Brassica nigra	β Sitosterol	Black mustard	-274.24	-103.4
	Apegenin		-300.36	-95.9
	Campesterol		-290.83	-103.5
	Chalcone		-212.03	-106.2
	Sinalbin		-211.94	-117.6
	Sinalexin		-150.23	-111.3
Bridella micrantha	Borneol	Coast gold leaf	-177.14	-74.6
	Camphor		-173.57	-73.9
	Cineole		-168.51	-83.4
	Pinene		-166.3	-80.7
Camellia sinensis	EGCG	Green Tea	-336.12	-118.8
	Gallic acid		-208.37	-73.5
	Methylxanthine		-204.92	-81.1
	Theobromine		-222.44	-85.2
	caffeine		-211.16	-76
	xanthine		-179.48	-78.8
	theophylline		-200.48	-85.4
Curcuma domestica	Curcumin	Long turmeric	-324.14	-96.6
	tumerone		-260.84	-71.7
	zingiberone		-272.1	-74.3

PLANTS/ Chemotherapeutics	Phyto compounds	Commmon Names	Primary Docking Using HEX 8.0 E value (Kcal/mol)	Scondary Docking Using iGemDock E value (Kcal/mol)
Glycyrrhizin glabra	coumarin	Licorice	-179.89	-91.9
	glabrin		-192.64	-81.2
	glycyrrhizin		-444.9	-135.3
	isoglabrolide		-269.33	-210.8
Ocimum Sanctum	Anisic acid	Holy Basil	-170.41	-89.6
	cineole		-168.51	-83.4
	eugenol		-220.41	-89.2
	Linolool		-209.19	-73.8
Pongmea pinnata	Glabrin	Karanj	-192.64	-81.2
	Kaempferol		-289.17	-107.4
	Kanujin		-305.17	-138.8
	Karanjin		-283.93	-103.2
	Pinnatin		-260.55	-127.4
	Pongamol		-261.39	-130.5
	Pongapin		-287.01	-142.4
	Quercitin		-277.33	-103.5
Syzygium Aromaticum	β Caryophyllene	Clove	-258.59	-91.6
	Crategolic acid		-368.45	-128.3
	Eugenol		-220.41	-89.2
	vanillin		-203.48	-76.7
Thymus vulgaris	Thujone	Green Thyme	-208.17	-267.3
	Thymol		-212.08	-90.7
	Terpinene		-192.8	-86.3
	Pinene		-166.3	-80.7
	Cymene		-192.8	-65.2
	Cavacrol		-198.24	-69.7
	β Caryophyllene		-258.59	-91.6
	camphene		-176.9	-65.9
Psidium guava	β caryophyllene	Guava	-258.59	-91.6
	β Sitosterol		-274.24	-103.4
	Maslinic acid		-283.66	-206.9
	Pinene		-166.3	-80.7
Mentha piperetta	β caryophyllene	*Mentha piperetta*	-258.59	-91.6
	Limonene		-192.63	-63.7
	Menthol		-200.45	-72.7
	Pinene		-166.3	-80.7
	Pulegone		-210.62	-74.8

PLANTS/ Chemotherapeutics	Phyto compounds	Commmon Names	Primary Docking Using HEX 8.0 E value (Kcal/mol)	Scondary Docking Using iGemDock E value (Kcal/mol)
Piper Nigrum	Dipiperamide	Black pepper	-388.95	-115.2
	Piperidine		-143.04	-56.6
	Piperine		-304.45	-112.2
	Wisanine		-250.75	-148
Punica Gratum	Anthyocyanidins	Pomegranate	-210.35	-108.8
	Anthyocyanins		-265.01	-94.6
	Ellagic acid		-299.27	-119.4
	Punicalagins		-396.69	-267.3
	Punicic acid		-260.21	-125.4
Rosmarinus officinalis	Borneol	Rosemary	-177.14	-74.6
	Camphene		-176.9	-65.9
	Camphor		-173.57	-73.9
	Cineole		-168.51	-83.4
	D Pinene		156.9	-79
Sessame spp	β Sitosterol	gingli, safed til	-274.24	-103.4
	β Stigmasterol		-278.78	-106.3
	Sesamin		-338.39	-103.4
	Sesamolin		-279.82	-125.5
	Stigmasterol glucoside		-318.79	-183.3
Termanalia Chebula	Chebulinic acid	Harad	-358.7	-267.3
	chebulagic acid		-358.7	-251.8
	Chebulic acid		-222.05	-155.4
	Ellagic acid		-299.27	-119.4
	Gallic acid		-208.37	-73.5

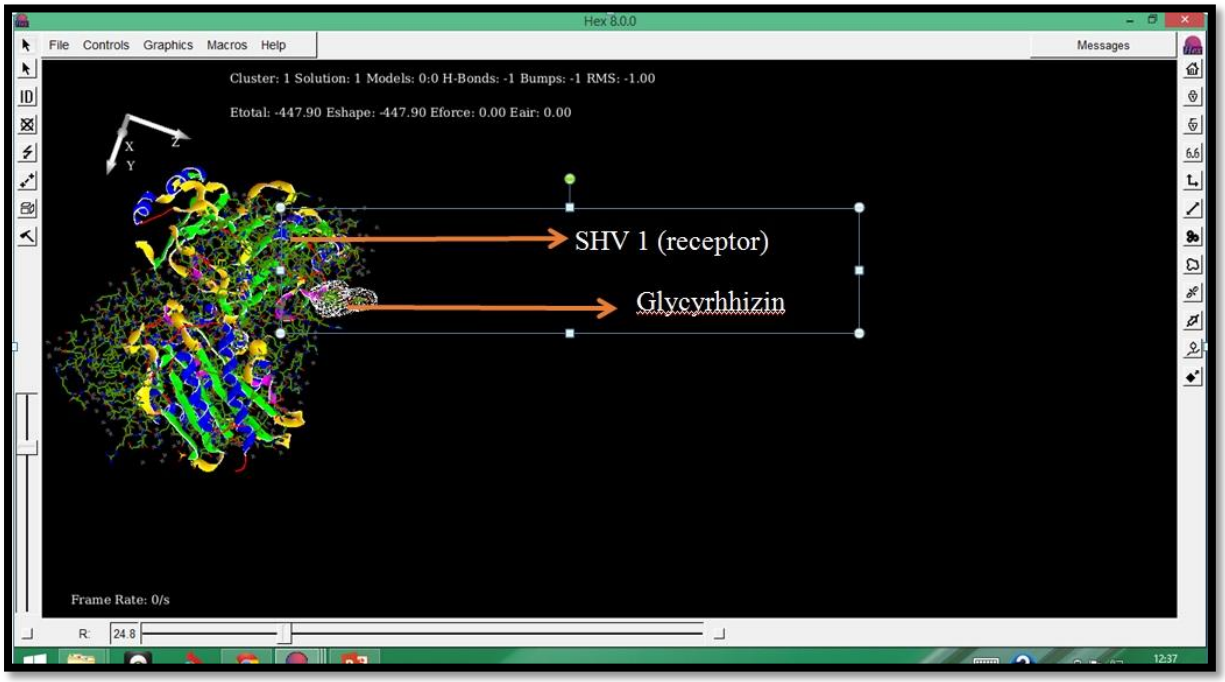

Figure 4.3 (A): Receptor- ligand docking of SHV 1 β lactamase with Glycyrrhizin using Hex 8.0

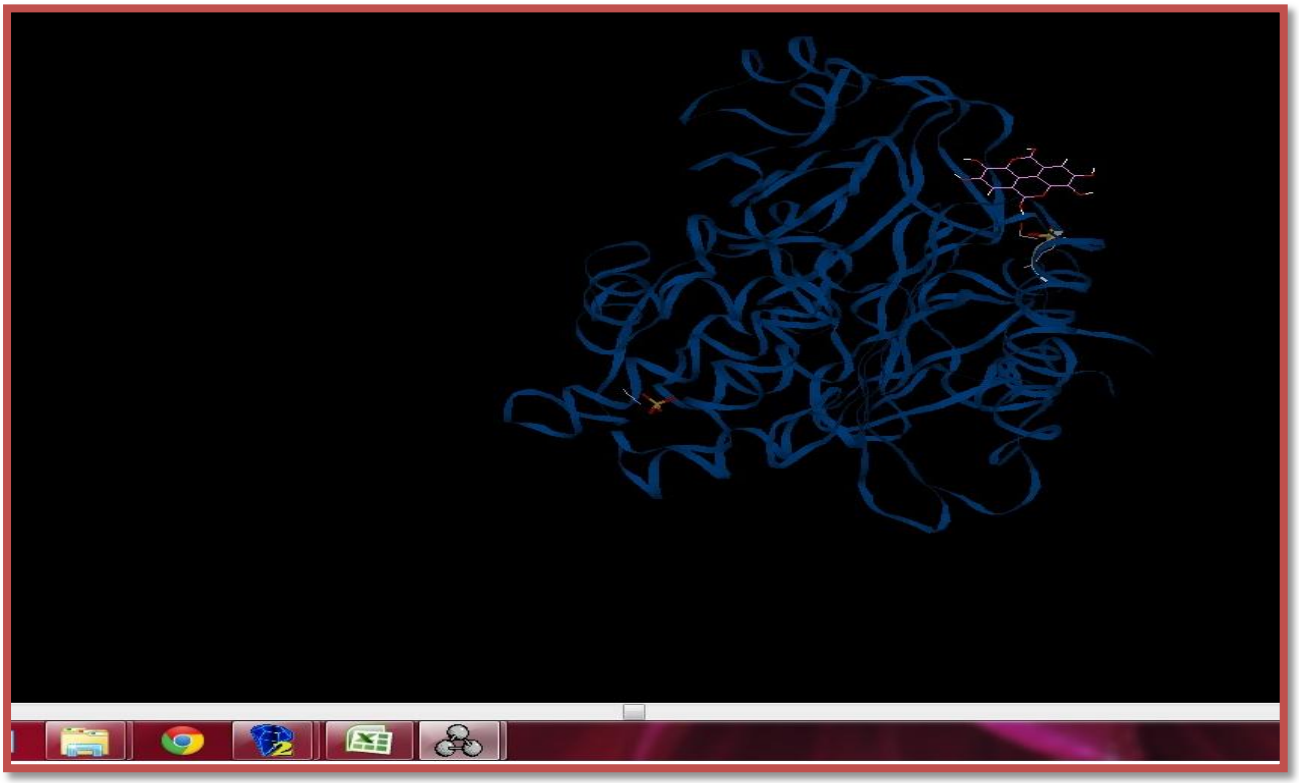

Figure 4.3 (B): Receptor- ligand docking of SHV 1 β lactamase with chebulinic acid using iGemDock

4.11 Toxicity Prediction Analysis

In silico toxicity prediction analysis revealed that 68% out of the selected phytoligands (95) were found to be nontoxic on the basis of their higher Lethal Dose (Oral rat LD_{50}> 1000 mg/kg). Highest LD_{50} was found to be in case of Epigallo ctaechin gallate (22,261.86mg/kg). More than one third of the selected phytoligands exhibited low bioaccumulation factor with lowest in case of chebulic acid (0.2 units). 27% of phytoligands were found to be non-toxic on the basis of their negligible development toxicity while 84% were found to be non-mutagenic, as given in Figure 4.5.

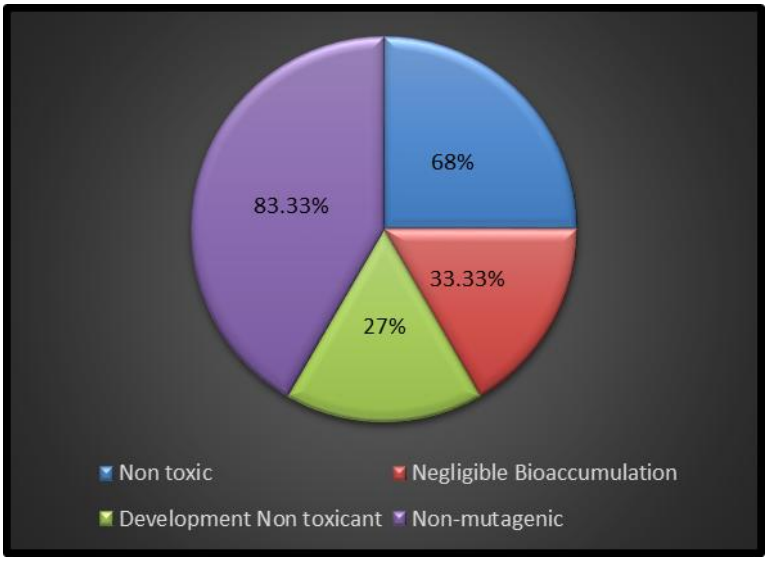

Figure 4.4: Toxicity distribution of the phyto-ligands

4.12 Quality Control Analysis of the plant extract

After filtration on the basis of E value and *in silico* toxicity parameters, *Termanalia chebula* was selected as the antibacterial. The aquo-alcoholic (30:70) herbal extract was extracted showed yield of 19.70%. A total of 10% of Ash content was found. The phyto-chemical analysis of the extract revealed presence of Flavonoids, Tannins, steroids, terpenoids and alkaloids. The total phenolic content was about 37% and the total phenolic content was about 96%. The reducing power assay revealed the reduction power of the

extract to be250 µg/mLin terms of ascorbic acid unit absorbance equivalence. Table 4.8 depicts quality control analysis as well as the quantitative and qualitative estimation.

Table 4.8: Quality control analysis as well as the quantitative and qualitative estimation of plant extracts RCTC-01

S. No.	Qualitative / Quantitative measures	*Terminalia chebula*(RCTC -01)
1.	% yield	19.70
2.	Foreign matter	0.010 %
3.	Total Ash	10 %
4.	Flavonoids	+
5.	Tannins	+
6.	Steroids	+
7.	Terpenoids	+
8.	Alkaloids	+
9.	Saponins	-
10.	Proteins	-
11.	Carbohydrates	-
12.	Glycosides	-
13.	Phenols	37.28±0.002%
14.	Flavonoids	96±0.001%

4.13 Biochemical characterization of *Klebsiella pneumoniae*

The biochemical fingerprinting profile of *Klebsiella pneumoniae*observed, proved the clinical strain as sucrose, glucose, lactose fermentor and TSI fermentor as well (Fig. 4.6). Table 4.9 depicts the biochemical fingerprinting of the bacterial species.

Table 4.9: biochemical fingerprinting of bacterial strain

SUGARS	**ACID**	**GAS**	**COLOUR**
Sucrose	+	-	Pinkish yellow
Lactose	+	+	Pinkish yellow
Glucose	+	+	Yellow
Mannose	-	-	Purple
TSI showed positive result with red to yellow colour change			

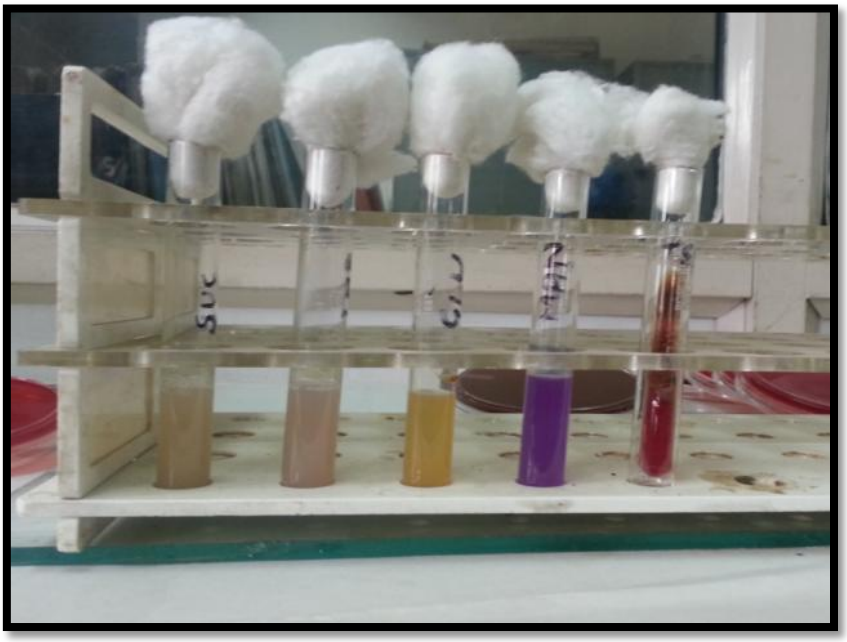

Figure 4.5: Fermentation profile of bacterial strain

4. 14 Kirby- Bauer disk- diffusion test

Kirby-Bauer susceptibility test was carried out and it was observed that Meropenem, Ertapenem and Imepenem were resistant to the bacterial lawn of *K. pneumoniae*thus exhibiting no zone of inhibition. Whereas, Colistin and Tigecycline exhibited, zone of inhibition of 18mm and 15mm respectively (Fig4.7). This proved that *E-coli* was susceptible to Colistin and showed an intermediary action towards Tigecycline. The measurement of zond of inhibition is revealed in Table 4.10.

Table 4.10: Kirby-Bauer disk diffusion analysis

S.No	Antibiotics	Diameter(mm)	CLSIStandard(mm)	Result
1.	Imipenem	0	≤14	Resistant
2.	Meropenem	0	≤14	Resistant
3.	Ertapenem	0	≤14	Resistant
4.	Colistin	18	≥18	Susceptible
5.	Tigecycline	15	15-18	Intermediate

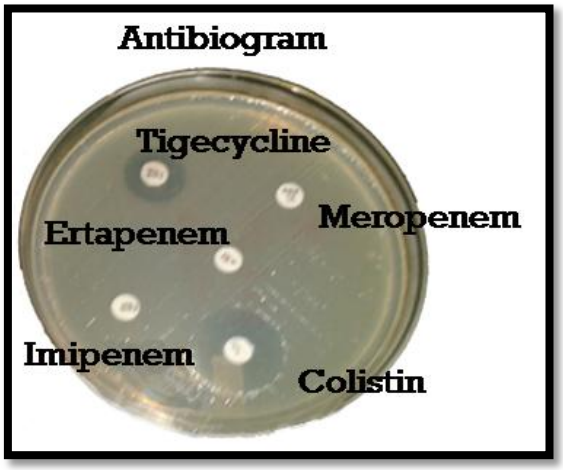

Fig 4.6 Antibiogram of*K. pneumoniae*

4.15 Modified Hodge Test

A clover leaf-like indentation of the *K. pneumoniae* growing along the test organism growth streak within the disk diffusion zone confirmed it to be a carbapenem producing microorganism (Fig. 4.8).

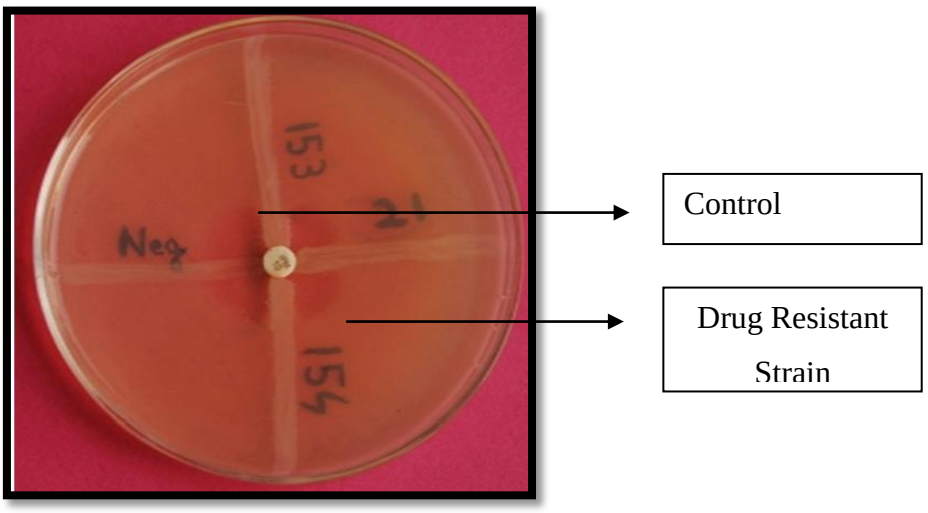

Figure 4.7: Modified Hodge test of K. pneumoniae

4.16 DIATABS test

The DIATABS test was carried out using Carbapenemase/Metallo- Confirmative Identification Kit. It was observed that there was zone of inhibition around Meropenem+ Dipicolinic acid suggests that the particular microorganism has carbepenems resistance, presence of KPC gene and all other genes are absent (Fig. 4.9).

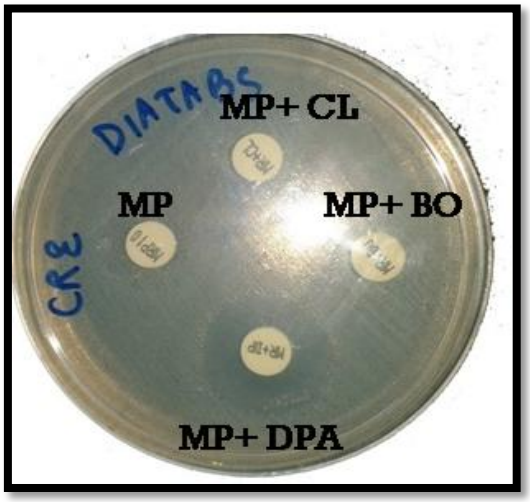

Fig 4.8: The DIATABS test

4.17Determination of Minimum Inhibitory Concentration (MIC)

The results of the antimicrobial determinations for all extracts are presented as plate counts at different concentrations in Table 4.11. The plant extract of RCTC 01 came out to be more potent than Polymyxin B with a MIC value at 100µg/ml and MBC value at 200µg/ml. The MIC and MBC value of Polymyxin B was observed as 500 and 1000 µg/ml respectively.

Table 4.11: Anti-bacterial activity of plant extracts

Plants Extracts/Atibiotic	Concentration (µg/ml)							
	6.25	12.5	25	50	100	200	500	1000
RCTC-01	63	55	39	17	2	0	0	0
Polymyxin B	51	57	40	35	21	16	7	0
Control	73	-	-	-	-	-	-	-

The study focuses to validate the *in silico* bioprospection model, previously fabricated with *Escherichia coli* as well asVancomycin Resistant *Enterococci (*Thakur *et al*, 2011 and 2013*)* on *K. pneumoniae* as the model organism. Molecular evolutionary relationship exists between *K. pneumoniae* and *Escherichia coli* as both of them belong to the same family i.e. *Enterobacteriaceae*. This phylogenetic relationship between them justifies the selection of *K. pneumoniae* as a test microorganism for validation studies of the bioprospection model. It was concluded that *in silico* bioprospection model successfully analyzed the potential of various natural plants or their products, based upon bioactivity parameters and presence of chemical constituents, using matrix based modeling, followed by optimization of herbal extracts. The Bioprospection model revealed that out of 44 herbal plants selected from herbal database, 18 herbal plants were shown to contain either 3 or more than 3 characteristic and hence illustrated a better score as compared to other herbs. These 18 plants when subjected to *in silico* molecular docking and toxicity studies against the SHV toxin of causative agent, revealed that 10 phytoligands (pongapin, wisanine, chebulic acid, stigmasterol, maslicnic acid,insoglaboride, chebulagic acid,thujone, punicalagins, chebulinic acid) were better than Polymyxin B (Last line drug against *K. pneumoniae*), both in terms of toxicity parameters (which included bioavailability and Oral LD_{50} in rat model) and docking simulation energy on the basis of affinity.

After filtration of the phyto-ligands and their respective herbal plants on the basis higher E value and nontoxic nature, *Termanalia chebula* (RCTC-01) was selected as the hebal lead for further validation. The aquo-alcoholic (30:70) herbal extract was extracted from the selected medicament and its efficacy was validated at *in vitro* levelagainst the multi-drug resistant isolates of *K. pneumoniae*.On the basis of Minimum Inhibitory Concentrations, the results revealed that the herbal extract inhibited the growth of *E. coil*at 100µg/ml

signifying that such plants have a potential to be used as anti-microbial agents and may require further testing for drug development as well as standardization process. The holistic approach of such medicinal herbals against bacterial strain exhibiting antibiotic resistance provides an advantage as compared to conventional antibiotics.The antibacterial potential of RCTC-01 indicated to be an augmentative therapeutic agent with significant bactericidal activity. This implicates that herbal is competent enough to have strong implications in providing therapeutic efficiency in mammalian system

Standard anti-microbial susceptibility testing methods including the agar diffusion and Kirby-Bauer may misinterpret the results, particularly for those extracts which exhibit low anti-microbial activity or in those cases where the active ingredient(s) may bind to the paper discs in an irreversible manner [Brown *et al*., 2004]. To overcome these drawbacks, the anti-microbial activity of the crude plant extracts was further confirmed using DIATABS analysis, where the presence of a particular inhibitory enzyme confirms the mechanism behind the acquired pattern of resistance. The consistency of results validated the above findings

This study provides indications to utilize the potential of such plants as agents for the treatment of infections caused by other agents. Besides, the plants may be effective while in combination with other medicinal plants or in combination with conventional antibiotics but further research to support this opinion is needed to confirm the same. Further studies like Maximum tolerable dose assessment of Plant extracts, *In vivo* efficacy testing as well as Pharmacokinetics studies of the extract are warranted to establish the efficacy at pre-clinical level.

CONCLUSION

This study has provided an insight into a systematic collection and analysis of literary data to obtain a logical output for ascertaining a desired biological activity in various phytoconstituents targeted against multi-drug resistant*Klebsiella pneumoniae*.It also relates to herbal formulation which is easy to prepare, core herbal plant of the formulation is native to Indian subcontinent, easily available and economic. Also, the therapeutic efficacy of the herbal extracts is found to be 5 folds better than the present available last line chemotherapeutic modality (Polymyxin B), as indicated by the *in vitro* studies. Also, the radical scavenging activity and the presence of secondary metabolites like flavonoids, alkaloids and polyphenols bequeath the herbal formulation to act as a broad spectrum antimicrobial formulation.

FUTURE PERSPECTIVES

- **To optimize the effective dose of the herbal extract and perform chronic toxicity studies of herbal extract:** Dose optimization studies of the plant extracts using in vivo Sprague Dawley Rat model by means of which the toxicity index of the prepared extracts would be calculated.

- **The *in vivo* Efficacy testing of the Herbal Extractwith honey used as a supplement**: Efficacy studies using the herbal extract so as to determine the exact therapeutic dose of the herbal extract used with honey to be used as a supplement.

- **Pharmacokinetic studies**: Pharmacokinetic probing of the given predominant pure compound isolated from the herbal extract using Pharmacoscintigraphy

- **To replicate the results in higher animals (Guinea pig and rabbits)**

- **Identification of Lead compound(s) from the herbal extract.**

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