# QUEST FOR ANTIMICROBIAL COMPOUNDS USING BOTH *In vitro* AND *In silico* APPROACHES


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# LIST OF ABBREVIATIONS

| | |
|---|---|
| AA | Amino Acid |
| ABC | ATP-Binding Cassette |
| ACE | Angiotensin-Converting Enzyme |
| ACT | Actinorhodin |
| ADME | Absorption Distribution Metabolism Excretion |
| ADT | Autodock Tools |
| AG | Aminoglycosides |
| AST | Antimicrobial Susceptibility Testing |
| CADD | Computer Aided Drug Discovery |
| CDBT | Central Department Of Biotechnology |
| CSRs | Cluster-Situated Regulators |
| Dam | DNA Adenine Methylase |
| DHFR | Dihydrofolate Reductase |
| DNA | Deoxyribonucleic Acid |
| DPPH | 1,1-Diphenyl-2-Picryl Hydrazyl |
| DPS | Dihydropteroate Synthase |
| EP tubes | Eppendorf Tubes |
| EPS | Extracellular Polymeric Substance |
| ESBL | Extended Spectrum Beta-Lactamase |
| FDA | Food And Drug Administration |
| GC | Guanine And Cytosine |
| GCMS | Gas Chromatography/ Mass Spectroscopy |
| HIV | Human Immunodeficiency Virus |
| HTS | High Throughput Screening |
| ISP | International *Streptomyces* Project |
| LB | Luria Bertani |
| MDR | Multi-Drug Resistance |
| MHB | Mueller Hilton Broth |
| MLS | Macrolide-Lincosamide-Streptogramin B |
| MRSA | Methicillin Resistance *Staphylococcus aureus* |
| ORF | Open Reading Frame |
| PABA | Para-Aminobenzoic Acid |
| PCR | Polymerase Chain Reaction |
| PDB | Protein Databank |
| PG | Peptidoglycan |

| | |
|---|---|
| PKSs | Polyketide Synthases |
| PSA | Polar Surface Area |
| QS | Quorum Sensing |
| QSAR | Quantitative Structure-Activity Relationship |
| R and D | Research And Development |
| RMSD | Root-Mean-Square Deviation |
| RNA | Ribonucleic Acid |
| RND | Resistance Nodulation Cell Division |
| ROS | Reactive Oxygen Species |
| RT | Room Temperature |
| SAM | S-Adenosylmethionine |
| SBVS | Structure Based Virtual Screening |
| SOD | Superoxide Dismutase |
| TGF | Transforming Growth Factor |
| VS | Virtual Screening |
| VRSA | Vancomycin Resistance *Staphylococcus aureus* |
| VRE | Vancomycin Resistance Enterococci |
| WHO | World Health Organization |

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# ABSTRACT

The rapid emergence of bacterial resistance and decreased efficacy of most antibiotics is a burning issue in the present context. Novel classes of potent antibiotics are a must for the effective treatment of infections caused by multi-drug resistance (MDR) strains. Simultaneously, the resistance problem must be dealt with by also preventing the possible emergence of resistance in the near future and for which lead drug candidates are sought. Thus, the new drug candidate should have the potential to inhibit multiple essential proteins/pathways in the bacterial pathogen. Streptomycetes, world's two-third antibiotic producers, have been explored from various geographical regions of Nepal with the aim to identify those that have potential to produce desired secondary metabolites against the WHO prioritized pathogens, especially the carbapenemase resistant pathogens categorized as critical by WHO. A total of 141 putative Streptomycetes were isolated, characterized and tested for their antimicrobial with optimized robust and rapid resazurin antimicrobial assay. Isolate named LAf4 showed broad spectrum potential of inhibiting all the carbapenemase producing test pathogens *Klebsiella pneumoniae, Pseudomonas aeruginosa, Acinetobacter baumanii* and colistin resistant *Salmonella sp.* Similarly, KH8 strain showed the best DPPH free radical scavenging activity and thus considered the best antioxidant. Partially purified KH8 extract and crude extract of LAf4 were further subjected to GCMS analysis to know the probable compounds responsible for the respective activity. Furthermore, *in silico* molecular docking approach was taken into account to identify the lead molecules against carbapenemases and some essential proteins of these bacteria, MetK and Dam proteins, based on earlier works. Virtual screening of a database of 1355 FDA approved drugs along with 6 promising indole derivatives based on the earlier works identified three lead drug candidates, Ziprasidone hydrochloride, (1R,4R)-4-(1H-indol-3-ylmethyl)-1-methyl-2,4-dihydro-1H-pyrazino[5,4-b]quinazoline-3,6-dione and 3-benzyl-6-(1H-indol-3-ylmethyl)piperazine-2,5-dione, against these targets, indicating that these could potentially be developed as drugs to overcome the carbapenem resistance in the pathogens.



Keywords: Carbapenem, Drug resistance, antimicrobials, Resazurin, *Streptomyces*

# 1. **INTRODUCTION**

## 1.1 Background

Antibiotics are the life savers that help to treat serious infectious diseases caused by bacteria, parasites, viruses and fungi by inhibiting their growth. In addition, they are also prescribed prophylactically with immunosuppressants to prevent secondary infections (Anon, 2016) implying that without antibiotics, the present world cannot survive. The rapid spread of multidrug resistant *Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa and Enterobacter* sp (ESKAPE) pathogens is of serious concern (Bionda *et al.*, 2013). Thus, the excitement about antibiotics is being alleviated by the spread of drug resistance all over the world (Laxminarayan *et al.*, 2013).

The apparent inexorable emergence of Antimicrobial resistance (AMR) strains among different pathogens is making a serious threat worldwide for the effective prevention and treatment of an ever-increasing ranges of infections by MDR strains that are presumed to have developed because of unmonitored health care practices resulting in inappropriate antimicrobial consumption, the misuse of antimicrobials in livestocks and the spread of antibiotic resistance genes in the environment (Ogawa *et al.*, 2018). The studies in Health Affairs have shown that antibiotics resistance of bacterial infections has doubled in past decade and so, rising from 5.2 percent in 2002 to 11 percent in 2014, costing additional billions of dollars annually (Scott, 2018). The concern of medical community is that, eventually, this may result in decreased efficacy and withdrawal of the available antibiotics and potentially could make the world vulnerable to numerous pathogens.

Some troublesome developments of resistance with major concern are as follows (WHO, 2018):

1. Resistance of *Klebsiella pneumoniae* against carbapenem antibiotics: last resort treatment.
2. Resistance in *Escherichia coli* to fluroquinolones
3. Treatment failure of gonorrhea to third generation cephalosporins: the last resort of medicine
4. Resistance to first line drugs in *Staphylococcus aureus*
5. Resistance of Enterobacteriaceae to last resort treatment: Colistin

Despite the increase in incidences of antibiotic resistance at an alarming rate, the number of novel antibiotics is growing painfully at a slow rate (Russell, 2002). Since 2000, only two novel classes of antibiotics, the oxazolidones (Barbachyn and Ford, 2003)

and cyclic lipopetides (Kern, 2006) have been introduced into the market which is definitely not enough in the present context.

Since antibiotic resistance continues as a natural process in evolving bacteria (José L. Martínez, 2012), it can be slowed down but not stopped. Therefore, there will always be a need of novel families of antibiotics to keep up with the resistance development in bacteria, preferably with novel modes of action. The most promising approaches for the identification of novel drugs involve the involvement of natural product sources (Cragg and Newman, 2013). *Streptomyces* spp are soil dwelling bacteria which are the largest group of antibiotic producers as world's two third of antibiotics are produced by them (Hasani *et al.*, 2014). In addition, they are known to produce antioxidants (D. R. Lee *et al.*, 2014).

Since Oxidative stress can play a role in the pathophysiology of many neuropsychiatric disorders such as schizophrenia, bipolar disorder, major depression etc (Ng *et al.*, 2008). Both genetic and non-genetic factors have been found to cause increased cellular levels of reactive oxygen species (ROS) beyond the capacity of antioxidant defense mechanism in patients of psychiatric disorders (Pandya *et al.*, 2013). These factors trigger oxidative cellular damage to lipids, proteins and DNA in leading to abnormal neural growth and differentiation (Salim, 2014). Therefore, novel therapeutic strategies such as supplementation with antioxidants could be effective for long-term treatment management of neuropsychiatric disorder. Thus exploring *Streptomyces* spp could be looked from both antibiotics and antioxidants since ROS has role in immune system (Torraca *et al.*, 2014) to prevent infections along with major antibiotics generating ROS in killing bacteria (Dwyer *et al.*, 2009).

Nepal is a land locked country with huge geographical diversity extending from the lower plains to the higher Himalayas which is created by upliftment of seabed during Pleistocene epoch (Tenzing *et al.*, 2018) thus could be a source of novelty, both for the microorganisms and their metabolites, in addressing the present diseases and antibiotic resistance problems. In addition, computational approaches could be employed to identify already available compounds as a time and cost effective method to narrow down the drugs with specific mode of action against the resistant strains as well along with the search of novel antibiotics from natural sources. The quest for new antimicrobials is so strong thus, integrating both *in vitro* and *in silico* approaches could be a new strategy.

## 1.2 Current Studies

The emergence of AMR that has already compromised the effectiveness of preceding treatments and making the control of infectious pathogens problematic has become a major concern globally. Moreover, dual antimicrobial therapy that increases their therapeutic potency appears to be highly effective against MDR pathogens. Augmentin, which is the association of amoxicillin and clavulanic acid, has successfully treated soft tissue and skin infections caused by MRSA and other infections (Drawz and Bonomo, 2010). Furthermore, combination of ceftriaxone and azithromycin has been currently used for the treatment of gonorrhea (Unemo and Shafer, 2014). Administration of intravenous colistin, meropenem and ertapenem has been successfully used to treat the multidrug-resistant *Klebsiella pneumoniae* carbapenemases (KPC) expressing *K. pneumoniae* infections in elderly patient (Potter *et al.*, 2016; Caniaux *et al.*, 2017). Though the combinations of multiple drugs show a promising result, rapid development of novel classes of antibiotics and treatment strategies is a must to suppress the current MDR threat.

Currently, some new drug candidates with activity against MDR pathogens are queued in the research and development (R&D) pipeline in various stages of clinical development. For instance, Ceftobiprole is in Phase III clinical trial for MRSA that exhibits resistance against the action of class A and class C β-lactamses (Assis *et al.*, 2017). Solithromycin, Zoliflodacin and Gepotidacin for gonorrhea treatment are in advanced stages of clinical trial evaluation (Alirol *et al.*, 2017; Wi *et al.*, 2017). But, still more classes of antimicrobials are required to have advanced armament with diverse antimicromical arsenals.

Daptomycin, a product of *Streptomyces roseoporus* was successfully used for the treatment of methicillin resistant *Staphylococcus aureus* (MRSA), vancomycin resistant *Staphylococcus aureus* (VRSA) and *Enterococci* (VRE) (Schneider *et al.*, 2014). An antimicrobial agent, 2-propenal 3-(1-aziridinyl)-3-dimethylamino, was produced by *Streptomyces cyaneofuscatus,* reported for the first time from the phylum *Actinobacteria* (Zothanpuia *et al.*, 2017).

Thus, quest for new antimicrobials could be explored in the family of soil dwelling bacteria Streptomycetes that are renowned for the production of multitude of specialized secondary metabolites having functions ranging from nutrient acquisition (e.g. siderophores for iron uptake) to antimicrobial compounds (Jones and Elliot, 2017). Hence, seeking diverse types within this genus from various geographical regions all over

the world are being conducted in search for novel antimicrobial and antioxidant compounds for eradication of serious threats.

However, the conventional approaches of search for novel antibiotics are estimated to take over 15 years and more than a billion US dollars to develop one drug out of at least 10,000 candidates from the laboratory to market (Prada-Gracia *et al.*, 2016). Thus, *in silico* approaches have been receiving more attention in the present context to untangle the scarcity of effective antibiotics. Computational approaches have been of utter importance for developing faster and accurate lead candidates against the identified targets or finding new potential drug targets by playing the set of low molecular weight compounds (Rao and Srinivas, 2011).

Some examples of approved drugs that owe their discovery largely to the tools of computer aided drug discovery (CADD) include the following: three therapeutics for the treatment of human immunodeficiency virus (HIV): saquinavir (approved in 1995), ritonavir and indinavir (both approved in 1996) (Van Drie, 2007), carbonic anhydrase inhibitor dorzolamide (approved in 1995) (Vijayakrishnan, 2009), the angiotensin-converting enzyme (ACE) inhibitor captopril (approved in 1981) as an antihypertensive drug (Talele *et al.*, 2010); and so on.

A striking example of the potential of CADD was illustrated in 2003 with the search for novel transforming growth factor (TGF)- β1 receptor kinase inhibitors. This protein is of major interest in cancer research that that regulates cellular proliferation, chemotaxis, differentiation, immune response, and angiogenesis (Wan *et al.*, 2012). One group at Eli Lilly used a traditional *in vitro* HTS approaches to find the inhibitor (Sawyer *et al.*, 2003), whereas a group at Biogen Idec used an *in silico* CADD approach using software from San Diego-based Accelrys and built a ligand model based on a known weak inhibitor of the receptor kinase (Singh *et al.*, 2003). The virtual HTS of 200,000 compounds identified 87 hits, the best hit being identical in structure to the lead compound discovered at Eli Lilly. CADD that involves reduced cost and workload was capable of producing the similar leads as a full-scale *in vitro* HTS thus could be used for efficient drug discovery in lesser time and with lesser manpower. Identification of leads is carried out either by random screening or directed design approach, both having equal importance in drug designing (Prathipati, 2005). The computational strategies come into play in assisting drug discovery and development in an efficient manner with the available *in vitro* techniques (Sliwoski *et al.*, 2014).

Thus, the present study integrates both screening of antimicrobial producing strains and CADD to support in development of potential leads that could be further taken to develop as antibiotics.

## 1.3 Hypothesis

### 1.3.1 Null Hypothesis:

Secondary metabolites effective against MDR pathogens could not be produced by isolated strains.

### 1.3.2 Alternative Hypothesis:

Secondary metabolites effective against MDR pathogens could be produced by isolated strains.

## 1.4 Objectives

### 1.4.1 General Objective

- To isolate bacteria producing secondary metabolites against MDR pathogens prioritized by WHO.

### 1.4.2 Specific Objective

- To screen Streptomycetes strains isolated from different soil samples.
- To perform Antimicrobial assay and Antioxidant assay of crude extracts from putative Streptomycetes.
- To identify the compounds responsible for antimicrobial and antioxidant properties.
- To perform CADD against various drug targets.

## 1.5 Rationale

The prevalence of life threatening MDR pathogens and decreased efficacy of the available antibiotics demand novel classes of potent antibiotics. The present study is focused on the identification of broad spectrum antimicrobials against the major WHO prioritized pathogens and also finding the natural antioxidants which could be useful against various human diseases. The attempt has been made to develop a robust and rapid method of screening antimicrobials even in the conventional methods of drug discovery.

## 1.6 Scope of Study

The present study focuses in identifying potential new antimicrobial and antioxidant compounds from Streptomycetes. Due to large number of extracts, a robust screening of antimicrobial and anti-oxidant assay was performed using 96 well microtiter plates. Identification of leads was performed by the use of FDA approved molecules and Indole derivatives against various drug targets in MDR pathogens.

# 2. LITERATURE REVIEW

## 2.1 Review of literature related to Streptomycetes

### 2.1.1 General characteristics

Streptomycetes are gram positive soil dwelling bacteria belonging to the phylum Actinobacteria and the family Streptomycetacea. They have large linear chromosomes, around 7,950 predicted genes with varying G+C content in their individual genes ranging from 61 to 80% (Wright and Bibb, 1992) and are of complex and saprophytic in nature, characteristic features distinct than all other bacteria. They have more than 65 sigma factors, a wealth of polysaccharide hydrolases and over 200 ABC transporters that allow the organism to rapidly respond to various environmental changes and utilize many complex carbon sources such as chitin, cellulose, lignin, mannan, xylan and agar ( Bentley *et al.*, 2002). The genus includes more than 500 species which are categorized on the basis of physiological, morphological and genetic characteristics (Farris *et al.*, 2011). Collectively, It is a group of chemoorganotrophic, aerobic, non-acid fast bacteria with filamentous form that resembles fungus (Zacharia and Traxler, 2017) and with an intricate life cycle.

The life cycle of Streptomycetes alternates between the filamentous vegetative mycelium stage and the spore bearing aerial hyphae stage. This makes them unique among all other bacteria (Zhou *et al.*, 2012). The spores are non-motile and the colonies are slow growing which form discrete and lichenoid, leathery or butyrous colonies. Initially they have relatively smooth surface but later on, they develop a weft of aerial mycelia that may appear floccose, granular, powdery or velvety. Also, they produce wide variety of pigments responsible for the color of vegetative and aerial mycelia (Kämpfer, 2015). Almost all of the bioactive compounds produced by Streptomycetes are initiated during the time coinciding with the aerial hyphal formation from the substrate mycelium (Stulberg *et al.*, 2016), perhaps also providing protection for the nutrients (solubilized plant materials) being released during symbiotic association with the host against the invaders.

These metabolites seem to be absolutely needless for the producers with no apparent function in their life cycle (Bérdy, 2005) but they show mutualistic interaction with the host protecting it from the pathogens. The compounds show an extreme chemical diversity like peptide compounds from simple amino acid derivatives to high molecular weight proteins (Karlovsky, 2008). In addition, the secondary metabolites produced by Streptomycetes give earthy odour due to the presence of volatile compounds like

geosmin and methylisoborneal (Komatsu *et al.*, 2008). Beside the production of metabolites, Streptomycetes degrade the insoluble remains of other organisms such as lignin, cellulose and chitin thus maintaining the balance of biosphere (Zhou *et al.*, 2012) and making it an important part of the ecosystem.

Streptomycetes have the ability to produce both primary metabolites and bioactive secondary metabolites with wide range of biological activities such as antivirals eg: Alanosine (Murthy *et al.*, 1966), insecticides eg: Prasinon (Box *et al.*, 1973), herbicides eg: herbimycin (Omura *et al.*, 1979), vasodilators eg: Triacsin A (Omura *et al.*, 1986), enzyme inhibitors eg: Leupeptin (Shewalet, 1988; Demain, 1999), antifungals eg: Amphotericin B (Caffrey *et al.*, 2001), antibiotics eg: Streptomycin (Ohnishi *et al.*, 2008), anti-tumoral eg: Mitomycin (Stulberg *et al.*, 2016), anti-parasitic eg: Nigericin (Leulmi *et al.*, 2018), and Immunosuppressives eg: Rapamycin (Bolourian and Mojtahedi, 2018).

### 2.1.2 Streptomycetes as Antibiotics producers

Due to their potential to produce large arrays of secondary metabolites with significance for human health, Streptomycetes are taken as the organism that have high potential to produce secondary metabolites like antibiotics with human health benefits and still being explored for new antimicrobials. The discovery of Streptothricin began the history of antibiotics production from Streptomycetes in 1942 (Waksman, 1943), followed by isolation of Streptomycin in 1943. Due to the availability of genetic information of this genus, screening for antibiotics became rapid and highly targeted. They produce over two thirds of the clinically useful antibiotics from natural origin (Lucas *et al.*, 2013).

**Table 2.1: List of some antibiotics produced by *Streptomyces* (Hasani *et al.*, 2014)**

| ***Streptomyces spp*** | **Antibiotics** | **Streptomyces *spp*** | **Antibiotics** |
|---|---|---|---|
| *S.orchidaccus* | Cycloserin | *S. erthyraeus* | Erythromycin |
| *S. oriantalis* | Vancomycin | *S. vensuella* | Chloramphenicol |
| *S. fradiae* | Neomycin<br>Actinomycin<br>Fosfomycin<br>Dekamycin | *S. aureofaciens* | Chlortetracycline<br>Dimethylchlortetracyclin |
| *S. nodosus* | Amphotericin B | *S. ambofaciens* | Spiramycin |
| *S. noursei* | Nistasin | *S. avermitilis* | Avermicin |
| *S. mediterranei* | Rifampicin | *S. alboniger* | Puromycin |
| *S. griseus* | Streptomycin | *S. niveus* | Novobicin |
| *S. kanamyceticus* | Kanamycin | *S. platensis* | Platenmycin |

| | | | |
|---|---|---|---|
| *S. tenebrarius* | Tobramycin | *S. roseosporus* | Daptomycin |
| *S. soectabilis* | Spectinomycin | *S. ribosidifieus* | Ribostamycin |
| *S. viridifaciens* | Tetracyclin | *S. garyphalus* | Cycloserine |
| *S. linolensis* | Lincomycin<br>Clindamycin | *S. vinaceus* | Viomycin |
| *S. rimosus* | Oxytetracyclin | *S. clavuligerus* | Cephalosporin |

## 2.2 Review of literature related to Antibiotic biosynthesis and its regulation in Streptomycetes

The biosynthesis of antibiotics is specified by large gene cluster including regulatory genes namely cluster-situated regulators (CSRs). The connection of these CSRs with conserved regulatory system monitor the organism's physiology, developmental state, population density, and environment to determine the onset and level of production of each antibiotics (Liu *et al.*, 2013). Streptomycetes have large linear chromosome, approximately 8-10 Mb (Bekker *et al.*, 2014), containing 20 secondary metabolic gene clusters.

These clusters encode the biosynthesis of polyketides by polyketide synthases (PKSs) (Yu *et al.*, 1998), bacteriocins, terpenoids, aminoglycosides, shikimate-derived metabolites and other natural products (Nett *et al.*, 2009) and peptides by non-ribosomal peptide synthases (NRPSs) (Gonsior *et al.*, 2015).

### 2.2.1 *Streptomyces coelicolor* A3 (2) as a model organism

*S. coelicolor* A3(2) is genetically the best known representative of the genus *Streptomyces* (Bentley *et al.*, 2002), and has been serving as a genetic model for the study of other Streptomycetes, mainly because of its production of diffusible pigmented metabolites which could serve as suitable genetic markers (Thompson *et al.*, 2002). all *S. coelicolor* A3(2) produced by antibiotics include the calcium-dependent acidic lipopeptide antibiotics (Hojati *et al.*, 2002), prodiginine, the red oligopyrrole antibiotic (Williamson *et al.*, 2006), and actinorhodin, the blue aromatic polyketide antibiotic (Zhang *et al.*, 2008). All the structures and biosynthetic gene clusters associated with these were known, which later on helped in genetic engineering to yield unnatural hybrid antibiotics (Bekker *et al.*, 2014) and engineering of small molecules through combinatorial biosynthesis (Kim *et al.*, 2015). The gene clusters for secondary metabolites in *S. coelicolor* A3 (2) including their location and products are enlisted in Appendix 8.1.

### 2.2.2 Role of *bld* gene and rare leucine codon in antibiotic production

In *S. coelicolor* the genes known to be responsible for aerial growth and production of secondary metabolites are *bld* genes. They are named so, because mutant colonies lack aerial growth and thus appear bald. The *bld*A mutant of *S. coelicolor* was found to be defective in the development of aerial hyphae and spores along with deficiency in antibiotics production (Lawlor *et al.*, 1987). Among the many *bld* genes, *bld*A is unusual and it doesn't code for protein but codes for tRNA which has the ability to translate UUA codon into the amino acid leucine (Fernández-Moreno *et al.*, 1991). Gene containing a TTA codon can be translated into protein when a functional bldA gene is expressed and a leu-tRNA$^{UAA}$ is provided. There are in total six different codons responsible for the integration of leucine in the growing protein chain, but UUA is the rarest of them.

The role of *bld*A could be crucial in the regulation of antibiotic production, most probably at the transcriptional level of gene expression. The *bld*A mutants in *S. coelicolor* were not only defective in aerial mycelial formation and sporulation but also in the ability to produce the wildtype antibiotics actinorhodin, undecylprodigiosin (red) and methylenomycin (Bibb, 1996).

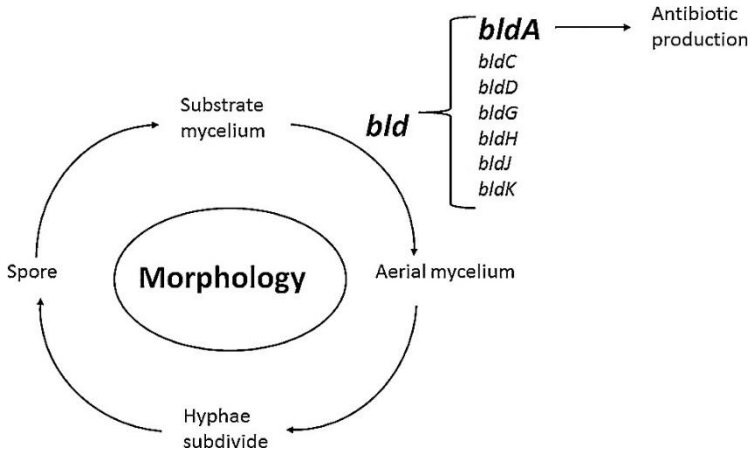



**Figure 2.1 Streptomycetes life cycle and the role of bld genes in morphological differentiation with supplemented function of bldA leading to antibiotic production (Hackl and Bechthold, 2015)**.

### 2.2.3 Activation of Actinorhodin biosynthesis in *S. coelicolor*

Actinorhodin (ACT) is responsible for the pH sensitive nature of the colonies, turning red in acidic conditions and blue under alkaline conditions (Bystrykh *et al.*, 1996). It is an aromatic polyketide derived Benzoisochromanequinone (Okamoto, *et al.*, 2009) which is a weak blue-pigmented antibiotic contributing to the blue colonies of *S. coelicolor* (Nass *et al.*, 2017).

There are five transcriptional units in the *act* gene cluster which determines ACT biosynthesis. One of the protein families that have high specificity for antibiotics production in actinomycetes is called Streptomycetes antibiotics regulatory proteins (SARPs) (Bibb, 2005). One of such SARP proteins is ActII-ORF4 which acts as transcription regulator whose N-terminal winged helix-turn-helix (HTH) domain binds to sequences of the targeted promoters and activate transcription through C-terminal domain of those genes that are dependent upon regulation of ActII-ORF4 protein (Liu *et al.*, 2013). ActII-ORF4 has a direct target for at least various eight known regulatory proteins including some involved in antibiotics production. Among them Xylose operon repressor ROK7B7 while DasR mediates the global response to N-acetylglucosamine indicating having role in carbon metabolism. Additionally, DraR and AsfQ1 are activators responding to nitrogen excess indicating role in nutrient sensing. LexA is a global regulator of DNA damage response. Those having role in antibiotics productions could be assumed to AbsA2 which is a global repressor of antibiotic synthesis. Moreover, AtrA is a transcriptional activator and an ACT precursor which binds to targets associated with metabolism of acetyl coenzyme A. Furthermore, AdpA is a pleiotropic regulator of antibiotic production and development which is regulated by BldA protein involved in antibiotic production of various *Streptomyces* strains ( Liu *et al.*, 2013). Thus, the role of AdpA seems to be more prominient in antibiotics production.

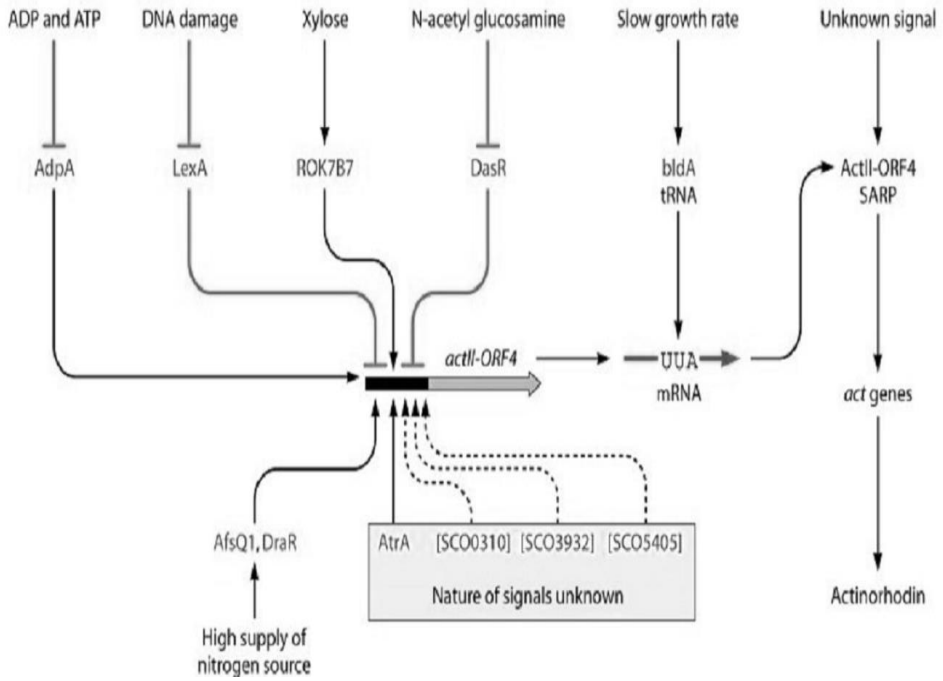



**Figure 2.2 General nature of signal inputs influencing the expression of actII-ORF4 and production of Actinorhodin** ( Liu *et al.*, 2013)

### 2.2.4 Function of AdpA (A-factor dependent protein) in BldA regulation in *Streptomyces griseus*

TTA codon containing *adp*A gene codes for a transcriptional regulator which starts a cascade of gene activations leading to morphological differentiation and secondary metabolites in *S. griseus* ( Lawlor *et al.*, 1987; Chater and Chandra, 2008). The transcriptional activator StrR activates streptomycin biosynthesis (Horinouchi and Beppu, 2007) which is under the regulation of AdpA. Thus, any strain development could look in this regulatory cascade for antibiotics production. Due to the rare TTA codon in the coding region of *adp*A gene, makes it a direct target of BldA regulation (Higo *et al.*, 2012). The gene sequence of *adp*A gene is conserved and a TTA codon is located in the same position in sequenced genomes of various *Streptomyces* strains. The AdpA cascade starts when the concentration of autoregulatory factor (A-factor) reaches a critical level and binds its receptor ArpA which subsequently dissociates from the promoter of *adp*A for its transcription. The production of A-factor requires AfsA, the A-factor biosynthesis enzyme. The translation of *adp*A into AdpA is only possible in the presence of a functioning *bld*A gene because Leu-tRNA$^{UUA}$ is necessary to express the *adpA* gene (Kalan *et al.*, 2013). The generated AdpA AraC/XylS family transcriptional regulator activates *bld*A transcription and at the same instant, starts a whole cascade of subsequent gene activation leading to morphological differentiation and the production of secondary metabolites. When AdpA reaches a critical level, it inhibits its transcription by binding to the promoter of its own gene as well as the transcription of *asf*A. Therefore, the production of A-factor by AsfA is repressed (Sidda, 2015) thus repressing antibiotics production cascade.

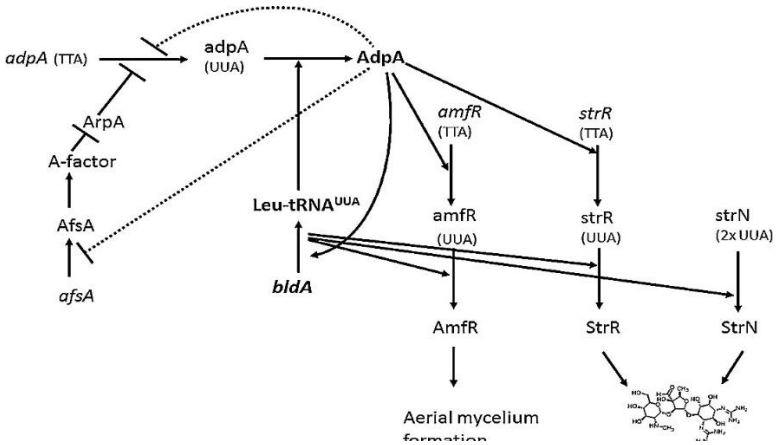



**Figure 2.3 AdpA–BldA feedback loop established for *S. griseus* with auto-regulatory mechanisms of AdpA** (Hackl and Bechthold, 2015)

## 2.3 Review of literature related to Antibiotics and their mechanisms of action

A primary interaction between an antibiotic and target site of the cell leads to the inhibition of essential cellular function causing bacterial cell death (Kohanski *et al.*, 2010). Many effective antimicrobials have been developed after the discovery of penicillin in 1929, by the study of drug target interaction (Cheng *et al.*, 2012) and by drug molecule modification (Černíková and Jampílek, 2014).

Five basic mechanisms of antibiotics associated with the nature of their structure and degree of affinity to target sites within bacterial cells are Inhibition of cell wall synthesis, inhibition of protein synthesis-translation, alteration of cell membranes, inhibition of nucleic acid synthesis and antimetabolite activity (Wright, 2010).

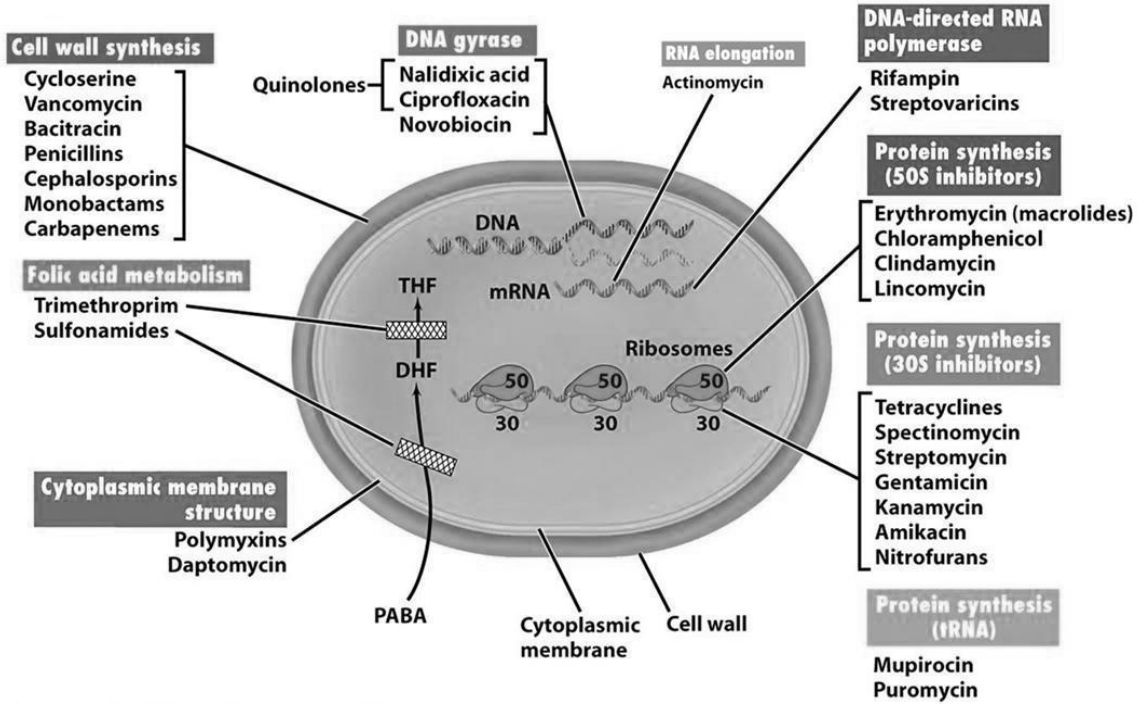



**Figure 2.4 Antibiotic target sites** (Etebu and Arikekpar, 2016)

### 2.3.1 Inhibition of cell wall synthesis

The bacterial cell is surrounded by peptidoglycan layers (PG) consisting of covalently cross linked glycan chains with attached peptides by transglycosylases and transpeptidases (penicillin binding proteins). This protective layer of the cell wall gives mechanical strength and stabilizes osmotically fragile protoplast membrane (Newton, 1965). β-lactams and glycopeptides inhibit the cell wall biosynthesis resulting in changes of cell shape and size, induce cellular stress responses and end up with cell lysis (Kohanski *et al.*, 2010).

Glycopeptide antibiotics such as vancomycin also inhibit PG synthesis through binding to D-alanyl D-alanine portion of the peptide chains and by blocking the activity of transglycosylase and transpeptidase (Reynolds, 1989). Moreover, the prominently usedβ-lactam antibiotics such as penicillins, cephalosporins and carbapenems primarily target the penicillin binding proteins (PBPs) to disrupt the PG biosynthesis. PBP interacts with β-lactam ring due to structure resemblance to D-alanyl D-alanine portion of the peptide chain (Tipper and Strominger, 1965). The enzymes are incapacitated to form the PG layers due to its inability to hydrolyze the peptide bond formed with the drug, thus are not available for the synthesis of new peptidoglycans (Kong *et al.*, 2010). They also perturb the activity of PG biosynthesis mechanism to induce futile cycle of PG synthesis thus resulting the degradation of cellular resources (Cho *et al.*, 2014).

### 2.3.2 Inhibition of protein synthesis-translation

Protein synthesis is an essential process for bacterial cells for their multiplication and survival because proteins are responsible for the structural composition, metabolic and physiological processes and response to adverse conditions (Ennis, 1965). Protein biosynthesis is catalyzed by ribosomes and cytoplasmic factors which shortly bind to the particles during the initiation phase, elongation phase and termination phase. Microbial 70S ribosome consist two ribonucleoprotein subunits; a free 30S subunit and 50S subunits which join at the initiation step of protein synthesis and separate at termination step. Each subunit contains RNA (5S and 23S rRNA in 50S subunits and 16S rRNA in 30S subunits) and ribosomal proteins (Cocito *et al.*, 1997). The function of ribosome is to translate the genetic message conveyed by messenger RNA (mRNA). Antimicrobial agents block different step in microbial protein biosynthesis interfering in the function of ribosomes and cytoplasmic factors (Liwa and Jaka, 2015). They can be divided into 50S inhibitors and 30S inhibitors.

50S inhibitors like macrolides, lincosamides, streptogramins, amphenicols and oxazolidinones inhibit protein synthesis by blocking either initiation of protein translation or translocation of peptidyl-tRNAs (Cocito *et al.*, 1997). Macrolide-lincosamide-streptogramin B (MLS) group of antibiotics target the conserved sequence of peptidyl transferase center of 23S rRNA and block the function resulting in a premature detachment of incomplete peptide chains (Tenson *et al.*, 2003). Oxazolidinones interfere with protein synthesis by binding at P site at 50S ribosomal subunit (Bozdogan and Appelbaum, 2004).

Similarly, 30S inhibitors such as aminoglycosides (AG) and tetracyclines interact with 16S rRNA of the 30S subunit near the A site through H-bonds and this cause misreading and premature termination of translocation of tRNA (Davies and Davis, 1968). AG binds to

the 16SrRNA which then induce the alteration in the conformation of the complex formed between an mRNA codon and its cognate aminoacyl-tRNA at the ribosome, leading to mismatching of tRNA and mistranslation of proteins (Cocito *et al.*, 1997). Positively charged aminoglycosides attached to negatively charged Outer Membrane leads to the formation of large pore thus allowing the penetration of antibiotic into the bacteria (Kapoor *et al.*, 2017). Tetracyclines reversibly bind to the 30S subunit to prevent the binding of acyl-tRNA to the A site of the ribosome (Petkovic *et al.*, 2017).

### 2.3.3 Alteration of cell membrane

Cell membranes are diffusion barriers that segregate and regulate intra and extracellular flow of molecules. The membrane forms an effective barrier to many antimicrobial agents. However, a number of drugs can cause disorganization of the membrane by increasing the permeability of the membrane thus allowing them and other substance to enter the cell and inhibition of specific metabolic processes (Liwa and Jaka, 2015). Colistin and polymyxin B are the best known compounds for the cell membrane inhibition. The polymyxins primarily act in the cell wall of gram negative bacteria which lead to rapid permeability changes in cytoplasmic membrane and ultimately cell death (Poirel *et al.*, 2017).

### 2.3.4 Inhibition of nucleic acid biosynthesis

DNA and RNA are essential for the replication of all organisms including bacteria. Any disturbances in nucleic acid synthesis can cause adverse effect in both survival and lineage of bacterial cell. Antibiotics inhibit nucleic acid synthesis by blocking replication or stopping transcription.

#### 2.3.4.1 Inhibitors of DNA topoisomerases

Topoisomerases, crucial enzyme for the survival of bacteria (Deweese and Osheroff, 2009) control the topology of DNA and are critical for protein translation and cell division (Kohanski *et al.*, 2010) thus making this an essential target for antimicrobial agents. DNA gyrase, Type II topoisomerase negatively supercoils DNA in presence of ATP, catenation and decatenation of double stranded circular DNA and resolves knot in DNA. Quinolones are broad spectrum antibacterial agents that rapidly bind to DNA-topoisomerase complexes and interfere with nick sealing activity of DNA gyrase and DNA Topoisomerase IV. This results in the trapping of the enzyme on DNA along with drug, forming quinolone-enzyme-DNA complexes. The subsequent release of DNA ends, leads to the production of cellular poison which ultimately causes cell death (Drlica *et al.*, 2008; Wang *et al.*, 2010).

### 2.3.4.2 Inhibitors of microbial RNA synthesis

Rifamycins inhibit DNA-dependent transcription by binding with high affinity to β-subunits of DNA-bound and actively transcribing RNA polymerase, thus inhibiting nascent RNA strand initialization (Floss and Yu, 2005).

## 2.3.5 Inhibition of microbial metabolic pathways

Sulfonamides (such as sulfanilamide) and diaminopyrimidine (such as trimethoprims) disrupt the folic acid metabolism in the microbial cell by competitively blocking the biosynthesis of tetrahydrofolate. Sulphonamides competitively inhibit the conversion of pteridine and p-aminobenzoic acid (PABA) to dihydrofolic acid by pteridine synthase due to high affinity for the enzyme. Trimethoprim act at later stage of folic acid synthesis by inhibiting dihydrofolate reductase (Yoneyama and Katsumata, 2006). Since, Folic acid is essential for the metabolism of nucleic acid and amino acids (Etebu and Arikekpar, 2016), the alteration in this particular metabolic pathway can lead to bacterial cell death.

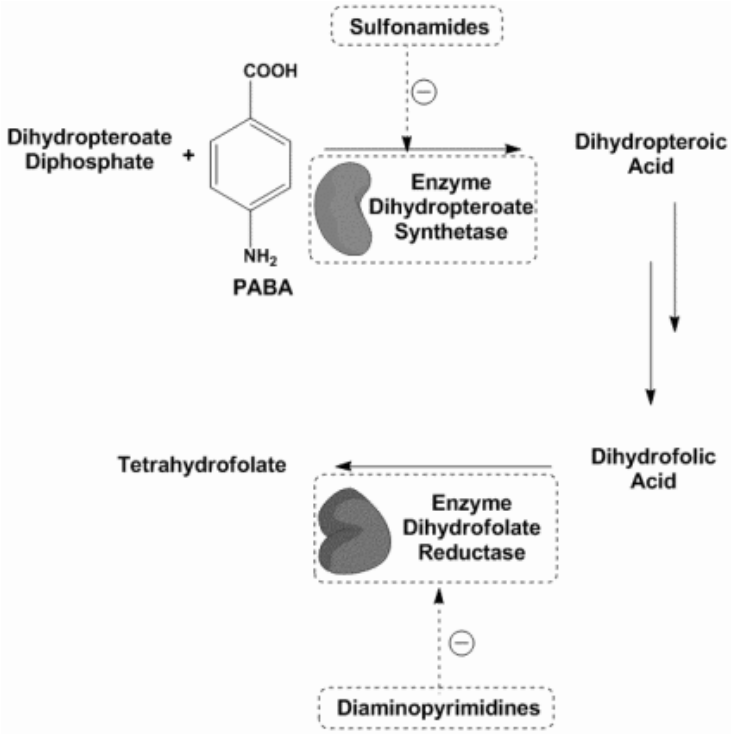



**Figure 2.5 Biosynthesis of tetrahydrofolate and mode of action of sulfonamides and diamonipyrimidines** (Mehta, 2011)

## 2.4 Review of literature related to Reactive Oxygen Species (ROS)

Reactive oxygen species (ROS) are the reactive by-products of partial oxidation of molecular oxygen and are produced through a successive single electron reduction

(Finkel, 2001). Elevated level of these species cause the oxidative damage of different macromolecules (proteins, lipids and DNA), leading to loss of function, and ultimately cell death (Kashmiri and Mankar, 2014). However, bacteria are equipped with protective enzymes; catalase and superoxide dismutase (SOD) that detoxify ROS (SodA, SodB, SodC, KatG, KatE) and regulatory mechanisms (SoxRS, OxyRS and SOS regulons) to counter damage as a response to oxidative stress (Zhao and Drlica, 2014). The naturally occurring species of ROS are superoxide, hydrogen peroxide and highly reactive hydroxyl radical which continuously arise inside cells grown aerobically through autoxidation of redox enzymes (Imlay, 2015). Bacteria also contain small redox protein thioredoxin and peroxiredoxin and molecules glutathione and NADPH that maintain an intracellular reducing environment or scavenge chemically reactive oxygens (Belikov *et al.*, 2015). Although bacteria may use ROS to self-destruct when stress (like antibiotic) is severe (Hong *et al.*, 2017). In E.coli, OxyR and Sox RS are oxidative stress regulators of catalases and SOD in response to $H_2O_2$ and $O_2^{\bullet-}$ (Memar *et al.*, 2018). Drugs that can induce ROS and overcome these defensive mechanism could be of high interest.

### 2.4.1 Role of ROS in antibiotic mediated killing of Bacteria

The generation of hydrogen radical contributes to the killing efficiency of lethal bactericidal drugs. Several bactericidal agents such as β-lactams, quinolones and aminoglycosides induce ROS generation (Kohanski *et al.*, 2007). These antibiotics trigger hydroxyl radical formation and cell death through disruption of the Cpx and Arc, the two-component system signaling. (Kohanski *et al.*, 2010). Similarly, these drug classes utilize internal iron from iron-sulphur clusters to induce Fenton-mediated hydroxyl radicals which is mediated by TCA cycle and a transient depletion of NADH (Fang, 2011).

Lethal stress causes an undefined redox imbalance upon the treatment of bactericidal antibiotics. For example: accumulation of intracellular superoxides (Wang and Zhao, 2009). These accumulated superoxides damage iron-Sulphur clusters in proteins and destabilize ferrous ($Fe^{2+}$) iron. The released iron reacts with hydrogen peroxide in Fenton reaction ($Fe^{2+} + H_2O_2 = Fe^{3+} + HO^{\bullet} + OH^-$) to generate hydroxyl radicals which are highly deleterious to cells and can directly damage DNA, lipids, and proteins or can specifically oxidize the deoxynucleotide pool and its subsequent incorporation into RNA and DNA (Acker *et al.*, 2016).

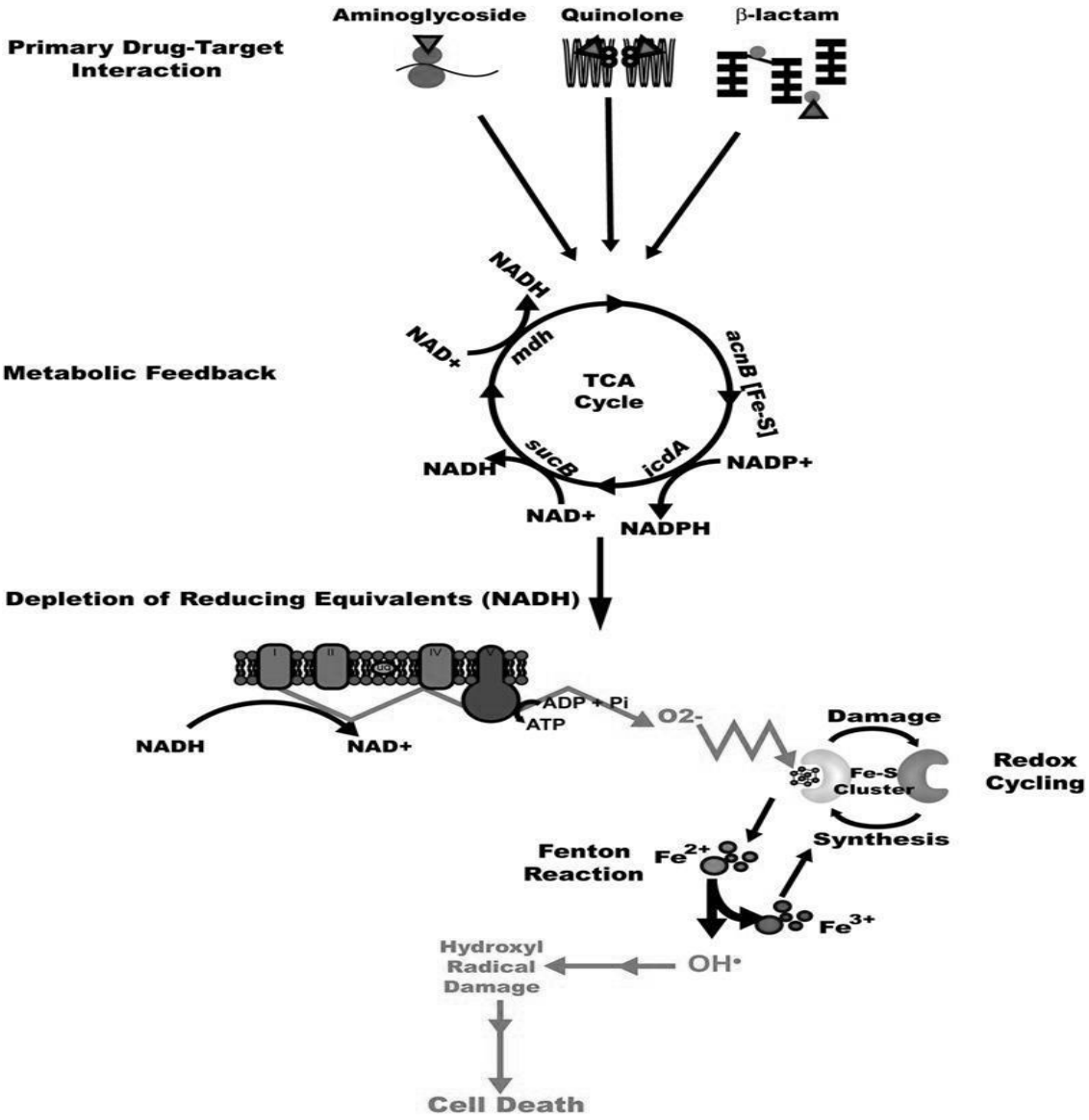



**Figure 2.6 Mechanism leading to ROS mediated Antibiotic killing (Kohanski *et al.*, 2007)**

## 2.5 Review of literature related to Antibiotic Resistance

Antibiotic drugs that have metamorphosed medical sciences in treatment of infectious diseases (Moore, 1948) are also used in non-medical applications like promoting growth in livestocks (Adjiri-Awere and Lunen, 2005). However, such non-clinical application along with other limiting factors have resulted in the rapid emergence and spread of antibiotic resistant in human pathogens worldwide and is causing relevant problems for human health, threatening the efficacy of antibiotics (Ventola, 2015).

Antibiotics are usually effective against microbes. However, the bacteria can develop resistance and this resistance to antibiotics can either be either intrinsic due to the mechanisms present in all strains of a given bacterial species or acquired due to the

acquisition of specific mechanism of resistance (figure 5) because of the consequence of either mutation or recombination or horizontal gene transfer (Martinez *et al.*, 2009) or combinations of these. The level of antibiotic-resistant infections and the degree of antibiotic consumption are strongly interrelated (Zaman *et al.*, 2017).

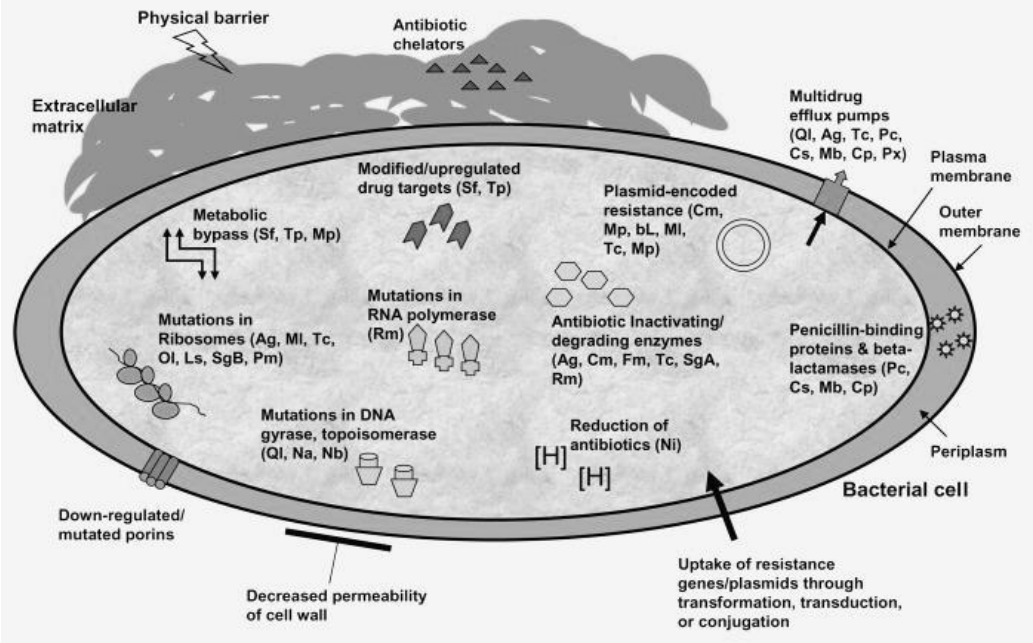



**Figure 2.7 Innate and acquired antibiotic-resistance mechanisms in bacteria (Sriramulu and Sriramulu, 2013)**

Note: Ag, aminoglycosides; Ml, macrolides; Tc, Tetracyclines; Ol, Oxazolidinones; Ls, Lincosamides; SgA, Streptogramin A; SgB, Streptogramin B; Pm, Pleuromutilins; Ql, quinolones; Na, nalidixic acid; Nb, novobiocin; Sf, sulfonamides; Tp, trimethoprim; Mp, mupirocin; Cm, chloramphenicol; Fm, fosfomycin; Rm, rifamycins; Ni, nitroimidazoles; Pc, penicillins; Cs, cephalosporins; Mb, monobactams; Cp, carbapenems; Px, polymyxins; Fa, fusidic acid; bL, beta-lactams; Bt, bacitracin; As, antiseptics.

High concentration of the same drug is required to have an effect on less sensitive or resistant pathogens. One of the adverse consequences of excessive antibiotic use is the disruption of the natural microbial ecology that supports the evolution of the bacteria either different from the previous ones or drug resistant strains of the same (Levy, 1997). In addition, the antibiotic producing strains possess a resistance gene to detoxify the antibiotics produced by themselves and upon contact with other bacteria the same resistance gene when transferred to a formerly susceptible microorganism by gene transfer mechanisms serve to bypass the effects of exogenously added antibiotics (Baquero *et al.*, 2009).

However, the evolution of antibiotic resistance determinants and endogenous antibiotic selective pressure in the antibiotic producing organisms are not always functionally correlated (Baquero *et al.*, 2009). They primarily exert other roles appropriate to the habitat and behavior of bacteria despite causing antimicrobial resistance. For example, antibiotic inactivating enzymes such as beta-lactamases or aminoglycoside acetyltransferases are primarily responsible for modification of peptidoglycan layer (Massova and Mobashery, 1998; Li *et al.*, 2015).

Also, Multidrug efflux pumps were primarily involved in extruding the toxic compounds present in the bacterial rhizosphere and genes like Qnrs provided resistance only to synthetic antibiotics like quinolones (Jacoby, 2005) since these were not present in natural ecosystems. All these, later on evolved to become responsible for antibiotic resistance.

Antibiotic resistance is usually an outcome of natural selection. Nature awards microorganisms with some degree of low level of resistance. Thus, one in millions of fraction of bacteria becomes naturally resistant to the antibiotics (Laxminarayan and Brown, 2001; Zaman *et al.*, 2017). All these resistance mechanisms are based on either modifying the target or reducing the concentration of free antibiotics that can access the target.

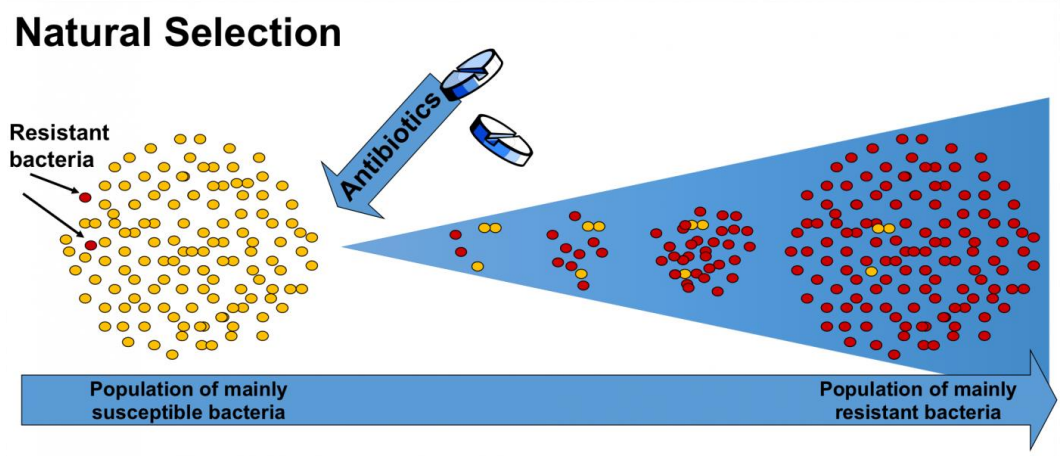



**Figure 2.8 Natural selection of antibiotic resistant bacteria.**

### 2.5.1 Mechanisms of antibiotic resistance

Although the manner of acquisition of resistance may vary, they could be potential be grouped in four primary mechanisms that create resistance to bacteria. They are antibiotic inactivation, target modifications, changes in membrane permeability and efflux pumps (Silva, 1996) .

### 2.5.1.1 Antibiotic inactivation

One of the common primary mechanisms of resistance is antibiotic inactivation by enzymatic inhibitions that modify or degrade the drug itself via hydrolysis, transference of functional groups and redox mechanisms (Kon and Rai, 2016 ).

#### 2.5.1.1.1 Hydrolysis

Hydrolysis of many antibiotics, mainly beta lactam agents (Fig 6a) that are responsible for bacterial cell wall synthesis (Macheboeuf *et al.*, 2006), occur by the enzymes produced by bacteria that target and destroy hydrolytically susceptible chemical bonds (Dzidic *et al.*, 2008).

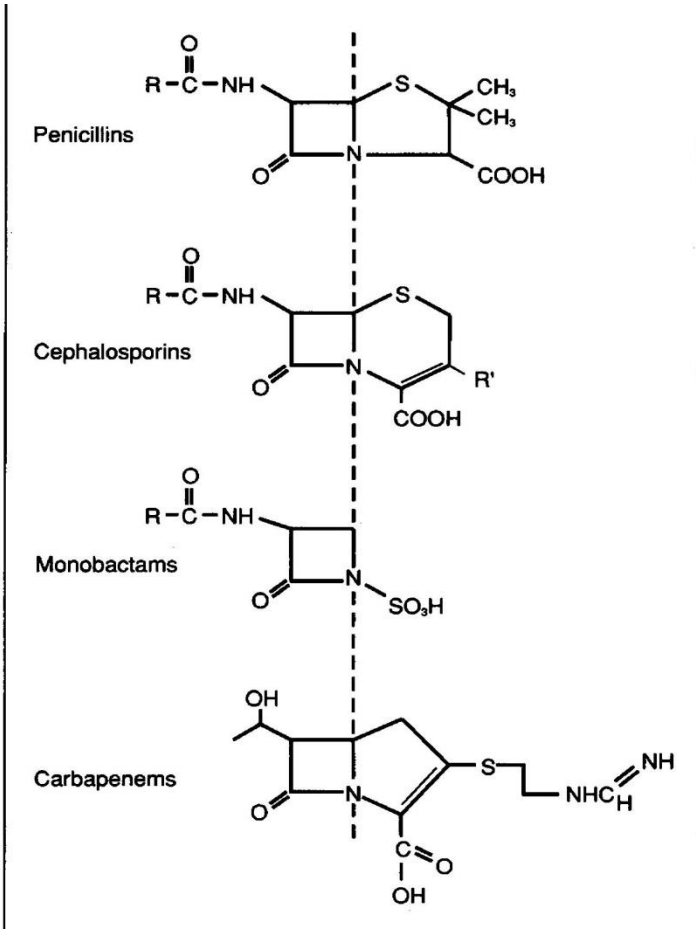



**Figure 2.9a: Structures of β-lactam antibiotics. The β-lactam ring (to the left of the dashed line) is shared by all of these compounds** (Dever and Dermody, 1991)

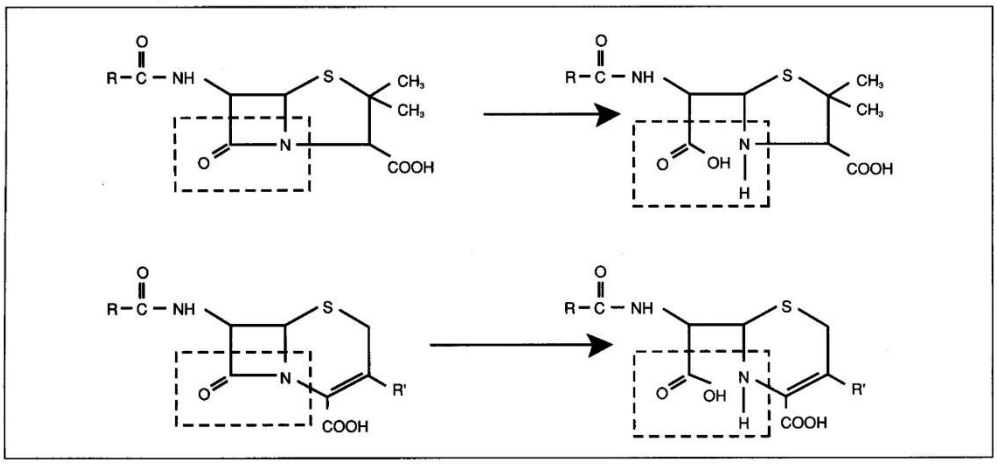


**Figure 2.9b: Reactions catalyzed by β-lactamases resulting in the degradation of penicillins (top) and cephalosporins (bottom)** (Dever and Dermody, 1991)

Resistance to β-lactam antibiotics is imparted by β-lactamase enzymes. The enzymes include penicillinases, AmpC cephaloporinases like MOXs, MIR, FOX family, and CMI family, extended-spectrum β-lactamases (ESBLs) like SHV1, TEM1, TEM2, and CTX-Ms. These enzymes confer resistance to penicillin and all cephalosporins, including cefotaxime and ceftazidime. In addition, the producer of TEM and SHV show co-resistance to tetracycline, sulfonamide and aminoglycosides (Morosini *et al.*, 2006).

These enzymes are produced by all gram negative bacteria and some gram positive bacteria and catalyze the hydrolysis of β-lactam bonds to acidic derivatives that do not have antibacterial properties as shown in figure 6b (Dever and Dermody, 1991). ESBLs have ability to inactivate newer cephalosporins with broad spectrum antibiotic activity (Shaikh *et al.*, 2015).

#### 2.5.1.1.2 Group transfers

Group transfer is another mode of antibiotic inactivation which is carried out by transferases enzymes like adenyltransferases, acetyltransferases, glycotransferases, and kinases (Wright, 2011). These enzymes inactivate antibiotics such as aminoglycosides, chloramphenicol, streptogramin, macrolides or rifampicin by addition of functional groups like adenylyl, phosphoryl, or acetyl group to the periphery of the antibiotic molecules (Kelechi and Uchechukwu, 2015). These enzymes are usually found in low concentration in the periplasmic space of bacteria, although the modification of aminoglycosides is efficient due to the slow uptake of antibiotics into periplasm and high affinity of group transferring enzymes towards aminoglycosides (Garneau-Tsodikova and Labby, 2016)

### 2.5.1.2 Target modification / alteration

The modification of antibiotic target site is another mode of mechanism by which antibiotic is unable to bind the target site properly conferring resistance to it (Dzidic *et al.*, 2008). The noticeable mechanism is point mutation in selected genes, resulting in resistance with minimal impact in microbial fitness. For example, single mutations in target genes such as gyrA indulge high level of resistance and further monotonous mutations in same gene result in increment in level of resistance in fluoroquinolone ( Tankovic *et.al*., 2003; Kon and Rai, 2016 )

Catalytic enzymes result target modification via highly efficient and region-selective modification. For example, ribosome methyltransferases (Erm) modify the 23S rRNA of large subunit of the ribosome at position A2058 *(E.coli* numbering), leading resistance to three structurally different classes of antibiotics, macrolides, lincosamide and streptogramin B (Lambert, 2005; Vester and Long, 2009). Erm enzymes catalyse S-adenosylmethionine (SAM) dependent mono and di-methylation of N6 with loss of H-bond properties, creating steric block of the antibiotic binding site (Wright, 2011) inhibiting antibiotics including macrolides such as erythromycin, lincosamides such as clindamycin and type B streptogramins like quinuspristin. All these antibiotics tend to bind to the peptide exit tunnel region of large subunit of the ribosome (Gaillard, *et al.*, 2016).

In Gram positive bacteria, glycopeptides (eg: vancomycin) inhibit cell wall synthesis by forming complex with C-terminal acyl-D-alanyl-D-alanine (acyl-D-ala-D-ala) containing residues in peptidoglycan precursors through a series of five hydrogen bonds. When acyl-D-ala-D-ala is modified to acyl-D-ala-D-Lactate (acyl-D-ala-D-Lac) or acyl-D-ala-D-Serine (acyl-D-ala-D-Ser) with replacement of amide bond to ester linkages, eliminating a key H-donor and leading to electronic repulsion. Thus, glycopeptides cannot bind with these modified complex hence achieving resistance against vancomycin (Allen and Nicas, 2003; Kapoor *et al.*, 2017).

Some bacteria are unresponsive to specific antibiotics as they bypass the inactivation of a given enzyme. For instance, the two enzymes dihydrofolate reductase (DHFR) and dihydropteroate synthase (DPS) are responsible for tetrahydrofolate biosynthesis. DPS converts para-aminobenzoic acid (PABA) into dihydropteroic acid as PABA is toxic to the cell when accumulated (Silva, 1996). DHFR transforms dihyrofolate into tetrahydrofolate. These enzymes are inhibited by sulfonamides and trimethoprim, respectively. In trimethoprim and sulfonamide resistant bacteria, the inhibition of DPS and DHFR are eluded (Dzidic *et al.*, 2008) by production of these enzymes (DPS and

DHFR) with decreased affinity to their inhibitors and also by overproduction of enzymes (Hoek *et al.*, 2011).

### 2.5.1.3 Efflux pumps

Efflux pumps are proteinaceous membrane transporters involved in expulsion of toxic substrates including antibiotics out of the cells (Webber and Piddock, 2003). Prokaryotes efflux transporter are classified into five families. They are major facilitators (MFs), small multi-drug resistance (SMR), resistance nodulation cell division (RND), ATP-binding cassette (ABC), and multi-drug and toxic extrusion (MATE). All these systems use proton motive force as an energy source except ABC family which utilizes ATP hydrolysis to conduct the export of substrates (Blanco *et al.*, 2016)

Inducible multidrug efflux pumps are responsible for intrinsic antibiotic resistance of many organisms and mutation of the regulatory elements controlling the production of efflux pumps may lead to an increase in antibiotic resistance (Poole, 2007). In addition, antibiotic resistance by efflux systems has been studied in a number of clinically important microbes such as *Campylobacter jejuni* (CmeABC), *E. coli* (AcrAB-TolC, AcrEF-TolC, EmrB, EmrD), *Pseudomonas aeruginosa* (MexAB-OprM, MexCD-OprJ, MexEF-OprN and MexXY-OprM), *Streptococcus pneumoniae* (PmrA), *Salmonella typhimurium* (AcrB) and *Staphylococcus aureus* (NorA) (S. Chen *et al.*, 2007). Multidrug efflux pumps are involved in intercellular signaling like thrusting of quorum-sensing signals in *Pseudomonas aeruginosa* (Martínez and Baquero, 2014). RND pumps are also reported to export multiple antibiotics (Sun *et al.*, 2014).

The tripartite efflux pumps of Gram-negative bacteria are formed by an integral membrane protein, whose activity is commonly linked to the membrane proton motive force, an outer membrane protein and a periplasmic protein (Martinez *et al.*, 2009).

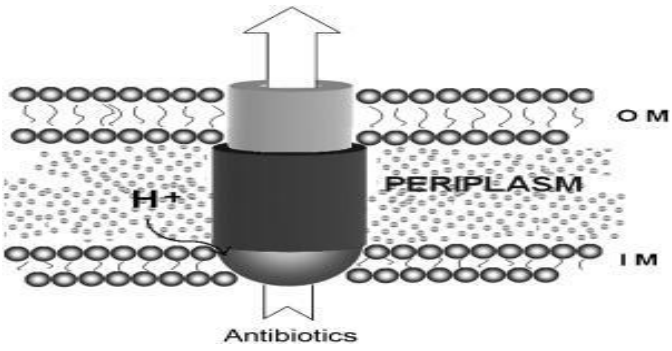



**Figure 2.10: Structure of a tripartite efflux pump (Martinez *et al.*, 2009)**

The tripartite RND family is the most intriguing and salient to resistance in Gram negative bacteria. AcrAB-TolC from *Escherichia coli*, and MexAB-OprM from *Pseudomonas aeruginosa* are most studied of RND family members. This system consists

of a cell membrane-spanning pump (AcrB and MexB), an outer membrane pore (TolC and OprM), and a periplasmic adapter protein (AcrA and MexA) that connect both. Antibiotic efflux is coupled with a vectorial proton influx into the cell (Borges-Walmsley, *et al.*, 2003).The transporter AcrB is a large protein domain containing more than 1000 residues. They show a broad array of substrate specificity and are trimers which capture the drug molecules from periplasm. For example: AcrAB-TolC can extrude basic dyes such as acriflavine and ethidium or antibiotics such as β-lactams, tetracyclines, chloramphenicol, and rifampin, except aminoglycosides or detergents like sodium dodecyl sulfate (SDS) and Triton X-100 and even simple solvents such as hexane and heptanes (Takatsuka *et al.*, 2010).

**Table 2.2: Mechanisms of drug resistance of common antibiotics** (Zaman *et al.*, 2017)

| Antibiotic class | Example(s) | Mode(s) of resistance |
|---|---|---|
| P-Lactams | Penicillins, Cephalosporins, Penems, Monobactams | Hydrolysis, efflux, altered target |
| Aminoglycosides | Gentamicin, Streptomycin, Spectinomycin | Phosphorylation, acetylation, nucleotidylation, efflux, altered target |
| Glycopeptides | Vancomycin, Teicoplanin | Reprogramming peptidoglycan biosynthesis |
| Tetracyclines | Minocycline, Tigecycline | Monooxygenation, efflux, altered target |
| Macrolides | Erythromycin, azithromycin | Hydrolysis, glycosylation, phosphorylation, efflux, altered target |
| Lincosamides | Clindamycin | Nucleotidylation, efflux, altered target |
| Streptogramins | Synercid | Carbon-Oxygen lyase, acetylation, efflux, altered target |
| Oxazolidinones | Linezolid | Efflux, altered target |
| Phenicols | Chloramphenicol | Acetylation, efflux, altered target |
| Quinolones | Ciprofloxacin | Acetylation, efflux, altered target |
| Pyrimidines | Trimethoprim | Efflux, altered target |
| Sulfonamides | Sulfamethoxazole | Efflux, altered target |
| Rifamycins | Rifampin | ADP-ribosylation, efflux, altered target |
| Lipopeptides | Daptomycin | Altered target |
| Cationic peptides | Colistin | Altered target, efflux |

## 2.5.2 Additional mechanisms of antibiotic resistance

### 2.5.2.1. Quorum Sensing

Microorganisms can sense the changes in the surrounding environment, integrate the signals and thus adapt their physiology. Such mechanisms that involve the ability to cell to cell communication in a microbial population is called Quorum Sensing (QS) (Waters and Bassler, 2005). The process of QS is similar in both Gram positive and Gram negative bacteria and uses wide range of auto-inducers: Acylhomoserine lactones (AHLs), oligopeptides, furanosyl borate (auto-inducer-2), and fatty acids (McDougald *et al.*, 2007; Kumar *et al.*, 2017).

QS involves cell-density-dependent production of small signal molecules called auto-inducers through which bacteria functionally coordinate various activities including production and expression of virulence factors, antibiotic production, drug resistance, bioluminescence, plasmid conjugation and biofilm formation (Zhang and Dong, 2004; Antunes *et al.*, 2010). The unique characteristic of pathogenic bacteria is that concentration of QS-mediated genes constitutes around 10% of its total genome. Even at low cell densities , they can evade the host's defense (Kalia *et al.*, 2014).

Most of the microorganism use QS to gain the virulence and antibiotic resistance. When the number of cells increases within population, there is also increment in auto-inducer molecules. Once the minimal threshold level crosses, they sense the quorum and respond by triggering the signal transduction cascades resulting in the production of virulence factors and biofilms (Castillo-Juárez *et al.*, 2015). It has a key role in antibiotic resistance as it enhances replication and transfer of plasmids (carrier of antibiotic resistance genes). It is vital for intra and inter-bacterial gene regulation and for keeping microcolonies (Raffa *et al.*, 2005; Waters and Bassler, 2005). Microcolony is a small cluster of bacterial cells, typically on the order of ~100 cells, representing an early stage in biofilm formation which later on forms macrocolonies in which the cells are held together by extracellular polymeric substance (EPS).

Multi drug resistance is achieved through a variety of mechanisms and QS appear to be the regulator of it by two ways - Regulation of biofilm associated EPS matrix and regulation of efflux pump genes. The upregulation of efflux pump is controlled by QS. For example: in *E. coli*, overexpression of luxR homologue SdiA (a quorum sensing regulator) leads to overexpression of AcrAB efflux pump which positively influences multidrug resistance (Rahmati *et al.*, 2002). EPS prevents the access of antimicrobial agents into the bacterial community. In *Acinetobacter*, blaPER-1, antibiotic determinant which is the first step of biofilm formation cycle, is more critical for cell adherence and

has ability to withstand stress like exposure to high antibiotics (Prashanth, Vasanth, Saranathan, R., and Pagal, 2012). Similarly, In *P. aeruginosa*, when cell population density is high, QS system is activated and auto-inducer c4-HSL is generated which solely induces the expression of mexAB-orpM operon directly or inactivates MexR repressor, thus results in enhancement of the transcription of MexAB-OrpM efflux pump (Subhadra *et al.*, 2016).

#### 2.5.2.2 Biofilm formation

Biofilm is a microbially derived sessile community wherein the cells are embedded in a self-produced matrix of extracellular polymeric substance (EPS) and exhibit an altered phenotype with respect to growth rate and gene transcription (Prashanth *et al.*, 2012). The EPS is made up of carbohydrates, nucleic acids, proteins and other macromolecules. Biofilm preserving microbes adhere to each other or to surfaces.

Production of biofilm is the dominant virulence factor that correlates with increase in antibiotic resistance, natural surfactant resistance and reduced phagocytosis (Costerton *et al.*, 1995). A pathogen with a biofilm phenotype leads to increased colonization and endurance which is the leading cause of infections. The nature of biofilm structure and physiological characteristics of biofilm producers confer inherent resistance to antimicrobial agents. There are three mechanisms responsible for resistance in biofilm producers: i) delayed penetration of the antimicrobial agent through biofilm matrix, ii) altered growth rate of biofilm organisms, and iii) other physiological changes due to the biofilm mode of growth (Donlan and Costerton, 2002).

Biofilm matrix that is made up from polymeric substances is known to retard the diffusion of antibiotics. When the diffusion barrier is established, the antimicrobial agent is deactivated faster in the outer layer of the biofilm than it diffuses (Chen and Stewart, 1996). Similarly, during nutrient limitation and environmental stress conditions, biofilm producing sessile microorganisms grow slowly. As a result, they take up antimicrobial agents more slowly. Slow growing cells are not very susceptible to many antimicrobial agent thus contributing in antibiotic resistance (Olson *et al.*, 2002; Costerton *et al.,* 2011).

Likewise, when bacterial growth rate is minimal and slow growth activated gene (rosS) is activated (effect of stress conditions such as nutrient limitation and accumulation of toxic metabolites) which favors more biofilm formation (Adams *et al.*, 2002).

## 2.6 Review of literature related to DNA adenine methylase (Dam)

### 2.6.1 DNA adenine methylase (Dam) as potential drug target

Dam methylase is an enzyme of DNA methyltransferases family that methylates the N-6 in adenine of the sequence 5'-GATC-3' in newly synthesized DNA (Horton*et al.*, 2006), which protects the DNA from cleavage by the restriction endonuclease MboI (http://www.uniprot.org/uniprot/P0AEE8).

Other important functions include their roles in mismatch repair in newly synthesized DNA strand during DNA synthesis (Barras and Marinus, 1989), regulation of protein expression (Seshasayee, 2007) and regulation of DNA replication (Skarstad and Katayama, 2013).

Dam methylation is not essential for viability of *E.coli* (Bale*et al.*, 1979), however, it is found essential in terms of viability and pathogenesis in *Salmonella typhimurium* (Portillo *et al.*, 1999), *Vibrio cholera* and *Yersinia pseudotuberculosis* (Julio *et al.*, 2001) which makes it a probable drug targ*et* against other pathogens as well. Dam inhibitors have been sought for its potential in drug development since humans do not produce this enzyme (Mashhoon*et al.*, 2006) and also because the bacterial GATC methylomes are believed to have an important role in antibiotic stress survival (Cohen *et al.*, 2016).

### 2.6.2 Structure of *dam* gene

The *dam* gene is a part of transcriptional unit of 834 bp long, that contains at least four genes and perhaps 6 or 7 genes (Lyngstadaas *et al.*, 1995). Promoters P1 and P2 (3kb upstream of *dam*) and P3 (2kb upstream of *dam*) are the most important for transcription of *dam* gene, the order of promoter strength being P2 > P1 > P3 > P4 > P5 (Rasmussen *et al.*, 1994).

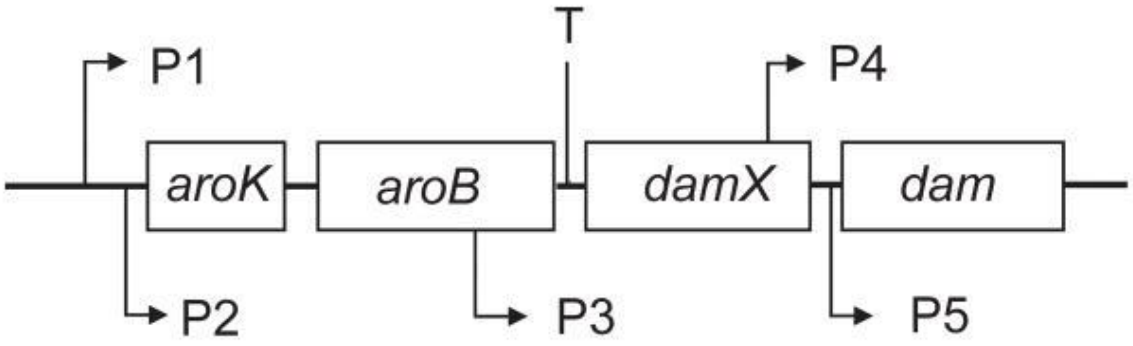



**Figure 2.11: Organization of the dam transcriptional unit in *E. coli*** (Marinus and Løbner-Olesen, 2014)

## 2.7 Review of literature related to Antioxidants

Oxidative metabolism is an essential process of cell survival. When reactive species are produced in excess and cannot be consumed, these accumulate in the body thus generating a stress. This stress characterized by the imbalance between the production of reactive species and antioxidant defense activity is the oxidative stress (McCord, 2000). It has a major role in the development of several chronic and degenerative illnesses. It is apparently valid that the accumulation of free radicals results in various modification or damages to biological macromolecules such as proteins, lipids and DNA which then cause DNA mutation and increase cancer risks (Chaturvedi, 2010).

Apart from modification and damaging the protein and cells, free radicals also interfere with the expression of number of genes and signal transduction pathways including stimulation in cell signaling pathways and inhibition of cell death signaling pathways. These changes are commonly seen in various human cancers (Valko *et al.*, 2006). All these strengthen the role of antioxidants in human body.

Antioxidants are the chemical scavengers that delay or inhibit the chain reaction formed by free radicals. The benefit of antioxidants is to defend the cells from the damage caused by the potentially harmful molecules 'reactive species'. They greatly reduce the adverse effect of free radicals by breaking them before these radicals attack the biological targets which prevent chain reaction or prevent the activation of oxygen to highly reactive products (Azzi *et al.*, 2004). The effectiveness of antioxidant compounds and the number of hydroxyl groups present in their aromatic ring is proportional to each other (White *et al.*, 2014).

Potentially useful candidates that exhibit antioxidant properties are vitamins C and E, carotenoids, flavonoids which are naturally present in foods like berries, tomato, potato, etc. whereas synthetic antioxidants like butylated hydroxyl anisole and butylated hydroxyl toluene are also available but they are reported to have deleterious effects to human health (Gupta *et al.*, 2013). Besides plants, microorganisms are also reported to produce strong antioxidants and thus could be developed as natural antioxidant drugs (Elnahas *et al.*, 2017).

Since Streptomycetes have the potential to produce 10-20 different bioactive metabolites (Sosio *et al.*, 2000) including antioxidants (Revathy *et al.*, 2013), these could be explored for various antioxidants which could be used to cure the diseases related to oxidative stress. Asthaxanthin is the most powerful quencher of singlet oxygens. It enhances both the specific and non-specific immune system and protects the cell membrane. It also protects the skin from ROS (https://www.nutrex-

hawaii.com/blogs/learn/benefits-of-antioxidants). The natural antioxidants Resveratrol and Sesamin prevent intracellular oxidation and improved cell survival by maintaining mitochondrial membrane potential and reducing mitochondrial ROS generation (Maharjan *et al.*, 2014). Dietary antioxidants can be used to terminate the site specific ROS which will be beneficial to prevent the ROS-related cell death mediated aging and the pathogenesis of neurodegenerative disorders like Alzheimer's and Parkinson's diseases (Maharjan *et al.*, 2016).

Since oxidation involves the production of free radicals which could lead to chain reactions and in turn damage the cells, antioxidants which could mitigate such effects, are immensely important since these could be used as preservatives in cosmetics (Polati *et al.*, 2007) and food (Santos-Sánchez *et al.*, 2017), oxidation inhibitors in fuels (Varatharajan and Pushparani, 2018) and more importantly as drug candidates. Due to the higher cost of natural antioxidants, synthetic antioxidants have dominated the market however, due to the toxic side-effects, these have consequently been restricted from use (Kulisic *et al.*, 2004). Thus, more focus towards natural antioxidants is a must.

The antioxidant activity of extracts is evaluated in terms of their free radical scavenging capacity using DPPH (2,2-diphenyl-1-picrylhydrazyl) assay which was preferred over other methods since this is easy, fast and among the most reliable method of determining the radical scavenging effects of antioxidants (Kedare and Singh, 2011; Kumawat *et al.,* 2012). DPPH solution is prepared in methanol since it is a stable, synthetic radical which does not disintegrate in water, methanol, or ethanol (Shimada *et al.*, 1992). The principle is based on the ability of DPPH, a stable free radical to easily receive an electron or hydrogen atom from the antioxidant molecules in forming a stable diamagnetic molecule thus decolorizing from purple to yellow color (Soare *et al.*, 1997). DPPH radicals dissolved in methanol gets converted into DPPH-H (diphenyl hydrazine) molecules upon contact with antioxidant agents as shown in the following equation. This causes the reduction in DPPH radicals in the environment thus resulting discoloration (Bhaigyabati *et al.*, 2014) which reflects the radical scavenging activity of the extracts under study. Because of the DPPH radical's ability to bind H, it is considered to have a radical scavenging property.

$DPPH^{\cdot} + A\text{-}H \dashrightarrow DPPH\text{-}H + A^{\cdot}$

## 2.8 Review of literature related to computer aided drug discovery (CADD)

High throughput screening (HTS) was a traditional process used in drug discovery to find novel therapeutic molecules by testing a large number of diverse chemical structures against targets to identify hits (Liu *et al.*, 2004). The traditional process of bringing a pharmaceutical drug to the market is a challenging, time consuming and expensive process that costs billions of dollars. About 90% of the developed drugs entering to the clinical trials fail to get FDA approval and reach to the market. Thus, to assay thousands of molecules with robotic automation, CADD plays a vital role to minimize human labour associated with screening of compounds and potentially reduce the cost and time of research and development (Szymański *et al.*, 2012). CADD is able to explain the molecular basis of therapeutic activity and also predict variable possibility to improve its activity, thus increasing the hit rate of novel drug compounds (Sliwoski *et al.*, 2014). However, both HTS and CADD can be used parallelly for the additional benefit for CADD in drug discovery project.

### 2.8.1 Virtual Screening (VS)

Virtual screening also called *in silico* screening is extensively used in CADD. The basic goal of VS is to reduce virtual space of chemical compounds to a more manageable number for further synthesis and screening against biological target and could lead to potential drug candidates (Vyas *et al.*, 2008). VS becomes effective method when combined with organisms' metabolic network analysis and subsequent experimental verification for the simultaneous identification of novel therapeutic targets and small inhibitory molecules against them (Shen *et al.*, 2010). The discovery of approved novel drug from the tools of CADD are carbonic anhydrase inhibitor dorzolamide (1995), angiotensin-converting enzyme (ACE) inhibitor captopril (1981), a frbrinogen antagonist tirofiban (1998) and three therapeutic agents for the treatment of human immunodeficiency virus (HIV): saquinavir (1995), ritonavir (1996) and indinavir (1996) (Talele *et al.*, 2010 ).

VS approaches can be categorized into two groups:

i) Ligand based virtual screening
ii) Structure based virtual screening.

#### 2.8.1.1 Ligand based virtual screening (LBVS)

These techniques are based on the analysis of ligands known to interact with a target of interest. This uses a set of reference structures collected from compounds known to interact with target and analyze their 2D or 3D structures (Sliwoski *et al.*, 2014). Some

popular approaches of this technique are Pharmacophore modelling, Molecular similarity techniques and quantitative structure-activity relationship (QSAR) modeling. Pharmacophore modeling is based on the common structural features of ligands that bind to a target for screening. In molecular similarity methods, the molecular fingerprint of known ligands that binds to a target is used for finding molecules with similar fingerprints through screening molecular libraries. QSAR modeling predicts the biological activity from the corresponding structural features of ligands that bind to a target (Leelananda and Lindert, 2016). CADD mediated drug design was successfully applied against casein kinase II (CK2) inhibitors (Oprea and Matter, 2004) and human epidermal growth factor receptor 2, HER2, a commonly over-expressed tyrosine kinase receptor found in various carcinomas (Tai *et al.*, 2010).

Thus, in drug discovery, LBVS is increasingly applied to complement HTS and also in cases where biological screening is difficult or has failed (Bajorath, 2009). Despite the increasing availability of crystal structure data for biological targets, ligand-based methods still is an attractive VS option particularly with the growing number of ligands to produce rather promising results (Kurczyk *et al.*, 2015).

#### 2.8.1.2 Structure based virtual screening (SBVS)

Structure based approaches rely on the ability to determine and analyze 3D structures of biological molecules by X-ray crystallography or NMR or homology modelling (Villoutreix *et al.* 2007). Computational methods that predict the 3D structure of a protein ligand complex are often referred as molecular docking approaches. It is considered as one of the most innovative and powerful approaches in drug design (Baldi, 2010). It is a large scale *in silico* screening of drug molecules in the databases of small molecules against a target of interest. It calculates the interaction between the target receptor protein and ligands and uses the scoring/energy function to rank the docked pose into the active site to identify best hits (Dror *et al.*, 2004). The active site pocket or binding site of receptor is used for identification of new ligands through docking.

Computer aided structure based virtual screen was performed against a natural chemical database to determine the availability of plant based ERβ-selective ligands. ERβ has been associated with estrogen induced promotion of memory function and neuronal survival (Zhao and Brinton, 2005). This technique is mature enough to benefit from an aggressive programme of experimental testing as it has emerged as a reliable, cost-effective and timesaving technique for discovery of lead compounds.

#### 2.8.1.2.1 Steps for SBVS

SBVS comes into account through several steps including target and compound library preparation, running the actual docking algorithms, post-processing and ranking the results by predefined scoring function for bioassay (Cheng *et al.*, 2012).

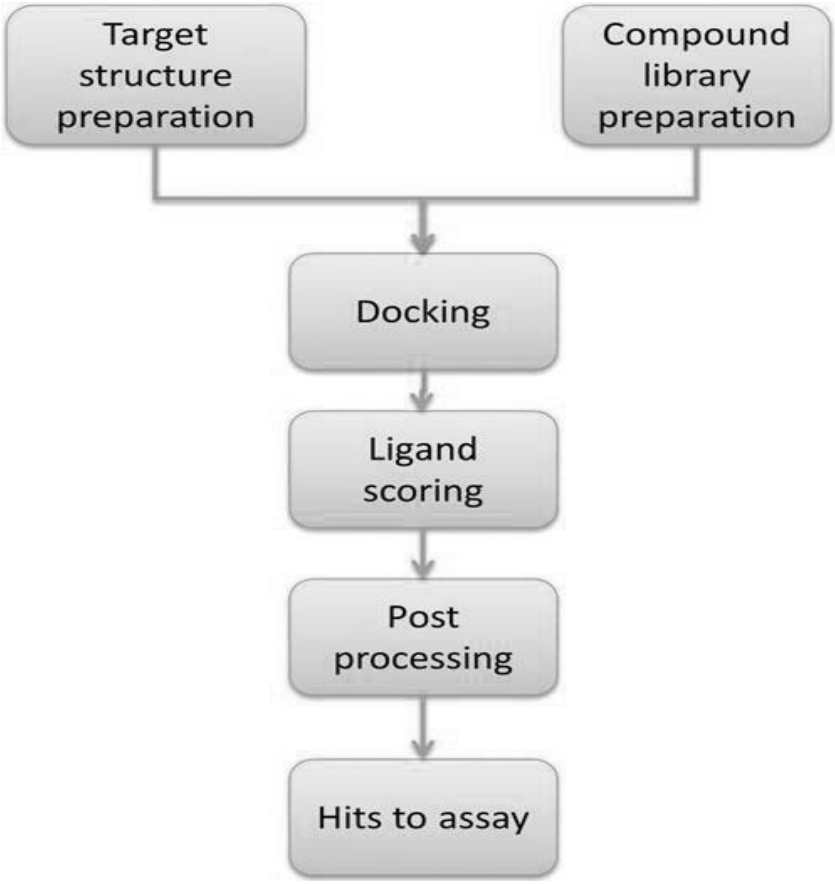



**Figure 12: Flowchart of structure based virtual screening (Cheng *et al.*, 2012)**

**i. Protein (target) structure determination**

All the structure based methods are based on the three-dimensional target structure. X-ray crystallography and NMR spectroscopy are mostly used to determine a protein structure. When 3D structure is not available through the experimental methods, homology modeling is used to predict the 3D coordinates of structure (Xiang, 2006). The protein structure having similar amino acids with the target sequence of interest helps in predicting the target structure, function and even possible binding and functional sites of the structure (Kristoffer and Arne, 2009). However, these methods have cost and time constraints and are also limited by experimental challenges.

There are many repositories that collect and store the information about drug molecules and target structure. The Protein databank (PDB) (http://www.rcsb.org/pdb/), is a global resource that contains millions of 3D information about experimentally determined biological macromolecules (Berman *et al.*, 2000). Structure file obtained from PDB consists of heavy atoms, water molecules, cofactors, activators, ligands, metal ions and several protein subunits with or without whole loop regions. This may cause steric clashes. To improve the docking performance, these aspects must be efficiently addressed by available softwares (JAWS, PROPKA) for protein preparation (Lionta *et al.*, 2014).

**ii. Binding pocket identification**

Once a 3D structure of a protein is known, finding binding pockets on that protein is an important step in structure based drug discovery. It gives the indication of where the small molecules can bind to target structures associated with increase or decrease of their activity (Leelananda and Lindert, 2016). It is usually based on the analyzing of amino acids interacting with protein and its ligands through the visualization of its structure. Computational methods use geometric algorithms and energetic considerations in identifying the binding pockets and softwares used include POCKET, SURNET, and so on (Sliwoski *et al.*, 2014).

**iii. Selection of compound database**

The success of docking depends on the content and quality of a compound library. There are number of chemical databases which are available publicly and commercially. Some of the commonly used databases are PubChem (30 million compounds) (http://pubchem.ncbi.nlm.nih.gov), ZINC (230 million compounds in 3D format) (http://zinc15.docking.org/). NIC set (3 million) (https://dtp.cancer.gov/default.htm).

**iv. *In silico* ADME/TOX filtering for drug-likeness**

Blind docking of such huge database is possible due to modern speed computers. But it becomes tedious and leads to a waste of time and computer resources. So, it is better to select relevant compounds from the library. Absorption, distribution, metabolism, excretion and toxicity (ADME-TOX) properties of compounds along with their physic-chemical properties are considered to screen out promising drug candidates. Small molecules usually go through one or several *in silico* ADME/tox filtering steps in an attempt to generate a database of molecules with drug-like molecules. The ultimate goal of *in silico* modeling of ADMET properties is to predict in vivo deposition behavior of potential drug molecules in the human body by assembling all kinetic processes into one inclusive model (Alqahtani, 2017).

Common filtering protocols include application of physiochemical filters by the Lipinski's rule of five which can be used to ensure relevance of biology or drug-likeness (Lipinski *et al.*, 2001). This rule states that the drug-like compounds should have following properties:

i) Molecular weight < 500 Daltons
ii) Calculated logP < 5 (or MlogP <4.15)
iii) Hydrogen bond donors < 5 (expressed as the sums of OHs and NHs)
iv) Hydrogen bond acceptors <10 (expressed as the sums of Os and Ns)

In this rule, any oxygen and nitrogen are defined as hydrogen bond acceptors and N-H or O-H group are considered as hydrogen bond donors. logP refers to octanol or water partition coefficient of a compound and is used to measure the lipophilicity. Lipophilicity (logP) of a drug is a single physiochemical property to predict its permeability to biological systems (Clark and Pickett, 2000). Other rules involve removing compounds containing specific chemical substructure associated with poor chemical stability, reactivity or toxicity like epoxides, anhydrides, frequent hitters and promiscuous inhibitors. Several online approaches use a molecule in sdf, or Mol2 or SMILES formats. The right balance between lipophilicity and polar surface area (PSA) can be achieved by introduction or replacement of heteroatoms, polar groups and other solubilizing groups. Minimizing the molecular weight leads to good oral bioavailability in these lead candidates (Rondeau and Schreuder, 2015).

**v. Pose prediction**

A correct docking is an essential feature in SBVS as incorrect orientation in the binding pocket leads to false results. Cluster algorithm can be used to differentiate between the similar and dissimilar docked poses improving pose prediction. Several techniques like root-mean-square deviation (RMSD), K-means are effective for clustering the dissimilar poses during the docking simulations (Danishuddin and Khan, 2015).

**vi. Docking and scoring**

Molecular docking is one of the most commonly used SBVS approach. It consists of sampling the ligand pose within the binding site of the target to form stable complex. Significant outcomes of the docking results greatly depends on the capability of method in reproducing experimentally solved ligand poses and scoring functions to rank the poses (Danishuddin and Khan, 2015). Many docking programs are developed. Some of them are:

**GOLD (Genetic Optimization of ligand docking)**: characterizes the evolutionary algorithm to explore the full range of ligand conformational flexibility with partial

flexibility of the protein. Advantage: can handle water and ion molecules in the active site of proteins easily (Jones *et. al.*, 1997).

**AutoDock**: a flexible automated and random search docking algorithm that uses Monti-Carlo simulated annealing, evolutionary genetic and Lamarckian genetic algorithm methods. It is also used with AutoDock Tools (ADT) (Cosconati *et. al.,* 2010).

Other tools are GLIDE (Grid-based Ligand Docking with Energetic), DOCK, FRED (Fast Rigid Exhaustive Docking), etc.

Scoring function is widely used for computing the fitness of receptor-ligand binding affinity. The main aim of this function is to estimate the free energy of binding of a ligand to a specific targ*et af*ter docking different ligands of a database. Commonly used scoring functions are force field based function, empirical scoring function and knowledge-based function (Lavecchia and Giovanni, 2013). The force field score functions estimate the binding free energy as a sum of the strength of intermolecular Vander Waals, electrostatic interaction and hydrogen bonding between all the atoms of two binding partners within a complex. It also accounts for solvation and entropic factors that occur upon binding (Lionta *et al.*, 2014).

### vii. Improvement of compound selection after docking (post-processing)

Before selecting the compounds for further trails, it is essential to post-process the compounds resulted from molecular docking. Sometimes, simplified scoring functions and inadequate sampling of the conformational space for ligand may lead to unrealistic poses, intra-ligand steric clashes, twisted amides, E/Z esters, imperfect hydrogen bonding network which poses unreasonably high score and need to be discarded.

To tackle the inefficiency of traditional clustering of docking poses, the Automatic analysis of poses using self-organizing map method is used for pose ranking with careful analysis of interatomic contacts between docked ligands and target (Bouvier *et al.*, 2009). Another approach is consensus induced fit docking (cIFD) which improve the probability of identifying accurately docked poses (Lionta *et al.*, 2014)

### viii. Experimental Validation:

The potential inhibitors are then tested experimentally. The common *in vitro* assays include:

a) *In vitro* enzyme activity assay
b) *In vivo* viability assay
c) *In vivo* cytotoxicity assay

# 3. MATERIALS AND METHODOLOGY

## 3.1 Study area

The study area of my thesis was different parts of Nepal. Nepal has a unique ecological niche and is a favorable habitat for variety of microorganisms. So, there could be possibility of screening out novel species of *Streptomyces* and therefore novel secondary metabolites which could be antimicrobials or antioxidants (primary focus of this study).

### 3.1.1 Sample collection

Since various national and international researches regarding Streptomycetes have already been done, soil sample collection was done accordingly with a view of isolating novel Streptomycetes strains. The soil samples which were collected from various regions of Nepal (Appendix 8.2) and brought at the Central Department of Biotechnology (CDBT), TU were used.

Based on earlier studies that the rare tree ferns habitat is found around Panchase in the western Nepal (2500 meters above sea level) and assuming that the soil around its rhizosphere could be rare and possibly there could be rare and novel Streptomycetes in that soil, the samples were collected in February, 2017. Furthermore, soil sample was collected in Hetauda, Southern Nepal (around 300 meters above sea level) from forest area at site with decayed leaves and from a river bank presuming that Streptomycetes living under stress conditions could produce the desired and effective metabolites. All soil samples were collected from a depth of 5 to 10 cm in clean plastic bags and tagged accordingly. The samples were preserved at 4°C at CDBT. A total of seventy four soil samples are stored in CDBT.

## 3.2 Isolation and morphological characterization of Streptomycetes

The isolation of Streptomycetes was performed by serial dilution method. The media used for screening process were ISP2 and ISP4 (Appendix 8.3) among all the ISP medias recommended by Shirling and Gottlieb (Shirling and Gottleib, 1996). ISP2 is a general media with glucose as a common carbon source and yeast and malt extracts as other nutrient sources whereas ISP4 is more selective for Streptomycetes screening. The agar plates were supplemented with 50µg/ml cycloheximide, an antifungal agent.

For screening of the organism 1 gm of soil sample was added to 10 ml of sterile distilled water in test tube and mixed well. 1 ml was then transferred to 9ml of sterile distilled water to make $10^{-2}$ dilution and likewise, diluted to $10^{-4}$. Then, 100µl sample was then taken from $10^{-4}$ dilution and spread plated on the ISP agar media and finally dried. The plates were incubated at 28°C for around 15 days. The plates were regularly checked

from 4 to 5 days of incubation for possible growth of colonies. The colonies of putative Streptomycetes were picked on the basis of colony characteristics like glabrous, chalky, heaped, etc. and subcultured in ISP agar media by streaking. Cultural characteristics such as color of aerial mycelium, color of substrate mycelium, morphological characteristics and pigmentation were recorded.

### 3.2.1 Gram Staining

A drop of water was first added on the slide in which a minute amount of colony from a fresh cultured petriplate was transferred to make a thin smear (nearly 1-1.5 cm in diameter) as much as possible. The smear was air dried and heat fixed for cell adhesion on the glass slide and to prevent significant loss of culture during further steps. Few drops of crystal violet was added on the slide and stood for 60 seconds. The excess stain was removed with water. Few drops of Gram's iodine was added to cover the fixed culture and stood for 30 seconds. After rinsing with water, few drops of decolorizer was added for a few seconds until the solvent flowing over the slide did not show color. Finally, the smear was counterstained with a few drops of safranin for 20 seconds, washed with water and blot dried or air dried before observing under a microscope at 100X magnifications to view the cells.

### 3.2.2 Biochemical tests

#### 3.2.2.1 Oxidase test

Strip of What man's No.1 filter paper was soaked in a freshly prepared 1% solution of tetramethyl-p-phenylene-diamine dihydrochloride. Then the paper was moistened with sterile distilled water laid in a petridish. The colony to be tested was picked with inoculating loop and smeared over the moist area. A positive result is indicated by color change from dark blue to maroon to almost black within 10-30 seconds. If the color change does not occur within 3 minutes, the result is negative.

#### 3.2.2.2 Citrate utilization

The Simmons citrate agar was autoclaved at 121 °C under 15 psi pressure for 15 minutes and cooled in slanted position (long slant, shallow butt). The medium will be a deep forest green due to the pH of the substrate and the bromothymol blue. A light inoculum picked from the center of a well-isolated colony was stabbed in butt and the slant was streaked back and forth. It was then incubated aerobically at 28°C for up to 4-7 days. The color change was observed from green to blue along the slant in case of positive result.

#### 3.2.2.3 Methyl Red – Voges-Proskauer (MR-VP) test

Prior to inoculation, medium was allowed to equilibrate to room temperature. Pure culture of test organism was inoculated in the medium. They were then incubated aerobically at 28°C for 48-72 hours.

For methyl red, 2 to 3 drops of methyl red indicator was added to the medium through the sides of culture tube. And the red color was observed immediately.

For Voges-Proskauer test, 6 drops of 5% alpha-naphthol was added, and mixed well to aerate. Then 2 drops of 40% potassium hydroxide was added, and mixed well to aerate. The tube was left undisturbed for 10-15 minutes. A pink-red color at the surface is observed.

### 3.2.3 Molecular characteristics: DNA Isolation and 16S rDNA Sequencing

DNA extraction of KH8 and LAf4 were done by TIANGEN DNA secure kit (www.tiangen.com/en). Universal 16s rRNA bacterial primers were used to amplify the gene from the isolated genomic DNA using the PCR program as mentioned in table 3.1. PCR products were visualized on a 1% agarose gel stained with ethidium bromide under UV light to confirm the band. The PCR products were cryopreserved and sent for sequencing in Xcelris Labs Limited, India.

**Table 3.1: PCR program**

| STEPS | TEMPERATURE (◦C) | TIME | CYCLE |
|---|---|---|---|
| Initial Denaturation | 95 | 2 mins | 1 |
| Denaturation | 95 | 30 sec | 35 |
| Annealing | 52 | 35 sec | |
| Extension | 72 | 60 sec | |
| Final Extension | 72 | 10 min | 1 |
| Hold | 4 | ∞ | |

## 3.3 Extraction of secondary metabolites

The putative Streptomycetes were cultured in 100ml ISP2 broth at 30°C for 7 to 10 days in a shaker at 150rpm for secondary metabolites production. After incubation, all the cultures were subjected to solvent extraction using ethyl acetate. Equal volume of 1:1 ratio of cultured broth and ethyl acetate were taken in a conical flask, agitated for about 30 minutes and separated with the help of separating funnel. The pellet was discarded and remaining solution was evaporated to dryness at 40°C under vacuum in rotavapour

for extract concentration. Then crude extract was dissolved in methanol and stored at 4°C until further use.

## 3.4 Antimicrobial Susceptibility Testing

### 3.4.1 Test organisms

The current critical and high prioritized pathogens by World Health Organization (WHO) against which research and development of antibiotics are pursued, have been the major focus of this study. The test organisms on the basis of WHO priority list are:

Priority: Critical

*Acinetobacter baumannii*, carbapenem-resistant

*Pseudomonas aeruginosa*, carbapenem-resistant

*Klebsiella pneumoniae*, carbapenem-resistant, ESBL-producing

Gram positive bacteria *Staphylococcus aureus* and Salmonella sp. under high priority by WHO were also taken for study. All the experiments were performed under aseptic conditions.

### 3.4.2 Disc Diffusion Assay

0.5 cm Whatman filter discs were sterilized and added with 20 µl of 15 mg/ml crude extracts of each samples. 100 µl of culture broth of test organisms maintained at McFarland 0.5 standard was spread on MHA plates with the help of glass spreader. The extract disks were added on the surface of MHA plates after about 30 minutes Incubation of inoculated AST plate. Four different extract discs and a negative control methanol were kept in each plate. The plates were then incubated at 37°C overnight (16-24 hrs.) in inverted position. After the incubation period, the diameters of zone of inhibition were measured with a ruler.

### 3.4.3 Resazurin Antimicrobial Assay

The test organism was grown overnight in a non-selective media Luria Bertani (LB) or MHB. Necessary dilutions were done on the next day to maintain McFarland 0.5 standard prior to inoculation. In a 96-well microtiter plates, 83 µl of culture along with 15 µl extract and 2 µl of 1% resazurin solution was kept in each well such that the final volume becomes 100 µl in each well and final resazurin concentration as 0.02%. The wells containing only resazurin and test organism without the addition of antimicrobials were considered negative controls for AST (no bacterial inhibition) and the wells containing only MHB broth and resazurin without the addition of bacteria were

considered as positive controls for AST. Both the controls were essential to validate the experiment. The plates were incubated at 37◦C and duration as per the test organisms.

The strength of the extracts was evaluated by calculating the percentage inhibiton of the test pathogens. This was done by the spectrophotometric analysis(551nm) and calculated using the formula as given below.

$$\% \text{ inhibition } = \frac{\text{O.D reading with test extract}}{(\text{Average of positive controls} - \text{Average of negative controls})} \text{ X } 100\ \%$$

## 3.5 Antioxidant test by DPPH assay

All the extracts were tested for antioxidant properties. 0.5mM of stock solution of DPPH (1, 1-diphenyl-2-picryl hydrazyl) was prepared in methanol. 80 µl DPPH was mixed with 20 µl of each extracts, well mixed and were incubated in dark at room temperature (RT) for 15 minutes. The colour change was observed from purple to yellow depicting the presence of antioxidants in the extracts and the absorbance was taken at 550 nm.

## 3.6 Purification

KH8 strain (one of the best antioxidants) isolated from the soil sample Khunargi, Rolpa was selected for solid state fermentation, extracted using ethyl acetate, concentrated using rotavapour and then taken for purification.

### 3.6.1 Bulk culture

Pure culture of isolate KH8 was used for the preparation of seed culture. One loopful of bacterial colony was inoculated in 50ml ISP2 broth and incubated for 3 days at 28°C and 150 rpm for seed culture. A fermenter tank (5 liters) was used for bulk culture in which 3 liters of ISP2 media was poured and autoclaved at 121°C and 15 lbs for 15 mins. Then the seed culture was transferred to the fermenter under aseptic conditions. The tank was maintained at 30°C under aerobic condition, pH maintained around 7 and agitated at 150 rpm for 10 days.

After fermentation, the culture broth was aseptically transferred to a sterilized bottle. The extraction was performed by taking equal volume of culture broth and ethyl acetate in separating funnel. The mixture was agitated for 10 minutes at 120 rpm and allowed to separate the layers in a separating funnel. The extraction process was repeated twice to extract any remaining metabolites from the culture broth. After separation, the organic (upper) phase was concentrated in a rotary evaporator. The extract weight was recorded and stored at 4°C for further experiments.

## 3.6.2 Chromatography

Chromatography was the first step used to purify the concentrated extracts from KH8 isolate.

Chromatography is mainly used for the separation or analysis of complex mixtures on the basis of their size, molecular weight, ionic mobility, polarity, etc. The separation of mixture in column chromatography is based mainly on differences between the solubilities of sample components in the mobile phase and stationary phase (Mallik *et al.*, 2016). When the mixture of mobile phase and sample to be separated are introduced to the column, the individual components of mixture move at different rates. The compounds which have lower affinity to stationary phase move faster and eluted out first whereas the compounds with greater affinity move slower and eluted accordingly. In this study, the compounds present in the extract eluted out last which indicated that the compound have higher affinity to Sephadex matrix. Sephadex-20 is used as it gives good separation and provides better means of purification of compounds and also stable in all solvents except acidic and oxidizing agents. It is prepared by hydroxypropylation of Sephadex G-25 to deliver a dextran gel with both hydrophilic and lipophilic properties (Murphy and D'Aux, 1975).

### 3.6.2.1 Thin Layer chromatography (TLC)

TLC of active extract was carried out on 0.2 mm thick silica gel coated aluminum plates.

For TLC analysis, the crude extract was dissolved in methanol in order to dilute the mixture. Then the plate was marked 1cm from both top and bottom. . 1 µl of extract was loaded in the silica gel plate at the center just above 1 cm line from bottom. The various solvent systems such as methanol, ethyl acetate, hexane, dichloromethane and benzene; and their mixtures at different concentrations were used to observe the spots under the UV absorption and thus the solvent optimization was done. The solvent optimization is very important prior to column chromatography in order to g*et* all good separation of the components in the mixture and also to know the polarity of our desired compound (antioxidant in this study) before the purification process. The combination of ethyl acetate and hexane in the ratio of 1:9 was used to distinguish the five different spots.

The mixture of solvent system was poured in development tank. Then the plate was placed in the tank and kept still. The solvent was allowed to run up to the mark at the top and again dried. Then the spots were visualized using ultraviolet light at 254 nm and marked. All the markings were done with a pencil to avoid the reaction with the solvent

if inked pen were used. DPPH reagent was added in each spot to check the anti-oxidant property. Then the RF value was calculated by using the formula:

$$Rf = \frac{distance\ from\ baseline\ travelled\ by\ solute}{distance\ from\ baseline\ travelled\ by\ solvent}$$

#### 3.6.2.2 Column chromatography

Sephadex LH 20 was used for column chromatography for initial purification of KH8 extract. 50 gm of Sephadex LH 20 was mixed with 200 ml of methanol for at least three hours (or overnight) at room temperature (RT) to make it swollen. The slurry was prepared by settled medium and solvent in the ratio of 3:1. The column was filled with slurry in a continuous manner without trapping air bubbles with the help of a glass rod if needed, and completely filled with a small space at the top for addition of methanol at later stages of the procedure. The crude exact was then dissolved in methanol and loaded onto the column. Fresh methanol was added to the top to begin the elution process. The colored bands were observed as the extracts were being eluded towards the bottom. Each fraction were collected sequentially and labelled for further analysis.

### 3.6.3 GCMS Analysis

Gas Chromatography-Mass Spectrometry (GC-MS) is one of the most accurate tools for analyzing the samples in which chemical mixtures (the GC component) are separated and the compounds are identified at molecular level (the MS component) (http://www.cpeo.org/techtree/ttdescript/msgc.htm). GC separates the mixture into individual volatile and semi-volatile compounds when heated. The heated gases are then carried out through the column with an inert gas such as helium. As the separated substances exit from the column, they flow into the MS where individual components are identified by the mass of the analyte molecules (Sneddon *et al.*, 2007).

The fraction which showed antioxidant positive and antimicrobial positive was analyzed through the GCMS for the identification of compounds responsible for the activity. The GC-MS was outsourced to Nepal academy of science and technology (NAST), Khumaltar.

## 3.7 Virtual screening

The lead proteins MetK and Dam were studied as the major antimicrobial targets for the drug discovery since these methyl transferases have been found to be essential to bacteria based on previous works.

### 3.7.1 Preparation of target proteins

The 3-D structures of target proteins were downloaded from rcsb.org in PDB file format. Crystal structure of a S-adenosylmethionine (SAM) synthase from *Neisseria gonorrhoeae* with bound S-adenosylmethionine (5T8S) (Dranow *et al.,* not published yet) and DNA adenine methyltransferase (Dam) from *Streptococcus pneumonia* complexed with SAM (2DPM) were retrieved from RCSB Protein Data Bank. The MetK proteins for other pathogens were retrieved from earlier works (Tha *et al.*, 2018).

The native ligand SAM was removed from the target proteins using Discovery Studio and the file was saved in pdb format. Then these proteins were prepared using Autodock vina in mgltools. At first, the molecule in pdb format was loaded. Then water molecules were removed to prevent hindrance during molecular docking, followed by addition of hydrogen and merging non- polar hydrogen atoms and finally Gasteiger charges were added. The file was then saved as pdbqt file format usable for molecular docking.

### 3.7.2 Ligand database preparation

The FDA approved ligands were selected as probable lead candidates that could be used as drugs since these are already in use for medical purposes and need not worry about failures in later clinical stages of development like other molecules. These were obtained from virtual database (ZINC15.docking.org) and saved as pdbqt files.

### 3.7.3 ADME/Tox Screening

In order to reduce the docking time and prevent possible future failures, all the selected ligands were ADME/Tox screened. The toxic profile and druglikeness of the screened ligands were predicted using OSIRIS data warrior software where druglikeness, mutagenicity and physicochemical properties of the ligands can be computed.

### 3.7.4 Docking by PyRx

PyRx, virtual screening software docked the selected ligand files against the target protein. First, macromolecule (target protein) and ligands were added. Upon continuation, a grid box would appear which is placed on the ligand binding site of the protein based upon the amino acid (AA) residues bonded with the binding site as retrieved from rcsb.org or 3D ligandsite computationally. The computed values show binding energies of all the docked ligands in the target proteins in KJ/mol. The more the negative value of energy with which the ligands bind, the stronger is the bond between ligand and active site. Thus, the negative energy values higher than that of the native ligand were searched for.

# 4. RESULTS AND DISCUSSION

Streptomycetes is the largest genus of Actinobacteria and produce two third of clinically useful bioactive compound 'antibiotics', used for treating infectious diseases. Due to emergence of multidrug resistant pathogens, there has been an increasing interest in the search for novel and effective antimicrobials from soil Streptomycetes in diversified ecological niche.

## 4.1 Sampling of Streptomycetes

Most of the Streptomycetes that have been explored are terrestrial. A wide range of Streptomycetes discovered in marine environments distinct from terrestrial species have currently increased the antimicrobials research towards marine species (Dharmaraj, 2010). However, due to the difficulty in retrieving marine soil, isolation of marine Streptomycetes have mostly been limited to marine sediments (Shaik *et al.*, 2017).

Nepal is an ecologically rich and geographically diverse place and a potential habitat for novel antimicrobials producers. Since it was built from the upliftment of sea bed (from marine to terrestrial) (Tenzing *et al.*, 2018), it could be a probable source for marine Streptomycetes species as well. So the focus in this study was to isolate both terrestrial and probable marine Streptomycetes species which could produce potent antimicrobials against the emerging pathogens.

Different 74 soil samples were collected and stored at CDBT-TU and among them, 48 soil samples were used for isolation of Streptomycetes, such that all the geographical regions with variable environmental conditions were covered with a view to isolate distinct strains. Sample collection was expanded to Panchase area at an altitude of 2500 meters, which is reported to have inhabited rare tree ferns, presuming that it could inhabit novel Streptomycetes strains with the desired metabolites producing capability. Matatirtha, a popular village in Kathmandu district for the availability of distinct edible as well as wild mushrooms, was also visited for soil sampling purpose. The saprophytic mushrooms inhabited soil could also be the source of different types of saprophytic Streptomycetes strains. Furthermore, sampling was also done from river bank and forest area with decayed leaves in Hetauda (345 meters altitude) and forest area in Shivapuri (1800 meters altitude).

## 4.2 Isolation and Characterization

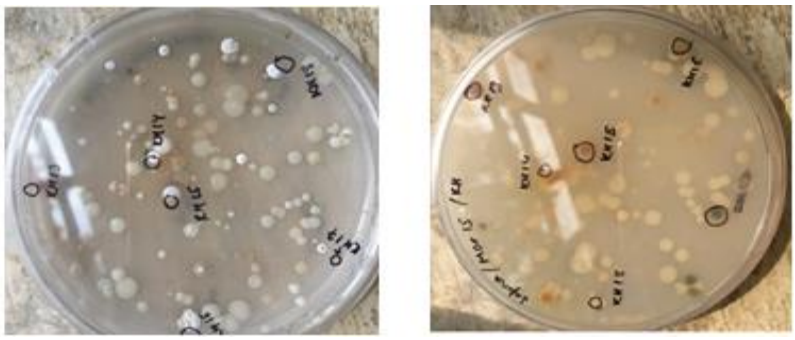


**Figure 4.1 Primary cultures of soil sample from Khunargi in ISP4 growth media Substrate mycelia at the right and Aerial mycelial growth at the left.**

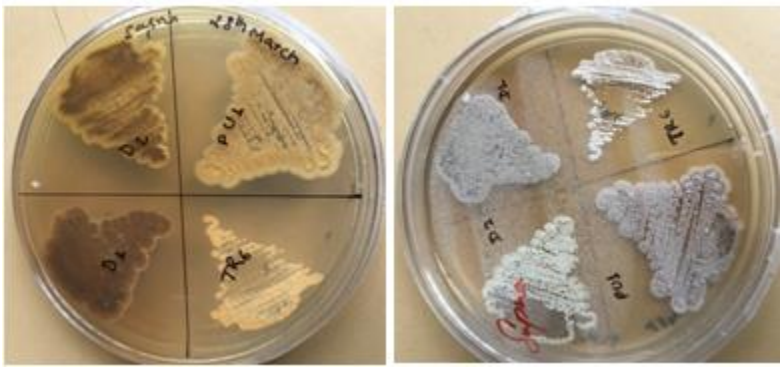


**Figure 4.2 Pure culture of 4 putative Streptomycetes isolates- Substrate mycelia at the left and Aerial mycelial growth at the right.**

Streptomycetes are gram positive, filamentous and slow growing bacteria. A total of 141 different types of putative Streptomycetes species were isolated from 48 different soil samples using ISP media for Streptomycetes (Shirling and Gottleib, 1996). All these isolates were grown on ISP2 and ISP4 culture media supplemented with 50 µg/ml of cycloheximide, an antifungal agent (Figure 4.1). After around 15 days of culture, the putative Streptomycetes based upon the morphology (chalky non-mucoid colonies with different colored aerial and substrate mycelia) were sub-cultured in new ISP plates (Figure 4.2) and the pure isolates were characterized based on the differences in their cultural and morphological characteristics (Table 4.1). Most strains possessed the earthy odor, one of the characteristic feature of Streptomycetes group (Gerber, 1967). However, because of their slow growing nature, contamination of various micro-organisms including bacteria and fungi were observed during incubation period. Media optimization could be done to make it more selective for Streptomycetes growth alone. Also, optimization during pretreatment of soil samples before culture could be done to reduce and/or stop bacterial contamination in the growth media used.

**Table 4.1 Cultural characteristics and morphological characteristics of the different color imparting Streptomycetes isolates**

| S. N. | Soil sample | Media used | Isolate code | Colour of substrate mycelium | Colour of aerial mycelium | Colony growth |
|---|---|---|---|---|---|---|
| 1 | Ghodagau (Rolpa) | ISP2 | GH2 | Orange | White | Moderate |
| 2 | Ghodagau | ISP2 | GH5 | Orange | Creamy | Moderate |
| 3 | Gorkha | ISP2 | GOb2 | Yellow | Pink | Abundant |
| 4 | Gorkha | ISP4 | GOb2 | Pink | Light pink | Abundant |
| 5 | Gorkha | ISP2 | GOb3 | Brown | Bluish | Abundant |
| 6 | Gorkha | ISP4 | GOb3 | Gray | Black | Moderate |
| 7 | Gorkha | ISP2 | GOb4 | Yellow | Gray | Abundant |
| 8 | Gorkha | ISP4 | GOb4 | Black | Gray | Abundant |
| 9 | Gorkha | ISP2 | GOb6 | Black | Blue | Abundant |
| 10 | Panchase | ISP2 | D1 | Yellow | White | Less |
| 11 | Panchase | ISP4 | D1 | Black | Gray | Moderate |
| 12 | Bhojpur | ISP2 | BHb3 | Yellow | Gray | Abundant |
| 13 | Bhojpur | ISP4 | BHb3 | White | Gray | Moderate |
| 14 | Nepalgunj | ISP2 | NEd3 | Dark orange | Creamy | Abundant |
| 15 | Nepalgunj | ISP4 | NEd3 | Yellow | White | Abundant |
| 16 | Lamjung | ISP2 | LAa1 | Yellow | White | Moderate |
| 17 | Lamjung | ISP2 | LAa5 | Dark pink | Gray | Abundant |
| 18 | Lamjung | ISP2 | LAa8 | Orange | Pink | Moderate |
| 19 | Lamjung | ISP2 | Lab9 | Brown | Gray | Abundant |
| 20 | Lamjung | ISP2 | LAd1 | Yellow | White | Abundant |
| 21 | Lamjung | ISP4 | LAd1 | Creamy | White | Moderate |
| 22 | Lamjung | ISP2 | LAe1 | Black | Gray | Abundant |
| 23 | Lamjung | ISP4 | LAe1 | Yellow | Gray | Less |
| 24 | Lamjung | ISP2 | LAe2 | Yellow | Creamy | Moderate |
| 25 | Lamjung | ISP2 | LAe3 | Brown | Gray | Abundant |
| 26 | Lamjung | ISP2 | LAe5 | Pink | Gray | Moderate |
| 27 | Lamjung | ISP2 | LAf4 | Black | Gray | Moderate |
| 28 | Lamjung | ISP4 | LAf4 | Brownish | White | Moderate |
| 29 | Ghorepani | ISP2 | GHO1 | Black | Gray | Less |
| 30 | Ghorepani | ISP4 | GHO1 | Black | Gray | Abundant |
| 31 | Kanchanpur | ISP2 | KA9 | Yellow | Gray | Less |
| 32 | Kanchanpur | ISP2 | KA11 | Yellow | White | Moderate |
| 33 | Khungri, Rolpa | ISP2 | KH8 | Brown | Gray | Abundant |

| | | | | | | |
|---|---|---|---|---|---|---|
| 34 | Khungri, Rolpa | ISP2 | KH17 | Brown | White | Moderate |
| 35 | Pidalna | ISP4 | PI3 | Gray | Grayish white | Abundant |
| 36 | Hatiya (Lamjung) | ISP2 | HA5 | Brown | White | Moderate |
| 37 | | ISP2 | PU1 | Yellow | Gray | Moderate |
| 38 | Gosaikunda | ISP2 | GK1 | Brown | White | Abundant |
| 39 | Mahendrapul Rolpa | ISP2 | MR1 | Yellow | White | Abundant |
| 40 | Mahendrapul Rolpa | ISP2 | MR2 | Yellow | Gray | Moderate |

Furthermore, Gram staining and methylene blue tests were performed for preliminary identification of the isolates (Figure 4.3). The isolated putative Streptomycetes strains were confirmed as gram positive by gram staining. Methylene blue test was performed to observe the short to long mycelia under 40X magnifications in inverted microscope. The gram negative and mycelia non-formers were ruled out from further experiments since Streptomycetes are Gram positive and mycelia formers (Manteca and Sanchez, 2009).

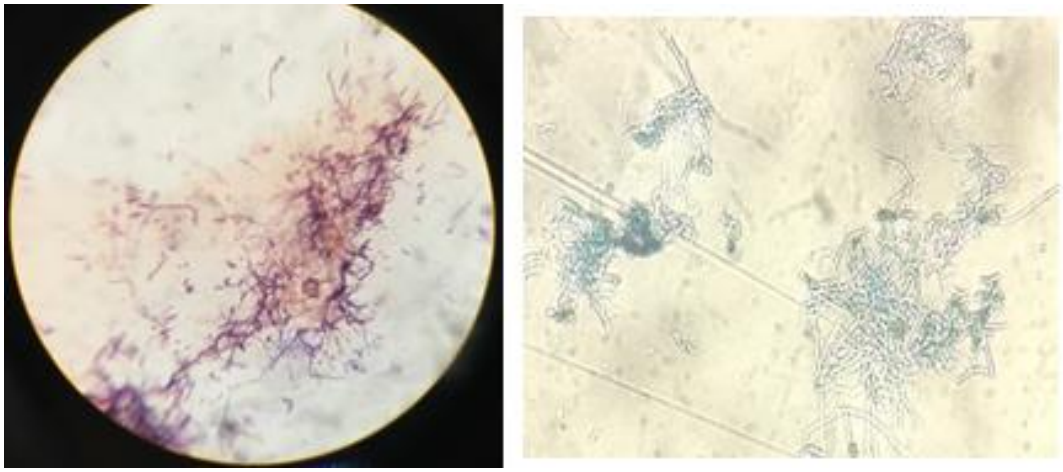


**Figure 4.3 Gram staining (left) and Methylene blue test (right) of isolated strain KH8**

### 4.2.1 Biochemical Tests

The biochemical tests were performed in some putative isolates of Streptomycetes (Table 4.2) that showed good results in our experiments, one being KH8 – the strongest antioxidant and LAf4 – the strongest and broad spectrum antimicrobial in this study. Both isolates showed positive methyl red, catalase test and casein test in ISP2 medium and negative result in citrate utilization test.

**Table 4.2 Biochemical tests of isolates KH8 and LAF4**

| Tests | KH8 | LAf4 |
|---|---|---|
| Gram staining | Gram positive | Gram positive |
| Methyl red | Positive | Positive |

| | | |
|---|---|---|
| Citrate utilization test | Negative | Negative |
| Catalase test | Positive | Positive |
| Casein test | Positive | Positive |

### 4.2.2 Molecular Identification of isolated strain LAf4

The putative *Streptomyces* LAf4 with the best antimicrobial potential in this study was taken for 16s rRNA sequencing. Upon comparing the determined sequences (first 312 bp) with other sequences of different *Streptomyces* species using BLAST tool of NCBI, it revealed high similarity with Uncultured *Streptomyces* sp. clone RSS38 (Accession number: KY796223). BLAST run against the sequences of all the organisms available in NCBI showed high similarity with *Stenotrophomonas sp.* which was not considered due to its structural dissimilarity with the isolated strain LAf4.

## 4.3 Screening for secondary metabolites

The morphologically look-alike isolates were ruled out from bioactive metabolites extraction. A total of 98 putative Streptomycetes strains were cultured in ISP2 broths for the extraction of secondary metabolites. The crude extracts were labeled and stored at 4°C for further use. The extraction of secondary metabolites was carried out using ethyl acetate as solvent and concentrated using rotavapour. Ethyl acetate was used due to its medium polarity which could extract both polar and non-polar biological compounds and also because of its minimum toxicity on test organisms (Wilbanks and Trinh, 2017). Furthermore, the boiling point of this solvent (77.1°C) is relatively lower than that of most other solvents and under vacuum, the bp drops to 9.1◦C. So this would be useful in extracting the heat sensitive metabolites without altering their properties.

## 4.4 Bioassay of the crude extracts

Bioassay was performed against various test organisms by disc diffusion method and qualitative resazurin antimicrobial assay. Some of the randomly selected crude extracts were randomly tested for the presence of antimicrobials against gram positive *Staphylococcous aureus* and gram negative *Pseudomonas aeruginosa* and *Klebsiella pneumonia* (K52), all available in the Streptomycetes laboratory at CDBT, TU.

### 4.4.1 Disc diffusion method

Clear zones of inhibition were observed around some disks after incubation with inoculated test organism (Figure 4.4), which indicates the presence of antibacterial activity. However, significant zones of inhibition were not observed upon testing with

other 20 crude extracts (Table 4.3). The lesser zones of inhibition could be due to the diffusion of the extracts into the agar plate thus reducing its concentration and efficacy or these extracts could have lesser and/or ineffective antimicrobials to begin with. Also, since disc diffusion assays require the diffusion of the extracts on the agar plate and agar being a polar substance, the presence of non-polar antimicrobials in these putative Streptomycetes extracts might not be detected using this method due to lack of diffusion which would give false results. All these demanded a robust antimicrobial screening assay mainly because of time and resource constraints to test the antimicrobial properties of all the 98 crude extracts.

**Table 4.3 Zones of inhibition of some extracts against the test pathogens**

| Isolate names | Zone of Inhibition (mm) | | |
|---|---|---|---|
| | *Pseudomonas aeruginosa* | *Klebsiella pneumonia* (K52) | *Staphylococcus aureus* |
| MR1 | 13 | - | - |
| GOc1 | 8 | - | - |
| GOc2 | 12 | - | - |
| Tl1 | 7 | - | - |
| GOa1 | - | 3 | - |
| KA2 | - | - | 3 |

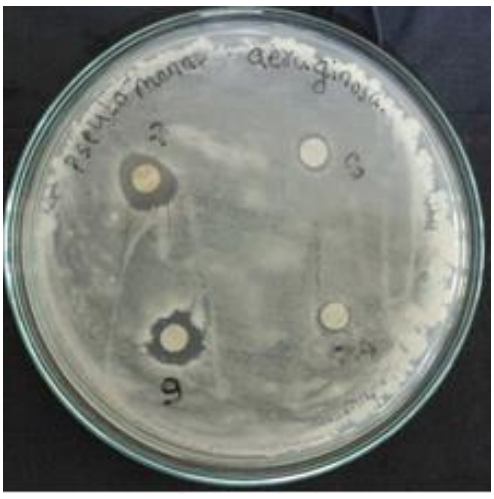


**Figure 4.4 Disc diffusion assay of some extracts against *Pseudomonas aeruginosa***

### 4.4.2 Resazurin antimicrobial assay

After the undesired disc diffusion assay results, the focus was mainly on the robust antimicrobial assay for faster and efficient screening of antimicrobials for which

resazurin assay developed by Drummond and Weigh in 2000 (Sarker *et al.*, 2007) has been modified.

Resazurin (7-hydroxy-10-oxidophenoxazin-10-ium-3-one) is a non-toxic, water soluble blue non- fluorescent dye which is converted into pink fluorescent resorufin (7-hydroxy-3H-phenoxazin-3-one) in the presence of viable cells with active metabolism (Riss *et al.*, 2013a). Reductases of viable cells reduce resazurin resulting in the formation of its highly fluorescent metabolic product resorufin (Scheme 1.0). This reduction is proportional to the number of metabolically active cells present (Driessche *et al.*, 2014).

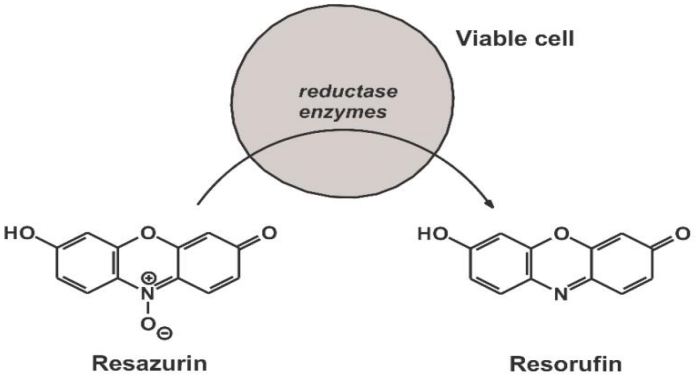



**Figure 4.5: Chemical structures of resazurin and resorufin (Csepregi *et al.*, 2018).**

The primary focus was in finding novel and effective Indole derivatives, promising scaffolds of drug development which have exhibited antibacterial, anticancer, anti-oxidants, anti-inflammatory, anti-diabetic, antiviral, antituberculosis activities and so on (Sravanthi and Manju, 2016). Indirubin and Isatin which are both the indole derivatives (Figure 4.6) have been reported as the major constituents of plant and microbial extracts with potent antimicrobial properties (Krivogorsky *et al.*, 2008; Al-Dhabi *et al.*, 2012; Fogaça *et al.*, 2017). Since these are both colored compounds, the primary focus was on the colored extracts with a view of screening novel Indirubin derivatives.

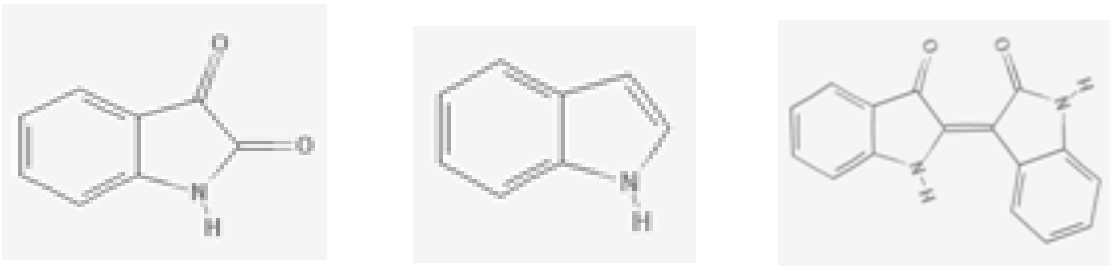



**Figure 4.6 2D structures of Indirubin, Indole and Isatin.**

Only the intense colored extracts were thus selected in the present study to test their antimicrobial properties against all the available test pathogens. The experiments were

designed to test the antimicrobial properties of the 20 mg/ml extracts, the final volume in each wells being 100 µl including extract, test organism and resazurin such that the final concentration of the extracts in each wells become 0.33 mg/ml. The effects of different concentrations of Ampicillin and Kanamycin were also tested against the test organisms, the concentrations being 50 mg/ml, 25 mg/ml, 12.5 mg/ml, 6.25 mg/ml, 3.125 mg/ml, 1.56 mg/ml, 0.781 mg/ml and 0.39 mg/ml. The positive antimicrobial assay was depicted by the retaining of the violet colour of resazurin after the test duration because it was not reduced indicating no cell growth while negative antimicrobial assay by the change of color to pink colored resorufin or non-colored hydroresorufin due to the reduction (Riss *et al.*, 2013b). The strengths of the antimicrobials in the extracts were studied based on the absorbance readings at 551nm. The tests with absorbance readings close to the negative controls (Test organism + Resazurin and no antimicrobials) were considered negative tests whereas those with absorbance readings close to the positive controls (Resazurin + Growth media and no bacteria) were considered positive tests. The strengths of the antimicrobials were measured accordingly.

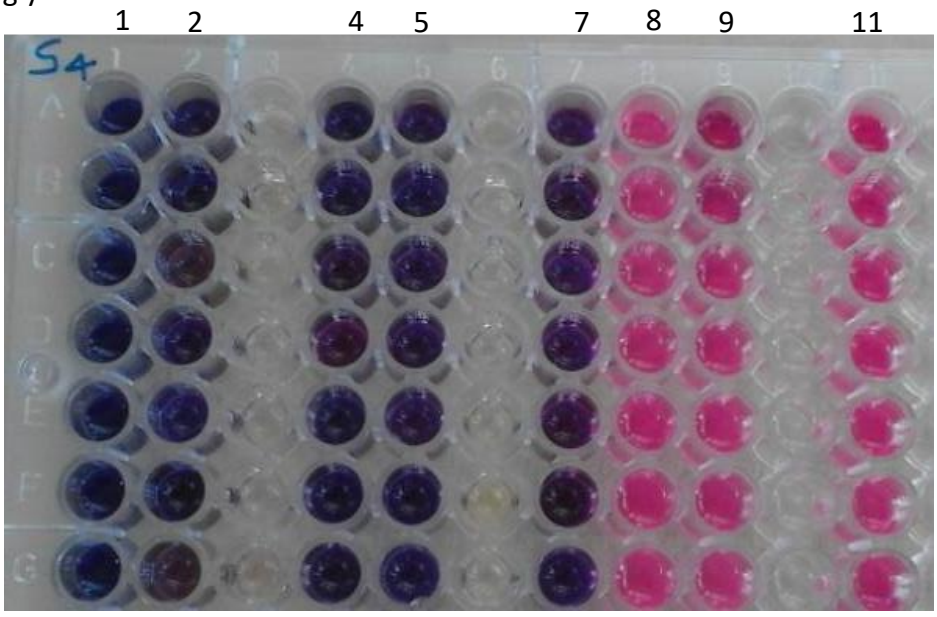



**Figure 4.7 Resazurin antimicrobial assay of the extracts against *Salmonella sp* S4**

Column 1- Positive Controls (2 µl Resazurin + 98 µl MHB)

Columns 2, 4, 5 and 7 – Tests (83 µl Test organism + 2 µl Resazurin + 15 µl extract)

Column 8 – Negative Controls (83 µl Test organism + 2 µl Resazurin + 15 µl MHB)

Column 9 –Tests with Ampicillin (50 mg/ml in 1$^{st}$ well and 1:2 dilutions to respective wells)

Column 11- Tests with Kanamycin (50 mg/ml in 1st well and 1:2 dilutions in the respective wells)

Among the 16 extracts (Appendix 8.4) tested for their antimicrobial properties against *Klebsiella pneumonia* K52, total of 13 extracts showed good antimicrobial properties among which extracts LAf4, GH5, KH6, LAd1, and LAe5 showed strong antimicrobial properties evaluated based on the spectrophotometric analysis (Appendix 8.5). The resazurin antimicrobial assays were further expanded to *Salmonella spp* S4 (Figure 4.7), *Pseudomonas aeruginosa* P39 and *Acinetobacter baumanii* Ab. The extracts LAf4, LAd1, LAe5 and HA5 showed better antibacterial properties against all these test pathogens (Figure 4.8) meaning these could have broad-spectrum antimicrobials in them. Also, KH8 showed the least antibacterial potential against all these four test pathogens. Similarly, neither Ampicillin nor Kanamycin at different concentrations showed any antimicrobial activity against these test pathogens. All the experiments were performed in triplicates at different working days thus further validating the consistency of the results obtained.

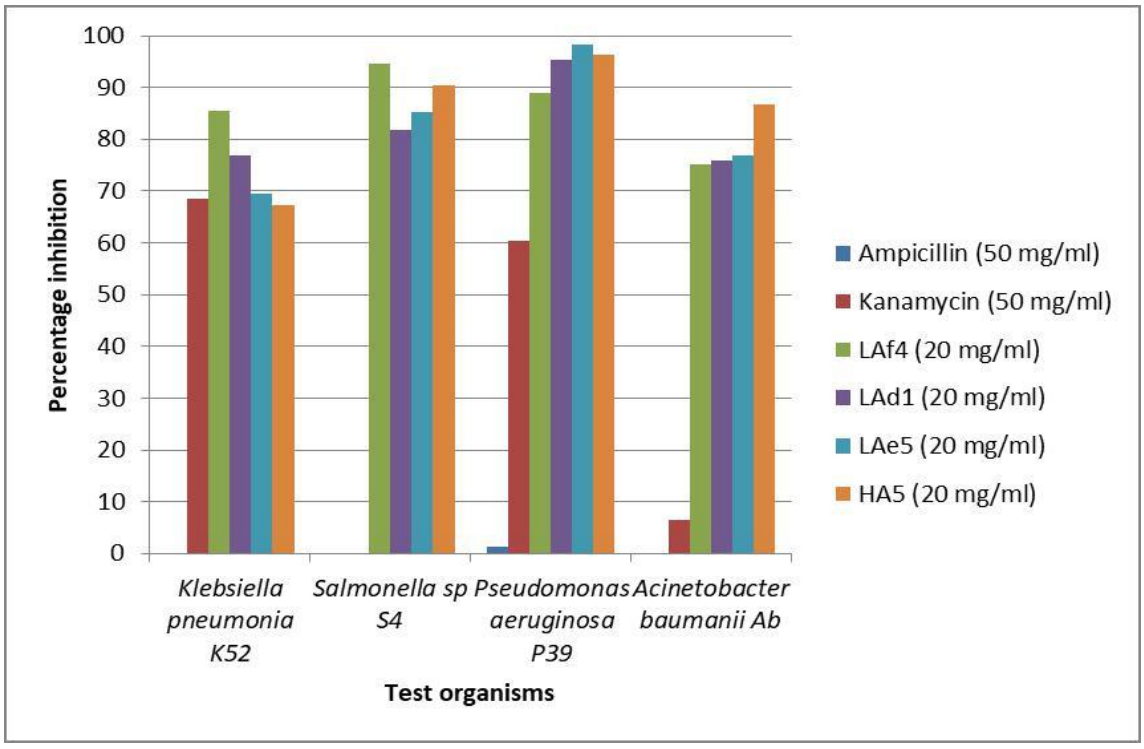



**Figure 4.8 Resazurin antimicrobial assay of four extracts against *Klebsiella pneumonia* K52, *Salmonella spp* S4, *Pseudomonas aeruginosa* P39 and *Acinetobacter baumanii* Ab**

This method depends on quick recognition of color change from blue color of resazurin (no bacterial growth) to pink color of resorufin (bacterial growth) indicating no inhibition. In the present study, this method proved to be rapid, sensitive and showed better results than in disc diffusion agar method. This showed to be a reliable method

for detection of antimicrobial secondary metabolites in the extracts even at low concentration. Moreover, results were easily distinguished by reading the color change from blue to stable pink color. The bacterial overgrowth would change the pink color of resorufin to non-colored hydroresorufin due to further reduction.

## 4.5 Antioxidant assay by testing DPPH free radical scavenging activity

In the present study, the qualitative antioxidant assay was done for all 98 crude extracts based upon the change in deep violet color of DPPH to colorless or pale yellow when neutralized. Among these, 54 crude extracts of different isolates showed positive DPPH scavenging activities (Figure 4.9). The crude extracts of two isolates NEd3 and KH8 showed the strongest antioxidants. The results were noticeably visible as a change in color from purple to yellow. Based on the time taken for color change (the faster the color change, the stronger is the antioxidants present in the extracts), and the intensity of the yellow color depicting radical scavenging activity, the extracts KH8 and NEd3 were considered the best among all the antioxidants. Also, the AST results depict KH8 as the extract with the least antimicrobial potential which could be because of the mitigation of the ROS generated to kill the bacteria by the presence of stronger antioxidants in the extract (Van Acker and Coenye, 2017). Thus, KH8 strain was selected for further evaluation of its antioxidant properties.

**Figure 4.9 DPPH assay with yellow colour indicating the positive antioxidant tests**

## 4.6 Fermentation and Purification of KH8 strains metabolites

The large scale fermentation of putative Streptomycetes strain KH8 was performed using ISP2 broth medium on a fermenter for 7 days maintaining pH at 7.0, 200 rpm agitation and proper aeration throughout the incubation period. A total of 2.1 gm crude extract was collected from 12 liters of culture broth after harvesting, cell separation, solvent extraction and subsequent concentration using rotavapour at 38°C.

### 4.6.1 Chromatography:

Chromatography is an analytical approach, used for the separation of mixture of chemical substances into its analytes so that the analysis of each analyte could be done accordingly. In present study, thin layer and column chromatography were performed with a view to purify the extracts isolated from putative Streptomycetes KH8 strain.

#### 4.6.1.1 Thin Layer Chromatography

The concentrated crude extract was studied by preparative thin layer chromatography (TLC) and purified by column chromatography using liphophilic sephadex (LH-20).

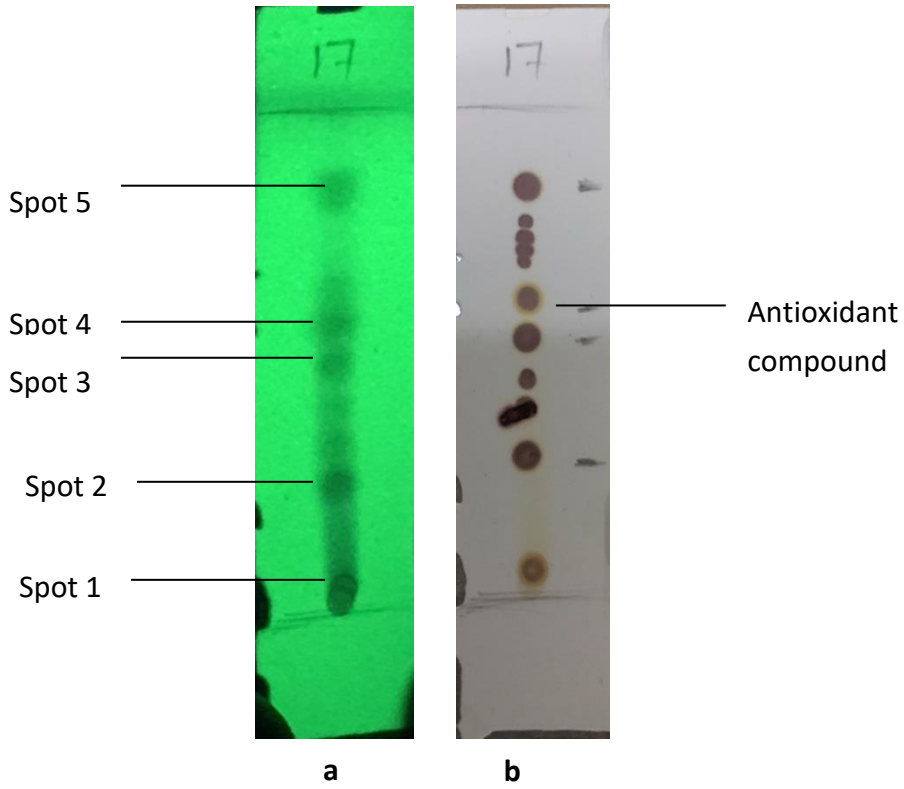



**Figure 4.10 a) Visualization of different spots on TLC plate under UV light**

**b) DPPH assay on the TLC plate**

The observed spots were marked in the developed TLC plates and were tested for their antioxidant potential by addition of one drop of DPPH reagent onto the marked spots. The fourth spot showed the antioxidant activity exhibited by the color change of the added DPPH from purple to light yellow (Figure 4.10b). The Rf value was found to be 0.61.

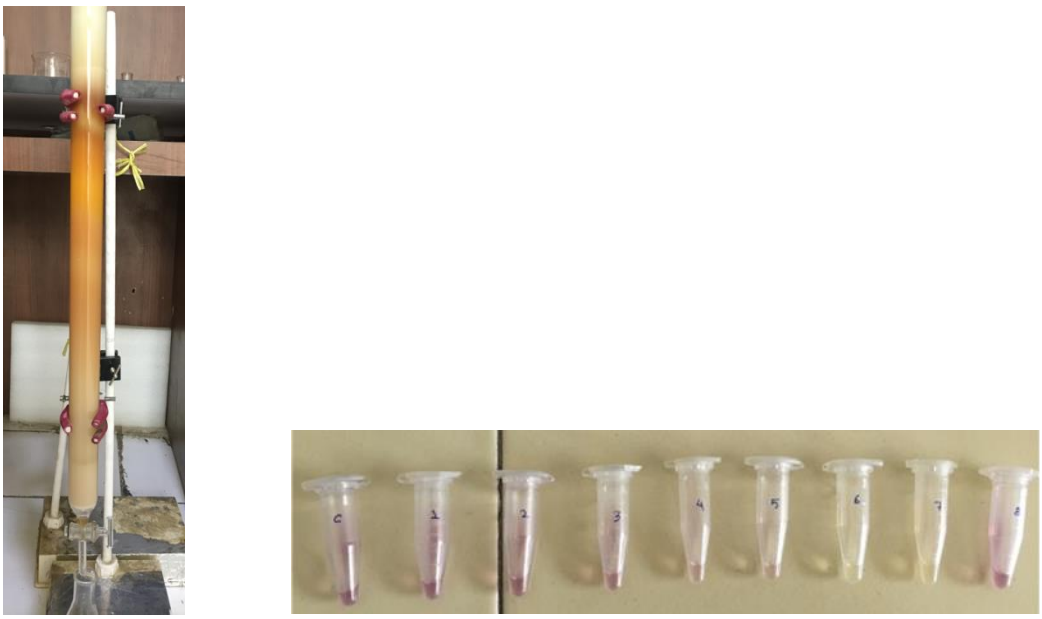
By the analysis of TLC, the properties of compound seemed to be non-polar in nature. The compound tends to be more soluble in solvent phase as it interacted weakly with

the polar absorbent (silica) on the TLC plate and had risen up faster with less polar solvent.

#### 4.6.1.2 Column chromatography

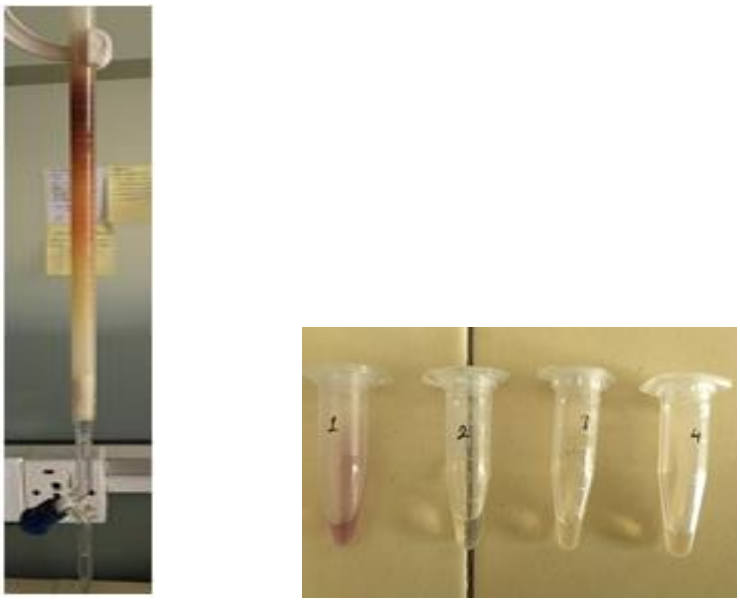
The crude extract of strain KH8 was then subjected to column chromatography on Sephadex LH-20 using methanol, a polar solvent due to its availability and methanol being economic compared to other solvents. This would mean the desired antioxidant which is non-polar, would be recovered at the latter phases of separation since the extracting solvent is polar and the desired compound is non-polar i.e.; polar compounds get eluted out first.

At the earlier phase of column chromatography, five different bands were observed. As the extract moved along with the addition of solvent, a total of seven different bands were observed and collected accordingly. Only the $6^{th}$ and $7^{th}$ fractions of the extract showed the antioxidant property upon performing DPPH radical scavenging assay. These two fractions were mixed and upon running TLC for the resulting mixture, four different spots were observed so column chromatography was run for the second time. As seen in TLC, four separate spots were observed and collected in different vessels among which $3^{rd}$ fraction again showed strongest antioxidant activity. Each of these were concentrated, weighed and stored at 4°C until further use.



**a** **b**

**Figure 4.11 a) First phase of Column chromatography with seven different bands**
**b) DPPH assay for seven aliquots of the extract.**



a b

**Figure 4.12 a) Second phase of Column chromatography with four different bands**
**b) DPPH assay for four aliquots of the extract.**

Thus obtained individual extracts were then subjected for GCMS analysis to know the types of molecules/functional groups present in the mixture of compounds.

### 4.6.2 GC-MS analysis

In this study, GC-MS technique was carried out in NAST for two extracts LAf4 and KH8 due to their potential activities to investigate the types of compounds there are, in the extracts (Figure 4.13 )for literature mining to narrow down the compound(s) that could potentially be responsible for the activity shown in AST and antioxidant assay, respectively (Table 4.4 and Table 4.5).

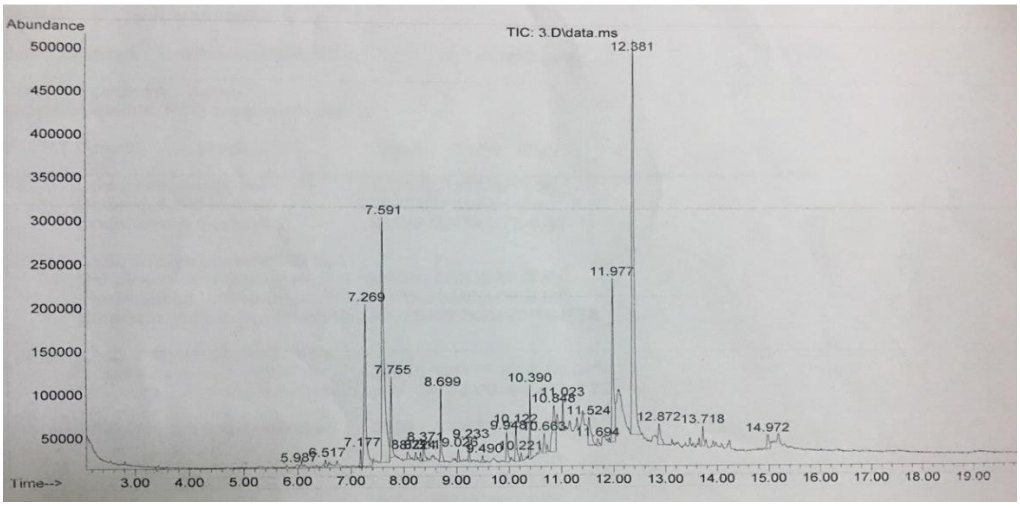



**Figure 4.13 GCMS spectra of isolate KH8**

The retention time indicates the time spent by the compounds on the column indicating size differences of the compounds. The area of a peak is proportional to the amount of the compound present in the sample. Quality measures indicate the probable match of the spectrum of unknown compounds in the sample with the spectrum library compounds. This data allows for optimization of solvent for further extraction of respective componds. The higher the quality, the better is the chances of reducing in the extraction of false compounds. Only those with 90 % and above quality scores were emphasized in this study. In addition, the numbers of probable compounds reported for the extracts were found to possess some antimicrobial and antioxidant activities by literature mining (respective references in table 4.4 and 4.5).

**Table 4.4 GC-MS analysis results of crude extract LAf4**

| **Compound name** | **Retention time (min)** | **Area (%)** | **Quality (%)** | **Activity** | **Reference** |
|---|---|---|---|---|---|
| 2,3-butanediol | 3.025 | 30.61 | 91 | Antibacterial and Antiseptic | (Ragasa *et al.*, 2004) |
| Naphthalene | 9.566 | 0.42 | 96 | Antibacterial | (Rokade and Sayyed, 2009) |
| 2,4-Di-tert-butylphenol | 10.39 | 1.07 | 95 | Antioxidant<br>Quorum sensing inhibitor | (Yoon *et al.*, 2006)<br>(Padmavathi *et al.*, 2014) |
| Tributyl phosphate | 11.476 | 2.82 | 87 | Antimicrobial | (Woolford, 1984) |
| Pyrrolo (1, 2-a) pyrazine -1, 4-dione, hexahydro-3-(2-methylpropyl) | 13.587 | 5.32 | 94 | Anticancer activity<br>Antioxidant | (Lalitha *et al.*, 2016)<br>(Ser *et al.*, 2015) |

**Table 4.5 GC-MS analysis results of crude extract KH8**

| SN | Compound name | Retention time (min) | Area (%) | Quality (%) | Activity | Reference |
|---|---|---|---|---|---|---|
| 1 | Benzoic acid | 7.269 | 10.50 | 95 | Antioxidant | (Velika and Kron, 2012) |
| 2 | 1,2-Benzenediol | 7.591 | 20.12 | 95 | Antioxidant | (Venkateswarlu *et al.*, 2003) |
| 3 | 3-methoxy-1,2-benzenediol | 8.224 | 0.34 | 92 | Antioxidant | (Mathew *et al.*, 2015) |
| 4 | 2-Methoxy-4-vinylphenol | 8.699 | 2.63 | 90 | Antioxidant | (Fukai *et al.*, 2009) |
| 5 | 2,6-dimethoxy phenol | 9.026 | 0.49 | 91 | Antioxidant | (Madeira *et al.*, 2011) |
| 6. | Methyl paraben | 9.948 | 1.65 | 91 | Antioxidant, used as preservatives in food, cosmetics and pharmaceuticals | (Lee *et al.*, 2006) |
| 7. | 1,3-dihydro-2H-indol-2-one | 10.122 | 1.88 | 93 | Antioxidant | (Rindhe *et al.*, 2011) |
| 8. | 4(1H)-Quinazolinone | 11.977 | 9.19 | 96 | Antioxidant | (Asif, 2014) |

## 4.7 Computational tools

Emerging and spreading antimicrobial resistance has become the global threat to public health (Senok *et al.*, 2012). So there is an urge for the new antibiotic development to prevent and treat the bacterial infections. Drug discovery from the traditional methods is very time consuming and expensive process and there is chances of failure of new drugs in various phases of clinical trials (Norris Posey *et al.*, 2014). So, virtual screening or virtual high throughput screening becomes an effective tool (Polgar and M. Keseru, 2011) for the fulfilment of new potential antimicrobial drugs, offering drugs at a lower time, cost and manpower (Sawyer *et al.*, 2003; Singh *et al.*, 2003).

### 4.7.1 Molecular docking

#### 4.7.1.1 Drug target selection

S-adenosylmethionine synthase (*metK*) and DNA adenosine methyltransferase (*dam*) were used as potential drug targets in this study for *in silico* screening. Based on earlier works, *metK* and *dam* were found to be essential genes in bacteria. As it is known *metK* gives the product S-adenosylmethionine (SAM) by catalyzing methionine and ATP (http://www.uniprot.org/uniprot/P0A817) and SAM is found in every living cells and acts as methyl group donor for methylation reactions (Fontecave, 2011; Loenen, 2010). In addition, it is a precursor molecule for aminopropylation pathway leading to synthesis of polyamines and for transulfuration pathway leading to synthesis of glutathione (Bottiglieri, 2002).

Similarly, *dam* methylates N6 position of adenine at all GATC sequences in bacteria (Messer and Noyer-Weidner, 1988) whereas DNMT1 (DNA cytosine-5 methyltransferase) methylates at N5 of cytosine at CpG islands in humans (Jair *et al.*, 2006). The dissimilarity in *dam* genes in pathogenic bacteria and humans make it an even better target. Since *dam* plays a vital role in bacterial processes such as timing of DNA replication (Boye and Løbner-Olesen, 1990), mismatch repairs (Schlagman *et al*, 1986), segregation of chromosomal DNA, and transposition (Guyot *et al*, 1993). All the methylase use SAM as a methyl donor (Low *et al.*, 2001). Thus interference in *metK*, a SAM producer and *dam* gene which uses SAM as methyl donor; could be used to block the DNA replication and mRNA transcription which could consequently be lethal to bacterial survival.

#### 4.7.1.2 Ligand database and protein target preparation

FDA approved drugs were selected as the ligands of choice for this study. Since these have already been approved by FDA, it can be presumed that they could be used for

human application with lesser side effects. Thus, their potential implications against newer emerging resistance pathogens could be explored, which are less likely to fail clinical trials if applicable against the target pathogens. As FDA approved drugs can be readily used for therapeutic purposes and due to the dire necessity of finding new drugs against emerging MDR pathogens, repurposing of these ligands was a major objective in this study. A database of 1355 purchasable ligands from a total of 1379 FDA approved drugs (DrugBank) were downloaded from ZINC database (http://zinc15.docking.org/substances/subsets/FDA/). Ligand and protein preparations were done as mentioned in earlier works.

#### 4.7.1.3 Virtual Screening of FDA approved drugs

For virtual screening of the ligands, the binding energy of the natural native ligand was determined by docking the ligand in the active site. Docking of native ligand into its binding site of the corresponding protein was carried out as a first step to know the energy with which the native ligand binds to its target.

Although these are FDA approved drugs but new parameters have emerged in drug discovery to make the drug candidates more efficient and prevent failure even after the use. Prior to docking, carrying ADMET tests and accessing pharmacokinetic parameters of the ligands would support in narrowing down the prospective ligands to further screen as putative drug candidate. Most of the drug candidates have failed due to the problem with metabolism and toxicity. The essential features of drugs are good absorption, distribution, metabolism, excretion and minimal toxicity. So, all candidates were analyzed using ORISIS program (Sander *et al*, 2015) for their druglikeness and pharmacokinetics properties using a list of parameters (Table 4.6), including Lipinski's rule of five.

**Table 4.6 Parameters used to evaluate the druglikeness of the ligands.**

| | |
|---|---|
| Molecular weight | 200 to 500 Daltons |
| cLogP | -3 to 6 |
| cLogS | -4 to -2 |
| Hydrogen bond donors | 0 to 5 |
| Hydrogen bond acceptors | 0 to 1 |
| Topological Polar Surface Area | 0 to 140 |

ORISIS calculates the essential features of drugs like molecular weight, cLogP, cLogS, druglikeness and toxicities like mutagenicity, tumorigenicity, reproductive effects and irritant effects. Many of these ligands, on the basis of these parameters failed as drug candidates. Some showed negative druglikeness values. Some showed high liphohilicity

though having positive druglikeness score. High cLogP value means the drug cannot be absorbed properly.

Out of 1379 ligands, 337 passed all the filters used. Only 121 ligands out of 337 had pdbqt files required for docking in ZINC database and so were first taken for virtual screening against MetK of *Neisseria gonorrhea* (Figure 4.14). Docking was carried out using Autodock vina in Pyrx interface for the selected ligands against SAM binding pocket.

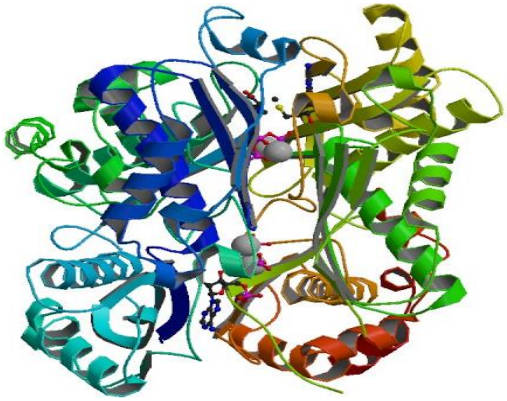


**Figure 4.14 Crystal structure of S-adenosylmethionine Synthase (MetK) from *Neisseria gonorrhoea* (https://www.rcsb.org/structure/5t8s)**

Upon docking with MetK of *N. gonorrhoea* (Figure 4.14), 24 out of 121 ligands exhibited higher B.E than native ligand SAM of MetK (Table 4.7). The higher the negative value of binding energy, the stronger is the ligand binding in the protein (Du *et al.*, 2016). The native ligand SAM was bound to the binding site of MetK in *N. gonorrhoea* with a B.E of -9.1 Kcal/mol. All the ligands with B.E stronger than SAM in MetK were considered MetK inhibitors. In this study, only B.E lesser and equal to -9.5 was taken to ensure the selected ligands to be strong MetK inhibitors.

#### 4.7.1.4 Cross reactivity to other eight pathogens

These 24 ligands were further docked with computationally modeled MetK structures of all other eight pathogens (Figure 4.15 and Appendix 8.6). Out of 24 ligands, only two ligands i.e. ZINC00009073 and ZINC00538550 showed promising results with higher binding energy than native ligand of MetK proteins in all nine of the pathogens (Table 4.7). These two drug candidates could be used as potential inhibitors of MetK activity in all pathogens.

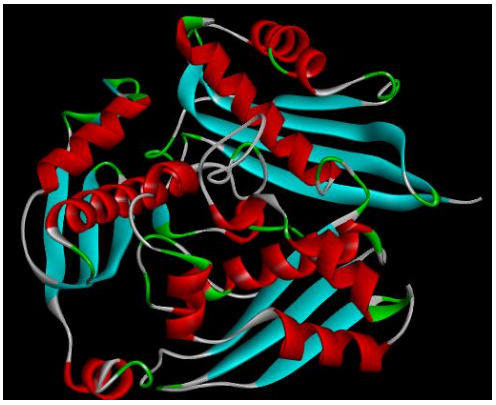


**Figure 4.15 MetK of *Pseudomonas aeruginosa* as given by Phyre2**

**Table 4.7 Respective binding energies (Kcal/mol) of the ligands in MetK of the target pathogens**

| Ligand | *N. gonorrhoea* | *H. pylori* | *P. aeruginosa* | *S. aureus* | *S. typhi* | *K. pneumoniae* | *A. baumanii* | *C. coli* | *E. faecium* |
|---|---|---|---|---|---|---|---|---|---|
| SAM | -9.1 | -5.6 | -6.6 | -6.1 | -5.6 | -6 | -5.9 | -7.1 | -6.2 |
| ZINC00000416 | -10.3 | -4.6 | -6.1 | -5.8 | -6.8 | -6.6 | -6.5 | -7.8 | -6.2 |
| ZINC00000596 | -9.7 | -4.8 | -6.5 | -5.9 | -6.2 | -4.9 | -5.7 | -6.8 | -6.5 |
| ZINC00000850 | -10 | -5 | -5.9 | -6.2 | -5.7 | -5.1 | -5.1 | -7.2 | -5.8 |
| ZINC00000856 | -9.6 | -5.2 | -6.6 | -5.1 | -6.1 | -6.8 | -6.2 | -7.4 | -6.6 |
| ZINC00003911 | -10.1 | -5.5 | -6 | -6.7 | -6.2 | -6.6 | -5.7 | -7.2 | -6.1 |
| ZINC00004319 | -10.1 | -5.7 | -6.3 | -5.8 | -6.8 | -6.9 | -6.6 | -7.6 | -6.6 |
| ZINC00005823 | -11.1 | -5.4 | -6.8 | -7.3 | -6.5 | -7 | -5.8 | -7.7 | -6.7 |
| **ZINC00009073** | -10.3 | -5.7 | -7.6 | -7 | -6.9 | -7.2 | -6.3 | -8.4 | -7.1 |
| ZINC00018635 | -9.5 | -5.3 | -6.4 | -6.6 | -6 | -6.8 | -5.9 | -7.1 | -6.1 |
| ZINC00020240 | -9.7 | -4.9 | -5.8 | -5.7 | -5.4 | -6.6 | -5.2 | -6.8 | -6.6 |
| ZINC00020251 | -10.1 | -5.3 | -6.8 | -5.9 | -5.9 | -5.1 | -5.4 | -7 | -6.3 |
| ZINC00056556 | -9.5 | -5 | -6 | -6.5 | -5.3 | -6.3 | -5.6 | -6.9 | -6.7 |
| ZINC00057253 | -9.6 | -4.5 | -6 | -6.5 | -5.5 | -6.2 | -4.8 | -6.3 | -5.9 |
| ZINC00057278 | -9.9 | -5.5 | -6.6 | -5.3 | -5.6 | -6.4 | -5.9 | -7.3 | -6.5 |
| ZINC00403010 | -10.2 | -5.9 | -6.2 | -5.8 | -6.6 | -6.9 | -6.3 | -7.6 | -6.5 |
| ZINC00403011 | -10.4 | -5 | -6.1 | -5.5 | -6.5 | -6.9 | -5.7 | -7.7 | -6.2 |
| **ZINC00538550** | -10.2 | -6.2 | -7.1 | -7.2 | -7.2 | -6.9 | -7.3 | -8.2 | -7.5 |
| ZINC00601281 | -9.9 | -5.1 | -6.4 | -5.8 | -6.1 | -5.6 | -5.7 | -6.6 | -5.9 |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| ZINC00897256 | -9.6 | -5.3 | -6.5 | -5.1 | -6.1 | -6.8 | -6.1 | -7.5 | -6.2 |
| ZINC00897258 | -9.7 | -5.6 | -5.8 | -5.2 | -5.7 | -6.5 | -5.6 | -7.3 | -6.2 |
| ZINC01530947 | -10.5 | -4.9 | -6.8 | -7.2 | -6.4 | -5.7 | -5.9 | -7.8 | -7.1 |
| ZINC01530948 | -10.3 | -5.6 | -6.6 | -6 | -6.5 | -5.6 | -5.6 | -7.9 | -7 |
| ZINC02599970 | -9.8 | -5.9 | -6.2 | -5.8 | -6.3 | -6.5 | -6.5 | -7.5 | -6.2 |
| ZINC03830339 | -9.6 | -5.3 | -6.3 | -5.6 | -5.7 | -6.8 | -5.8 | -6.8 | -6.3 |

#### 4.7.1.5 Cross reactivity with Dam, DNA adenosyl methyltransferase

In bacteria, Dam is responsible for DNA methylation and the main function of DNA methylation is to protect the cell from the effect of foreign DNA introduction as a defense mechanism. It means that restriction endonucleases distinguishes the endogenous and foreign DNA by its methylated pattern and eliminates the foreign DNA by cleavage as it is not protected by methylation (Noyer-Weidner and Trautner, 1993). Dam was chosen as target protein as it functions in many essential cellular functions such as DNA replication which are crucial for bacterial survival.

The narrowed down two ligands were docked against Dam protein which showed higher binding energy than SAM (Table 4.8). So these could be used to inhibit the activity of *dam* gene.

**Table 4.8 Binding energies of screened ligands from MetK docking in Dam protein of *Streptococcus pneumonia***

| Ligands | Binding Energy (kcal/mol) |
|---|---|
| SAM | -9 |
| **ZINC00009073** | -9.7 |
| **ZINC00538550** | -9.8 |

So, these two were further docked against the human homologue of MetK protein.

Binding energies of these two lead FDA approved ligands along with SAM with S-adenosylmethionine synthetase isoform type-1 (RCSB id: 2OBV) and S-adenosylmethionine synthetase isoform type-2 (RCSB id: 4KTT) in humans were carried similar to that of bacteria (Table 4.9). Both the ligands exhibited greater binding energies than the native ligand SAM meaning these could inhibit SAM producers in humans as well. However, SAM can be supplemented from external source since human have SAM transporters (Agrimi *et al.*, 2004) unlike bacteria which is not yet reported to have external SAM transporters.

**Table 4.9 Binding energies of the screened ligands from MetK and Dam protein docking in human homologue 1 and human homologue 2 of MetK**

| Ligands | Binding Energy (kcal/mol) of ligands in human homologue 1 of MetK | Binding Energy (kcal/mol) of ligands in human homologue 2 of MetK |
|---|---|---|
| SAM | -6.3 | -8.2 |
| **ZINC00009073** | -7.7 | -10.2 |
| **ZINC00538550** | -8.1 | -8.6 |

One of the ligands, **ZINC00009073**, Apomorphine hydrochloride (Figure 4.16a), is a powerful emetic and used during acute poisoning. The drug is a potent antioxidant and free radical scavenger which might have neuroprotective effects (Boyle and Ondo, 2015). This drug is used for the treatment of Parkinson's disease. It is also used for erectile dysfunction treatment. Similarly, another ligand **ZINC00538550**, Ziprasidone hydrochloride (Figure 4.16b), has been used for schizophrenia treatment (Mattei *et al.*, 2011).

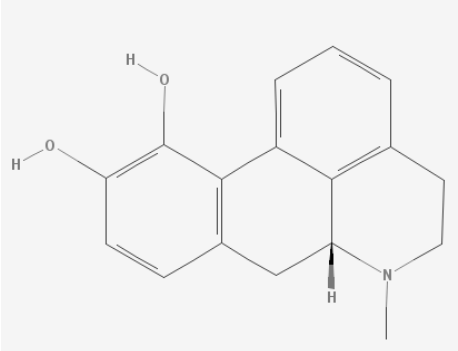



**Figure 4.16a Structure of ZINC00009073 (Apomorphine hydrochloride)**

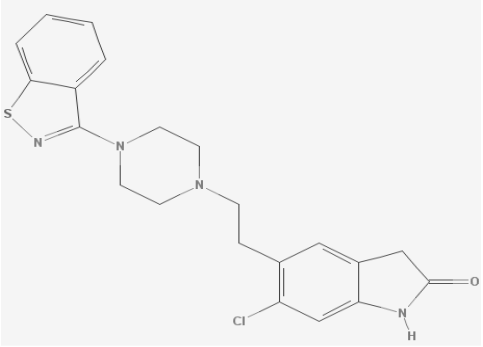



**Figure 4.16b Structure of ZINC00538550 (Ziprasidone hydrochloride)**

#### 4.7.1.6 Molecular docking against the resistance causing Carbapenemases

Several types of β-lactam containing antibiotics have been developed based on the lactam ring (Holten and Onusko, 2000). Among them, carbapenem groups (Figure 4.17) are taken as potential drugs to treat resistance to earlier lactam containing antibiotics (Hawkey and Livermore, 2012) like penicillin, cephalosporins. However, acquisition of carbapenemases, beta-lactamases having flexible hydrolytic abilities can hydrolyze a variety of antibiotics including penicillins, cephalosporins, monobactams and even highly effective carbapenems (Queenan and Bush, 2007) which is now considered one of the major challenges of managing infections by resistance pathogens.

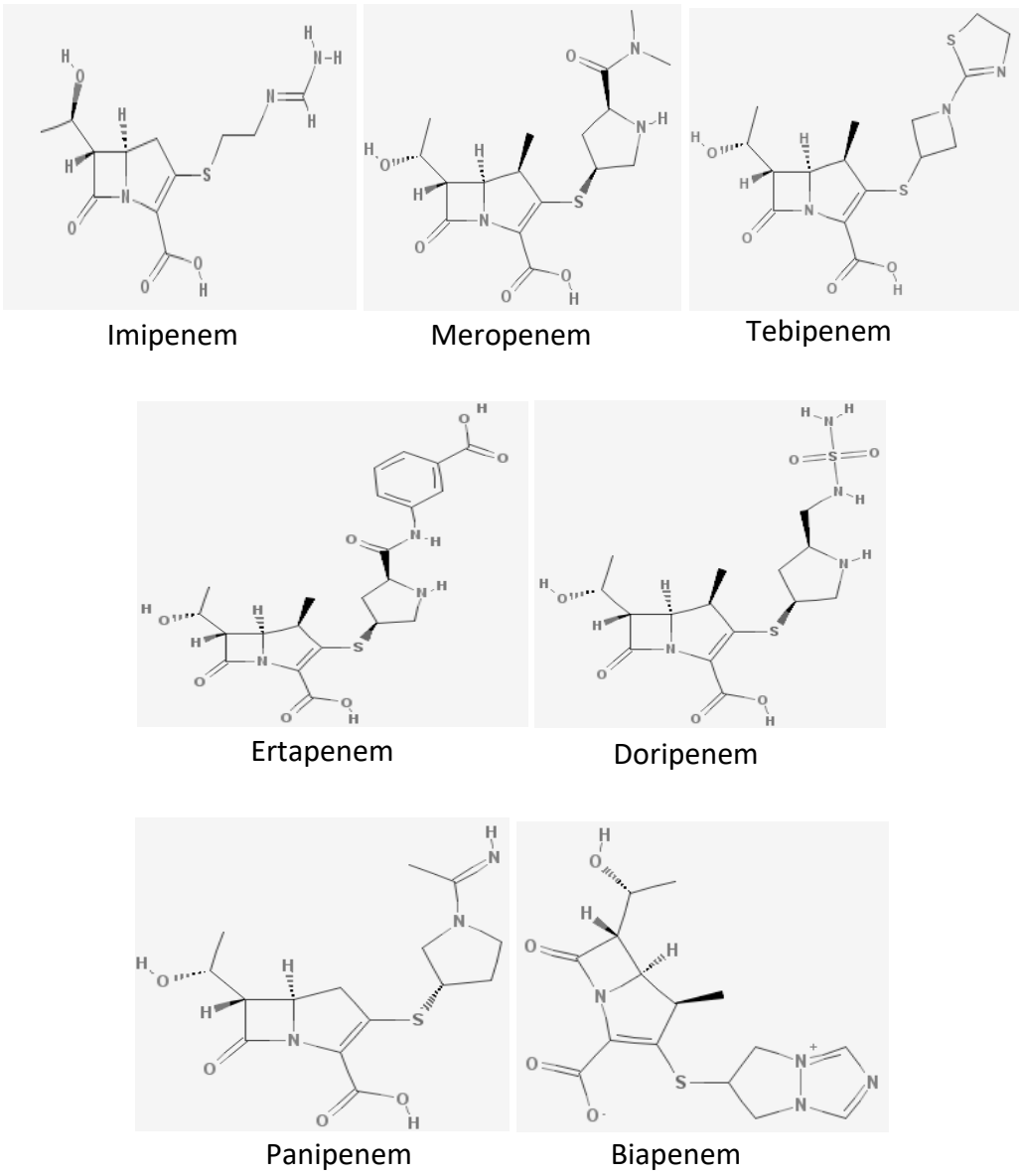



**Figure 4.17 Molecular structures of different carbapenem type antibiotics**

Carbapenemases have been reported to be produced by bacteria causing serious infections leading to serious need of new antibiotics against these. Hence, the virtual screening of these 121 druggable ligands were done against the carbapenem binding pocket of the carbapenemases enzyme also with the view of finding the ligands which could competitively inhibit the binding of the antibiotic in these enzymes thus allowing the antibiotic to function against the target pathogens upon co-administration with these developed ligands.

The carbapenemases enzymes of the WHO prioritized critical pathogens including *K. pneumoniae; P. aeruginosa and A. baumanii* were taken for study from rcsb.org. Since indole derivatives are being considered as important scaffolds of drug development

(Welsch *et al.*, 2010) six of the indole derivatives screened from the earlier computational works in finding leads that could act against the WHO prioritized pathogens were taken for docking purposes including the 121 FDA approved ligands that passed ADME/T tests in this study.

Thus, a total of 127 ligands along with 7 clinically approved carbapenems namely, Imipenem, Meropenem, Ertapenem, Doripenem, Panipenem, Biapenem and Tebipenem were taken for molecular docking against the GES-5 carbapenemase in *K. pneumonia* PDB id: 4H8R (Smith *et al.*, 2012), GES-5 carbapenemase in *P. aeruginosa* PDB id: 5F83 (Smith *et al.*, 2016) and OXA-239, a class D carbapenemase in *A. baumanii* PDB id: 5WI7 (Harper *et al.*, 2017).

Those ligands with higher binding energies than all of those carbapenems in the active binding site of the enzyme were considered as the enzyme inhibitors and thus could act as probable solutions to the carbapenem resistance as these ligands show strong binding to the enzyme binding site. Thus, these could render the enzymes unavailable for carbapenem binding and thus prevents the deactivation of carbapenems.

**Table 4.10 Binding energies of probable inhibitors of carbapenemase in *A. baumanii* along with that of a carbapenem with highest B.E compared to other carbapenems**

| Ligands | Binding Energy (kcal/mol) |
|---|---|
| ZINC6096622* | -9.4 |
| **ZINC00538550** | -8.9 |
| ZINC15219763* | -8.9 |
| ZINC49171024* | -8.8 |
| Ertapenem | -8.7 |
| *ZINC2009222* | -7.7 |

**Table 4.11 Binding energies of probable inhibitors of carbapenemase in *K. pneumonia* along with that of a carbapenem with highest B.E compared to other carbapenems**

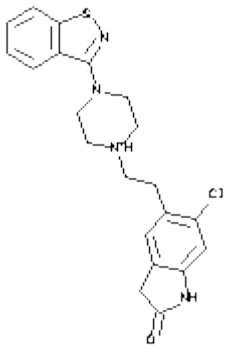
| Ligands | Binding Energy (kcal/mol) |
|---|---|
| ZINC 6096622* | -9 |
| ZINC15219763* | -9 |
| ZINC00020253 | -8.9 |
| ZINC00057255 | -8.9 |
| ZINC 4899565* | -8.9 |
| Ertapenem | -8.6 |
| *ZINC2009222* | -8.5 |

**Table 4.12 Binding energies of probable inhibitors of carbapenemase in *P.aeruginosa* along with that of a carbapenem with highest B.E compared to other carbapenems**

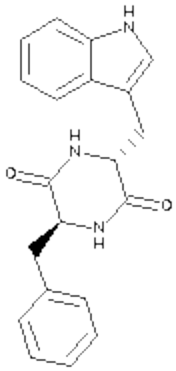
| Ligands | Binding Energy (kcal/mol) |
|---|---|
| ZINC15219763* | -9.4 |
| ZINC 4899565* | -8.9 |
| **ZINC00009073** | -8.8 |
| ZINC00020253 | -8.8 |
| ZINC00896569 | -8.7 |
| **ZINC00538550** | -8.6 |
| ZINC 6096622* | -8.5 |
| ZINC03812869 | -8.5 |
| Biapenem | -8.3 |
| *ZINC2009222* | -7.8 |

***** - Indole derivatives (among the six taken from earlier works from (Tha *et al.*, 2018)

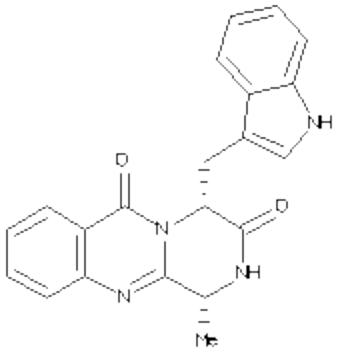
As per the various molecular docking studies (Table 4.8, 4.9 and 4.10) and based on earlier works, ZINC_6096622, ZINC_15219763, ZINC_00538550 were considered as the promising ligands that could potentially inhibit the target proteins and also carbapenemase enzyme - the major problem causing bacterial resistance in the present context (Figure 4.13).



ZINC id - **ZINC00538550**
Molecule name –Ziprasidone



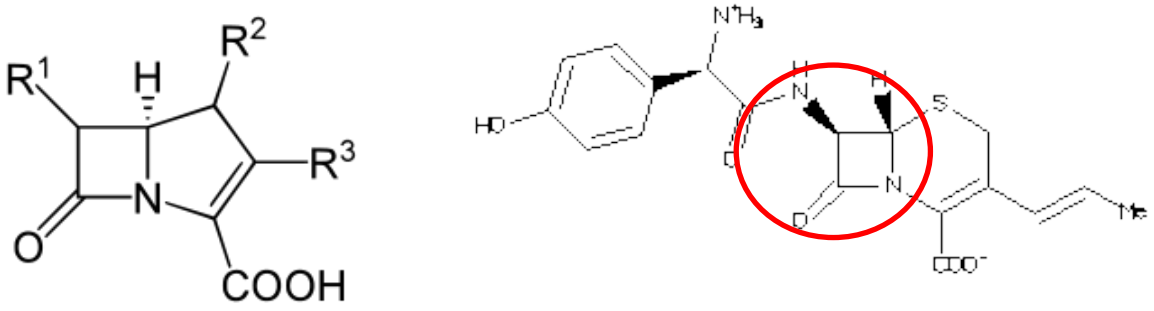
ZINC id - Zinc_6096622
Molecule name –3-benzyl-6-(1H-indol-3-ylmethyl)piperazine-



ZINC id - ZINC_15219763 Molecule name –- (1R,4R)-4-(1H-indol-3-ylmethyl)-1-methyl-2,4-dihydro-1H-pyrazino[5,4-

**Figure 4.18 Structures of the three promising lead drug candidates**

In addition, ZINC2009222 ligand has lower binding energy than the antibiotic against the Carbapenemases enzyme. It means it does not bind with the carbapenemase. However this ligand has backbone structure of a carbapenem in it as shown in figure 4.19. So, its structural similarity with the carbapenems makes it a probable coadministrating drug with carbapenems where the enzyme would bind with carbapenems and this drug would be free from enzyme function of hydrolysis which could potentially act as carbapenems blocking peptidoglycan biosynthesis (Papp-Wallace *et al.*, 2011) due to its beta-lactam ring structure. Thus, this molecule could be further explored.




a b

**Figure 4.19** **a. Backbone structure of a carbapenem**
**b. Structure of ZINC2009222 [(6S,7R)-7-[[(2R)-2-ammonio-2-(4-hydroxyphenyl)acetyl]amino]-8-keto-3-[(E)-prop-1-enyl]-5- thia-1-azab – a FDA approved drug**

# 5. SUMMARY

Streptomycetes are the ideal organisms for natural products discovery and were explored in this study hoping to find the novel strains and novel secondary metabolites of interest. The putative Streptomycetes were screened through the traditional methods using ISP media. The preliminary identification of 141 putative Streptomycetes isolates was done by observing their cultural and morphological characteristic, staining techniques and biochemical tests. The isolates were subjected to fermentation for secondary metabolites production in ISP2 broth. The growth media was filtered after incubation period and the secondary metabolites were extracted using ethyl acetate as the solvent. Then the extracts were concentrated using rotary evaporator to concentrate the crude extracts. These extracts were subjected to the antimicrobial and antioxidant assays. Upon performing disc diffusion antibiotic susceptibility tests, the crude extracts didn't show promising zones of inhibition against *S. aureus, P. aeruginosa and K. pneumoniae*. Since this method was time and resource consuming so robust antimicrobials screening was done by modifying resazurin microbial identification test to develop as robust antimicrobial assays. The extracts from putative Streptomycetes isolate named LAf4 showed positive antimicrobial activity against all the test organisms indicating the potential presence of broad spectrum antimicrobials in the extract. Thus, this organism could be either subjected to strain development for higher secondary metabolite production and also could be explored for modifying the media to further enhance the metabolite production. Similarly, upon performing DPPH assay to access the antioxidant property among 98 extracts, extracts from cultures of two isolates, KH8 and NEd3, showed good antioxidant property with immediate change of color from purple DPPH to reduced yellow 1, 1-diphenyl-2-picrylhydrazine in TLC plates indicating the scavenging of DPPH free radicals by the antioxidants present in the extract. The isolate KH8 was taken for fermentation in a 5l fermenter that produced 2 gm of crude extracts after filtration, extraction and concentration. TLC was performed for solvent optimization and the crude extract was subjected to partial purification . Using 1:9 ratios of ethyl acetate and hexane as the carrier solvent, Column Chromatography was performed and four fractions from the extracts were collected after separation. One of these fractions showed immediate color change while performing the DPPH assay indicating the presence of potent antioxidants in it. It was finally taken for GCMS analysis to identify the nature of compounds present in the extract.

Furthermore, the rapid increase in antibiotic resistance globally that demands the rapid discovery of novel classes of antibiotics to cope against these problems. Thus, the time and cost-effective computational approaches that have been used for more efficient

drug discovery in the current era has also been the major focus in the present study. Molecular docking of the FDA approved ligands and the indole derivatives from the previous works against the various target proteins including *metK, dam* and carbapenemases were performed and ligand ZINC00538550 (Ziprasidone hydrochloride) had higher binding energies than the native ligands against MetK and Dam of nine WHO prioritized pathogens, ZINC_6096622 (3-benzyl-6-(1H-indol-3-ylmethyl)piperazine-2,5-dione) and ZINC_15219763 (1R,4R)-4-(1H-indol-3-ylmethyl)-1-methyl-2,4-dihydro-1H-pyrazino[5,4-b]quinazoline-3,6-dione that have shown higher binding energies against several SAM utilizing methyl transferases showed higher binding energies than the carbapenem class of antibiotics against the carbapenemases of three organisms WHO prioritization of pathogens as critical were virtually screened potential drug target leads for coadministration along with the respective carbapenem antibiotics. In addition, ZINC2009222 [(6S,7R)-7-[[(2R)-2-ammonio-2-(4-hydroxyphenyl)acetyl]amino]-8-keto-3-[(E)-prop-1-enyl]-5- thia-1-azab was identified as beta-lactam containing molecule that had lower binding energies than the carbapenem class of antibiotics towards carbapenemases of respective pathogens indicating that it could not be metabolized in the presence of carbapenem class of antibiotics due to lower affinity of the molecule and this could also be potentially be coadministered along with carbapenem antibiotics if this molecule can inhibit peptidoglycan biosynthesis. The present research suggests that coadministration of Ziprasidone, carbapenem and [(6S,7R)-7-[[(2R)-2-ammonio-2-(4-hydroxyphenyl)acetyl]amino]-8-keto-3-[(E)-prop-1-enyl]-5- thia-1-azab could be teste for efficacy immediately with the notion that the higher binding energy of Ziprasidone with carbapenemases and MetK and Dam would have higher binding affinity than the native ligands hence the bacteria has to be engaged in clearing this molecule first and could lead in poor clearance of carbapenem making it available for acting against the penicillin binding proteins thus inhibiting bacterial cell wall synthesis, and also exhibiting its ROS inducing potential for additionally killing the bacteria. Even if the bacteria overexpresses carbapenemases to engage both Ziprasidone and carbapenem then [(6S,7R)-7-[[(2R)-2-ammonio-2-(4-hydroxyphenyl)acetyl]amino]-8-keto-3-[(E)-prop-1-enyl]-5- thia-1-azab could act as bactericidal antibiotics due to its lower binding energy to carbapenemases. Other two indole derivatives could be also explored for coadministration with carbapenem so that the dual action of these compounds could inhibit bacterial growth. Thus, it is presumed that the computational biology could come in handy for developing the lead candidates as an effective solution to the current resistance problem.

# 6. CONCLUSION

Increasing resistance problems in the pathogenic bacteria have rendered all the currently available antibiotics ineffective. This demands the need of novel class of potent antibiotics if future generations are to survive.

Based on the present study, a total of 141 putative Streptomycetes strains were isolated from 48 different soil samples taken from different parts of Nepal. Secondary metabolite production and respective antibiotic susceptibility tests from all of these strains would take a lot of time, labor and resources and could be a limiting factor in the present context in our research laboratory. The present study emphasized on the time effective protocols with the minimal use of resources. The secondary metabolite productions was carried out on a small volume of growth media and modified resazurin antimicrobial assay was used to test the antimicrobial properties of the extracts. This saved a lot of resources and was around four times faster than the usual disc or well diffusion antimicrobial assays. Also, the lesser concentration and polarity of the antimicrobials didn't affect the results in resazurin antimicrobial assay unlike the disc or well diffusion antimicrobial assays since the diffusion in agar further decreases the concentration of the extracts used and non-polar antimicrobials if present in the extracts would not diffuse in the polar agar thus would indicate the results as negative even for antimicrobial containing extracts. The putative Streptomycetes extract LAf4 among the 90 extracts tested inhibited all four of the test pathogens thus LAf4 was one of the major strains with broad spectrum antimicrobials.

The disc diffusion assays with the same extracts on the same test pathogens did not show promising results which was due to either the lesser concentration of antimicrobials produced, or due to the lack of efficient diffusion in the agar plates. So it was concluded that resazurin antimicrobial assay could be better and robust method for screening antimicrobials compared to the traditional disc or well diffusion assays. Also, the growth media used for secondary metabolites production could be modified since Carbon Catabolite Repression (CCR) could play a major role in the activation of genes involved in the desired secondary metabolites production.

Moreover, virtual screening of FDA approved drugs and indole derivatives employed in other study was done to screen putative drug candidate for further exploration as antimicrobials, especially antibacterial antibiotics. The present molecular docking studies revealed the three molecules namely, Ziprasidone hydrochloride, (1R,4R)-4-(1H-indol-3-ylmethyl)-1-methyl-2,4-dihydro-1H-pyrazino[5,4-b]quinazoline-3,6-dione and 3-benzyl-6-(1H-indol-3-ylmethyl)piperazine-2,5-dione as potential inhibitors of *metK,* an essential

gene in all of the 9 target pathogens and also the carbapenemases of all 3 critical pathogens as prioritized by WHO. These three indole derivatives (important scaffolds of drug development) molecules, could be very vital to be pursued further for potential novel drug development.

Similarly, antioxidant properties of all the putative Streptomycetes isolates were explored with a view to find novel and potent natural antioxidants since natural antioxidants with lesser side effects are very scarce in the present context and antioxidants have a major role in treating major human diseases including Alzheimer's disease, cancer and so on. DPPH free radical scavenging assay was performed for all the extracts in this study since this is one of the best method for screening antioxidants and also being time and cost effective compared to other methods. The isolated strain KH8 showed the best antioxidant property of all the strains tested which was taken for purification using TLC and column chromatography. The GCMS results showed 1, 2-Benzenediol being the most abundant in the antioxidant containing portion of the extract KH8 and 2, 3-butanediol being the most abundant probable antimicrobial compounds in LAf4 extract.

# RECOMMENDATIONS

LAf4, LAd1, LAe5 and HA5 which showed positive AST against four MDR pathogens could be further studied. The screened lead drug candidates identified through virtual screening could be tested *in vitro* for verification. With additional data these could be taken for animal testing and toxicity testing for drug development.

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# 8. APPENDIX

## 8.1 Secondary metabolite gene clusters in *Streptomyces coelicolor* A3(2) (Nett *et al.*, 2009)

| S. No. | Cluster location | Actual or predicted product* |
|---|---|---|
| 1 | SCO0124-0129 | Eicosapentaenoic acid |
| 2 | SCO0185-0191 | **Isorenieratene** |
| 3 | SCO0267-0270 | Lantibiotic |
| 4 | SCO0381-0401 | Deoxy sugar |
| 5 | SCO0489-0499 | **Coelichelin** |
| 6 | SCO0753-0756 | Bacteriocin |
| 7 | SCO1206-1208 | **THN**, **flaviolin** |
| 8 | SCO1265-1273 | Aromatic polyketide |
| 9 | SCO1864-1867 | **5-Hydroxyectoine** |
| 10 | SCO2700-2701 | Melanin |
| 11 | SCO2782-2785 | **Desferrioxamine** |
| 12 | SCO3210-3249 | **Calcium-dep. Antibiotic** |
| 13 | SCO5071-5092 | **Actinorhodin** |
| 14 | SCO5222-5223 | **Albaflavenone** |
| 15 | SCO5314-5320 | **Gray spore pigment** |
| 16 | SCO5799-5801 | Siderophore |
| 17 | SCO5877-5898 | **Prodiginine** |
| 18 | SCO6073 | **Geosmin** |
| 19 | SCO6266 | **γ-Butyrolactone Scb1** |
| 20 | SCO6273-6288 | Hexaketide |
| 21 | SCO6429-6438 | Dipeptide |
| 22 | SCO6681-6685 | Lanthionine-cont. peptide SapB |
| 23 | SCO6759-6771 | **Hopene** |
| 24 | SCO6826-6827 | Polyketide |
| 25 | SCO6927-6932 | Lantibiotic |
| 26 | SCO7221 | **Germicidin** |
| 27 | SCO7669-7671 | Aromatic polyketide |
| 28 | SCO7681-7691 | Coelibactin |
| 29 | SCO7700-7701 | **2-Methylisoborneol** |
| 30 | SCP1.228c-246 | **Methylenomycin** , **2-alkyl-4-(hydroxymethyl) furan-3-carboxylic acids** |

* The highlighted products are observed ones (actual).

## 8.2 List of Soil Sample Used

| SN | Soil Sample | SN | Soil Sample | SN | Soil Sample |
|---|---|---|---|---|---|
| 1. | Khungri, Rolpa | 14. | Kalinchok | 27. | Dudhkoshi |
| 2. | Fulchoki | 15. | Shivapuri | 28. | Hatiya, Lamjung |
| 3. | Gorkha | 16. | Tanahu | 29. | Khopasi |
| 4. | Manang | 17. | Mahendrapul | 30. | Butwal |
| 5. | Poonhill | 18. | Dolpa (3) | 31. | Kailali |
| 6. | Sundarijal | 19. | Lantang | 32. | Doleshwor |
| 7. | Dhading | 20. | Matatirtha | 33. | Dolkha (4) |
| 8. | Lamjung | 21. | T.U. construction site | 34. | Sindhupalchok |
| 9. | Ghorepani, Rolpa | 22. | Panchase | 35. | Nala |
| 10. | Kanchanpur | 23. | Chitwan | 36. | Halesi |
| 11. | Khotang | 24. | Trishuli | 37. | Baglung |
| 12. | Panauti | 25. | Dharan | 38. | Bhojpur |
| 13. | Nagarkot (2) | 26. | Nepalgunj (3) | 40. | Pidalna |

## 8.3 Media Composition

- **ISP2 media (gm/l)**
    1. Glucose 4
    2. Yeast extract 4
    3. Malt extract 10
    4. Agar 20

    pH: $7.3^{+}_{-}0.2$ at 25◦C

- **ISP4 Media (gm/l)**
    1. Soluble starch 10
    2. Calcium carbonate 2
    3. Ammonium sulphate 2
    4. Dipotassium hydrogen phosphate 1
    5. Magnesium sulphate 1
    6. Sodium chloride 1
    7. Ferrous sulphate 0.001
    8. Manganese chloride 0.001
    9. Zinc sulphate 0.001
    10. Agar 20

    pH: $7.3^{+}_{-}0.2$ at 25◦C

## 8.4 Extracts used in various wells of the microtiter plate during Resazurin antimicrobial assay against the target pathogens

| | 2 | 4 | 5 | 7 | 9 | 11 |
|---|---|---|---|---|---|---|
| A | LAb9 | HA5 | LAe1 | D1 | Amp 50 mg/ml | Kan 50 mg/ml |
| B | LAe5 | LAf4 | KA1 | GH2 | 25 mg/ml | 25 mg/ml |
| C | KH8 | GOb6 | LAd1 | GH5 | 12.5 mg/ml | 12.5 mg/ml |
| D | NEd3 | LAa1 | KH6 | GHO1 | 6.25 mg/ml | 6.25 mg/ml |
| E | LAb9 | HA5 | LAe1 | D1 | 3.125 mg/ml | 3.125 mg/ml |
| F | LAe5 | LAf4 | KA1 | GH2 | 1.56 mg/ml | 1.56 mg/ml |
| G | KH8 | GOb6 | LAd1 | GH5 | 0.781 mg/ml | 0.781 mg/ml |
| H | NEd3 | LAa1 | KH6 | GHO1 | 0.39 mg/ml | 0.39 mg/ml |

## 8.5 Absorbance readings at 550 nm after the incubation period of AST plates

Test organism – *Klebsiella pneumoniae*

| | Positive control | | | | | Negative control | | |
|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 4 | 5 | 7 | 8 | 9 | 11 |
| A | 2.044 | 1.448 | 1.365 | 0.929 | 0.16 | 0.19 | 0.203 | 1.656 |
| B | 2.265 | 1.691 | 1.834 | 1.757 | 1.706 | 0.2 | 0.203 | 1.502 |
| C | 2.416 | 1.071 | 0.987 | 1.672 | 1.838 | 0.204 | 0.215 | 1.602 |
| D | 2.403 | 1.386 | 1.218 | 1.77 | 1.22 | 0.203 | 0.216 | 1.612 |
| E | 2.425 | 1.858 | 1.892 | 0.714 | 0.859 | 0.216 | 0.221 | 1.437 |
| F | 2.456 | 1.947 | 2.189 | 1.679 | 1.486 | 0.218 | 0.223 | 1.784 |
| G | 1.979 | 0.704 | 1.508 | 1.989 | 1.876 | 0.244 | 0.219 | 0.513 |
| H | 2.551 | 1.354 | 1.506 | 1.942 | 1.708 | 0.223 | 0.214 | 0.18 |

Test organism – *Salmonella sp* S4

| | 1 | 2 | 4 | 5 | 7 | 8 | 9 | 11 |
|---|---|---|---|---|---|---|---|---|
| A | 2.312 | 1.879 | 2.166 | 1.914 | 1.557 | 0.164 | 0.177 | 0.164 |
| B | 2.371 | 1.831 | 2.068 | 1.981 | 1.911 | 0.174 | 0.183 | 0.158 |
| C | 2.343 | 1.183 | 1.796 | 2.001 | 1.537 | 0.198 | 0.166 | 0.177 |
| D | 2.303 | 1.837 | 1.356 | 1.889 | 1.729 | 0.173 | 0.168 | 0.175 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| E | 2.294 | 1.517 | 2.068 | 1.73 | 1.656 | 0.166 | 0.164 | 0.19 |
| F | 2.381 | 2.174 | 2.337 | 2.157 | 2.092 | 0.165 | 0.184 | 0.217 |
| G | 2.242 | 1.159 | 1.766 | 1.856 | 1.889 | 0.159 | 0.183 | 0.19 |
| H | 2.207 | 1.833 | 1.736 | 2.084 | 1.89 | 0.173 | 0.153 | 1.253 |

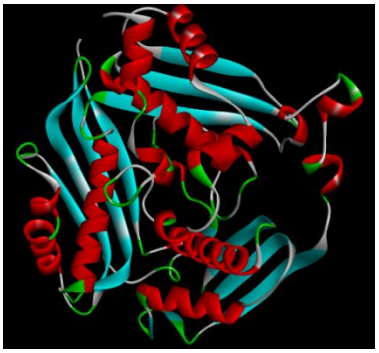
Test organism – *Pseudomonas aeruginosa* P39

| | 1 | 2 | 4 | 5 | 7 | 8 | 9 | 11 |
|---|---|---|---|---|---|---|---|---|
| A | 2.276 | 1.941 | 2.208 | 1.693 | 2.017 | 0.28 | 0.287 | 1.49 |
| B | 2.16 | 1.735 | 1.845 | 1.959 | 1.508 | 0.304 | 0.215 | 1.146 |
| C | 2.203 | 1.097 | 2.211 | 2.154 | 2.205 | 0.236 | 0.23 | 1.379 |
| D | 2.298 | 1.51 | 0.854 | 1.852 | 1.905 | 0.233 | 0.25 | 0.43 |
| E | 2.428 | 2.121 | 2.231 | 1.978 | 2.14 | 0.261 | 0.297 | 0.27 |
| F | 2.355 | 2.064 | 2.29 | 2.153 | 2.362 | 0.24 | 0.372 | 0.236 |
| G | 2.165 | 1.267 | 2.158 | 2.237 | 2.282 | 0.263 | 0.336 | 0.285 |
| H | 2.449 | 2.108 | 1.5 | 1.49 | 2.197 | 0.267 | 0.291 | 0.354 |

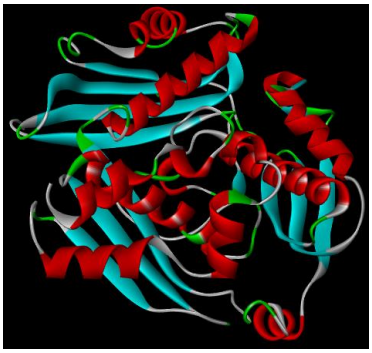
Test organism – *Acinetobacter baumanii* Ab

| | 1 | 2 | 4 | 5 | 7 | 8 | 9 | 11 |
|---|---|---|---|---|---|---|---|---|
| A | 1.487 | 1.132 | 1.296 | 0.994 | 0.971 | 0.155 | 0.129 | 0.218 |
| B | 1.546 | 1.178 | 1.174 | 1.082 | 1.157 | 0.148 | 0.128 | 0.135 |
| C | 1.444 | 0.842 | 1.095 | 1.112 | 1.04 | 0.129 | 0.12 | 0.125 |
| D | 1.468 | 1.038 | 0.869 | 0.085 | 0.999 | 0.12 | 0.114 | 0.127 |
| E | 1.459 | 1.207 | 1.317 | 0.921 | 0.942 | 0.12 | 0.115 | 0.128 |
| F | 1.428 | 1.172 | 1.128 | 1.061 | 1.076 | 0.125 | 0.105 | 0.119 |
| G | 1.523 | 0.654 | 1.173 | 1.209 | 1.074 | 0.13 | 0.105 | 0.109 |
| H | 1.55 | 0.086 | 0.881 | 0.084 | 0.97 | 0.119 | 0.141 | 0.119 |

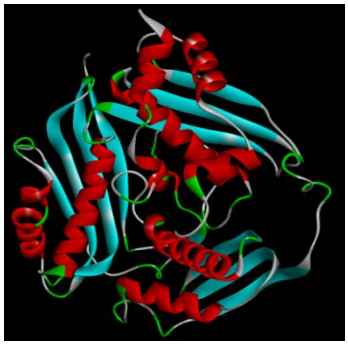
## 8.6 Computationally modelled MetK structures of all other eight pathogens



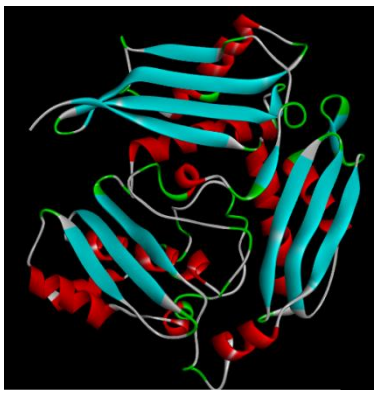
MetK *of Acinetobacter baumanii* as given by Phyre2



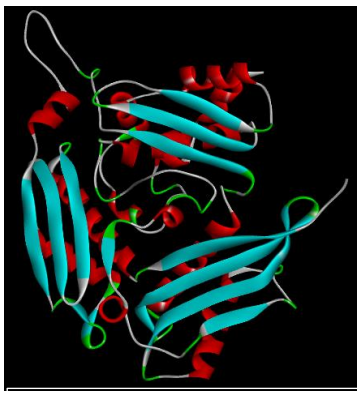
MetK of *Enterococcus faecium* as given by Phyre2



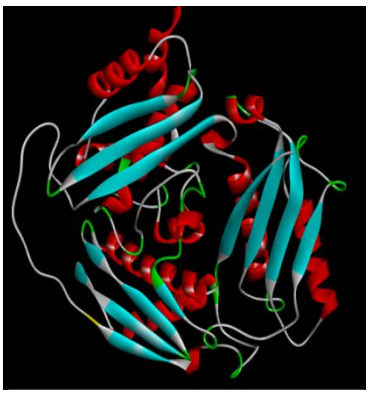
Metk of *Helicobacter pylori* as given by Phyre2



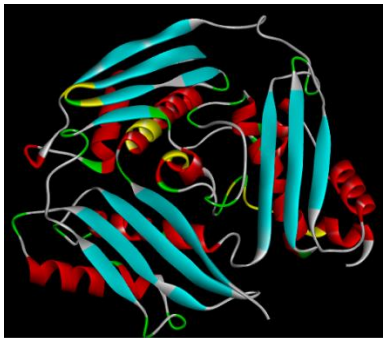
MetK of *Salmonella typhi* as given by Phyre2



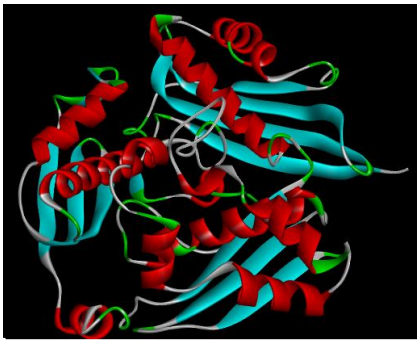
MetK of *Staphylococcus aureus* as given by ps2v2



MetK of *Campylobacter coli* as given by RaptorX



MetK of *Klebsiella pneumonia* as given by RaptorX



MetK of *Pseudomonas aeruginosa* as given by Phyre2