Understanding the growth inhibitory mechanism of action of celecoxib in combination with ampicillin in *Staphylococcus aureus*

Thesis Submitted for the degree of

DOCTOR OF PHILOSOPHY

By

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CERTIFICATE

This is to certify that the thesis entitled **"Understanding the growth inhibitory mechanism of action of celecoxib in combination with ampicillin in *Staphylococcus aureus*"** submitted by **Yajarla N Gajapati Varma** bearing registration number **11LAPH08** in partial fulfilment of the requirements for award of **Doctor of Philosophy** in the **Animal Sciences** is a bonafide work carried out by him under my supervision and guidance.

This thesis is free from plagiarism and has not been submitted previously in part or in full to this or any other University or Institution for award of any degree or diploma. Part of this thesis has been

A. Published in the following publication:

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DECLARATION

I hereby declare that the results of the study incorporated in the thesis entitled **"Understanding the growth inhibitory mechanism of action of celecoxib in combination with ampicillin in *Staphylococcus aureus*"** have been carried out by me under the supervision of **Dr. Arunasree MK**, Department of Animal Biology, School of Life Sciences, University of Hyderabad and this work has not been submitted for any degree or diploma of any other university earlier.

Dated:

YAJARLA N GAJAPATI VARMA

(Research Scholar)

This thesis is dedicated to
my brother Shri. Y. Chandra Varma for all
his hardships to bring me till this stage

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Y N Gajapati Varma 

CONTENTS

ABBREVIATIONS

μg	micro gram
µM	micro molar
6-AUs	6-Anilinouracils
7-AAD	7-aminoactinomycin D
ABC	ATP-binding cassette family
ABS	antibiotic stewardship (to reduce antibiotic resistance)
AHRQ	Agency for Healthcare Research and Quality
AIDS	acquired immune deficiency syndrome
AMR	Antimicrobial resistance
ATCC	American Type Culture Collection
ATP	Adenosine triphosphate
$CaCO_3$	Calcium carbonate
CDC	Centre for Disease Control and Prevention (USA)
cDNA	complementary DNA
CFU	colony-forming unit
CLSI	Clinical and Laboratory Standards Institute
COX	cyclooxygenase
Coxibs	selective COX-2 inhibitors
CRE	Carbapenem-resistant *Enterobacteriaceae*
CRKP	carbapenem-resistant *K. pneumoniae*
DEG	Database of Essential Genes
dGTP	deoxyguanosine triphosphate
DMSO	dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DSC	Differential Scanning Calorimetry
EF-G	elongation factor G
EF-Tu	elongation factor Tu
ESBLs	Extended-spectrum beta-lactamases

ESKAPE	*Enterococcus*, *Staphylococcus*, *Klebsiella*, *Acinetobacter*, *Pseudomonas* and *Enterobacter* species
FAP	Familial Adenomatous Polyposis
FDA	Food and Drug Administration
FTIR	Fourier Transform Infrared
GISA	glycopeptide intermediate *S. aureus*
GLASS	Global Antimicrobial Surveillance System
h	hour
H_2O_2	Hydrogen peroxide
HIV	Human Immunodeficiency Virus
HLA	hemolysin alpha
HRP	horseradish peroxidase
ICU	intensive care unit
IUPAC	International Union of Pure and Applied Chemistry
KPC	*Klebsiella pneumoniae* carbapenemase
LB	Luria Bertani
LPS	Lipopolysaccharide
MATE	multidrug and toxic compound extrusion family
MDR	multidrug resistant
MDRAB	multidrug-resistant *A. baumannii*
MFS	major facilitator superfamily
MIC	minimum inhibitory concentration
min	minutes
mg	milligram
mL	millilitre
mM	milli Molar
mRNA	messenger RNA
MRSA	Methicillin-resistant *S. aureus*
MSSA	Methicillin sensitive *S. aureus*
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
N	Normality

NaCl	Sodium chloride
NaOH	Sodium hydroxide
NDM-1	New Delhi Metallo β-lactamase-1
NK	Natural killer cells
nm	nanometre
NSA	non-*S. aureus* staphylococci
NSAID	Non-steroidal anti-inflammatory drug
OD	Optical Density
PAGE	polyacrylamide gel electrophoresis
PBP	penicillin-binding protein
PBS	phosphate-buffered saline
PCR	Polymerase chain reaction
PDR	Pan-drug resistant
PGE2	Prostaglandin E2
PGs	prostaglandins
PM	physical mixture
PMSF	phenylmethylsulphonyl fluoride
PXRD	Powder X-Ray Diffraction
qPCR	quantitative PCR
Rh123	Rhodamine 123
RNA	Ribonucleic acid
RND	resistance-nodulation-cell division family
rpm	revolutions per minute
rRNA	ribosomal RNA
SCFA	Short chain fatty acids
SDS	Sodium dodecyl sulphate
SEH	Staphylococcal enterotoxin H
SFP	Staphylococcal food poisoning
SIRT1	member of the sirtuin family
SMILES	simplified molecular-input line-entry system
SMR	small multidrug resistance family

TB	Tuberculosis
TBST	Tris-buffered saline-Tween
TMP-SMX	trimethoprim-sulfamethoxazole
Tns	Transposons
TRI	Total RNA isolation
tRNA	transfer RNA
TSB	Tryptic soy broth
TSS	toxic shock syndrome
UN	United Nations
UV-Vis	Ultra violet-Visible
VISA	Vancomycin-intermediate *S. aureus*
VRE	vancomycin-resistant *Enterococci*
VRSA	Vancomycin resistance *S. aureus*
WHO	World Health Organisation
XDR	extensively drug-resistant

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INTRODUCTION

1.Introduction

Bacteria were the first formed living creatures on Earth. Bacteria are prokaryotic, single celled microscopic organisms. Since their existence they have been one of the dominant forms. Antonie van Leeuwenhoek, for the first time, observed bacteria under microscope. Bacteria are cosmopolitan in distribution, can live in extreme temperatures, extreme pH conditions and even at extreme salt conditions. Bacteria are present in soil, water and in air. Bacteria live on the surface of animals and even inside the body of animals. Their biomass exceeds that of individual biomass of plants and animals [1].

Most of the bacteria residing in/on human are harmless. Some bacteria are beneficial as they are involved directly or indirectly in multitude of physiologic functions such as digestion, metabolism, immune regulation, etc. Some gut microbiota possess enzymes required for digestion of oligosaccharides and some other complex sugars which are absent in human cells thus aiding in generating nutrients [2, 3]. Some of the by-products of bacterial metabolism such as short chain fatty acids (SCFA) are an essential energy requirement for intestine and, are crucial for modulating immune responses [4-7]. On the other hand, many bacteria cause a wide range of diseases thus acting as pathogens. During infections, these bacteria attack the host cells and proliferate in tissues, which is often referred as colonization, as a result of which the host immune system recognizes and fights against these invaders [8, 9].

Some bacteria live in/on animals as commensals [10] and under normal conditions they do not cause infections. The same bacteria can cause infections to the host when they enter other parts of the body and can lead to fatal diseases.

Bacteria are classified into two groups namely Gram-positive and Gram-negative based on whether they can retain the Gram's stain or not. Gram-positive bacteria can retain the crystal Violet-Iodine complex due to their thick cell walls having less amount of lipids and hence appear purple. Gram-negative bacteria cannot retain the crystal violet- iodine complex due to presence of more lipid content in their cell walls and hence appear pink due to the counter stain safranin [11]. Bacteria may be in various shapes *viz*. rod, spherical, comma or spiral and they are called as bacilli, cocci, vibrio or spirilla, respectively. They can exist as single, pairs (diplo), chains (strepto), clusters (staphylo) or sarcina (cubes of eight) [1, 12]. Gram-positive bacteria

include *Bacillus*, *Streptococcus*, *Clostridium*, *Enterococcus and Staphylococcus*. Gram-negative bacteria include *Klebsiella*, *Salmonella*, *Haemophilus*, *Neisseria*, *Campylobacter* and *Pseudomonas*. Both Gram-positive and Gram-negative bacteria can cause a wide range of illness (Table 1).

Table 1: List of some diseases caused by different bacteria

Bacteria	**Diseases**
Bacillus	Anthrax, meningitis, ophthalmitis, etc.
Streptococcus	Pneumonia, glomerulonephritis, endocarditis, etc.
Clostridium	Botulism, tetanus, gastroenteritis, etc.
Enterococci	Urinary tract infections, cellulitis, endocarditis, etc.
Staphylococcus	Food poisoning, toxic shock syndrome, impetigo, boils, etc.
Klebsiella	Pneumonia, meningitis, bloodstream infections, etc.
Pseudomonas	Respiratory system infections, bone and joint infections, etc.
Neisseria	Meningococcemia, conjunctivitis, gonorrhoea, etc.
Salmonella	Typhoid, food poisoning, gastroenteritis, etc.

1.1 Antibiotics

Antibiotics are effective in controlling bacterial infections. Antibiotics are chemical molecules that are released by bacteria or fungi which can kill (cidal) or inhibit the growth (static) of other species of bacteria or fungi growing in their vicinity. Alexander Fleming, in 1928, discovered an antibiotic called penicillin from *Penicillium notatum*, a brush mould, which did not allow the growth of *Staphylococcus aureus* around it. Later, two scientists, Florey and Chain isolated and purified penicillin and finally made it available as injectable form of drug. The era of antibiotics was started with the discovery of penicillin and twenty new classes of antibiotics came into market up to 1962. After 1962, only two more classes of antibiotics were discovered [13]. Based on the mode of action, an antibiotic can be grouped as bactericidal, where bacteria are killed or bacteriostatic, where further growth of the bacteria is inhibited [14].

1.1.1 Classes of antibiotics

Antibiotics, based on the mechanism of action, are classified as inhibitors of cell wall synthesis, nucleic acid synthesis inhibitors, transcription and translation inhibitors or inhibitors of specific metabolic pathways (Figure 1).

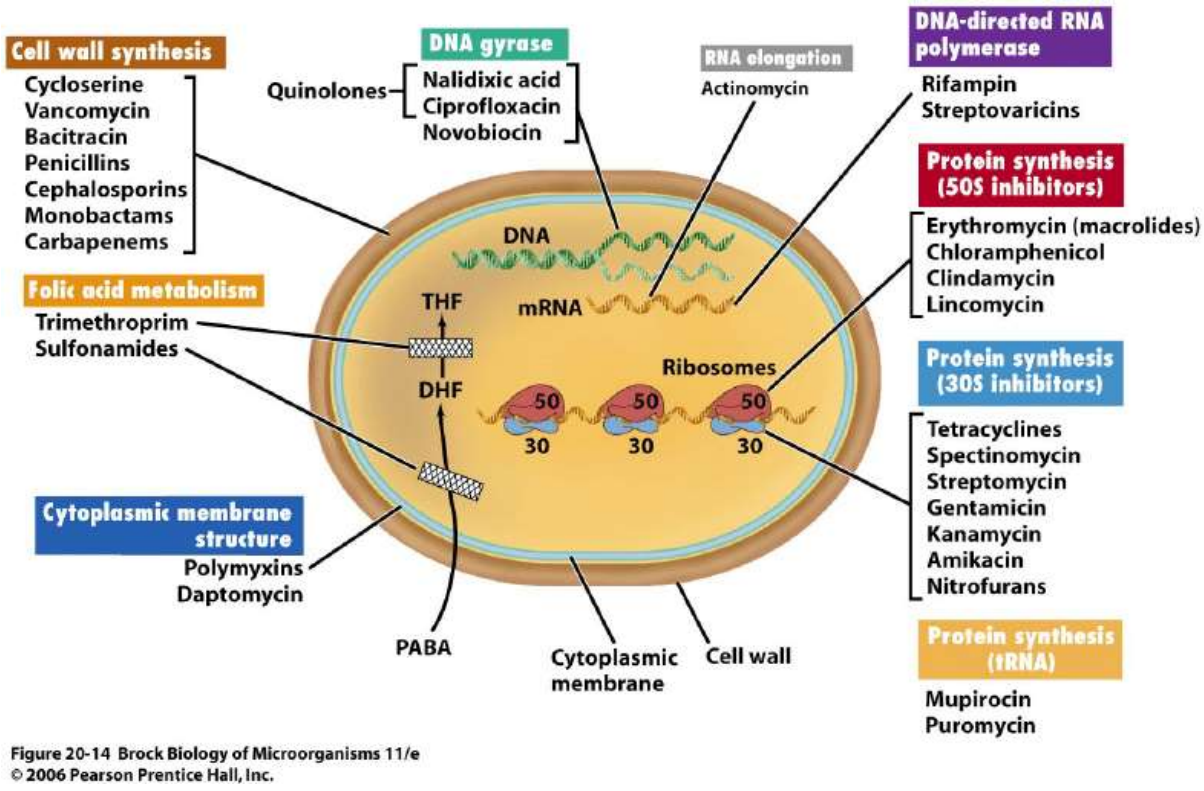


Figure 1: Classes of antibiotics

1.1.1.1 Inhibitors of cell wall synthesis

Cell wall of bacteria protect from osmotic lysis by covering the plasma membrane [15]. Gram-positive bacteria contain one layer of cell wall composed of peptidoglycan. In Gram-negative bacteria, the cell wall is composed of two layers; outer layer composed of lipopolysaccharides (LPS) and inner layer composed of peptidoglycans. The peptidoglycan layer present in Gram-positive bacteria is thicker when compared to Gram-negative bacteria [15].

The summary of various classes of antibiotics and their mode of action is shown in Table 2.

Table 2: Various modes of action of antibiotics and their targets in bacteria

Mode of Action	Target	Drug Class
Inhibit cell wall biosynthesis	Penicillin-binding proteins	β-lactams: penicillins, cephalosporins, monobactams, carbapenems
	Peptidoglycan subunits	Glycopeptides
	Peptidoglycan subunit transport	Bacitracin
Disrupt membranes	Lipopolysaccharide, inner and outer membranes	Polymyxin B, colistin, daptomycin
Inhibit nucleic acid synthesis	DNA	Fluoroquinolones
	RNA	Rifamycin
Inhibit biosynthesis of proteins	30S ribosomal subunit	Aminoglycosides, tetracyclines
	50S ribosomal subunit	Macrolides, lincosamides, chloramphenicol, oxazolidinones
Antimetabolites	Folic acid synthesis enzyme	Sulfonamides, trimethoprim
	Mycolic acid synthesis enzyme	Isonicotinic acid hydrazide (isoniazid)

Bacterial cell wall synthesis inhibitors include penicillins, cephalosporins, carbapenems, aztreonam (Figure 2) and vancomycin and they are classified as shown in Figure 3.

Introduction

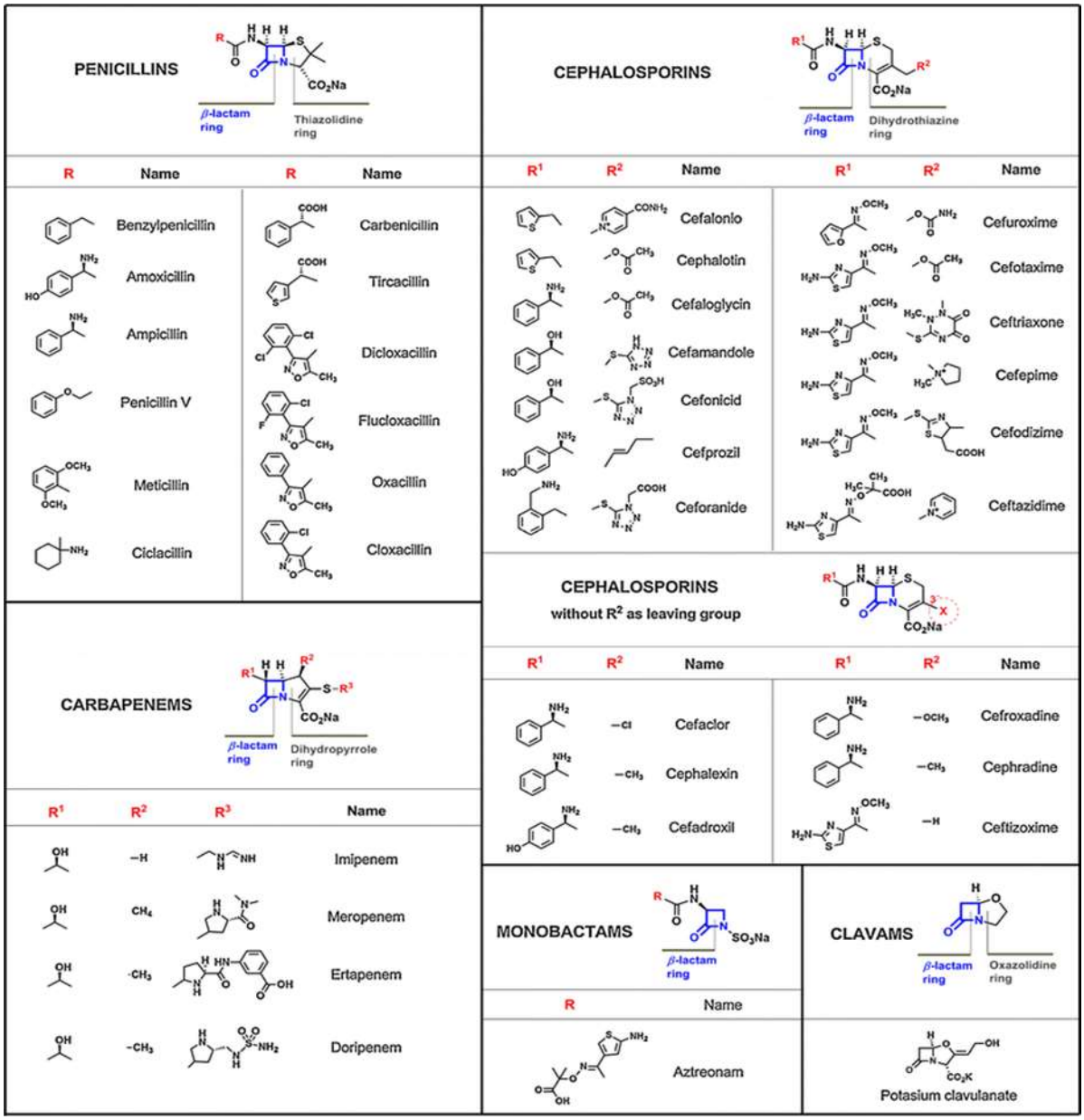


Image courtesy: Curr Treat Options Allergy (2015) 2:141–154 DOI 10.1007/s40521-015-0050-4

Figure 2: Chemical structures of different beta-lactams

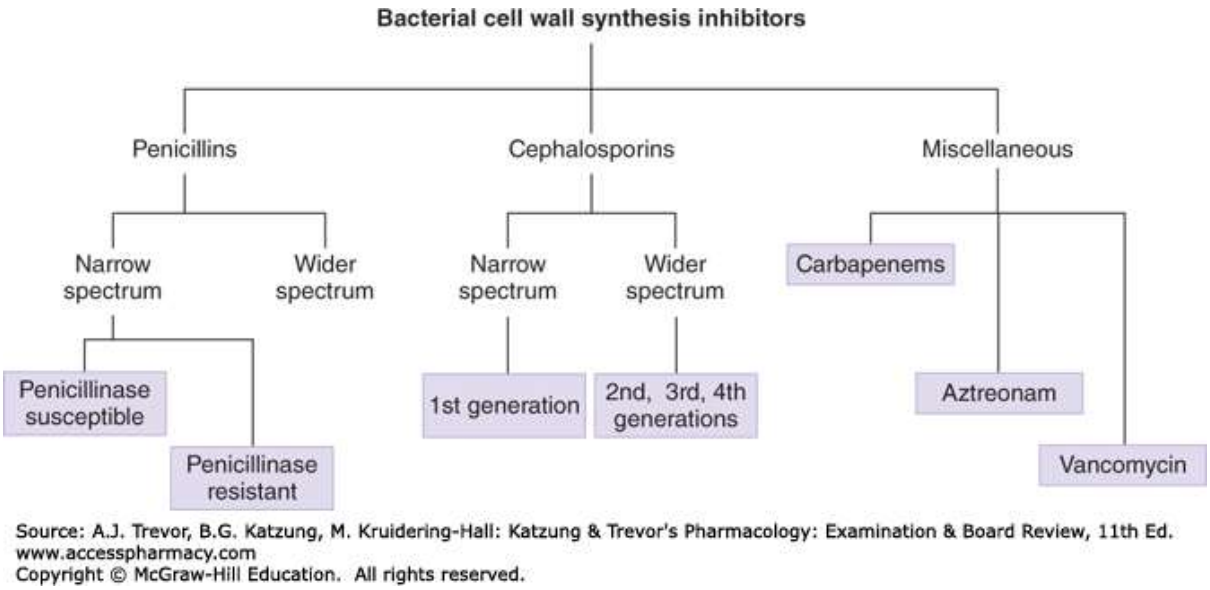


Figure 3: Classification of various bacterial cell wall synthesis inhibitors

1.1.1.2 Antibiotics disrupting the membranes

Antibiotics disrupting the bacterial membranes include polymyxins (Figure 4A) and daptomycin (Figure 4B).

Polymyxins are active on almost all Gram-negative bacteria. These are bactericidal in nature. These bind to bacterial cell membrane and change its structure. This increases the permeability of cell membrane. These also bind to outer cell wall of bacteria and destabilise it [16].

Daptomycin gets inserted into the cell membrane and then aggregates. This causes a curvature in the membrane, creating a hole, leakage of ions, and depolarisation of the membrane finally leading to the death of bacteria [17].

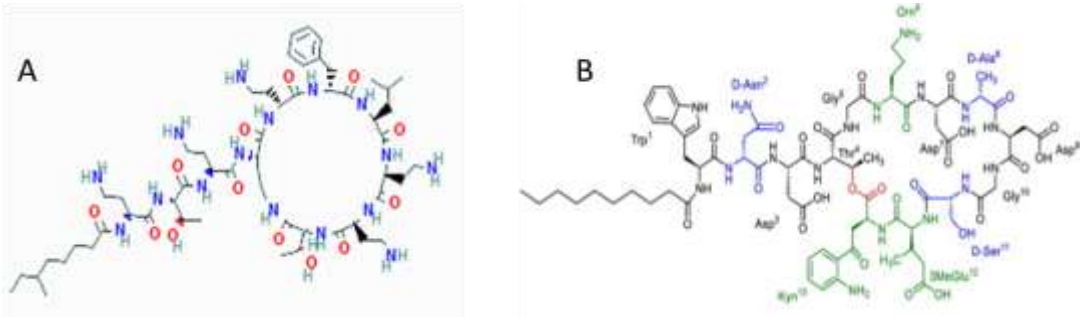


Figure 4: Structures of (A) Polymyxin B and (B) Daptomycin

1.1.1.3 Inhibitors of nucleic acid synthesis

Antibiotics may inhibit the synthesis of nucleic acids like DNA (deoxyribonucleic acid) or RNA (ribonucleic acid) and thereby control bacterial infections.

Inhibitors of DNA synthesis (Replication)

Quinolone antibiotics target the bacterial topoisomerase II (DNA gyrase) and inhibit the ligase function of this enzyme. Fluoroquinolones are produced by addition of fluorine to quinolones at C_6. Some examples of quinolones include nalidixic acid (Figure 5A), cinoxacin, norfloxacin, lomefloxacin, enoxacin, ofloxacin, ciprofloxacin, levofloxacin, sparfloxacin, gatifloxacin, moxifloxacin and trovafloxacin [18].

Aminocoumarins also target the DNA gyrase but at different site than that of quinolones. These antibiotics inhibit the ATPase reaction catalysed by the DNA gyrase. These antibiotics include novobiocin (Figure 5B), clorobiocin and coumermycin A1 [19].

dGTP (deoxyguanosine triphosphate) analogues, 6-anilinouracils (6-AUs) (Figure 5C), selectively inhibit major replicating enzyme i.e., DNA polymerase-III of bacteria [20].

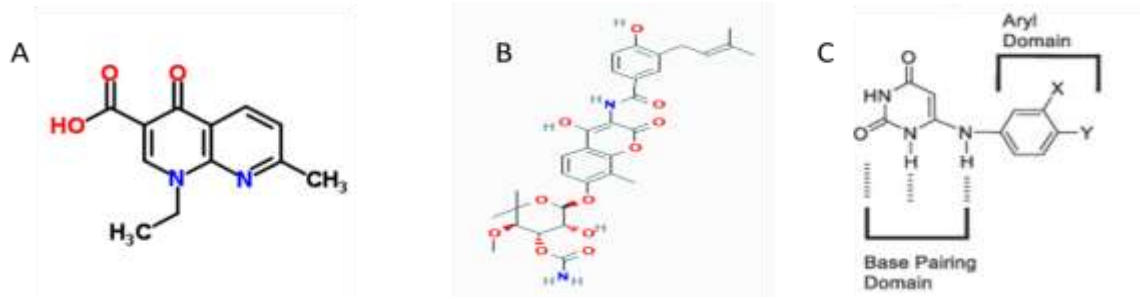


Figure 5: Structures of (A) Nalidixic acid (B) Novobiocin and (C) 6-Anilinouracil

Inhibitors of RNA synthesis (Transcription)

Rifamycins inhibit the transcription initiation by binding to RNA polymerase. This group includes rifamycin, rifampicin (or rifampin) (Figure 6A), rifalazil, rifapentine, rifabutin and rifaximin [21]. Myxopyronin (Figure 6B) targets the RNA polymerase at different site than that of rifamycins [22]. Fidaxomicin (Figure 6C) inhibits the opening of DNA strands which is necessary for RNA synthesis to occur [23].

Streptolydigin (Figure 6D) inhibits the elongation of transcription by binding to RNA polymerase [22].

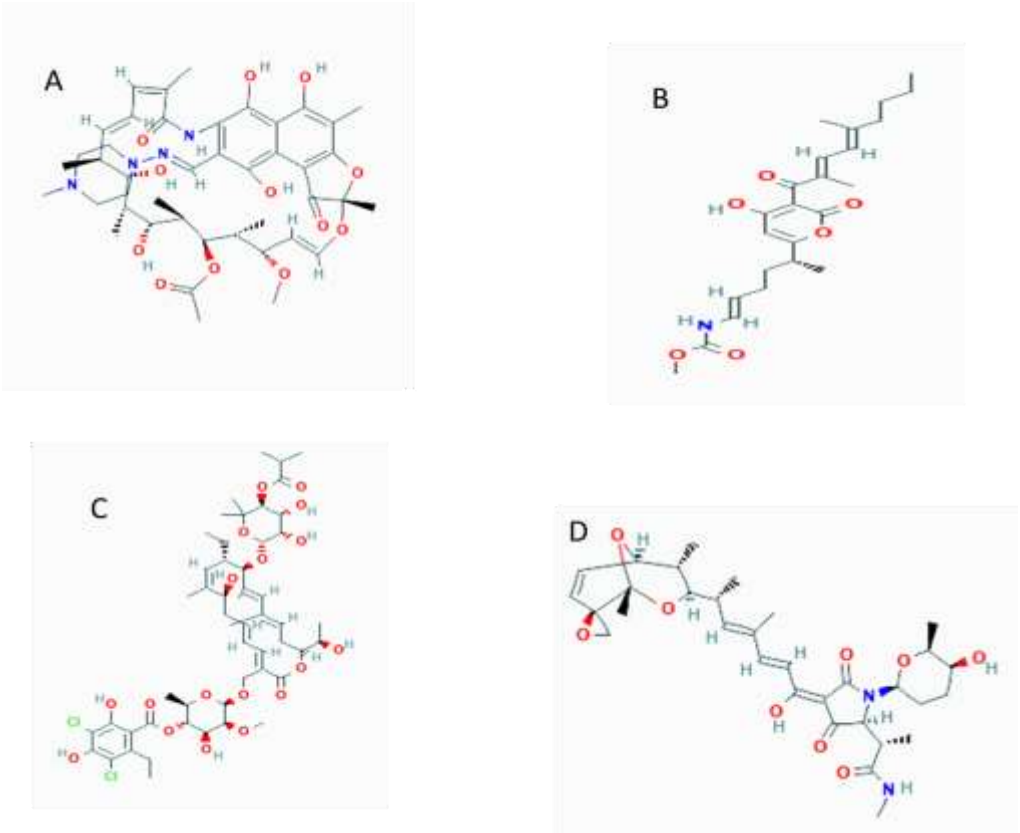


Figure 6: Structures of (A) Rifampicin, (B) Myxopyronin B, (C) Fidaxomicin and (D) Streptolydigin

1.1.1.4 Inhibitors of protein synthesis (Translation)

Linezolid (Figure 7A) inhibits the formation of initiation complex thereby halts polypeptide synthesis [24].

Anti-30 S ribosomal subunit antibiotics

Aminoglycosides (streptomycin, gentamicin (Figure 7B), kanamycin, neomycin, etc.) bind to 30S ribosomal subunit and cause the inaccurate synthesis of proteins via misreading of mRNA [25]. Tetracyclines [26] (Figure 7C) and tigecycline [27] block the A site of ribosome so that charged tRNA cannot be recruited and translation is terminated.

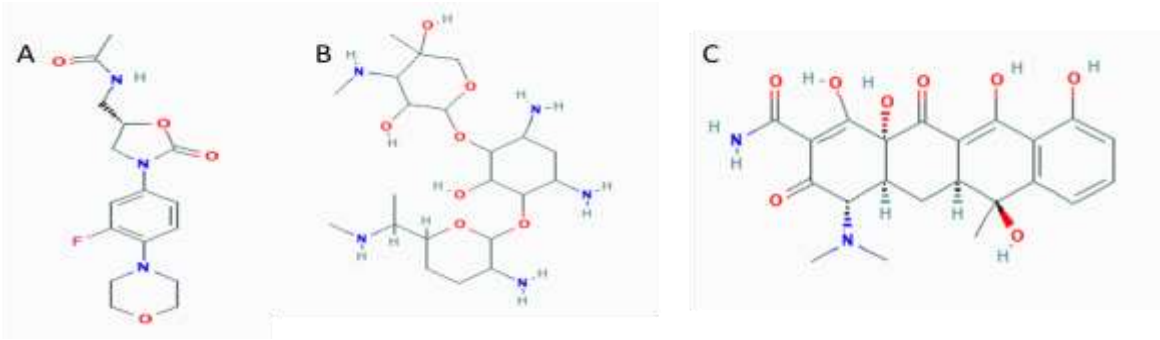


Figure 7: Structures of (A) Linezolid, (B) Gentamicin and (C) Tetracycline

Anti-50S ribosomal subunit antibiotics

Macrolides (erythromycin (Figure 8A), azithromycin, clarithromycin, etc.) bind to P site of 5 S ribosomal subunit and prevent the peptidyl transferase (ribozyme) function and hence synthesis of polypeptide [28]. Chloramphenicol (Figure 8B) binds to 23S rRNA and also inhibits peptidyl transferase activity [29]. Fusidic acid (Figure 8C) works by averting the translocation of elongation factor G (EF-G) [30].

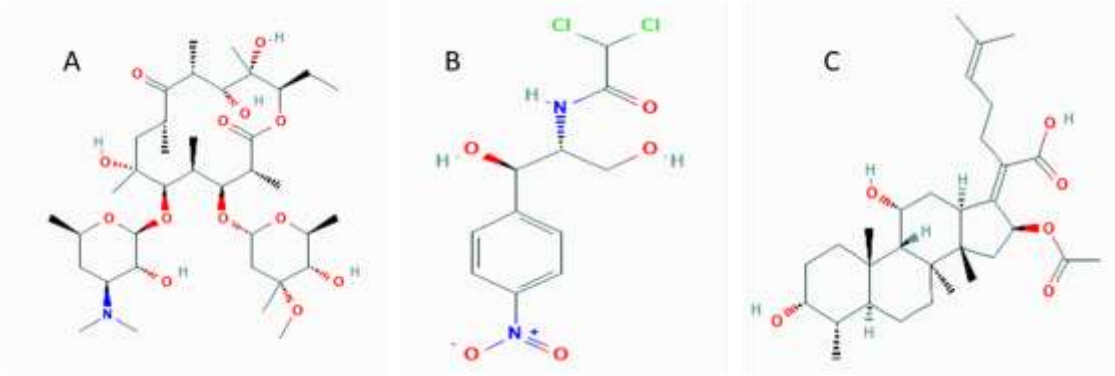


Figure 8: Structures of (A) Erythromycin, (B) Chloramphenicol and (C) Fusidic acid

1.1.1.5 Inhibitors of Metabolite Synthesis

These antibiotics usually target either the folic acid synthesis [sulphonamides (Figure 9A) and trimethoprim] [31] or mycolic acid synthesis [isoniazid (Figure 9B), triclosan and thiolactomycin] [32].

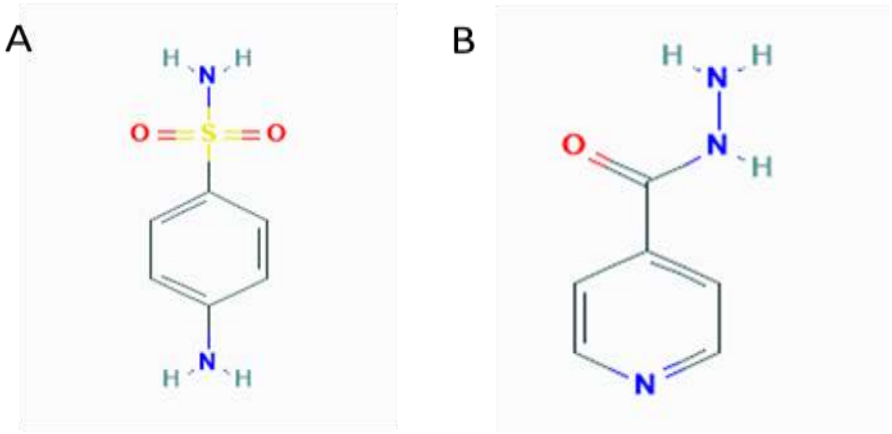


Figure 9: Structures of (A) Sulphonamide and (B) Isoniazid

1.2 Anti-Microbial resistance

Although antibiotics have been effective in treatment of bacterial infections, specifically during World War II [33], due to the improper (mis/over) use, the bacteria have adapted and developed antimicrobial resistance (AMR) to various classes of antibiotics by several different mechanisms. AMR is defined as the unresponsiveness of microbes to antimicrobial drugs (clinically relevant) at standard doses [34-36]. AMR further gets accelerated by the selective pressure or misuse of antibiotics. Alexander Fleming warned about AMR in his Noble prize lecture and this has become a reality in the current day, with evolution of several multidrug resistant (MDR) bacteria [37, 38]. Treating AMR infections is not only complicated and time consuming but causes severe economic burden on the patient and family. The discovery of every new antibiotic has been invariably followed by emergence of resistance. Furthermore, the transfer of resistance genes from one bacterium to another *via* horizontal gene transfer has also escalated AMR. The methods of horizontal gene transfer include *i*) transformation, *ii*) conjugation and, *iii*) transduction [39]. The major driving force in resistance development and dissemination could perhaps be from irrational usage of antimicrobial agents (Figure 10) [34, 40, 41].

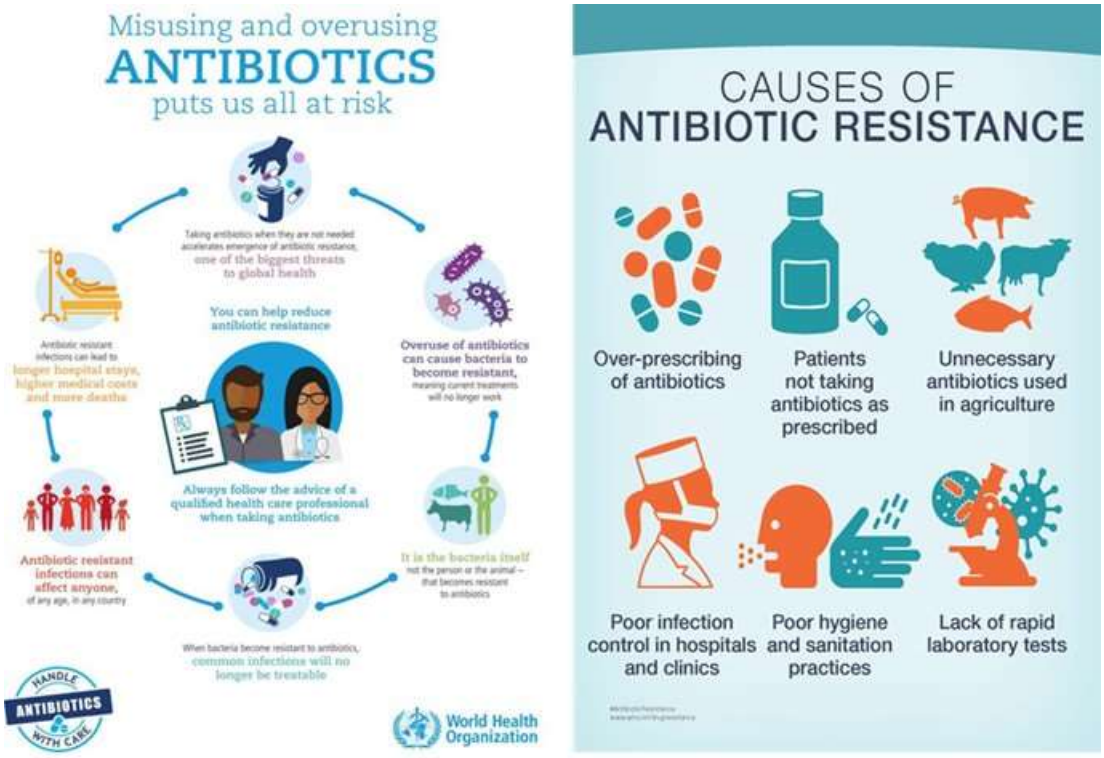


Figure 10: Causes of antibiotic resistance

1.2.1 Mechanisms of antibiotic resistance

Bacteria obtain antibiotic resistance by various mechanisms like modification of antibiotic molecule, decreasing the permeability of antibiotic into the cell, efflux of antibiotic to the outside or by changing the target sites (Figure 11).

1.2.1.1 Alterations of the antibiotic molecule

1.2.1.1.1 Chemical alterations of the antibiotic

In order to survive, bacteria have evolved different types of modifying enzymes, which can add various types of chemical groups to antibiotics and thereby inactivate the antibiotics. These modifications include i) acetylation (aminoglycosides), ii) adenylation (lincosamides), and iii) phosphorylation (chloramphenicol). Most protein synthesis inhibitors are affected by this mechanism. For example, chloramphenicol

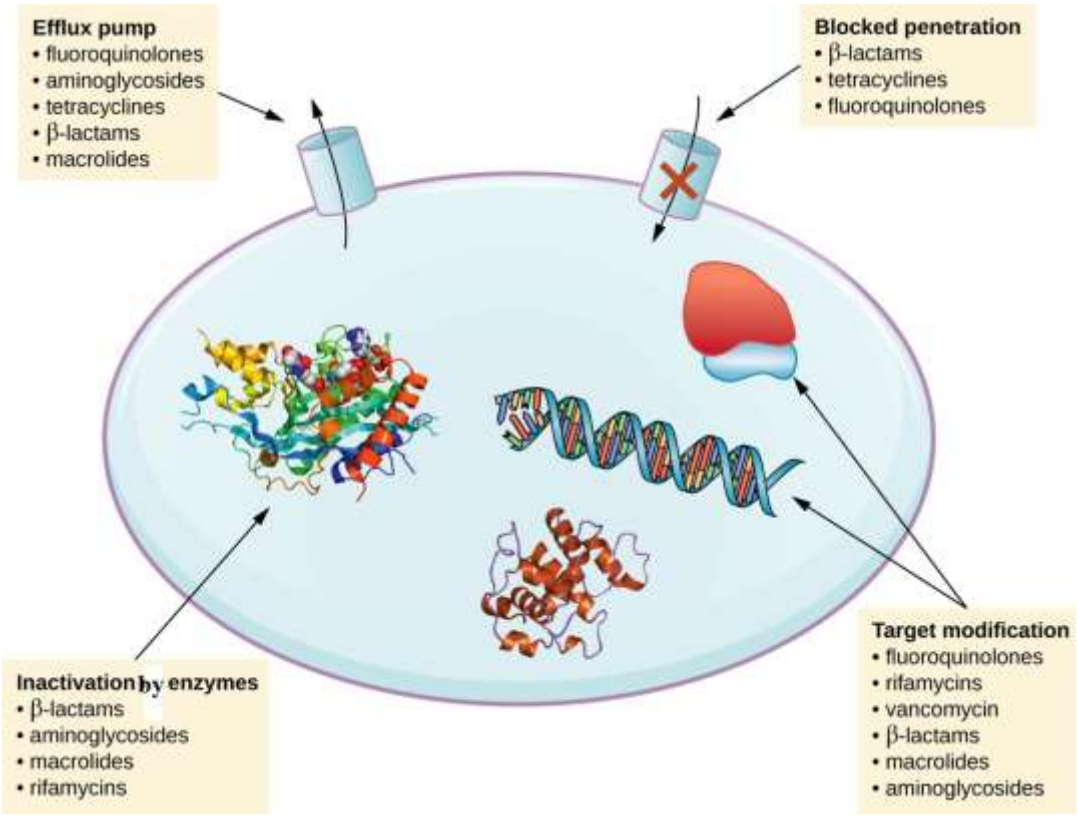


Figure 11: Various mechanisms of antibiotic resistance in bacteria

acetyltransferases are the enzymes that transfer acetyl group onto chloramphenicol and inactivate it [39].

1.2.1.1.2 Destruction of the antibiotic molecule

In this case, the bacteria inactivate the drug by disrupting or cleaving the antibiotics by producing protective enzymes. For example, β-lactamases break the amide bond of β-lactam ring present in β-lactam antibiotics such as penicillins. These disrupted antibiotics are inefficient to bind to their target and hence are not effective on bacteria [42-44]. Till date, greater than five hundred different β-lactamases have been identified. Extended-spectrum beta-lactamase (ESBL bacteria) are the bacteria that produce the beta-lactamase which affords resistance to commonly used antibiotics like penicillins and cephalosporins. Metallo-β-lactamases hydrolyse a broad range of β-lactam drugs including carbapenems and are not inhibited by clavulanic acid or tazobactam. These bacteria are usually referred as superbugs. NDM-1 (New Delhi Metallo β-lactamase-1) (carbapenemase) producing *K. pneumoniae* is an example [39].

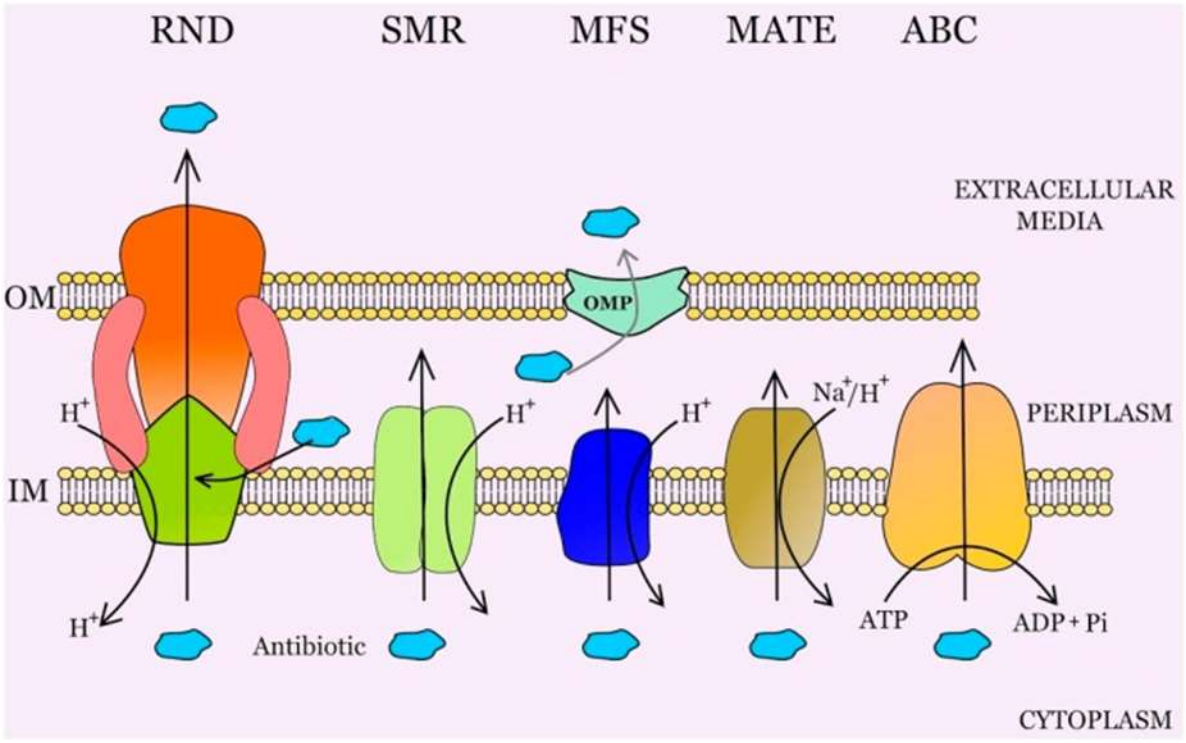
1.2.1.2 Decreased antibiotic penetration and efflux

1.2.1.2.1 Decreased permeability

Porins, water-filled diffusion channels present in the outer membrane of bacteria, allow the hydrophilic antibiotics like β-lactams, tetracyclines and fluoroquinolones to pass-through them. Alterations in i) type of porins, ii) level of porins, and iii) impairment of function cause porin-mediated antibiotic resistance. For example, β-lactam resistance in *E. coli* is caused due to alterations in OmpF, OmpC and PhoE porins and muations in *oprD* gene cause imipenem resistance in *P. aeruginosa* [39].

1.2.1.2.2 Efflux pumps

Efflux pumps are the transport proteins present in the cytoplasmic membrane of bacteria and involved in the extrusion of toxic substrates out of the cell. There are 5 major families of efflux pumps in bacteria: i) the major facilitator superfamily (MFS), ii) the small multidrug resistance family (SMR), iii) the resistance-nodulation-cell division family (RND), iv) the ATP-binding cassette family (ABC), and v) the multidrug and toxic compound extrusion family (MATE). Tet efflux pumps (belonging to MFS family) extrude tetracyclines and provide resistance. NorA of *Staphylococcus aureus* (an MFS) provides resistance to fluoroquinolones. EmrE (SMR family) of *E. coli* affords resistance to efflux of cationic dyes quaternary ammonium compounds, acriflavine and ethidium. AcrAB-TolC in *Enterobacteriaceae* (RND family) offer resistance to tetracyclines. MsrA (in *S. epidermidis*) and MsrC (*E. faecalis*) (both belong to the ABC transporter family) give resistance to macrolides. FepA (a MATE) in *Listeria monocytogenes* offers resistance to fluoroquinolones [39].



Microorganisms. 2016 Mar; 4(1): 14. doi: 10.3390/microorganisms4010014

Figure 12: Different types of efflux pumps present in bacteria

1.2.1.3 Changes in target sites

1.2.1.3.1 Target protection

In this mechanism of drug resistance, antibiotic resistance protein interacts with target protein and hence the antibiotic cannot bind to the target. TetM and TetO proteins which confer resistance to tetracyclines, are homologous to elongation factors (of translation) EF-G and EF-Tu, interact with ribosome and dislodge the antibiotic from ribosome. These also cause conformational change of ribosome so that antibiotic cannot bind to the ribosome. Another example, quinolone resistance protein, Qnr, competes with DNA gyrase so that quinolones cannot interact with DNA gyrase [39].

1.2.1.3.2 Modification of the target site

Target site modification is a common mechanism. It can occur when i) target site encoding gene may undergoes mutation, ii) enzymatically modifies the binding site or iii) replaces or bypasses the original target.

1.2.1.3.2.1 Mutations of the target site

Development of rifampin resistance is a classic example of mutation of target site. Rifampin binds to β-subunit and thereby inhibits the function of RNA polymerase ($\alpha_2\beta\beta'\sigma$). Point mutation in *rpoB* gene leads to the change in amino acid so that the mutated β-subunit has decreased affinity for rifampin and it can continue the transcription even though the drug is attached to it. Resistance to some fluoroquinolones and oxazolidinones (linezolid and tedizolid) is also by mutation of target site [39].

1.2.1.3.2.2 Enzymatic alteration of the target site

In this method of antibiotic resistance, the target site is altered by the addition of some chemical groups by modifying enzymes. The *erm* genes (erythromycin ribosomal methylation) produce the enzyme which methylates the adenine present at position 2058 in domain V of the 23 S rRNA present in larger 50 S ribosomal subunit. This methylation alters the target so that the antibiotic cannot bind to 50 S subunit. This modification can even offer cross resistance to macrolides along with erythromycin. *ermA* and *ermC* are found in plasmids in MSSA [39].

1.2.1.3.2.3 Complete replacement or bypass of the target site

In this type, the original target of antibiotic is replaced to form a new target molecule. The newly formed target will show biochemically similar function like original target but it cannot be inhibited by the antibiotic. *mecA* gene (present in methicillin resistant *S. aureus*), a part of staphylococcal chromosomal cassette mec (SCCmec), encodes PBP2a which is a penicillin binding protein (PBP) (or transpeptidase, needed for bacterial cell wall synthesis) instead of normal PBP and has lower affinity to all β-lactams, including penicillins, cephalosporins and carbapenems. Vancomycin resistance in enterococci involves a group of genes called as *van* gene clusters which alter the process of synthesis of peptidoglycan cell wall by changing the terminal amino acids of pentapeptide. When these terminal amino acids are changed, the interaction between pentapeptide and vancomycin will be lost and hence the bacterium acquires resistance to vancomycin. Vancomycin resistance is also seen in *S. aureus* (VRSA). Resistance to trimethoprim-sulfamethoxazole (TMP-SMX) also comes under the bypass mechanism [39].

1.2.2 Drug resistance by transposons

Transposons (Tns) are DNA elements that can move within DNA or between DNA molecules. So, these are also called as jumping genes. Transposons are mainly of two types: i) Class-I (retro) transposons which have RNA intermediate, and are present in eukaryotes; ii) Class-II (DNA) transposons, which are present in both prokaryotes and eukaryotes. Bacterial transposons belong to Tn family and usually these carry antibiotic resistance genes along with other genes. Transposons can move among the plasmids or between chromosomal DNA and plasmid and help in transmission of drug resistance genes. Some examples of antibiotic resistance which involve transposons are vancomycin resistance in *Enterococcus*; carbapenems and kanamycin resistance in *P. aeruginosa*; tetracycline resistance in *Klebsiella*; resistance to chloramphenicol, kanamycin and neomycin in *Salmonella typhimurium*; gentamicin, kanamycin, and tobramycin resistance in *S. aureus*. Vertical (from one generation to next) and horizontal (by transformation, conjugation or transduction) transfer of antibiotic resistance genes among the bacteria challenge us to treat the infections caused by them [45].

1.3 Reasons for antibiotic resistance

Use of millions of kilograms of antibiotics globally for prophylactic measures and for treatment of infections in people, animals and agriculture is the major cause for creation of selection pressure in bacteria, eliminating the susceptible bacteria and selecting the resistant ones [46]. Antibiotic residues in the food products of animals indicates over use of antibiotics [47]. The commensal microorganisms living in these animals are becoming resistant due to use of antibiotics. The excreta of these animals often contain antibiotic resistant bacteria and when they are released into the environment, transfer the genes of antibiotic resistance to other microbes via horizontal gene transfer [48]. The use of antibiotics without prescription is one of the major reasons for the misuse of antibiotics in many developing countries [49]. The misuse of antibiotics is mainly for treatment of viral infections like common cold, flu, etc. or by prescribing the antibiotics without having knowledge on causative agent and its resistance pattern [50]. Inappropriate antibiotic treatment strategy results in antibiotic resistance among 30-50% of patients [51]. Furthermore, treatment with antibiotics for longer durations can result in the emergence of resistant bacteria.

Antimicrobials in waste waters are creating selection pressure and leading to the evolution of antimicrobial resistant organisms [52]. The resistant bacteria produced in animals or environment can spread among the animals and even to the human beings as zoonotic diseases through food chain [53] or through animal handlers [54]. *Campylobacter*, *Enterococci, Listeria*, *Salmonella* and some strains of *E. coli* are able to spread in this manner. Low sanitation practices and poor hygiene are another major cause for the spread of nosocomial infections to the healthy individuals leading to the community acquired resistant bacteria [55]. The total amount of antibiotics being applied to defined number of individuals in a geographically defined setting like home, farm or hospital involves selection density. Each individual becomes a 'factory' of resistant bacteria that enter the environment.

1.4 Evolution of superbugs

If the bacteria acquire resistance to different class and generations of antibiotics, they emerge as superbugs. In Figure 13 the emergence of superbugs, mainly the development of *S. aureus* superbugs is shown and in Figure 14 the timeline of antibiotic resistance compared to antibiotic development is shown.

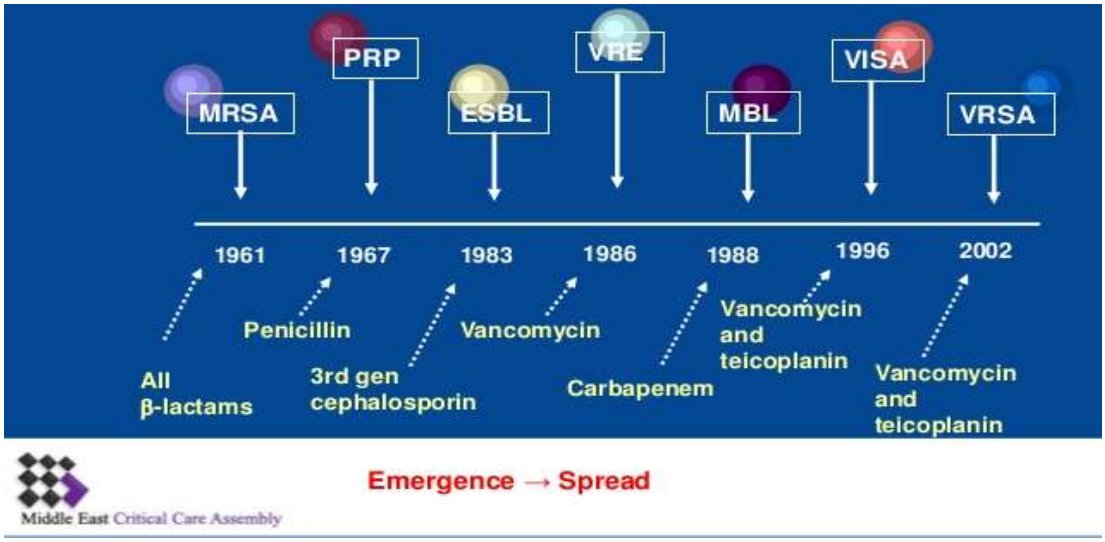


Figure 13: Emergence of superbugs

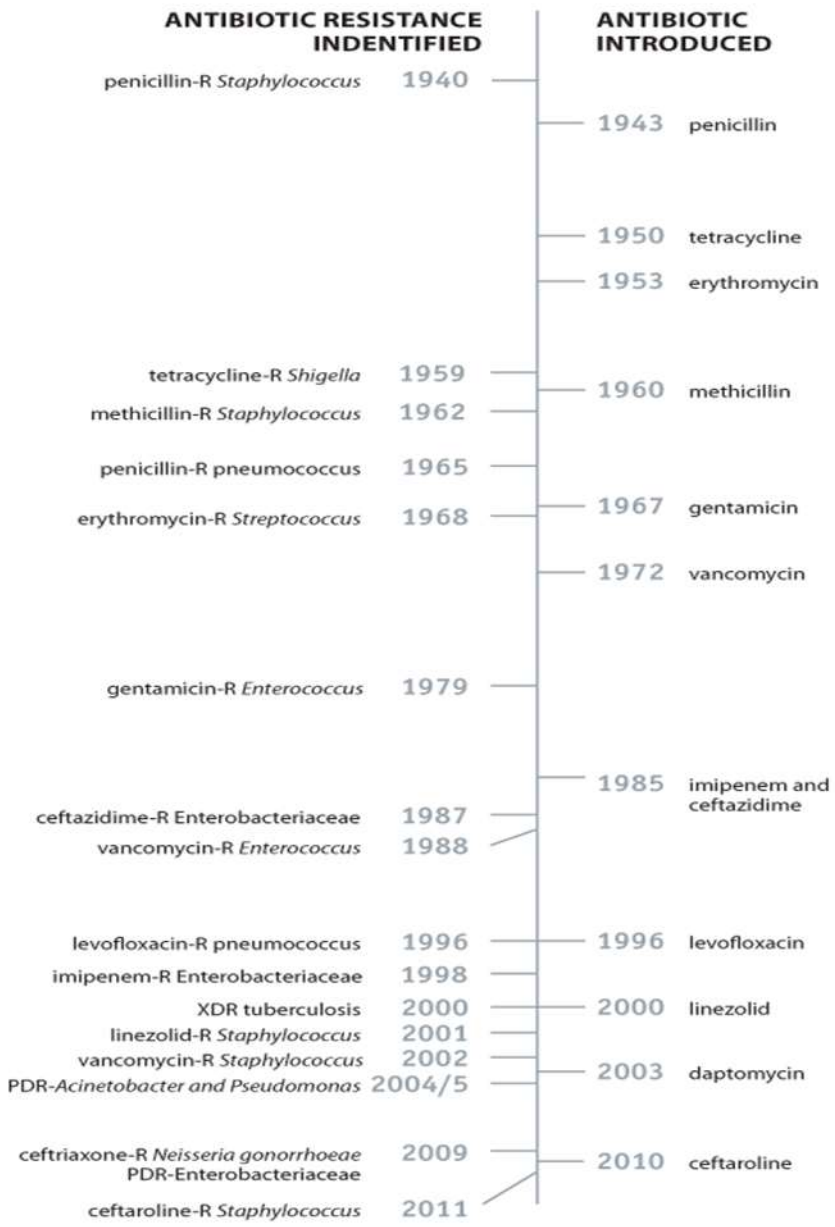


Figure 14: Timeline of antibiotic resistance compared to antibiotic development

Infections caused by superbugs are difficult to treat. Depending upon the number of classes of antibiotics to which the bacteria are resistant, there are three different types of drug-resistant bacteria: 1) multidrug-resistant (MDR), defined as "acquired non-susceptibility to at least one agent in three or more antimicrobial categories", e.g., *S. aureus*, 2) extensively drug-resistant (XDR) defined as "non-susceptibility to at least one agent in all, two or fewer anti-microbial categories (i.e. bacterial isolates remain susceptible to only one or two categories)", e.g., XDR-TB; 3) pandrug-resistant (PDR) bacteria defined as "non-susceptibility to all agents in all antimicrobial

categories", e.g., *Pseudomonas aeruginosa* [57]. ESKAPE pathogens that include *Enterococcus*, *Staphylococcus*, *Klebsiella*, *Acinetobacter*, *Pseudomonas* and *Enterobacter* species are the major cause of nosocomial (hospital acquired) infections throughout the world [58].

Enterococcus faecalis live as commensal in the gastrointestinal tract of poultry and humans [59]. In recent years, it has emerged as a major nosocomial pathogen. It can transfer antibiotic resistance genes (tetracycline resistance) to other species like *E. coli*. It also shows synergistic virulence in interaction with *E. coli* [60]. *E. faecalis* is even resistant to vancomycin (VRE-vancomycin resistant *Enterococci*) and hence it has emerged as superbug [61].

Staphylococcus aureus is a commensal in respiratory tract of 30% of the population [62]. Soon after the introduction of penicillins to control the *S. aureus*, penicillin resistance was observed in this organism. Methicillin, a beta-lactam antibiotic, which cannot be degraded by beta-lactamases was used to treat these resistant bacteria. Soon after the introduction of methicillin into clinic for the treatment of *S. aureus* infections, the organism developed resistance by producing a different penicillin-binding protein, PBP2a, from *mecA* gene, which has lesser affinity to methicillin. Next, vancomycin, a glycopeptide that stops the cell wall biosynthesis, became the drug of choice for the treatment of MRSA infections. However, *S. aureus* had gained vancomycin resistance by horizontal gene transfer through *vanA* gene containing transposon Tn1546 from *Enterococci*. Depending upon the minimum inhibitory concentration (MIC) of vancomycin for *S. aureus* these bacteria are classified into vancomycin-intermediate *Staphylococcus aureus* (VISA) and vancomycin-resistant *Staphylococcus aureus* (VRSA) [63].

Klebsiella pneumoniae is a commensal living in the intestine and transferred to the environment via the faeces. As an opportunistic pathogen it can cause several diseases including community and nosocomial infections. *Klebsiella* produce extended-spectrum beta-lactamases (ESBL) and are therefore resistant to all beta-lactam antibiotics, other than carbapenems. Some *Klebsiella* are even resistant to aminoglycosides, fluoroquinolones, tetracyclines, chloramphenicol, and trimethoprim/sulfamethoxazole making them PDR bacteria [64].

Introduction

Acinetobacter is present in soil, water and in animals. MDR *Acinetobacter* are resistant to all penicillins and cephalosporins. XDR *Acinetobacter* are resistant to all penicillins, cephalosporins and carbapenems. PDR *Acinetobacter* is even resistant to polymyxins and tigecycline along with the above-mentioned classes [65]. *Acinetobacter baumannii*, carbapenem-resistant is in priority 1 list released by WHO for priority pathogens (Table 3).

Pseudomonas aeruginosa can cause generalized inflammation and even fatal diseases when it colonizes lungs, the urinary tract, and kidneys [66]. By undergoing mutations and acquiring resistant genes through horizontal gene transfer this bacterium gained enormous resistance to various classes of antibiotics. Production of ESBLs, aminoglycoside-modifying enzymes, efflux pumps and modification of topoisomerases makes this a PDR bacterium [67].

Enterobacter cause nosocomial infections in immunocompromised patients. Carbapenems are the drugs of last resort for these infections. Carbapenem-resistant *Enterobacteriaceae* (CRE) are ESBL producing bacteria which are resistant to carbapenems. These bacteria usually produce two types of enzymes, KPC (*Klebsiella pneumoniae* carbapenemase) and NDM (New Delhi Metallo-beta-lactamase) [68]. CRE is also in priority 1 list released by WHO indicating its aggressiveness.

Mycobacterium tuberculosis causes tuberculosis (TB). The mismanagement of patients with multidrug resistant-TB (MDR-TB) to various classes of antibiotics lead to the development of extensively drug resistant TB (XDR-TB). The World Health Organization (WHO) defines XDR-TB as MDR-TB that is resistant to at least one fluoroquinolone and a second-line injectable drug (amikacin, capreomycin, or kanamycin) [69].

Escherichia coli is a common microbe in the intestine of human and animals. Some *E. coli* can cause serious food poisoning or infections in bloodstream or urinary tract. ESBL-*E. coli* is resistant to penicillins and even up to third generation cephalosporins [70].

Evolution of superbugs is challenging the scientists and doctors to discover new antibiotics or alternative treatment strategies.

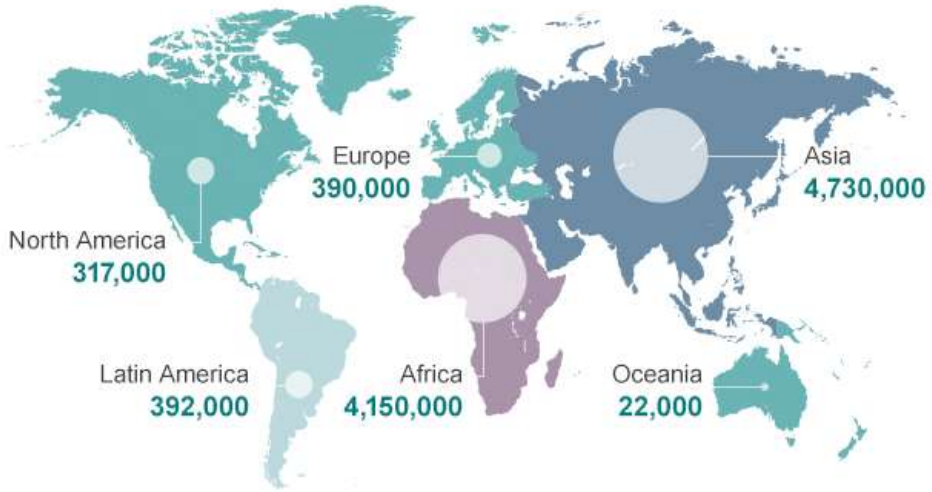
Table 3: The complete WHO (World Health Organisation) priority pathogens list

Priority 1: Critical	Priority 2: High	Priority 3: Medium
Acinetobacter baumannii, carbapenem-resistant	*Enterococcus faecium*, vancomycin-resistant	*Streptococcus pneumoniae*, penicillin-non-susceptible
Pseudomonas aeruginosa, carbapenem-resistant	*Staphylococcus aureus*, methicillin-resistant, vancomycin-intermediate and resistant	*Haemophilus influenzae*, ampicillin-resistant
Enterobacteriaceae, carbapenem-resistant, ESBL-producing	*Helicobacter pylori*, clarithromycin-resistant	*Shigella spp.*, fluoroquinolone-resistant
	Campylobacter spp., fluoroquinolone-resistant	
	Salmonellae, fluoroquinolone-resistant	
	Neisseria gonorrhoeae, cephalosporin-resistant, fluoroquinolone-resistant	

1.5 Statistics of antibiotic resistance

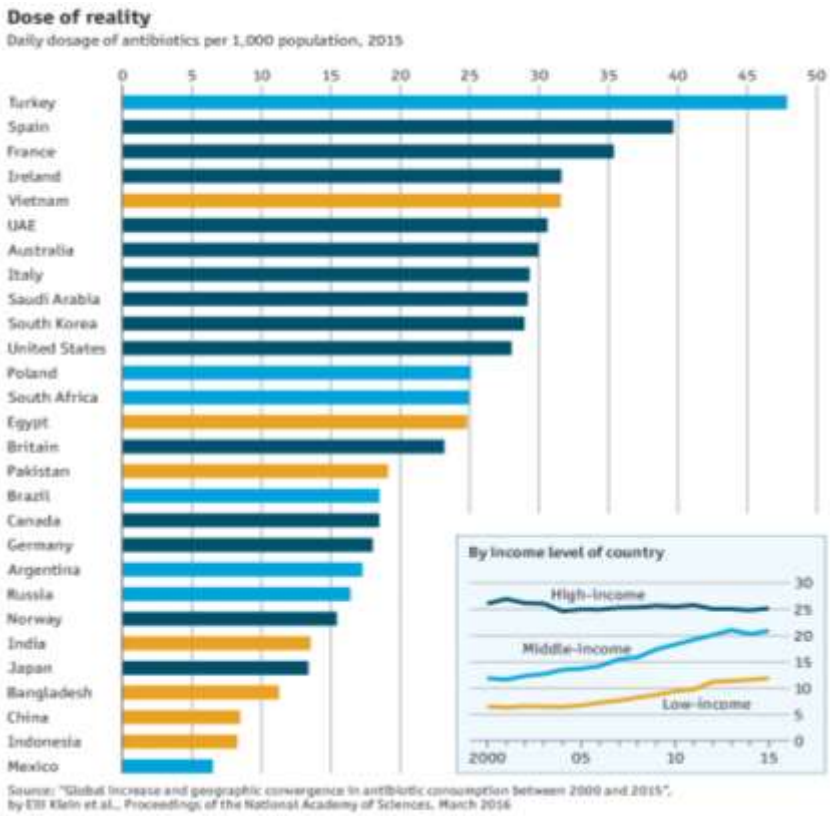
Antibiotic resistance is an emerging global problem. In 2014, WHO estimated that there were about 480,000 new cases of MDR-TB. It was also estimated that XDR-TB is present in 9.7% of people with MDR-TB. Currently, 7,00,000 people die per annum due to drug-resistant bacterial diseases. "WHO's new Global Antimicrobial Surveillance System (GLASS)" revealed that 5,00,000 people, across 22 countries, who are suspected with bacterial infections have antibiotic resistance. Deaths of new borns due to sepsis caused by pathogens resistant to first-line of antibiotics are more than 50,000 annually and is estimated to reach two million by 2050 [71]. UN Ad hoc Interagency Coordinating Group on Antimicrobial Resistance anticipated that has estimated deaths due to anti-microbial resistance will be 10 million per annum by 2050. Deaths attributable to antimicrobial resistance every year by 2050 in various continents are given in Figure 15 and daily dose of antibiotics in the year 2015 among

various countries is given in Figure 16. Approximately 24 million people will be forced into extreme poverty by 2030 because of antimicrobial resistance which needs costly treatment for infections. MRSA occurrence in India had increase from 29 % in 2008 to 47 % in 2014 [164].



Source: Review on Antimicrobial Resistance 2014

Figure 15: Deaths attributable to antimicrobial resistance every year by 2050



Source: "Global increase and geographic convergence in antibiotic consumption between 2000 and 2015", by Eili Klein et al., Proceedings of the National Academy of Sciences, March 2016

Figure 16: Daily dose of antibiotic per 1,000 population among various countries in the year 2015

1.6 Battling antibiotic resistance

In a step towards combating AMR, WHO is organising "Antibiotics: Handle with care" awareness week in every November since 2015 to bring awareness in people regarding the proper use of antibiotics and the adverse effects of antibiotic resistance by microbes. "In India, various actions have been taken including setting up of a National Task Force on AMR Containment (2010), "Chennai Declaration" by a consortium of the Indian Medical Societies (2012), Setting of Indian Council of Medical Research national surveillance network of laboratories, "Redline" campaign for educating public and National Action Plan on AMR 2017" [71]. Antibiotic stewardship programs are implemented to battle antibiotic resistance. One such program is 'One Health' a strategy promoted for innovative and efficient approaches for minimizing or stopping the occurrence and blowout of antibiotic resistance and usage on humans and animals by WHO in collaboration with other organisations. Some of the stewardship programs include: (i) Community-based treatment programs, (ii) Infection control programs, (iii) Vaccination programs and (iv) and Proper sanitation [51, 72].

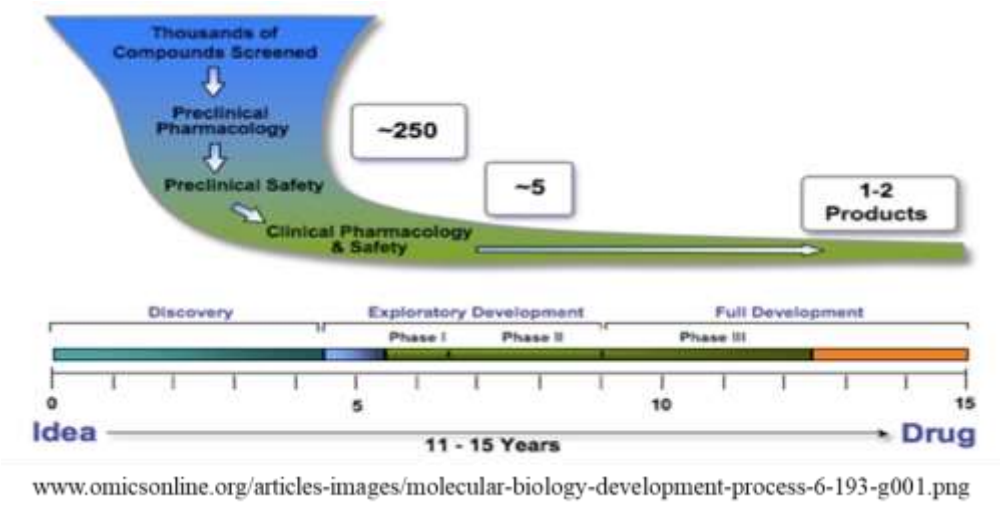
In a recent study it was demonstrated that the development of antibiotic stewardship (ABS) programs had significantly reduced the incidence of infections due to pathogenic bacteria. It was observed that these interventions were effective in haematology and oncology departments [73]. Combination of ABS programs with several intensified infection control measures, had an effective decrease of AMR up to a maximum of 70%, with hand hygiene compliance being the best and efficient practice [74-77].

Besides maintaining AMR stewardship programs, it is required to come up with novel approaches for combating AMR. Below are few of the strategies which have been employed for management of AMR.

1.6.1 Development of new antibiotics

The tremendous increase in the antibiotic resistance demands the discovery of new antibiotics. The discovery new antibiotics gradually decreased in past 3 decades (Figure 18) and at the same time antibiotic resistance has been increasing endlessly. Apart from that there are many factors for drying of antibiotic pipeline. A new drug

takes an approximately 12 years to come into market (Figure 17) and the approximate cost for each antibiotic discovery is $2.6 billion [78]. Huge loss in investments on anti-infectives sector by many pharmaceutical companies forced them to quit from the new drug discovery. In addition to this, most of the new antibiotics are reported to show adverse effects in clinical trials [79, 80]. As of December 2018, approximately 42 new antibiotics are in clinical development. Generally, one out of five from phase 1 clinical trials is expected to be approved for patients [81]. Hence, alternative methods along with antibiotic discovery to treat drug resistant bacterial infections should be adopted.



www.omicsonline.org/articles-images/molecular-biology-development-process-6-193-g001.png

Figure 17: Various phases in new drug discovery and approximate time for each phase

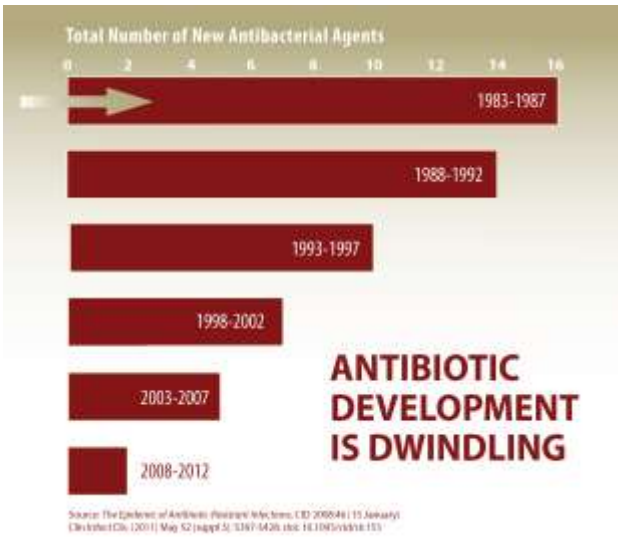


Figure 18: Pipeline of new drug discovery

1.6.2 Alternatives to new drug discovery/development

The important alternatives to the new drug discovery are drug combination therapy, drug repositioning (repurposing) and drug rescuing.

1.6.2.1 Drug combination therapy

In the drug combination therapy, more than one drug is given to the patients so that each drug will have different target and hence theoretically it is highly difficult for bacteria to acquire resistance. Cefixime plus azithromycin is recommended for gonococcal and chlamydial infections, but these bacteria developed resistance to these drugs [82]. Amoxicillin and clavulanic acid combination are routinely used for bacterial infections where clavulanic acid inhibits the beta-lactamase enzyme and amoxicillin binds to peptidyl transferase (PBP) and inhibits the bacterial cell wall synthesis [83]. Similarly, ampicillin-sulbactam combination [84] and piperacillin-tazobactam combinations [85] are used where the former is penicillin and later is an inhibitor of beta-lactamase. Rifampin, isoniazid, pyrazinamide and ethambutol combination are used to treat tuberculosis. Initially the combinations of different classes of antibiotics were effective on various drug resistant bacteria but gradually the bacteria acquired resistance to various drug combinations [86].

To limit MDR, XDR and PDR bacterial infections, the Food and Drug Administration (FDA), USA has suggested non-traditional treatment strategy with combination of non-antibiotic and antibiotic drugs. In one of these methods, the non-antibiotic drug may act as an immunomodulator and hence host immune system is activated to kill the pathogen [87] and the antibiotic that is used in combination also targets the bacteria. In another type, the non-antibiotic may act as a potentiator of antibiotic [88], possibly by increasing the half-life or by increasing the permeability of antibiotic, and hence antibiotic can show its action with greater potency.

1.6.2.2 Drug repurposing

Drug repurposing is also known as drug repositioning or drug reprofiling, where a drug approved for treatment of one disease is used to treat another disease. Since new drug development pipe line has dried up, this approach helps in the effective use of approved drugs and abandoned drugs which already passed through various clinical

trial phases for alternative treatment strategies. The advantages of drug repurposing are [89]

i) Safety: The approved drugs are already tested for safety and hence these drugs can be readily used to test on other diseases (Table 4).

ii) Money and time saving: Drug repurposing will save nearly 40% of the cost that is spent and time it requires on new drug discovery (Figure 19).

iii) The market potential: Some repositioned drugs like thalidomide and lenalidomide may have good market value for manufacturers.

Table 4: Repurposing of existing drugs to treat other diseases or infections

Drug	Original indication	New indication	Clinical trial stage
		Infectious Diseases	
		Anti-bacterial	
PNU-100480	MRSA	Tuberculosis	Phase I clinical trail
Sulphamethaxazole +Trimethoprim	Generic antibacterial	Tuberculosis	Clinical use
Raloxifen	Osteoporosis + breast cancer	*P. aeruginosa*	Preclinical
		Anti-protozoal	
Astemizole	Antihistamine	Malaria	preclinical
Dapsone	Leprosy	Malaria	phase 3 completed
Amphotericin	Antifungal	Leishmaniasis	phase 3 completed
DB289	Pneumocystis	Malaria and African trypanosomiasis	phase 2 completed
Eflornithine	Cancer	African trypanosomiasis	phase 3 completed
Fosmidomycin	Urinary-tract infections	Malaria	phase 2 completed
Harmine	Cancer	Malaria	preclinical
Miltefosine	Cancer	Visceral and cutaneous leishmaniasis	phase 2 completed
Paromomycin	Antiamebic	Visceral leishmaniasis	phase 4 completed
Pentamidine	Pneumonia (*Pneumocystis carinii*)	Trypanosomiasis and antimony-resistant leishmaniasis	phase 2 completed
Auranofin	Rheumatoid Arthritis	Amebiasis	Clinical use
		Anti-parasitic	
Closantel	Antihelminthic	Onchocerciasis	preclinical
		Anti-prion disease	
Quinacrine	Malaria	Creutzfeldt-Jakob Disease	phase 2 completed
		Others	
Arsenic	Tuberculosis and syphilis	Acute promyelocytic leukemia	phase 2 completed, phase 3 active
Digoxin	Congestive heart failure and arrhythmia	Cancer	phase 1 completed, recruiting subjects for phase 2
Fumagillin	Antiamebic	Cancer (angiogenesis inhibitor)	preclinical
Gemcitabine	Antiviral	Cancer	phase 2 active

Pharmaceuticals (Basel). 2013 Jan 28;6(2):124-60. doi: 10.3390/ph6020124.

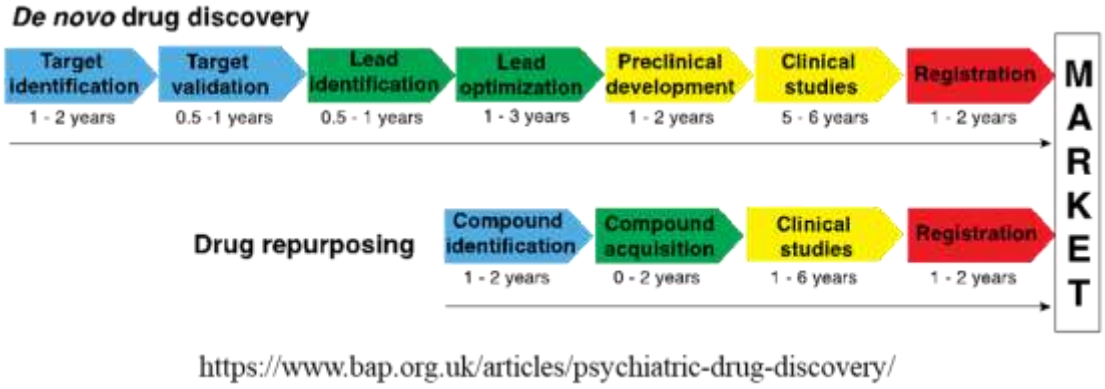


Figure 19: Advantage of drug repurposing in saving the time

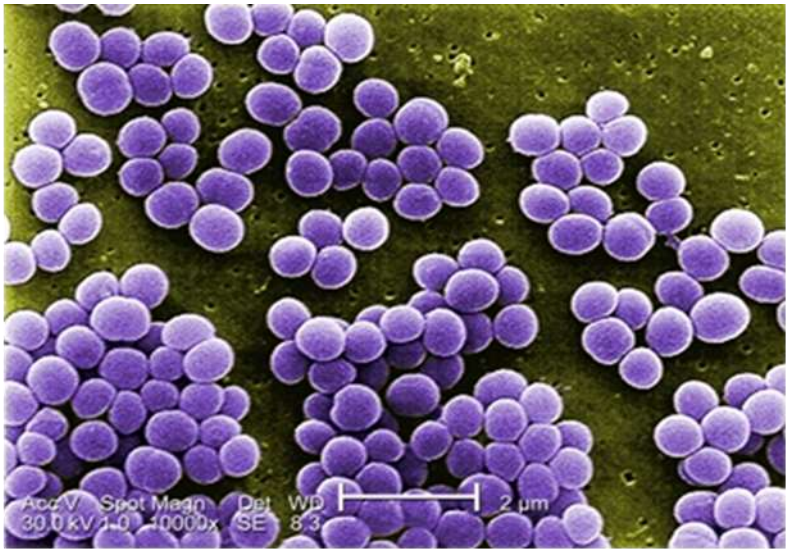
1.6.2.3 Drug rescuing

According to Dr Reed, "The ability to rescue a drug that was once studied for a specific use and found safe but ineffective and archived by its innovator and then rediscovered and repurposed for a different use" is called as drug rescuing [165]. Drug rescuing is also a good alternative to new drug discovery [90].

1.7 Combating *S. aureus* infections by drug repurposing

1.7.1 Introduction

According to the bacterial nomenclature [91], the genus *Staphylococcus* comprises of 47 species, and numerous subspecies. *S. aureus* (Figure 20) is part of human microbiota. It can cause a wide range of illnesses as an opportunistic pathogen. It is very important and an essential aspect to discriminate the meat contaminations of *S. aureus* and NSA (non-*S. aureus* staphylococci) in terms of public health significance [92, 93]. It causes both nosocomial and community acquired infections [94]. Many of the health problems which are difficult in treating are associated with the toxins produced by *S. aureus* [95]. In addition to this, the drug-resistance occurrences have been increasing in many strains of *S. aureus* [96, 97].



https://articles.extension.org/pages/28432/staphylococcus-aureus

Figure 20: *Staphylococcus aureus*

S. aureus can cross host defence system barriers and is very adaptable. Colonization of *S. aureus* is observed in almost half the population of healthy adults [98]. This bacterium can exist as a commensal without causing any pathogenesis to host cells and sometimes exists as a mild to dangerous pathogen causing severe infections such as pneumonia, endocarditis, skin abscesses, osteomyelitis and could even lead to toxic shock syndrome (TSS). Majority of infections related to respiration, joints, skin, bone, soft tissues and endovascular disorders are due to *S. aureus*. Antibiotic resistant strains cause severe infections which are very hard to treat [99]. Two major forms of antibiotic resistant *S. aureus* are observed, Methicillin-resistant *S. aureus* (MRSA) and Vancomycin resistant *S. aureus* (VRSA) [99-101]. In 1961, MRSA evolved by acquiring *mecA* gene that codes for a modified penicillin-binding protein (PBP2a) with less binding efficiency for β-lactam antibiotics [102]. Vancomycin became the choice of antibiotic to treat MRSA infections. However, in 1997, vancomycin-resistant *S. aureus* was reported with acquisition of enterococcal *vanA* cassette into *S. aureus*. Many of the hospital infections, in particular the device-related infections, are caused by this pathogen [103-106].

S. aureus causes food-borne diseases. When *S. aureus* grows in food (including dairy products) it produces 20 kinds of heat-resistant and food poisoning enterotoxins. The sources of Staphylococcal food poisoning (SFP) can be raw milk from mastitis-

affected cattle, dairy products, pork and also inadequate hygienic conditions [107] leading to serious economic loses.

1.7.2 MRSA and VISA

Since the time of first discovery of MRSA strains in 1961, cephalosporins are the only choice of antibiotics to treat MRSA. Later the glycopeptide, vancomycin, was found to be effective in treating MRSA infections. However, vancomycin-intermediate resistant *S. aureus* (VISA) was discovered in 1996 in Japan (initially known as glycopeptide intermediate *S. aureus* (GISA)).

Healthcare settings encounter MRSA infections frequently. In 1999, more than 50% infections were reported to be due to MRSA in intensive care unit patients. However chronologically a steady increase of 7.4 % in MRSA strains have been reported globally. In 2012, European Surveillance data indicated that MRSA has increased by 17.8% compared to 2011 report. Similarly, in 2013, the morbidity due to MRSA increased by 7%. In contrast, very few reports have shown a decline in MRSA infections, in particular CDC data indicates that during 5 years, hospital-onset and community-onset MRSA infections declined by 42% and 29% respectively. Sepsis and catheter associated urinary tract infections due to hospital acquired MRSA after the operation increased by 8 and 3.6 %, respectively, in the last decade. Even deaths from HIV/AIDS, were lower (17,000) than MRSA (18,800) on average in a year during the last decade. Also, the prevalence of paediatric MRSA musculoskeletal infections resulting in many adverse outcomes and longer hospitalizations were also high (11.8% in 2001 to 34.8% in 2009) [108].

Diabetic patients with MRSA infections, in particular the diabetic foot infections, are most difficult to treat. Studies have reported that 43% of diabetic patients have MRSA bacteraemia and with 20% of overall death rate due to MRSA [166].

Agency for Healthcare Research and Quality (AHRQ) has reported that despite all the efforts to lower the MRSA infections, the occurrence of resistant infections is constantly growing and is a global alarming threat (Figure 21). With the new antibiotic drug discovery pipeline being void and no success in clinical trials for new antibiotics during the last two decades, drug repurposing emerges as a viable alternative strategy for the treatment of resistant *S. aureus* infections.

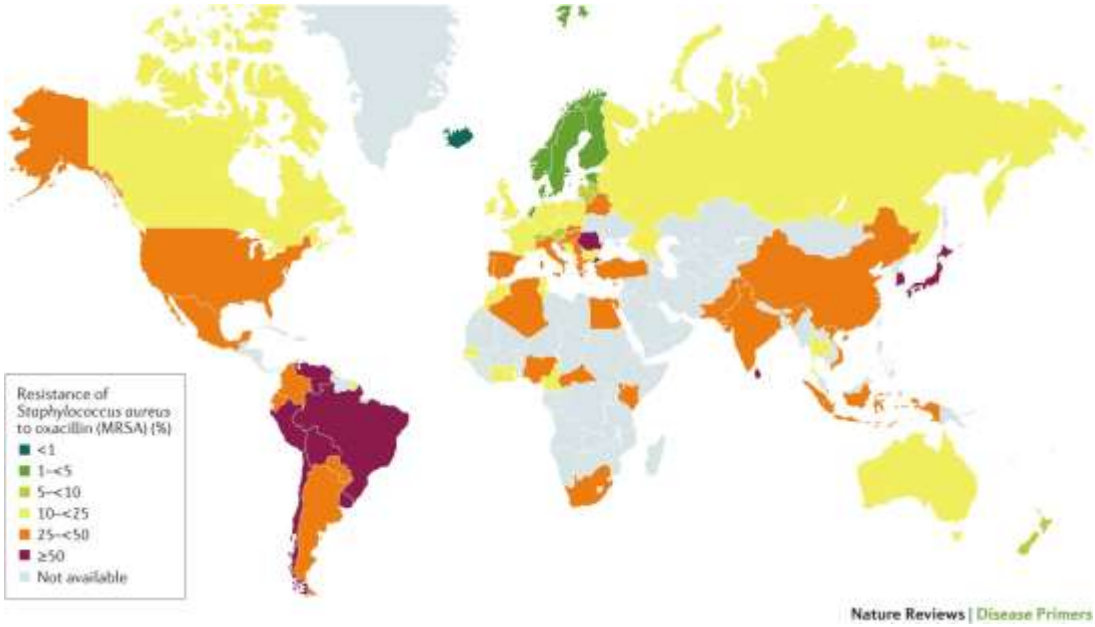


Figure 21: Global MRSA status

1.8 Celecoxib as a viable alternative

1.8.1 Celecoxib

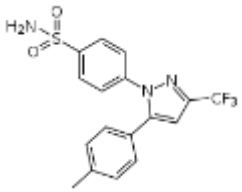
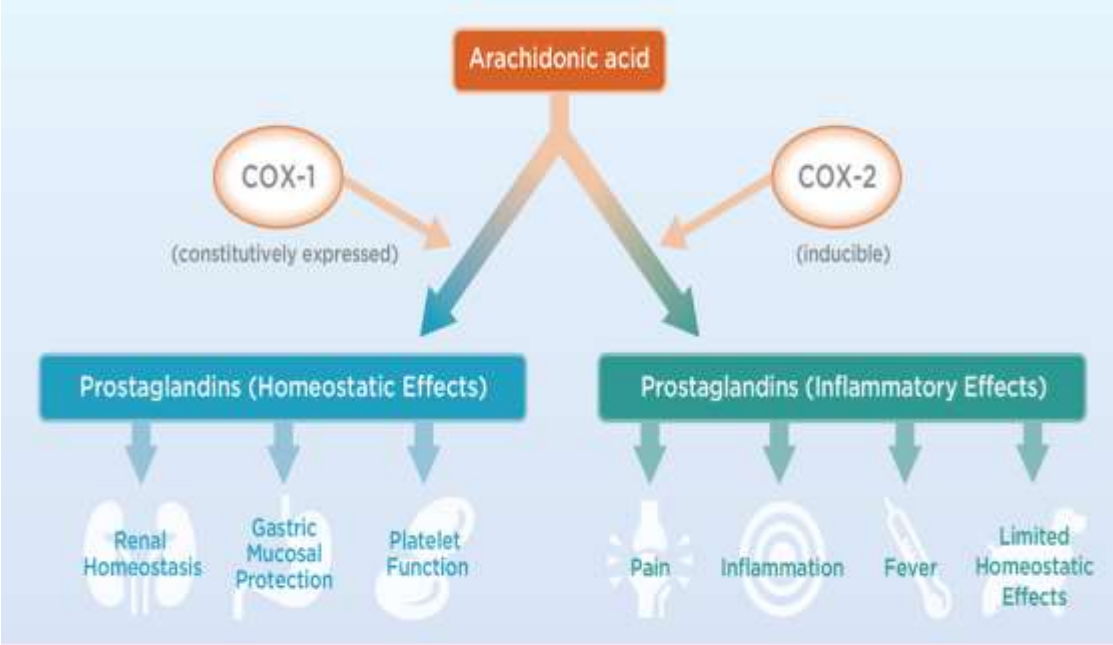


Figure 22: Structure of celecoxib

Celecoxib (IUPAC name: 4-[5-(4-methylphenyl)-3-(trifluoromethyl)-1H-pyrazol-1-yl] benzenesulfonamide) belongs to the class of non-steroidal anti-inflammatory drug (NSAID) (Figure 22). NSAIDs (such as aspirin, ibuprofen, naproxen, etc.) belong to the class of drugs that are used as antipyretic, pain-relieving and anti-inflammatory [109]. They are used in pain management and are active components in treating arthritis and other rheumatic diseases [110]. The mechanism of action of NSAIDs is inhibition of the enzyme cyclooxygenase (COX), which is involved in production of prostaglandins (PGs).

COX exists as two isoforms, COX-1 (constitutive isoform) which participates in cellular homeostasis, and COX-2 (inducible isoform) which is induced by

inflammatory mediators like cytokines, chemokines and produces prostaglandins (Figure 23). The classical NSAIDs inhibit the activity of both isoforms as a result of which protective activities of COX-1 are affected resulting in gastric side effects. This led to development of selective COX-2 inhibitors, coxibs [111]. One such coxib is celecoxib which is effective but with reduced side effects (gastric ulcers) of the NSAIDs. Celecoxib has also been approved by the FDA to treat Familial Adenomatous Polyposis (FAP) [112]. Several researchers reported that besides being effective drugs for pain and inflammation management, COX-2 inhibitors are beneficial in other disease conditions like cancer [113], Alzheimer's [114] etc. Many researchers have reported that use of conventional anticancer therapies combined with COX-2 inhibitors improved outcome of the cancer treatment [115]. Cancer treatment has also been associated with efflux pump associated MDR due to decrease in intracellular concentration of drugs. COX-2 has been reported to be involved in regulation of MDR1 efflux pump and thus COX-2 inhibitors are used in cancer treatment [116] along with other drugs.



www.previcox.com/for-vets/SelectiveScience.html

Figure 23: Homeostatic effects of cyclooxygenase-1 (COX-1) and inflammatory effects of cyclooxygenase-2 (COX-2)

1.8.2 COX-2 during bacterial infection

Prostaglandins are one of the important class of mediators of inflammation and infection. Gram positive and negative bacteria can enhance their release to control immune responses during their pathogenesis. Prostaglandins are also important in regulating activation, proliferation and migration of various innate immune mediators like macrophages, neutrophils, dendritic cells and NK cells [117]. When bacteria invade, macrophages are crucial cells of the immune system which destroy bacterial cells through phagocytosis. During a bacterial infection, the phagocytic activity of macrophages is diminished by increased PGE2.

With this background, our earlier studies have reported that celecoxib sensitizes bacteria to antibiotics and thus inhibiting bacterial growth [118]. The findings indicated that celecoxib alone does not affect bacterial growth, but when it is used in combination with antibiotic (at concentration lower than minimum inhibitory concentration (MIC)) it greatly reduces bacterial growth when compared with antibiotic alone at that concentration. Furthermore, the celecoxib combination test is effective with antibiotics of different classes [118]. From *in vitro* studies, we have also reported that this combination is effective in limiting intracellular *S. aureus* (macrophages infected with *S. aureus)* where celecoxib activated the host immune system by increased phagocytic activity of macrophages *via* decreasing PGE2 synthesis thus resulting in sensitizing the bacteria to antibiotic. Hence, celecoxib shows dual effect of immunomodulation of the host on one side and sensitizing the bacteria to lower doses of antibiotic on other side [119]. Furthermore, in *in vivo* studies, we have reported the efficacy of celecoxib and antibiotic combination in inhibiting the bacterial infection in polymicrobial sepsis model in mice along with demonstrating the efficacy of the co-treatment in clinical isolates of ESKAPE (*Enterococcus, Staphylococcus, Klebsiella, Acinetobacter, Pseudomonas and Enterobacter* species) pathogens [120]. Although the mechanism of celecoxib action in co-treatment to improve host immune response is determined, the exact mechanism through which celecoxib is sensitising the bacteria to antibiotic is not understood.

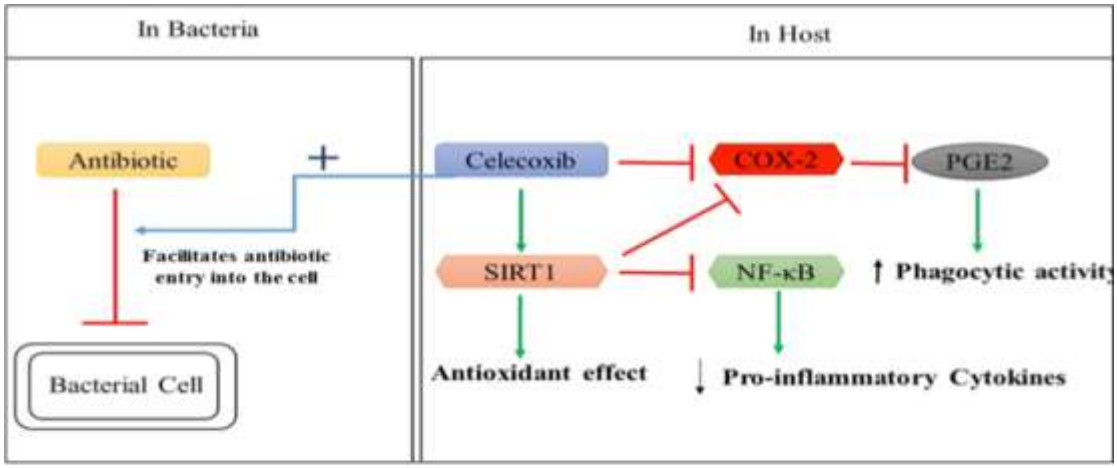


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Figure 24: Action of ampicillin-celecoxib combination in polymicrobial sepsis condition in mice

Previous studies from our laboratory demonstrated that celecoxib, in combination with antibiotics like kanamycin, gentamycin, ampicillin and chloramphenicol was effective in inhibiting the laboratory strains of *S. aureus*, *M. smegmatis*, *E. coli* [118]. Further, *in vitro* macrophage phagocytosis assays using *S. aureus* and ampicillin also confirmed our earlier results. In mouse model we have demonstrated the efficacy of combinatorial treatment of celecoxib and meropenem (antibiotic) [120]. The target organism for our experiments is methicillin resistant *S. aureus* since it is one of the major causative organisms of nosocomial infections. Though, most strains of *S. aureus* are resistant to ampicillin, we chose ampicillin for our experiments because it is second generation penicillin antibiotic and it is also present in the essential drug list of WHO. We would like to revive the old antibiotics; therefore, we chose the ampicillin.

Aim of the Study

With this background, the present study aims at understanding the growth inhibitory mechanism of action of celecoxib in combination with ampicillin in *Staphylococcus aureus*.

To accomplish this aim, the following specific objectives were framed.

Objectives

1. Study the changes in physiochemical properties of ampicillin in presence of celecoxib by co-crystal studies.

2. Study the gene expression changes in *S. aureus* in ampicillin, celecoxib and co-treated bacteria.

3. Evaluate the efficacy of ampicillin-celecoxib cotreatment in isolates of *S. aureus* obtained from bubaline mastitis.

Objective 1

Study the changes in physiochemical properties of ampicillin in presence of celecoxib by co-crystal studies

Objective 1: Study the changes in physiochemical properties of ampicillin in presence of celecoxib by co-crystal studies.

2.1 Materials and methods

2.1.1. Effect of NSAIDs and ampicillin on *S. aureus* growth

The growth inhibitory effect of NSAIDs like gallic acid (GA), ibuprofen (Ib), indomethacin (Ind), flurbiprofen (Fb) & celecoxib (Ce) and ampicillin (AMP) (antibiotic) were tested at different concentrations on *S. aureus*. Effect of drugs on bacterial growth was evaluated by MTT assay as described earlier [121]. The cell viability was calculated in comparison with the untreated control cells by considering their growth as 100 %. A graph was plotted between the various concentrations of drugs/antibiotic (on X-axis) against the growth of bacteria (on Y-axis).

2.1.2. Determination of minimum inhibitory concentration (MIC) for ampicillin by broth macro-dilution (tube) method

Using broth macro-dilution (tube) method, MIC of ampicillin for MSSA (ATCC 29213) and MRSA (ATCC 33591) was determined as described by Clinical and Laboratory Standards Institute (CLSI). Briefly, test tubes containing approximately 5 $X10^5$ colony forming units/mL (CFU/mL) of either MSSA or MRSA suspended in LB (Luria Bertani) broth were added with various concentrations of ampicillin ranging from 0.125 µg/mL to 32 µg/mL, in doubling dilution series and incubated at 37 ^{O}C for 16 h in orbital shaker. MIC was noted as the lowest concentration of drug which completely halted the bacterial growth when observed by naked eye.

2.1.3. Sodium chloride (NaCl) as control to induce stress

To eliminate the possibility that the growth inhibition in combination of ampicillin and celecoxib was due to the stress induced by addition of the two drugs, a control experiment was performed in which 1 mM NaCl (sodium chloride) was added to the test tube containing bacterial culture with ampicillin.

2.1.4. Growth kinetics of MSSA in combinatorial treatment

Growth curve of MSSA was plotted when cultured in presence or absence of ampicillin, celecoxib or both. The overnight bacterial culture was diluted to 0.03 OD and then the culture was split into four flasks and either treated with 25 µM celecoxib, 3 µg/mL ampicillin, both ampicillin (3 µg/mL) and celecoxib (25 µM) or left

untreated. The bacterial growth in all the flasks was monitored every 40 min by counting the colony forming units (CFU) formed on agar plates.

2.1.5. Bacterial ghost membrane preparation and drug entry into bacterial ghosts

Bacterial membrane ghosts were prepared as described earlier [122]. Briefly, *S. aureus* inoculum was added to a fresh TSB medium and allowed to grow at 37 ^{O}C on shaker incubator for 72 h and then centrifuged for 10 min at 5000 rpm. Cells were gently washed with 0.5 % saline and re-centrifuged. The bacterial pellet was washed again. Bacterial concentration of 10^6 CFU/mL was taken for preparation of ghosts.

1 mL each of 5X concentration of NaOH, SDS, $CaCO_3$ prepared in the TSB broth was added to the 2 mL of bacterial suspension at OD of 0.4. This total of 5 mL will have final concentration equal to 1X corresponding to every component. Final concentrations, respectively, of NaOH, SDS and $CaCO_3$ are 0.00231 N, 1.665 mg/mL and 1.05 µg/mL.

After 1 h incubation, the bacterial cells were collected by centrifugation. Bacterial pellet was suspended in 1 mL of H_2O_2 (final concentration of 40.8 µL/mL) and incubated for 30 min. After the incubation, the bacterial cell pellet was collected and washed with saline solution. Finally, the bacterial pellet was resuspended in 60% ethanol and left for 30 min at room temperature with gentle vortex for 30 seconds every 5 minutes. The cell pellets were collected and washed to obtain bacterial ghosts. These bacterial ghosts were suspended in phosphate-buffered saline (PBS) and treated with celecoxib alone, ampicillin alone and combination of both ampicillin and celecoxib. Untreated bacterial ghosts acted as control. The entry of drugs into the membrane ghosts was monitored by spectrophotometer at wavelength of 239 nm and 259 nm for ampicillin and celecoxib respectively.

2.1.6. Membrane permeability assay by 7-AAD

The membrane permeability using 7-aminoactinomycin D (7-AAD) provides a rapid analysis of intactness of the membrane that determines the cell viability. Cells with intact membrane do not show any fluorescence whereas cells with damaged membrane have red fluorescence as the dye penetrates into the cell. The membrane

permeability was analysed using 7-Aminoactinomycin D with as described earlier [123] with slight modifications.

Heat killed cells were used as positive control, where (7-AAD) enters the cells. In brief, the drug-treated and untreated cells were washed in cold PBS. Cells were resuspended in PBS to get the bacterial density of 1×10^6 cells/mL. To 1 mL of cells, 2.5 µL of fluorescent stock solution and 1 µL 7-AAD stock solution were added so that the final concentration of 7-AAD was 20 µg/mL. After 30 min incubation on ice, the stained cells were washed with ice-cold PBS, resuspended in sheath fluid and analysed by flow cytometry using violet light and 488 nm excitation and measuring the fluorescence emission using 440 nm and 670 nm bandpass filters.

2.1.7. Membrane Potential assay by Rhodamine 123

The membrane potential was measured using Rhodamine 123 (Rh123) by flow cytometry as described earlier [124]. In brief, 1 mL of drug-treated and untreated (control) cells were washed with PBS. Finally, the bacteria were resuspended in 5 mL PBS, pH 7.3, and containing 5 µg Rh123/mL. After 30 min incubation at 37 °C, cells were washed thrice with PBS. These bacteria were examined immediately by flow cytometry. Fluorescence observed is directly proportional to the membrane potential.

MSSA and MRSA were treated with AC01 (celecoxib alone), AC10 (ampicillin alone), AC21 (combination of ampicillin and celecoxib in 2:1 ratio for MSSA) and AC41 (combination of ampicillin and celecoxib in 4:1 ratio for MRSA) co-crystals.

2.1.8. Ampicillin-celecoxib (AC) co-crystal development

Ampicillin and celecoxib co-crystals were developed by co-grinding followed by slow solvent evaporation method using different proportions of the drugs, ampicillin:celecoxib, respectively (1:1, 1:2, 1:3, 1:4, 2:1, 3:1 and 4:1) as described earlier [125, 126]. For example, AC21 has 2 parts of ampicillin and 1 part of celecoxib. Ampicillin and celecoxib were weighed as per the ratios and were manually grounded for 30 min using a sterile ceramic mortar and pestle. These co-ground mixtures (labelled as AC11, AC12. AC13, AC14, AC21, AC31, AC41) and the pure compounds ampicillin (AC10) and celecoxib (AC01) were dissolved in hot methanol and were left at room temperature (RT) covered with a perforated aluminium foil until the solvent evaporated leaving the co-crystals.

Objective 1: Study the changes in physiochemical properties of ampicillin in presence of celecoxib by co-crystal studies.

2.1.9. Powder X-Ray Diffraction (PXRD)

Ampicillin-celecoxib co-crystals were powdered and that powder was used to characterize the co-crystals by Powder X-Ray Diffraction (PXRD) analysis. A 2θ angle of 5° to 70° was used. The graph was drawn with 2θ angle on X-axis and intensity on Y-axis.

2.1.10. Differential Scanning Calorimetry (DSC)

The stability and melting temperatures of the co-crystals were checked by Differential Scanning Calorimeter (DSC). The graph was prepared for each sample by taking temperature from 0 °C to 300 °C on X-axis and heat flow on Y-axis.

2.1.11. Effect of ampicillin-celecoxib co-crystals on MSSA and MRSA growth

The co-crystals were checked for their efficiency to inhibit the growth of MSSA and MRSA at various concentrations ranging from 122 ng/mL to 125 µg/mL.

2.1.12. Permeability of physical mixture and co-crystals into bacterial ghosts

Bacterial ghosts as described earlier were used to test the entry of co-crystals specifically with respect to co-crystal AC21 and the physical mixture (PM) having 2:1 ratio of ampicillin and celecoxib. Absorbance was measured at 239 nm and 259 nm for ampicillin and celecoxib, respectively.

2.1.13. Fourier Transform InfraRed (FTIR) spectroscopy

Ampicillin (AC10), celecoxib (AC01) and cocrystal AC21 were analysed by FTIR to see whether there are any interactions between ampicillin and celecoxib. A plot was prepared between wavenumbers (X-axis) and % transmittance (Y-axis).

2.1.14. Partition coefficient (LogP) determination

The n-octanol-water partition coefficient of ampicillin and cocrystal AC21 was determined by shake flask method as described earlier [127]. Briefly, equal volumes

of n-octanol (10 mL) and water (10 mL) are taken in a flask and were mutually saturated on a thermostat shaker at 100 rpm, 25 ºC for 24 h. After saturation, the two phases were allowed to separate on standing at room temperature. A calibration curve was prepared for serial dilutions of ampicillin in water and n-octanol phases by measuring the absorbance at 259 nm on a UV-Vis spectrophotometer (Shimadzu). The concentration of ampicillin of AC21 co-crystal in both the phases was determined using the calibration curve and the logP was calculated as log10 of the ratio of concentration of ampicillin in n-octanol phase to water phase. The logP value is average of a minimum of three replicates ± standard deviation.

Objective 1: Study the changes in physiochemical properties of ampicillin in presence of celecoxib by co-crystal studies.

Objective 1: Study the changes in physiochemical properties of ampicillin in presence of celecoxib by co-crystal studies.

2.2.1. Effect of NSAIDs and ampicillin on *S. aureus* growth

The effect of ampicillin, celecoxib and other non-steroidal anti-inflammatory drugs (NSAIDs) such as ibuprofen, indomethacin, gallic acid on *S. aureus* growth was studied by broth dilution method. The results clearly demonstrated that ampicillin inhibited bacterial growth at 5 µg/mL and NSAIDs and celecoxib did not show any effect on the growth of *S. aureus*. Celecoxib did not show any growth inhibitory effect even at 100 µM concentration (Fig. 25).

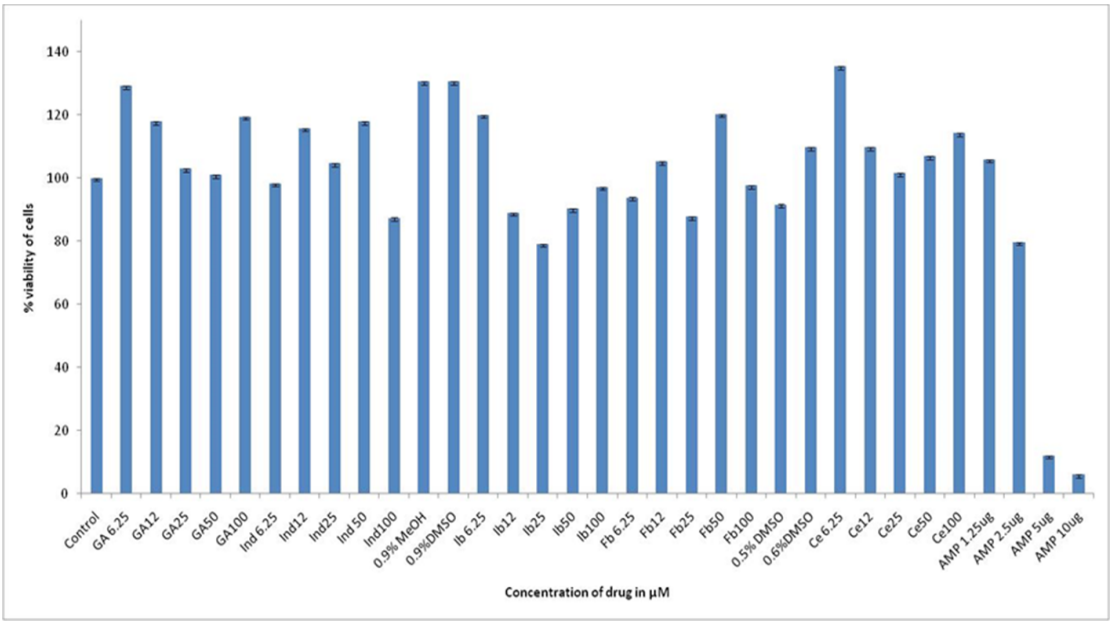


Figure 25: Effect of various concentrations of NSAIDs (in µM) on the growth of *S. aureus*. The NSAIDs used were GA (gallic acid), Ind (indomethacin), Ib (ibuprofen), Fb (flurbiprofen) and Ce (celecoxib). AMP, ampicillin (in µg/mL)

2.2.2. Determination of minimum inhibitory concentration (MIC) for ampicillin by broth macro dilution (tube) method

MIC of ampicillin for MSSA (Figure 26A) and MRSA (Figure 26B) were, respectively, 4 µg/mL and 8 µg/mL.

Objective 1: Study the changes in physiochemical properties of ampicillin in presence of celecoxib by co-crystal studies.

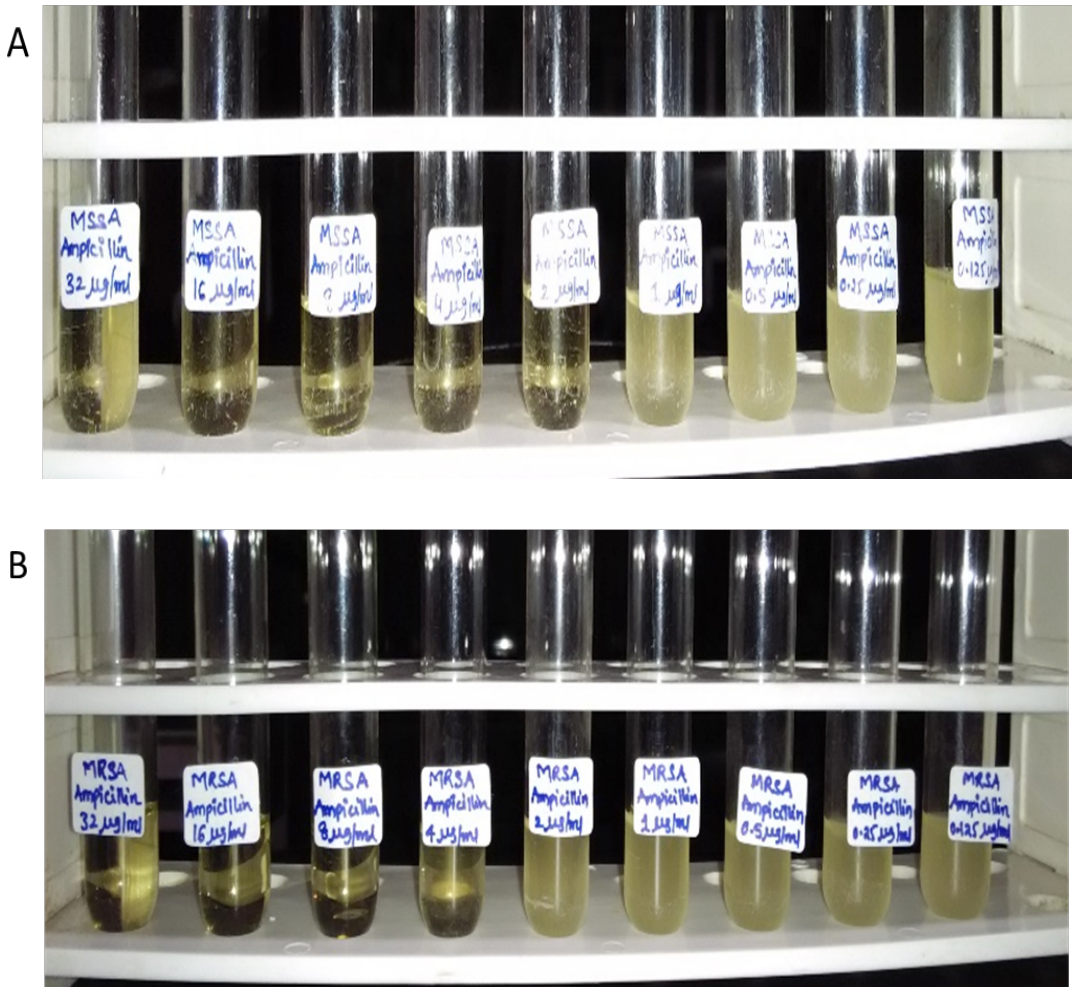


Figure 26: A) MIC determination of ampicillin for MSSA and B) MIC determination of ampicillin for MRSA

2.2.3 Sodium Chloride (NaCl) as control to induce stress

Next, we addressed the possibility that bacterial growth inhibition by ampicillin and celecoxib combination treatment was due to stress by adding 1 mM sodium chloride (1 mM NaCl) instead of celecoxib in the combination treatment. The macrodilution assay clearly demonstrated that although the combination of ampicillin and 1 mM NaCl caused stress and therefore less growth was observed compared to untreated or ampicillin-treated cells, the growth inhibition in combination of ampicillin and celecoxib is not due to salt-induced stress (Figure 27).

Objective 1: Study the changes in physiochemical properties of ampicillin in presence of celecoxib by co-crystal studies.

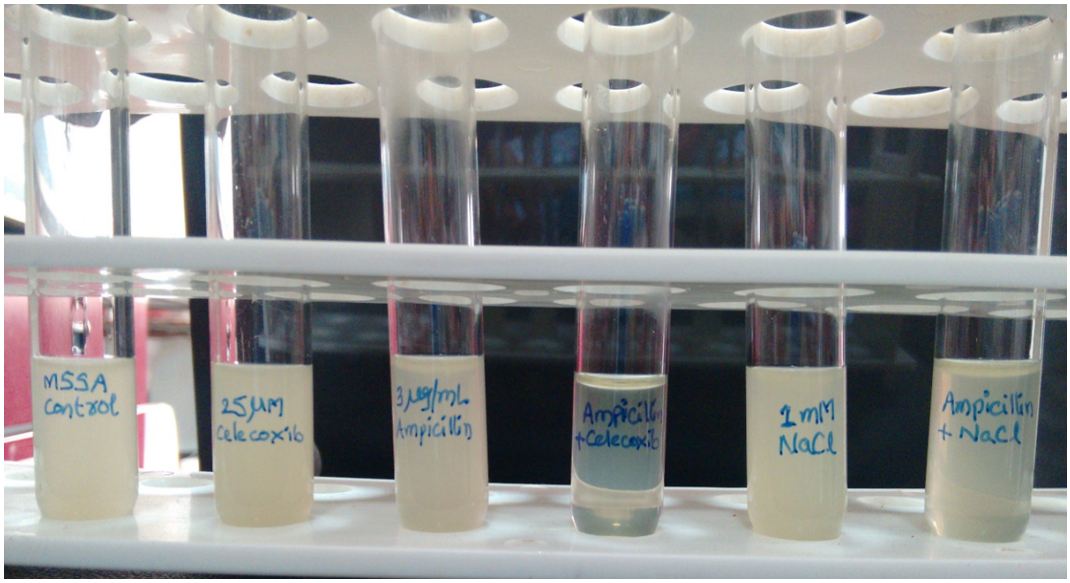


Figure 27: Sodium chloride (NaCl) as a control to induce stress

2.2.4. Study of the growth kinetics of MSSA in combinatorial treatment

The bacterial growth curve assessment by measuring absorbance at 600 nm may have interference by the dead bacteria. So, *S. aureus* ATCC29213 growth curve was graphed by counting the colony forming units (CFU) on agar plates (Table 5) for control, celecoxib alone treatment, ampicillin alone treatment and combination treatment. A plot was prepared between time (X-axis) and number of CFU (Y-axis). From this graph (Figure 28), it is very clear that ampicillin treatment had growth inhibitory effect initially for few generations of doubling, which was partially overcome by bacteria adapting to the new environment. Celecoxib did not show any growth inhibitory effect on bacteria and the kinetics were similar to that of untreated control cells. However, in celecoxib and ampicillin co-treated cells, the cells appeared to not recover as compared to ampicillin alone treatment.

Objective 1: Study the changes in physiochemical properties of ampicillin in presence of celecoxib by co-crystal studies.

Table 5: Number of CFU (x 10^8/mL) of MSSA in each sample for every 20 min after treatment

Time in min	CFU in Control	CFU in Celecoxib	CFU in Ampicillin	CFU in combination
0	80	90	100	82
20	100	108	90	72
40	160	120	92	92
60	190	200	66	72
80	220	210	90	70
100	270	250	160	66
120	260	260	162	60

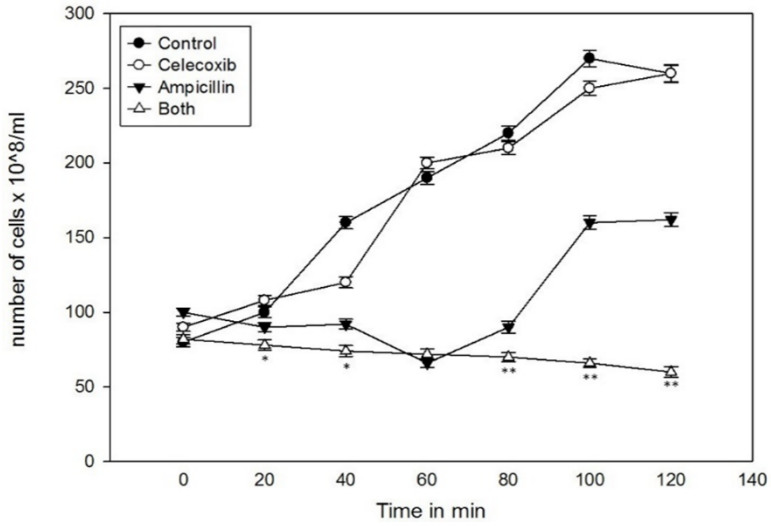


Figure 28: Graph showing the growth kinetics of *S. aureus* in presence or absence of celecoxib or ampicillin or both celecoxib and ampicillin

2.2.5. Bacterial ghost membrane preparation and drug entry into bacterial ghosts

Objective 1: Study the changes in physiochemical properties of ampicillin in presence of celecoxib by co-crystal studies.

Since celecoxib alone did not inhibit bacterial growth but was able to inhibit in combination with ampicillin, we next asked the question, is there an increased ampicillin penetration into the cell in presence of celecoxib? To address this question, we determined the concentrations of celecoxib and ampicillin inside bacteria using empty bacterial membrane vesicles. The results clearly indicated increased absorbance for ampicillin in presence of celecoxib in the membrane fraction suggesting increased entry of ampicillin (Figure 29A).

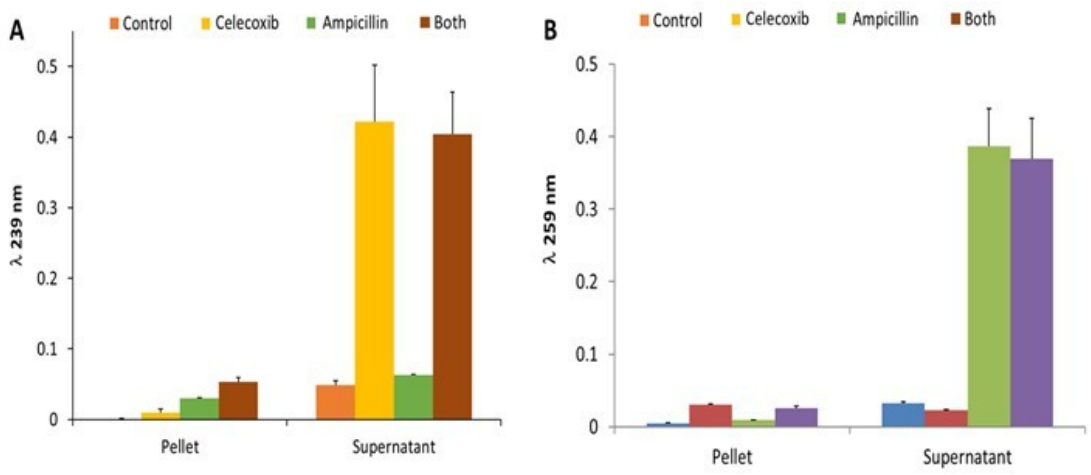


Figure 29: A) Graph showing the increase in absorbance at 239 nm of ampicillin in pellet fraction of bacterial ghost membranes in presence of celecoxib **B)** Graph showing the absorbance of celecoxib at 259 nm

2.2.6. Membrane permeability assay by 7-AAD

We further assessed the permeability of the membrane to the drugs in combination by flow cytometry. The results clearly indicated that there is no change in the membrane permeability by celecoxib treatment. However, there was an increased permeability in presence of ampicillin which further increased in cotreatment of ampicillin-celecoxib when compared with ampicillin alone treatment (Figure 30).

Objective 1: Study the changes in physiochemical properties of ampicillin in presence of celecoxib by co-crystal studies.

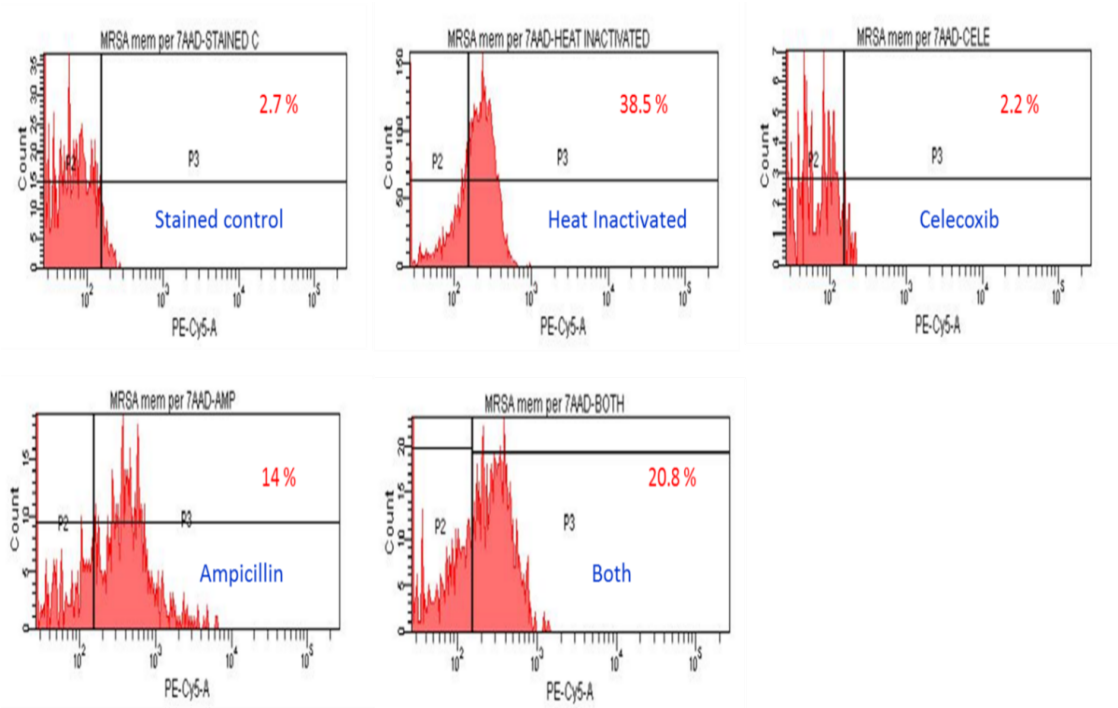


Figure 30: Flow cytometric analysis of membrane permeability of *S. aureus* using 7-AAD

2.2.7. Membrane Potential assay by Rhodamine 123

Recently, it has been suggested that decreased membrane potential (MP) is a mechanism by which bacteria attain resistance. Furthermore, membrane permeability and membrane potential are interlinked. Therefore, we have analyzed the MP by flow cytometer using Rhodamine 123 dye. For MSSA, significant change in MP in all treatment conditions (Figure 31A) was not observed. MP in MRSA was decreased in ampicillin treatment (Figure 31B) suggesting that MRSA might be attaining resistance to ampicillin via decrease in MP. Celecoxib treatment did not show any effect on MP.

Objective 1: Study the changes in physiochemical properties of ampicillin in presence of celecoxib by co-crystal studies.

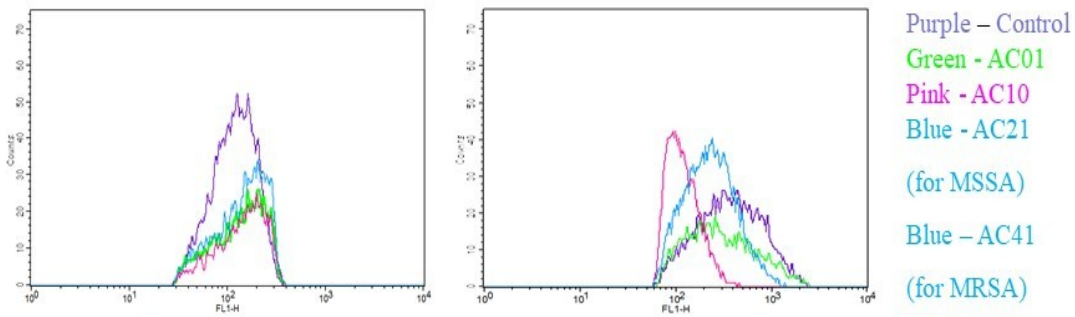


Figure 31: Flow cytometric analysis of membrane potential by Rhodamine 123. **A)** Membrane potential of MSSA **B)** Membrane potential of MRSA in different treatments. AC01 is celecoxib alone treated, AC10 is ampicillin alone treated, AC 21 (for MSSA) and AC 41 (for MRSA) are combination treated. Control is without any drug.

2.2.8. Ampicillin-celecoxib (AC) co-crystal development

The co-ground mixtures of ampicillin (A) and celecoxib (C) in different proportions (Table 6) were crystallized. AC01 is celecoxib pure compound and AC10 is ampicillin pure compound. Remaining compounds are co-crystals having various proportions of ampicillin and celecoxib. The numbering to the co-crystals are designated based on the proportion of ampicillin and celecoxib, respectively.

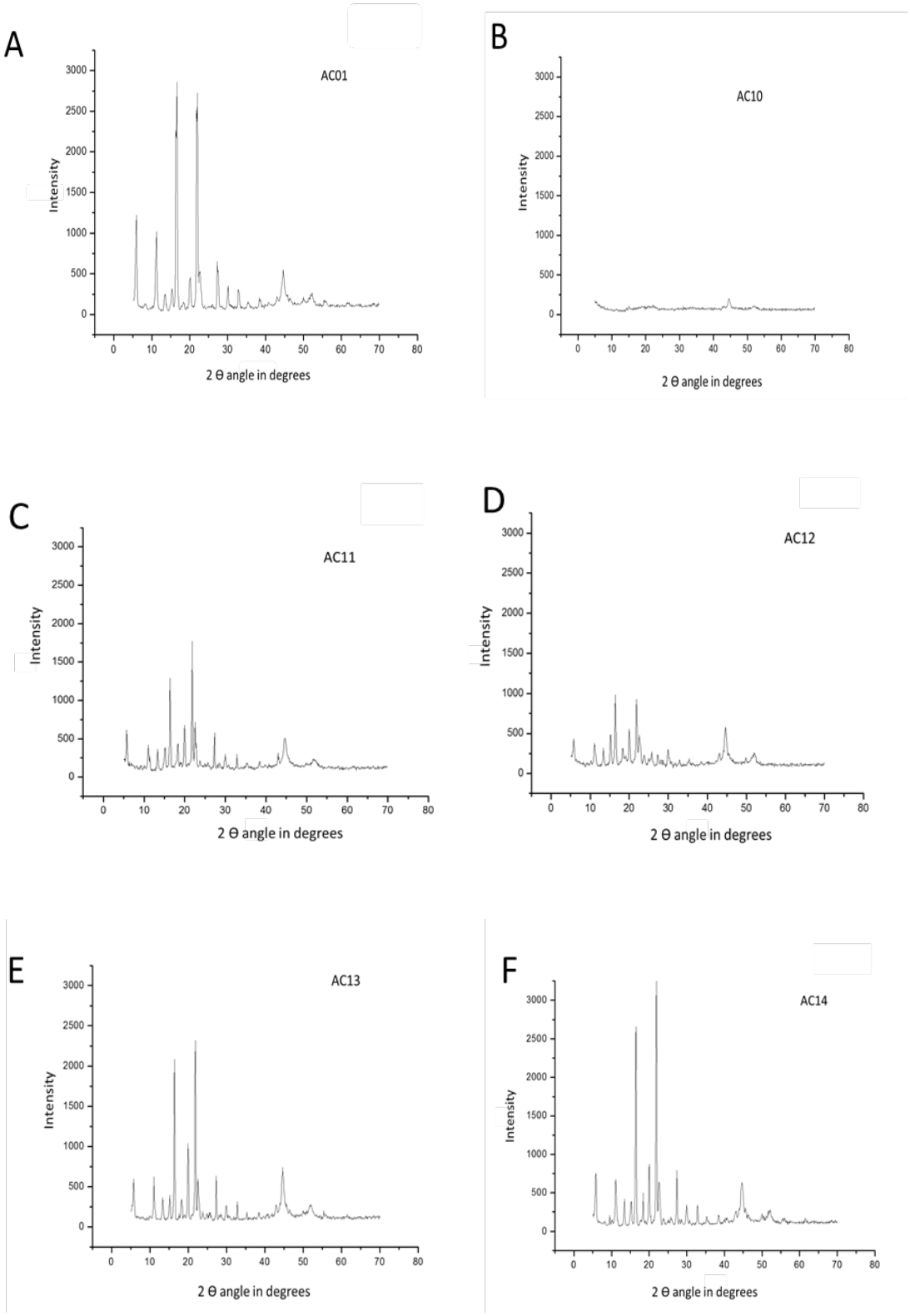
Table 6: Percentages of each drug in combination

	AC01	AC10	AC11	AC12	AC13	AC14	AC21	AC31	AC41
% of ampicillin	Nil	100	50	33	25	20	67	75	80
% of celecoxib	100	Nil	50	67	75	80	33	25	20

2.2.9 Powder X-Ray Diffraction (PXRD) study of the co-crystals

The powder XRD analysis of the cocrystals showed that there are weak interactions between ampicillin and celecoxib, which is evidenced from the new peaks observed/disappearance of existing peaks in cocrystals when compared with crystals made from pure compounds (AC01 and AC10) (Figures 32, 33A and 33B).

Objective 1: Study the changes in physiochemical properties of ampicillin in presence of celecoxib by co-crystal studies.



Objective 1: Study the changes in physiochemical properties of ampicillin in presence of celecoxib by co-crystal studies.

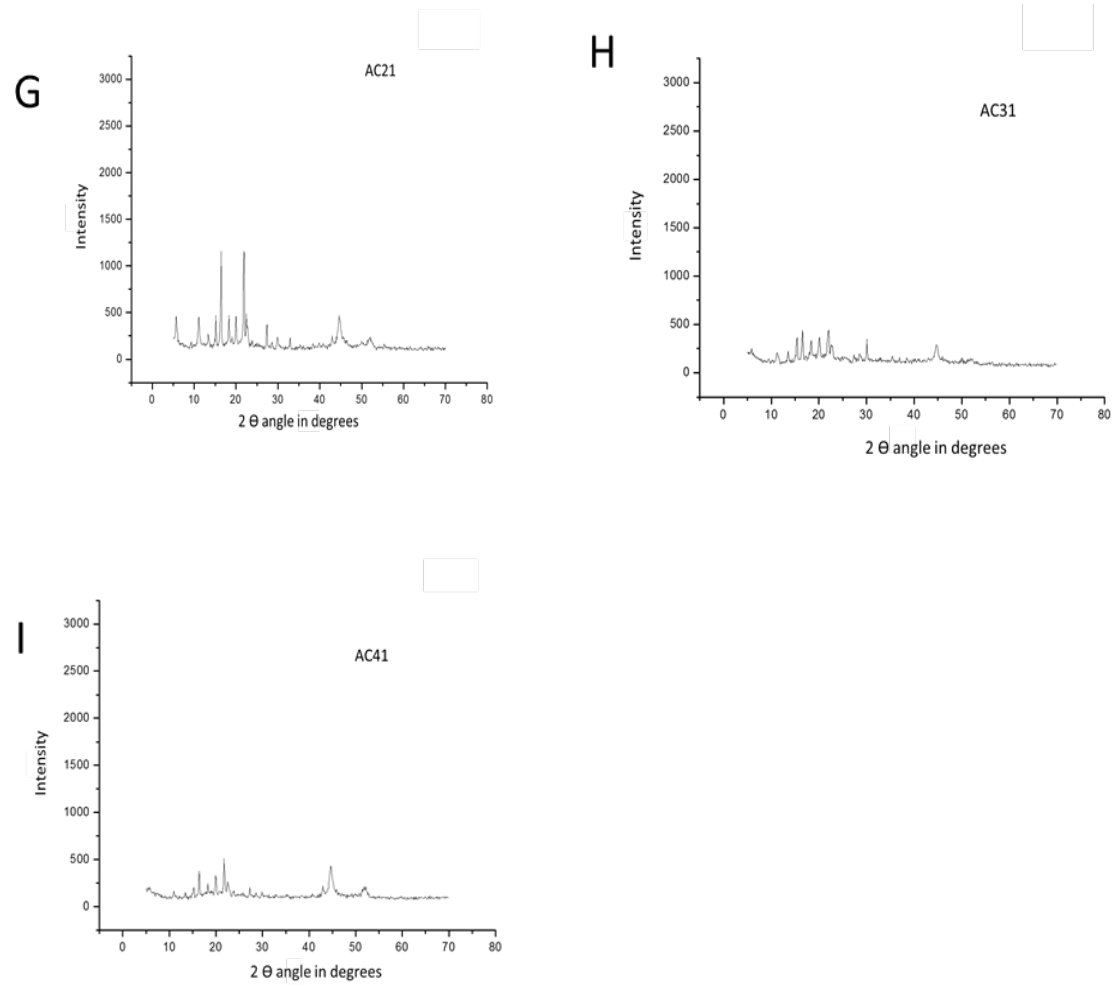


Figure 32: PXRD graphs for pure compounds (AC01 and AC10) and co-crystals (from A to I)

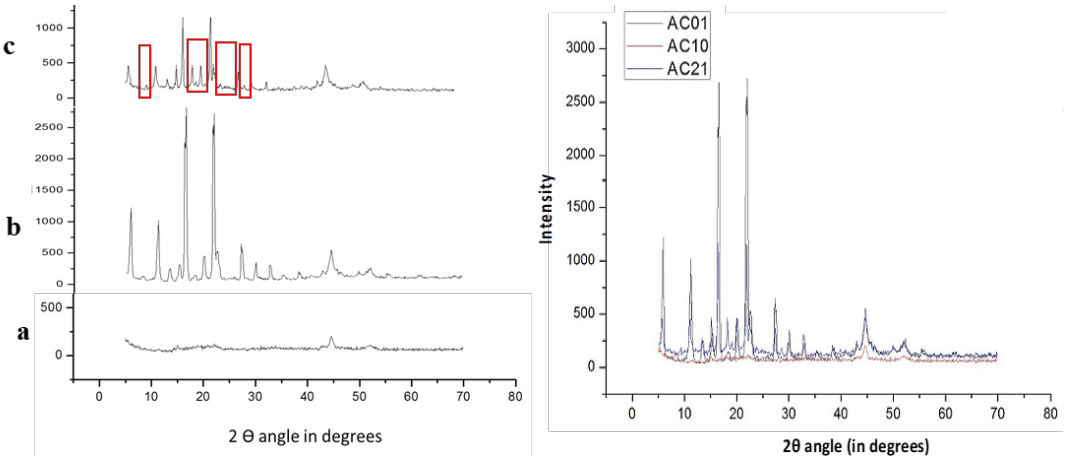
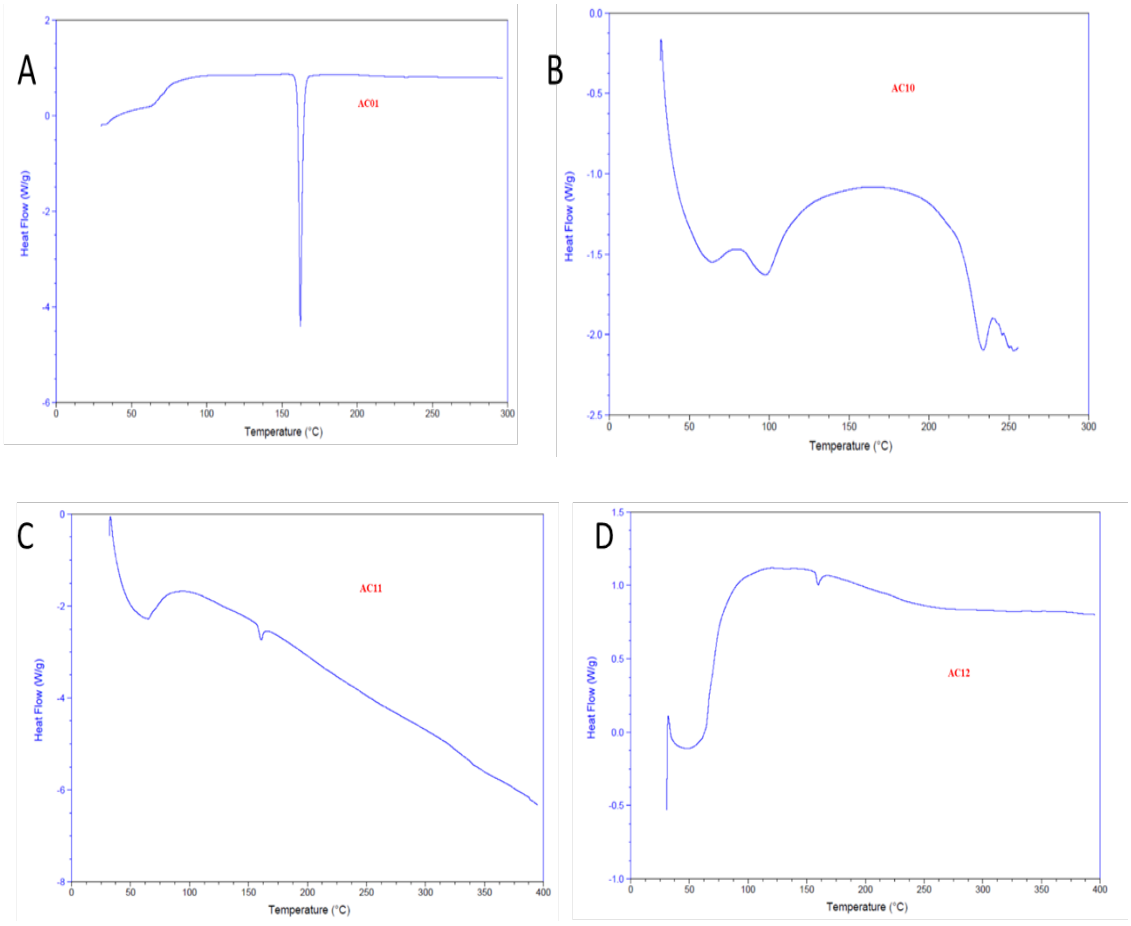


Figure 33: A) PXRD analysis of the crystals AC10 (ampicillin alone) (a), AC10 (celecoxib alone) (b), and co-crystal AC21 (c) **B)** Merged chromatograms of the PXRD

Objective 1: Study the changes in physiochemical properties of ampicillin in presence of celecoxib by co-crystal studies.

2.2.10. Stability of co-crystals by Differential Scanning Calorimetry (DSC)

Differential Scanning Calorimetric (DSC) analysis of co-crystals (Figure 34) showed that the crystalline nature of celecoxib was preserved in all combinations, but there is a phase transition in ampicillin in combination with celecoxib in AC21 when compared with amorphous form of pure compound (Figure 35). This indicates that ampicillin might be interacting with celecoxib by weak electrostatic interactions or hydrogen bonding or van der Waals forces. DSC and PXRD combined images of all co-crystals are given in Figure 36.



Objective 1: Study the changes in physiochemical properties of ampicillin in presence of celecoxib by co-crystal studies.

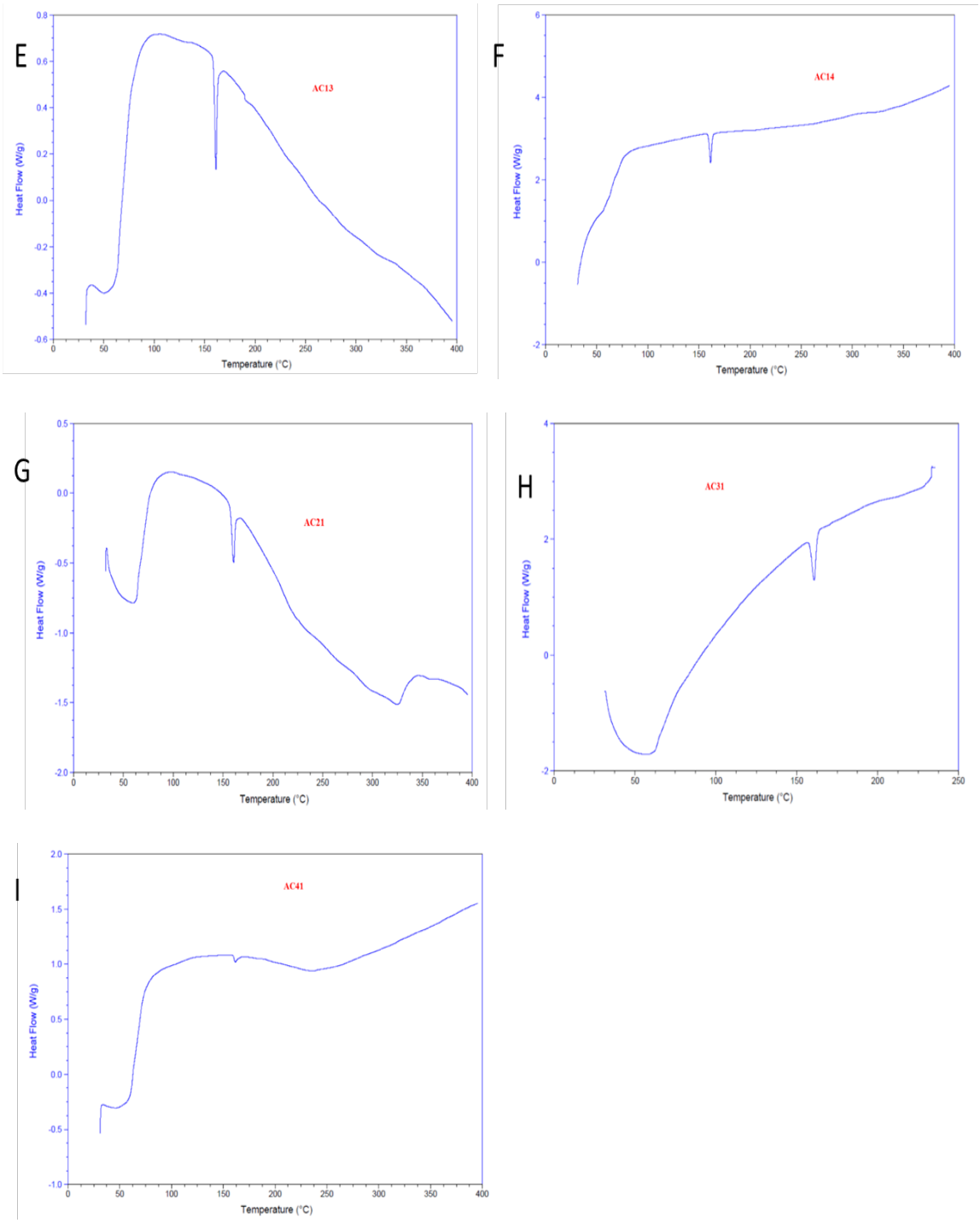


Figure 34: DSC graphs for pure compounds (AC01 and AC10) and co-crystals (from A to I)

Objective 1: Study the changes in physiochemical properties of ampicillin in presence of celecoxib by co-crystal studies.

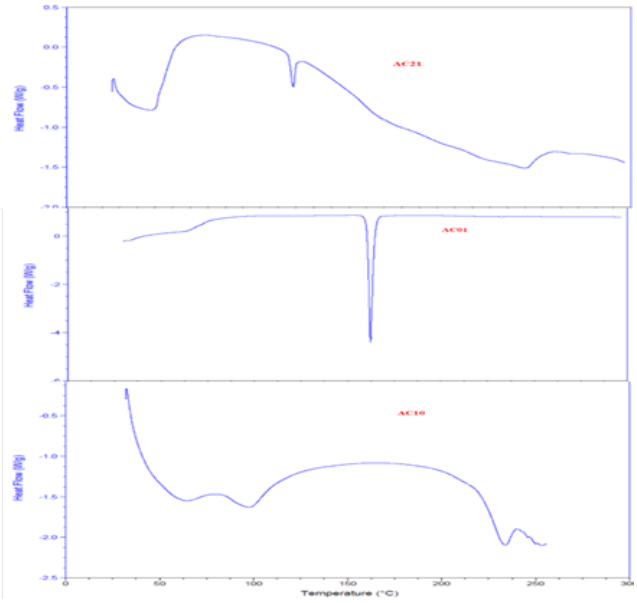


Figure 35: DSC analysis of the crystals AC10 (ampicillin alone) (a), AC01 (celecoxib alone) (b), and co-crystal AC21 (c)

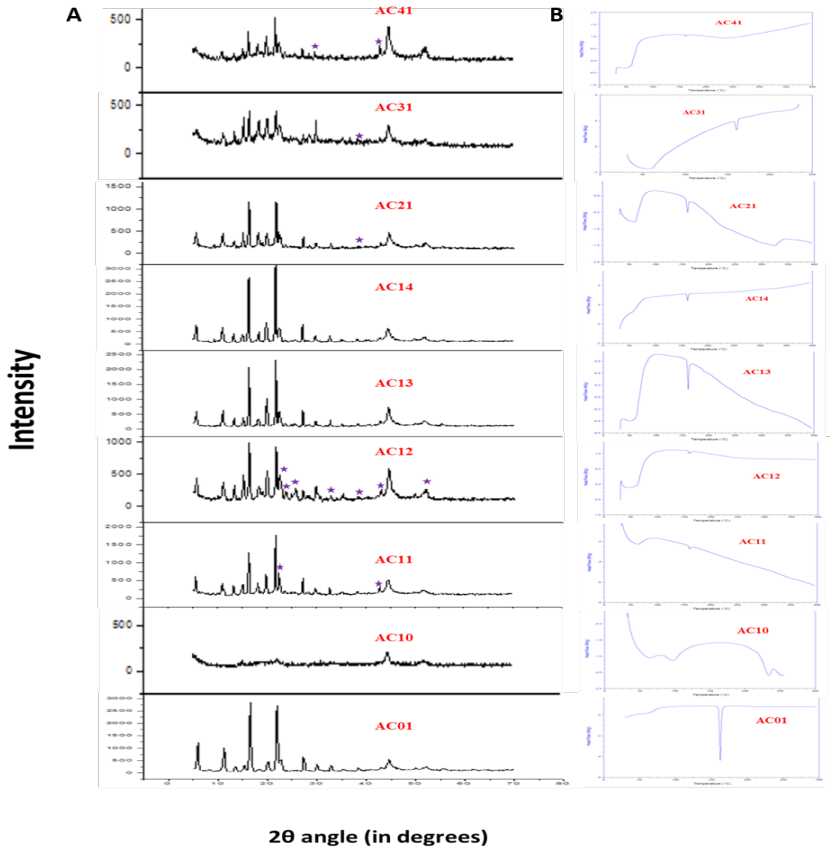


Figure 36: Figure showing the (A) powder XRD data and (B) DSC data of co-crystals

Objective 1: Study the changes in physiochemical properties of ampicillin in presence of celecoxib by co-crystal studies.

2.2.11. Effect of ampicillin-celecoxib co-crystals on MSSA and MRSA growth

It was observed that the death of MSSA and MRSA is high in the co-crystals prepared with less proportion of celecoxib. The results clearly demonstrated an increased death of bacteria by cocrystals (Figure 37 for MSSA and Figure 39 for MRSA). However, the cocrystal AC21 (ampicillin 2 parts and celecoxib 1 part) showed more potency on MSSA (Figure 38) and AC41 on MRSA (Figure 40).

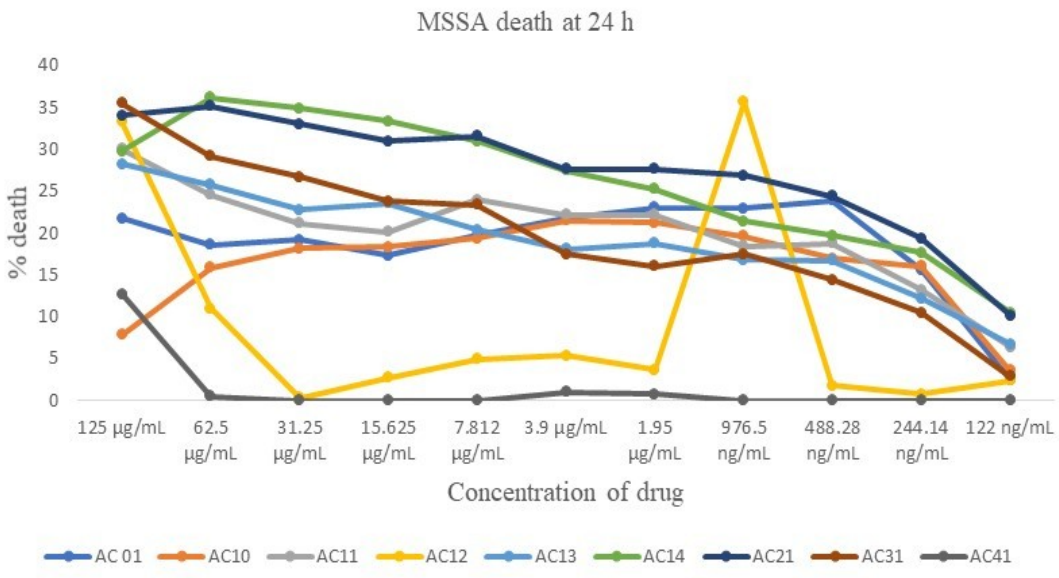


Figure 37: Cell viability of *S. aureus* (MSSA) in presence of ampicillin and celecoxib cocrystals at various concentrations

Objective 1: Study the changes in physiochemical properties of ampicillin in presence of celecoxib by co-crystal studies.

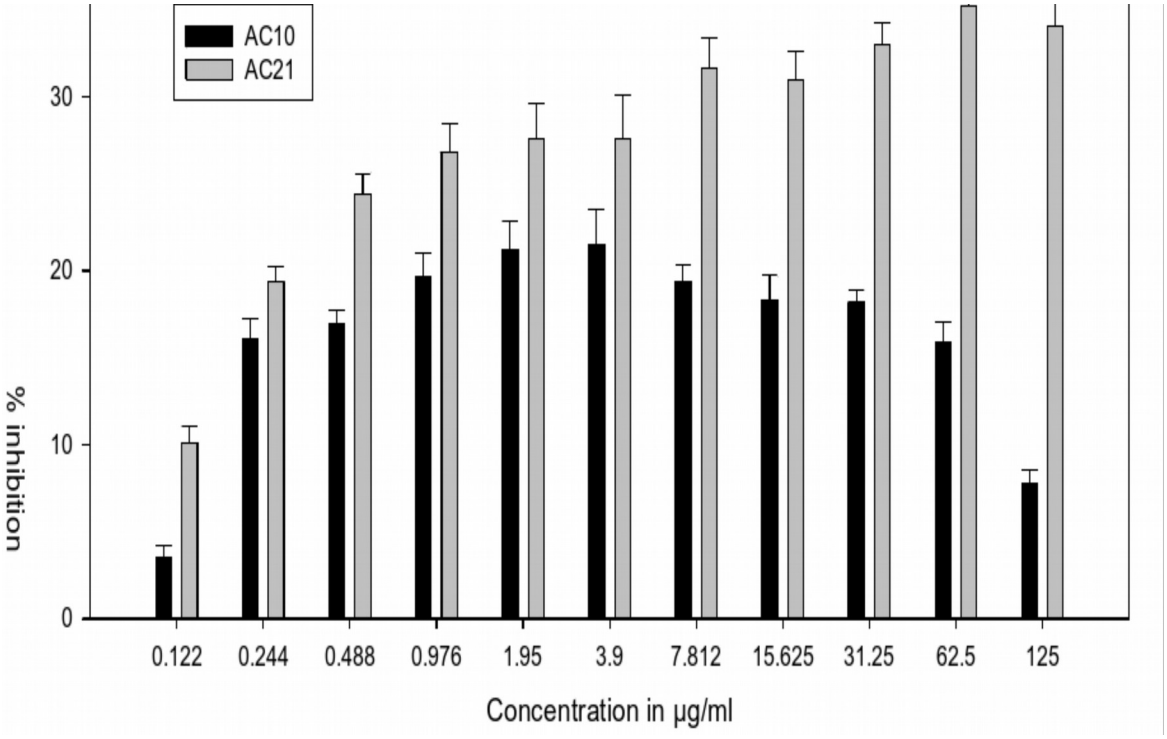


Figure 38: Graph showing efficacy of co-crystal AC21 against *S. aureus* (MSSA) when compared to ampicillin alone (AC10)

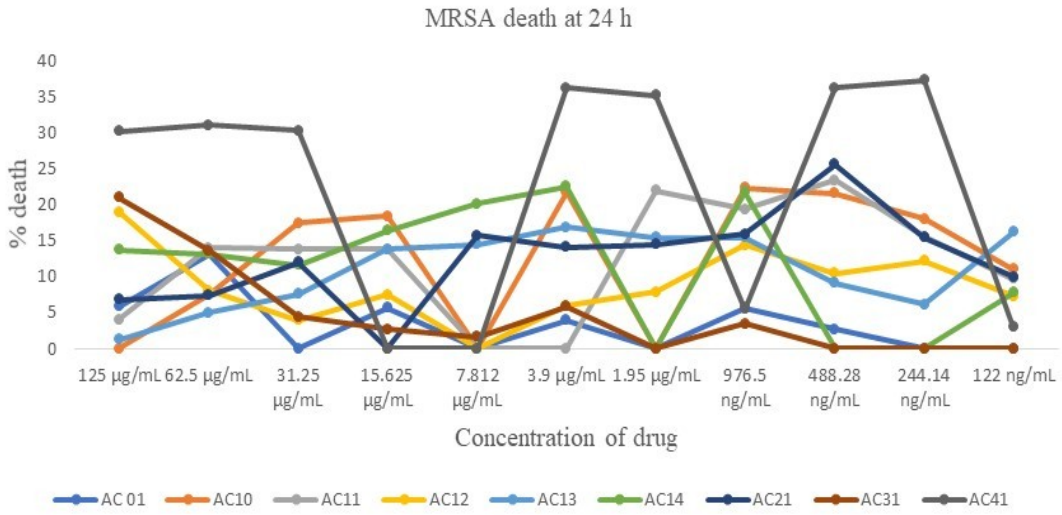


Figure 39: Cell viability of *S. aureus* (MRSA) in presence of ampicillin and celecoxib cocrystals at various concentrations

Objective 1: Study the changes in physiochemical properties of ampicillin in presence of celecoxib by co-crystal studies.

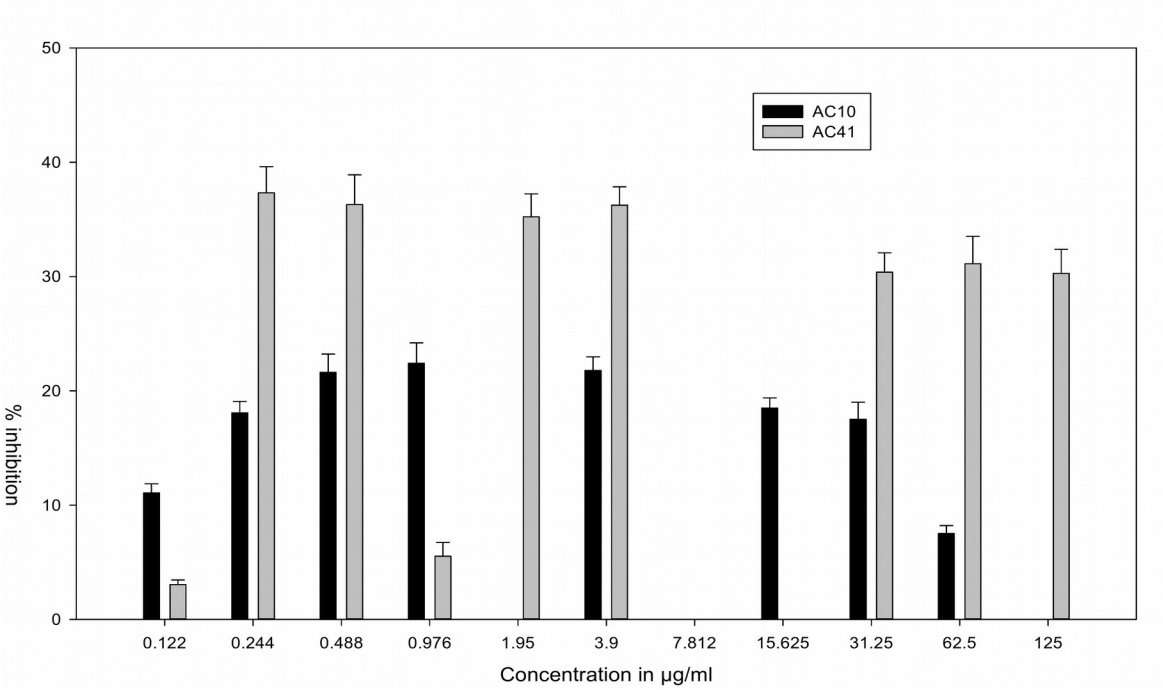


Figure 40: Graph showing efficacy of co-crystal AC41 against *S. aureus* (MRSA) when compared to ampicillin alone (AC10)

2.2.12. Permeability of physical mixture and co-crystals into bacterial ghosts

Further, to determine the effect of celecoxib on increasing the permeation of ampicillin in the cocrystal, we incubated the bacterial membrane ghosts with AC21 and physical mixture of both the drugs (without crystallization) in 2:1 ratio and measured the absorbance of ampicillin at 239 nm in the pellet (membrane) and supernatant fraction. The cocrystal AC21 showed more absorbance at 239 nm corresponding to ampicillin in pellet fraction suggesting increased entry of the ampicillin (Figure 41) compared to the physical mixture (PM).

Objective 1: Study the changes in physiochemical properties of ampicillin in presence of celecoxib by co-crystal studies.

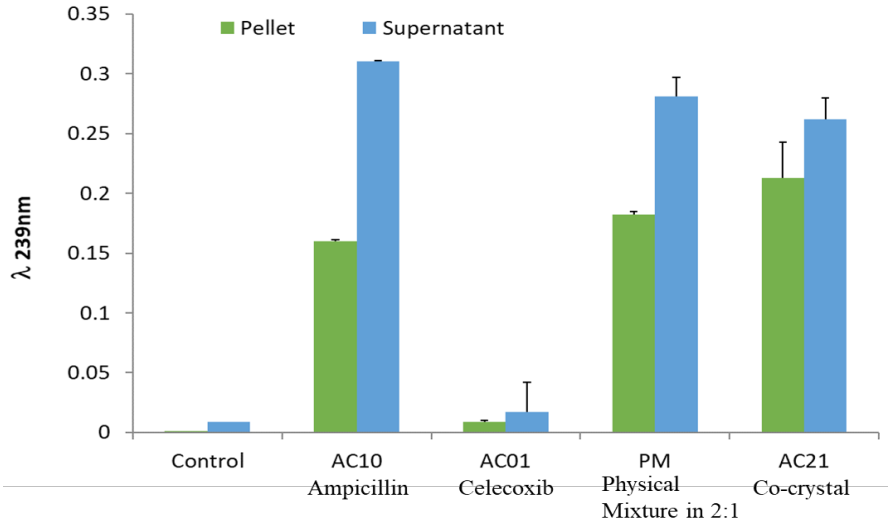


Figure 41: Graph showing the absorbance of ampicillin at 239 nm in membrane pellets and supernatant fractions of membrane ghosts treated with AC10 (ampicillin), AC01 (celecoxib), AC21 co-crystal and physical mixture (PM) of ampicillin and celecoxib in 2:1 ratio

2.2.13. Fourier Transform InfraRed (FTIR) spectroscopy

FTIR spectroscopy analysis was performed for AC01 (celecoxib), AC10 (ampicillin) and AC21 cocrystals. Formation of broad peaks in AC21 when compared with sharp peaks in AC01 indicates the interaction between ampicillin and celecoxib. Shift in the wave number from 1768 cm^{-1} to 1678 cm^{-1} for same functional group indicates the interaction between two drugs in cocrystals. Formation of new peak in AC21 also indicates the interactions between the two drugs in cocrystals (Figure 42).

Objective 1: Study the changes in physiochemical properties of ampicillin in presence of celecoxib by co-crystal studies.

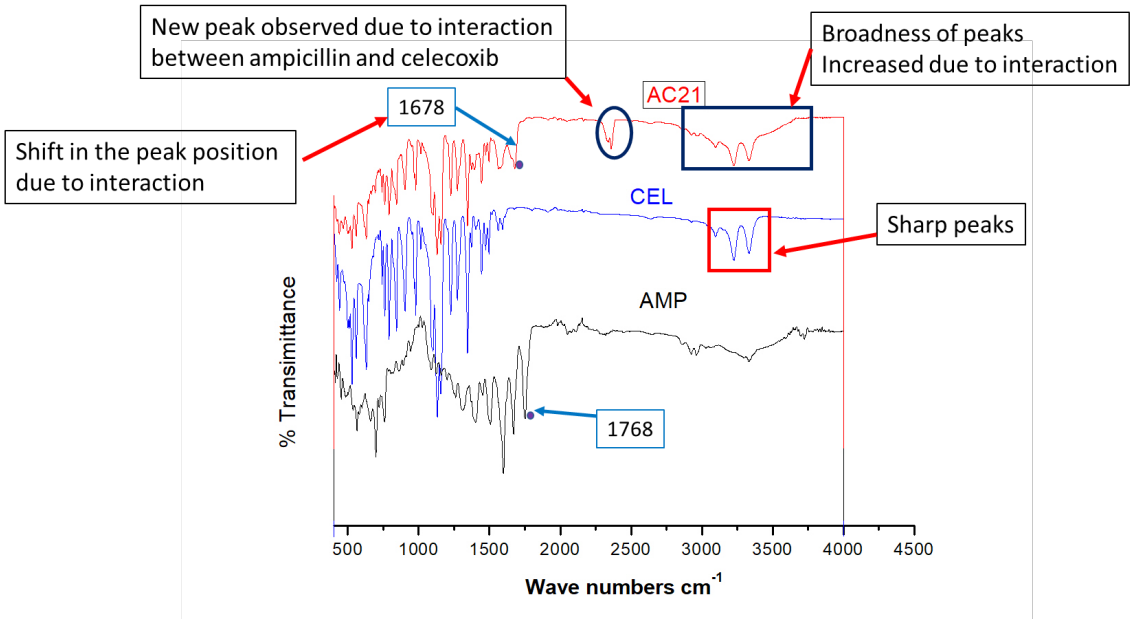


Figure 42: FTIR analysis of cocrystals AC01 (celecoxib), AC10 (ampicillin) and AC21 indicating the interactions between ampicillin and celecoxib in cocrystals

2.2.14. Partition coefficient (LogP) determination

We experimentally determined the logP value, indicating the permeation/solubility of the drug, for the cocrystal AC21 and ampicillin by shake flask method along with prediction of the logP using various softwares such as ALOGPS and Molinspiration by giving the SMILES (simplified molecular-input line-entry system) of both ampicillin and celecoxib together as structural input in ampicillin-celecoxib and celecoxib-ampicillin format since we do not have the structural information of the co-crystal. The experimental logP value was determined to be 2.08 $\pm$ 0.089 for AC21 and 1.16 $\pm$ 0.12 for ampicillin. The ALOGPS predicted the logP of the cocrystal as 2.01 and the Molinspiration predicted it to be 2.3. The logP value for ampicillin alone according to Pubchem database is 1.35 which is much lower than the AC21. The logP value indicates the solubility and permeation of a drug. A drug with logP between 1-3 shows moderate solubility and moderate permeation. Although ampicillin logP is 1.35

it is less when compared to 2.08 in cocrystal AC21 indicating an increased permeation of ampicillin in presence of celecoxib. This result is in line with our experimental permeability assay by flow cytometer using 7-AAD and bacterial ghost assay.

2.3 Discussion

Development of alternate treatment strategies for drug resistant bacterial infections is the major responsibility of scientific community. Drug repurposing is one of the methods FDA has suggested for identification of drugs to treat MDR bacteria. We [118] and others [128] had previously shown that celecoxib, selectively inhibits COX-2, and acts as an antibacterial agent when given in combination with an antibiotic. In the present study, we aimed at elucidating the mechanism of action of celecoxib on bacterial growth. To achieve this, first we have studied the growth kinetics of *S. aureus* ATCC29213 in presence or absence of ampicillin alone, celecoxib alone and a combination of both the drugs. As indicated in results, combinatorial treatment showed growth inhibition. Synergistic effect is defined as the joint action of drugs, that when taken together increases each other's effects. However, in the present study the synergistic definition does not hold good since celecoxib alone does not show any effect on the bacterial growth. Nevertheless, celecoxib in combination was enhancing ampicillin inhibitory effect. Growth inhibition of *S. aureus* by ampicillin was also shown to be increased in synergy when used in combination with other non-antibiotic compounds [129, 130].

With the ghost membrane drug uptake assay it was clear that indeed there was an increased uptake of ampicillin in presence of celecoxib. Therefore, we next addressed the question whether the increased uptake/entry of ampicillin into bacteria in presence of celecoxib is due to the modulation of membrane potential and permeability? It is well established that bacteria attain resistance by altering their membrane permeability and potential to stop entry of the antibiotics and is very well known with ampicillin [131, 132]. Our flow cytometric results where we show increased membrane permeability and potential to combinatorial drug treatment are in line with the established results.

However, it is not clear as to how celecoxib is able to potentiate antibiotic uptake. To address this question, we carried out co-crystal studies. In recent years, research

Objective 1: Study the changes in physiochemical properties of ampicillin in presence of celecoxib by co-crystal studies.

focussed in altering the physicochemical properties of a drug so as to improve its solubility, bioavailability, stability, permeability etc [133-136]. It was already established that the weak non-covalent interactions present in the amoxicillin-clavulanate co-crystals can alter the physicochemical properties of amoxicillin thus increasing its bioavailability. So, co-crystals of celecoxib and ampicillin were prepared by co-grinding method followed by solvent evaporation at room temperature. The crystals were analysed by PXRD, FTIR and DSC that confirmed the weak electrostatic, hydrogen bonding or van der Waals forces between the two drugs that might be enhancing the entry or permeation of ampicillin. This was also evident from the increase in logP value of ampicillin from 1.16 to 2.08. Such an increase in the permeation and dissolution properties of a drug in cocrystals has been well documented [137-139].

Objective 1: Study the changes in physiochemical properties of ampicillin in presence of celecoxib by co-crystal studies.

Objective 2

Study the changes in gene expression profile in ampicillin-celecoxib co-treatment to *S. aureus*

Objective 2: Study the changes in gene expression profile in ampicillin-celecoxib co-treatment to *S. aureus*

3.1 Materials and methods

3.1.1. Sample preparation: drug treatments

S. aureus (ATCC 29213) was grown in tryptic soy broth (TSB) overnight at 37 ºC in shaker incubator. This culture was used for further inoculations during sample preparation. For sample preparation, exponentially growing *S. aureus* ($OD_{600\ nm}$ = 0.4) cells were incubated with celecoxib (final conc. = 25 µM) alone or ampicillin (final conc. = 3 µg/mL) alone or both (combination) or neither (control) in 50 mL TSB for 40 min at 37 ºC in shaker incubator for 40 min. For combination of celecoxib and ampicillin, celecoxib was added after 20 min from the time of addition of ampicillin and incubated for another 20 min. All the cells were collected by centrifuging for 5 min at 4 ºC, 5000 rpm (revolutions per minute) and then washed with 25 mM Tris, pH 7.5, and frozen along with RNAlater (Sigma-Aldrich) in liquid nitrogen. The microarray analysis was out sourced to Genotypic Technology Private Limited, Bangalore, India. RNA isolation, labelling and scanning were performed by Genotypic Technology Private Limited for transcriptome analysis by microarray.

3.1.2. Microarray: Labelling, amplification and scanning

The samples were labelled using Agilent Quick Amp Kit (Part number: 5190-0442) according to the manufacturer's protocol. Using random hexamers as primers in reverse transcription, 500 ng of total RNA was converted to complementary DNA (cDNA). Single stranded cDNA was converted to double stranded cDNA and then to cRNA, *in vitro*, by T7 RNA polymerase. During this process, Cy3 dye was incorporated and finally its concentration was measured by Nanodrop. Two micrograms (2000 ng) of Cy3 labelled cRNA was fragmented and hybridized on Agilent Custom *S. aureus* 8x15k designed by Genotypic Technology Private Limited (AMADID: 27491) in Sure hybridization Chambers (Agilent) for 16 hours at 65º C using the Gene Expression Hybridization kit (Part Number 5190-0404; Agilent). This gene chip includes MW2, N315, COL and Mu50 (strains of *S. aureus*). The array covers 3613 genes for which 15150 probes were designed. Hybridized slides were washed using Agilent Gene Expression wash buffers (Part No: 5188-5327) and scanned at 5 µ resolution on a G2505C scanner (Agilent Technologies). Data extraction was done using Agilent Feature Extraction software Version 10.7.

Objective 2: Study the changes in gene expression profile in ampicillin-celecoxib co-treatment to *S. aureus*

3.1.2.1 Microarray Data Analysis

Feature extracted data was analysed using GeneSpring GX version 11.5 software from Agilent. Data normalisation was done in GeneSpring GX using the 75th percentile shift. Test samples were compared to control samples to obtain fold expression values. The differential expression of genes in test samples compared to control were identified and grouped using hierarchical clustering to identify significant gene expression patterns. Clustering analysis was performed using GeneSpring GX Software using Average Linkage rule with Pearson Uncentered Distance Metric. Microarray-related data was submitted to Gene Expression Omnibus with the accession number of **GSE56100**.

Microarray results were validated by real-time PCR for few genes and western blot analysis for few proteins.

3.1.3. RNA isolation

RNA was isolated using TRI reagent (Sigma) as per manufacturer's protocol. In brief, the samples were homogenized with TRI reagent followed by addition of chloroform. The samples were then centrifuged at 12000 g at 4 °C for 15 min. Aqueous phase was isolated into a fresh sterile tube and two volumes of isopropanol was added and incubated for 30 min on ice and then centrifuged. To the resulting pellet, 500 µL of 70% ethanol was added and washed in centrifuge at 8000 g for 5 min. The pellet was air dried and concentration of the obtained RNA was measured using NanoDrop™ spectrophotometer (Thermo Scientific™).

3.1.4. cDNA synthesis from RNA

Complementary DNA (cDNA) was synthesized from RNA using Roche cDNA synthesis kit according to the manufacturer's procedure.

Briefly, all the frozen reagents were thawed before use. Total reaction volume of 20 µL was set up by taking 1 µg total RNA and 2µL of 600 pmol/µL random hexamer primer, 4 µL of 5X reverse transcriptase reaction buffer, 0.5 µL of 40 U/µL RNase inhibitor, 2 µL of 10 mM each deoxyribonucleotide mix and 0.5 µL of 20 U/µL

reverse transcriptase. Total volume was made up to 20 µL with nuclease free, PCR-grade water. Reagents in the tube were mixed carefully. The reaction was carried out by incubating the tube at 25 ^{O}C for 10 min, followed by 30 min at 55 ^{O}C. Reverse transcriptase was inactivated by heating to 85 ^{O}C for 5 min. Reaction was stopped by placing the tube on ice.

3.1.5. Real-time polymerase chain reaction (qPCR)

Real-time PCR was performed to validate microarray data. Primers were designed using Primer3 software [140] for *spa, sei, sea, seg, sep, blaZ* to generate amplicon size of 100-150 bp (Table 1). 16S rRNA served as reference gene for normalization of the amount of RNA input and the efficiency of each cycle. cDNA was amplified using Maxima SYBR Green/ROX qPCR Master Mix (Fermentas) on 7500 fast *Real-Time PCR* Systems (Applied Biosystems) with an initial incubation of 95 °C for 10 mins followed by 40 cycles of 15 s at 95 °C, 30 s at 56 °C and 30 s at 72 °C. The Relative expression or fold change in expression of genes in the test samples was analyzed using comparative Ct method.

3.1.6. β – Lactamase activity assay using nitrocefin

The presence of β – lactamase in the *Staphylococcus aureus* culture media was evaluated by nitrocefin as substrate, a chromogenic β –lactam, as described earlier [141]. *Staphylococcus aureus* which were treated with celecoxib alone, ampicillin alone and combination of ampicillin and celecoxib were tested for amount of β – lactamase that was produced in various treatment conditions. Untreated bacteria were used as control. After the drug treatment, the bacteria were centrifuged at 3200 g for 30 min at 4 ^{O}C to separate them from medium. The experiment was performed at room temperature. The separated culture medium was assessed for β – lactamase by taking 1 mL of medium. 10 µL of 10 mM nitrocefin stock prepared in dimethyl sulfoxide (DMSO) was added. The hydrolysis of nitrocefin at room temperature was monitored for 2 min at 500 nm. The concentration of β – lactamase is directly proportional to the difference in the absorbance. A histogram was prepared by taking difference in the absorbance at 500 nm per minute for all the four samples.

Objective 2: Study the changes in gene expression profile in ampicillin-celecoxib co-treatment to *S. aureus*

3.1.7. SDS-PAGE

Cell lysate for 1D PAGE (one dimensional polyacrylamide gel electrophoresis) was prepared as described earlier [142]. Briefly, the cells were resuspended in 25 mM Tris pH 7.5 containing 2 mM phenylmethylsulphonyl fluoride (PMSF). Lysostaphin (50 µg/mL final concentration) was added and then incubated at 37 ºC for 20 min. Cells were sonicated and then the lysate was centrifuged at 14000 rpm for 1 h at 4 ºC. The soluble fraction was used for SDS-PAGE. 30 µg of total protein of all samples were loaded in wells of a 12 % SDS-polyacrylamide gel (SDS-PAGE). The gel was stained with Coomassie Brilliant Blue and the differences in protein expression were observed.

3.1.8. Western blot

Protein levels of enterotoxin H (SEH) and hemolysin alpha (HLA) were analyzed by Western blot. The growth conditions and treatments are same as described above. The culture supernatants were collected by centrifugation at 5000 rpm, 10 min, and 4 °C. The protein in the culture supernatant was precipitated by adding 4 volumes of acetone and incubating overnight at 4°C and collected by centrifugation for 30 min at 14000 rpm, 4 °C. Protein was air dried, dissolved in PBS and the concentration was determined by Bradford assay. The proteins were detected by western blot, as described earlier [141]. Briefly, 100 µg of protein was subjected to 15% SDS-PAGE and then proteins were transferred onto nitrocellulose membrane. For blocking the membrane 5% non-fat dry milk in Tris-buffered saline with 0.01% tween-20 (TBST) was used. The proteins were probed with primary antibody sheep polyclonal antibody to hemolysin alpha (Abcam ab15948) and rabbit polyclonal antibody to staphylococcal enterotoxin H (Abcam ab15902) diluted 1:1000 in TBST. After overnight incubation at 4 °C, horseradish peroxidase (HRP)-conjugated anti-sheep or anti-rabbit IgG were added. Finally, proteins were visualized by chemiluminescence detection kit using Kodak imaging system.

Objective 2: Study the changes in gene expression profile in ampicillin-celecoxib co-treatment to *S. aureus*

3.2 Results

Objective 2: Study the changes in gene expression profile in ampicillin-celecoxib co-treatment to *S. aureus*

3.2.1. Microarray data analysis

The microarray data was normalized to untreated cells (Control). The gene expression profiles of control cells and cells treated with celecoxib alone showed no significant difference. A huge change in the expression pattern of genes was observed in cells treated with ampicillin (Figure 43), as expected. Most of the genes upregulated upon ampicillin treatment were involved in antibiotic resistance, signal transduction, infection and pathogenesis, transposition and phage proteins etc. indicating a survival strategy of bacteria. Ampicillin not only inhibited cell wall synthesis in bacteria but also down regulated the genes involved in metabolic processes of carbohydrates, amino acids, lipids, nucleic acid etc. along with other transporter genes.

The microarray data analysis of the duplicate sets revealed that a total of 1223 genes were differentially expressed in cells treated with celecoxib and ampicillin when compared to ampicillin alone treated cells. Of these, 163 were essential genes according to the Database of Essential Genes (DEG) (Figure 44) and remaining 1060 were non-essential genes (Figure 45). Although many genes were down regulated in combinatorial treatment, most significant effect was seen in genes responsible for pathogenesis, drug resistance and upstream signal transduction when compared to ampicillin (Tables 7, 8 and 10). However, analysis of data for one set of treatment showed a significant change in expression of most of the virulence genes.

Objective 2: Study the changes in gene expression profile in ampicillin-celecoxib co-treatment to *S. aureus*

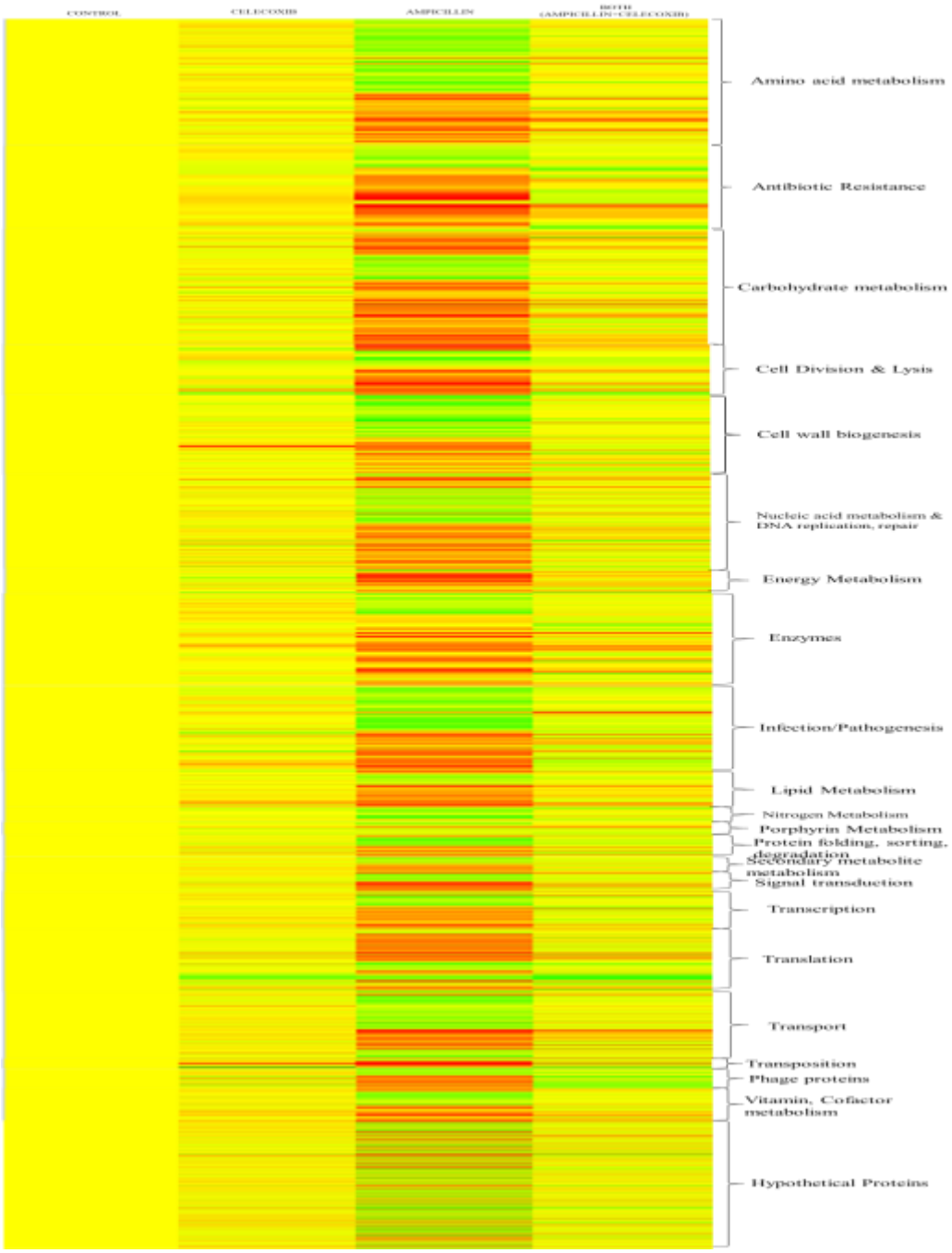


Figure 43: Heat map showing the differential gene expression of *S. aureus* treated with or without ampicillin, celecoxib or both after 4 h of addition of drugs. Expression in control cells is represented by yellow colour, more than two-fold increase by red and two-fold reduction in expression compared to control is represented by green colour

Objective 2: Study the changes in gene expression profile in ampicillin-celecoxib co-treatment to *S. aureus*

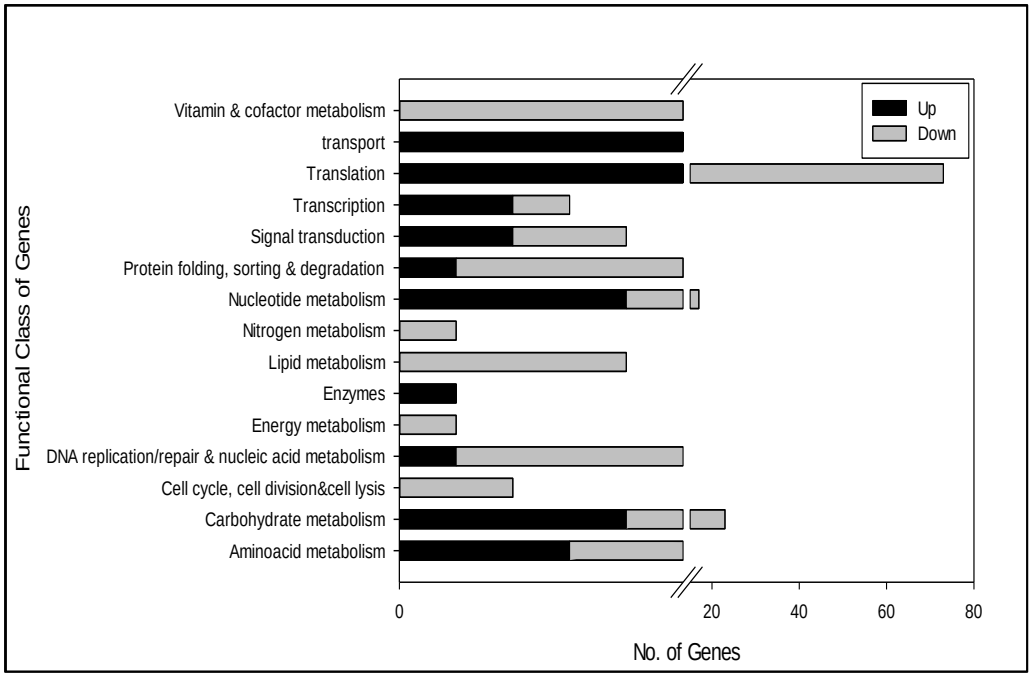


Figure 44: Graph showing the differentially expressed essential genes (according to the functional class) in cells treated with combination of celecoxib and ampicillin when compared to ampicillin alone

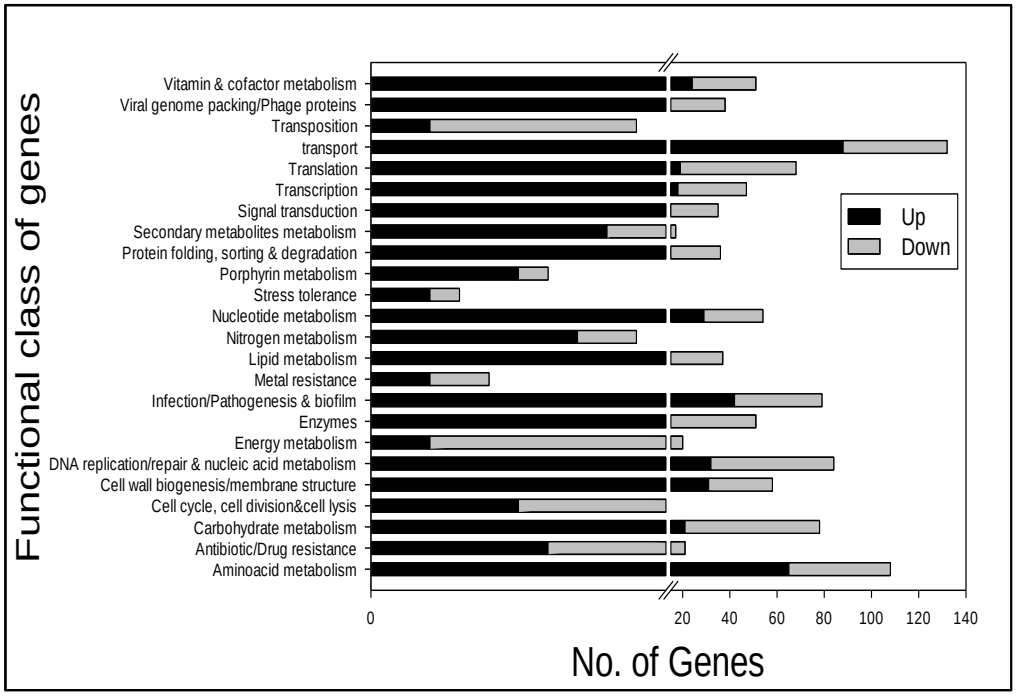


Figure 45: Graph showing the differentially expressed non-essential genes (according to the functional class) in cells treated with combination of celecoxib and ampicillin when compared to ampicillin alone

Objective 2: Study the changes in gene expression profile in ampicillin-celecoxib co-treatment to *S. aureus*

Table 7: List of differentially expressed genes in *S. aureus* treated with ampicillin alone or combination of ampicillin and celecoxib

S. No	Gene Name	Product	Fold change (Ampicillin) (Avg±SE)	Fold change (Both) (Avg±SE)	p-value
1	-	DNA polymerase	2.01±0.65	(-) 0.32±0.00	<0.0001
2	-	ABC transporter	(-) 0.89±0.89	(-) 0.27±0.17	<0.05
3	*adhE*	bifunctional acetaldehyde-CoA/alcohol dehydrogenase	1.21±0.24	(-) 0.18±0.00	<0.05
4	*aroA*	3-phosphoshikimate 1-carboxyvinyltransferase	2.22±1.95	(-) 0.10±0.05	<0.001
5	*ddl*	D-alanyl-alanine synthetase A	1.70±0.23	1.42±0.00	<0.05
6	*binL*	DNA-invertase	1.63±1.91	(-) 0.32±0.03	<0.01
7	-	FtsK/SpoIIIE family protein	4.25±3.03	(-) 0.07±0.03	<0.0001
8	*pre*	plasmid recombination enzyme	1.67±0.45	(-) 0.23±0.13	<0.01
9	-	truncated Pre protein	1.96±2.46	(-) 0.10±0.08	<0.001
10	*rep*	replication protein Rep	1.11±0.10	(-) 0.96±0.31	<0.001
11	-	replication-associated protein	0.38±0.39	1.98±2.38	<0.05
12	-	staphylococcal tandem lipoprotein	0.11±0.70	2.51±3.05	<0.0001
13	*kdpB*	potassium-transporting ATPase subunit B	2.36±2.89	(-) 0.24±0.12	<0.0001
14	*kdpC*	potassium-transporting ATPase subunit C	1.89±1.93	(-) 0.47±0.21	<0.001
15	*agrD*	AgrD	1.81±2.32	(-) 0.25±0.17	<0.001
16	-	putative ATP/GTP-binding protein	2.32±2.77	(-) 0.38±0.40	<0.0001
17	*aacA*	N-acetyltransferase	1.86±1.00	(-) 0.58±0.26	<0.0001
18	-	pathogenicity island protein	2.72±1.10	(-) 0.31±0.05	<0.0001
19	*set16*	superantigen-like protein	0.42±1.84	2.86±3.41	<0.0001
20	*set26*	superantigen-like protein	1.73±2.21	(-) 0.22±0.16	<0.01
21	*mecR1*	methicillin-resistance MecR1 regulatory protein	1.70±2.19	(-) 0.23±0.15	<0.01
22	-	holin	1.62±1.15	(-) 0.76±0.11	<0.0001
23	-	phage repressor	0.11±0.75	(-) 1.44±0.22	<0.05
24	-	portal protein	2.04±2.63	(-) 0.03±0.14	<0.001
25	*int*	prophage L54a, integrase	3.74±2.93	(-) 0.66±0.22	<0.0001
26	-	prophage L54a, major tail protein, putative	2.75±3.34	(-) 0.27±0.10	<0.0001
27	-	prophage L54a, N-6-adenine-methyltransferase	2.16±1.75	(-) 0.62±0.52	<0.0001
28	-	IS1272 transposase	1.64±2.12	(-) 0.17±0.13	<0.01

Objective 2: Study the changes in gene expression profile in ampicillin-celecoxib co-treatment to *S. aureus*

Table 8A: List of virulence factors and fold difference (compared to control) down regulated in combinatorial treatment of both celecoxib and ampicillin when compared to ampicillin treatment alone

Gene	Gene Product	Fold Difference	
		Ampicillin	Both
	Capsule Polysaccharides and Fibronectin binding factors		
cap8B	capsular polysaccharide synthesis enzyme Cap8B	0.26	0
cap8E	capsular polysaccharide synthesis enzyme Cap8E	1.07	0.17
cap8F	capsular polysaccharide synthesis enzyme Cap8F	0.55	-0.41
cap8H	capsular polysaccharide synthesis enzyme Cap8H	1.22	-0.05
cap8I	capsular polysaccharide synthesis enzyme Cap8I	4.56	-0.17
cap8J	capsular polysaccharide synthesis enzyme Cap8J	4.71	-0.35
cap8K	capsular polysaccharide synthesis enzyme Cap8K	4.65	-0.15
ebpS	elastin binding protein	1.8	0.36
clfA	fibrinogen-binding protein	0.45	-0.69
spa	Staphylococcal Protein A	0.97	-0.31
	Secreted proteins		
--	alpha-hemolysin	0.25	-0.52
hlgA	gamma-hemolysin, component A	0.74	0.67
hlgB	gamma-hemolysin component B	0.58	0.15
hlgC	gamma-hemolysin component C	1.02	0.57
lukS	Panton-Valentine leukocidin chain S precursor	1.01	0.81
tst	toxic shock syndrome toxin-1	0.86	-0.32
seb	staphylococcal enterotoxin B	1.47	-0.37
sep	enterotoxin P	1.18	-0.21
seg2	staphylococcal enterotoxin SeG	0.78	-0.91
sek2	staphylococcal enterotoxin Sek	1.45	-0.55
--	enterotoxin H	1.33	-0.45
set16	superantigen-like protein	1.27	-0.55
set21	superantigen-like protein	0.8	-0.2
set26	superantigen-like protein	3.94	-0.39
sak	staphylokinase precursor	0.84	-0.38

Objective 2: Study the changes in gene expression profile in ampicillin-celecoxib co-treatment to *S. aureus*

Table 8B: List of virulence factors and fold difference (compared to control) down regulated in combinatorial treatment of both celecoxib and ampicillin when compared to ampicillin treatment alone

Gene	Gene Product	Fold Difference	
		Ampicillin	Both
	Antibiotic Resistance Genes		
mecI	methicillin resistance regulatory protein	1.75	-0.29
mecA	penicillin binding protein 2 a	1.22	-0.65
mecR1	methicillin-resistance MecR1 regulatory protein	3.9	-0.39
blaZ	beta-lactamase	1.25	0.71
bleO	bleomycin resistance protein	0.99	0.6
fmtC	oxacillin resistance-related FmtC protein	1.08	0.66
femA	factor essential for expression of methicillin resistance	0.83	0.12
	Two-Component System		
agrA	accessory gene regulator protein A	1.44	0.19
agrD	accessory gene regulator protein D	1.21	-0.37
agrD	AgrD protein	1.23	0.27
sarA	staphylococcal accessory regulator A	1.85	0.06
-	RNAIII-activating protein TRAP	1.09	0.84
-	staphylocoagulase precursor	0.83	-0.82

Efflux pumps and membrane transporters differentially regulated in various treatment conditions were analyzed (Table 9A and 9B). Role played by specific efflux pumps in growth inhibition of bacteria was found to be negligible.

Objective 2: Study the changes in gene expression profile in ampicillin-celecoxib co-treatment to *S. aureus*

Table 9A: Table showing the efflux pumps/transporters differentially regulated in the various treatment conditions

AMP	BOTH	TRANSPORTERS
-1.79	-0.09	ABC transporter
1.05	0.31	ABC transporter ATP-binding protein
1.48	1.61	ABC transporter permease
-1.02	0.03	ABC transporter, ATP-binding protein
-0.06	0.35	ABC transporter, ATP-binding protein
-1.82	-0.21	ABC transporter, ATP-binding protein
-0.04	-0.02	ABC transporter, ATP-binding protein
-0.56	0.00	ABC transporter, ATP-binding protein
-0.01	0.16	ABC transporter, ATP-binding protein
-1.03	-0.04	ABC transporter, permease protein
0.96	0.51	ABC transporter, substrate-binding protein
0.74	0.34	ABC transporter, substrate-binding protein
1.67	0.49	amino acid ABC transporter, permease protein
-1.48	-0.39	amino acid ABC transporter, permease/substrate-binding protein
0.95	0.66	arsenic efflux pump protein
1.01	0.75	arsenic efflux pump protein
1.17	0.36	glycine betaine/carnitine/choline ABC transporter opuCA
1.74	0.27	glycine betaine/carnitine/choline ABC transporter opuCC (osmoprotec)
0.90	0.05	glycine betaine/carnitine/choline ABC transporter opuCD
-0.09	0.69	iron compound ABC transporter, ATP-binding protein, putative
0.52	0.51	iron compound ABC transporter, iron compound-binding protein, putative
-0.97	0.07	iron compound ABC transporter, permease protein
-0.38	0.17	iron compound ABC transporter, permease protein
-1.50	-0.55	major facilitator transporter

Table 9B: Table showing the efflux pumps/transporters differentially regulated in the various treatment conditions

AMP	BOTH	TRANSPORTERS
-1.13	-0.20	maltose ABC transporter, ATP-binding protein, putative
0.06	0.13	maltose ABC transporter, permease protein
-0.34	0.00	maltose ABC transporter, permease protein
-0.61	0.10	MATE efflux family protein
-0.88	-0.08	MmpL efflux pump, putative
-0.79	-0.48	molybdenum ABC transporter permease
-0.38	-0.06	molybdenum ABC transporter, ATP-binding protein ModC
-1.36	-0.16	molybdenum ABC transporter, molybdenum-binding protein ModA
-0.13	0.00	multi drug resistance protein (norA)
-1.86	-0.75	multidrug ABC transporter
0.56	0.29	oligopeptide ABC transporter, ATP-binding protein
-1.19	-0.23	oligopeptide ABC transporter, permease protein
0.94	-1.01	oligopeptide ABC transporter, permease protein
-0.94	-0.28	phosphate ABC transporter, ATP-binding protein
-1.03	-0.43	phosphate ABC transporter, permease protein
0.49	0.58	phosphate transporter family protein
-0.75	-0.06	phosphonate ABC transporter, ATP-binding protein
-0.77	-0.08	phosphonate ABC transporter, permease protein
1.00	-0.42	QacA protein
0.94	1.32	rarD protein
-1.08	-1.36	tetracycline resistance protein
0.55	-0.47	tetracycline resistance protein
1.22	-0.36	tetracycline resistance protein
-1.93	-0.07	transporter, putative
-0.57	0.17	transporter, putative
0.23	0.27	transporter, putative

Objective 2: Study the changes in gene expression profile in ampicillin-celecoxib co-treatment to *S. aureus*

3.2.2. RNA isolation

RNA was isolated and its integrity was checked by agarose gel electrophoresis (Figure 46). The isolated RNA showed clear bands of 23S and 16S rRNA indicating that RNA was in good condition. This RNA was used for cDNA synthesis.

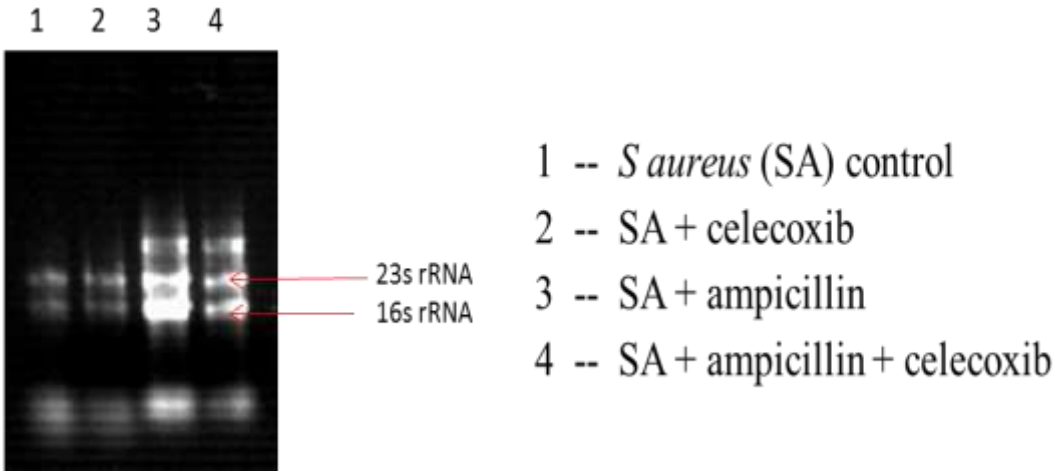


Figure 46: Integrity of RNA samples isolated from drug treated samples

3.2.3. cDNA synthesis from RNA

cDNA was synthesized from RNA. Real-time PCR was performed to validate the gene expression.

3.2.4. Real-time PCR to validate microarray data

Few genes belonging to each class described above were selected for the real-time quantification (Table 10 and Figure 47). The data confirmed the efficiency of celecoxib and ampicillin cotreatment in limiting *S. aureus* growth by down regulating all virulence genes. *blaZ* gene (which codes for beta-lactamase) expression was also measured by qRT-PCR (Figure 48) and it was observed that the *blaZ* expression was decreased in cotreatment when compared with ampicillin alone treatment.

Objective 2: Study the changes in gene expression profile in ampicillin-celecoxib co-treatment to *S. aureus*

Table 10: Real-time PCR data indicating $2^{-\Delta\Delta Ct}$ for few important virulence genes listed in Table 2 and 3 to validate microarray data (values indicate the fold increase in expression of genes when compared to the level of expression in control)

Gene	Gene Product	Fold Difference	
		Ampicillin	Both
spa	Staphylococcal Protein A	4.8	2.0
hla	Hemolysin Alpha	0.78	0.50
seA	Staphylococcal Enterotoxin A	0.96	0.57
seG	Staphylococcal Enterotoxin G	0.98	0.17
seP	Staphylococcal Enterotoxin P	1.25	0.23
mecA	Methicillin-resistant Protein A	2.69	0.41

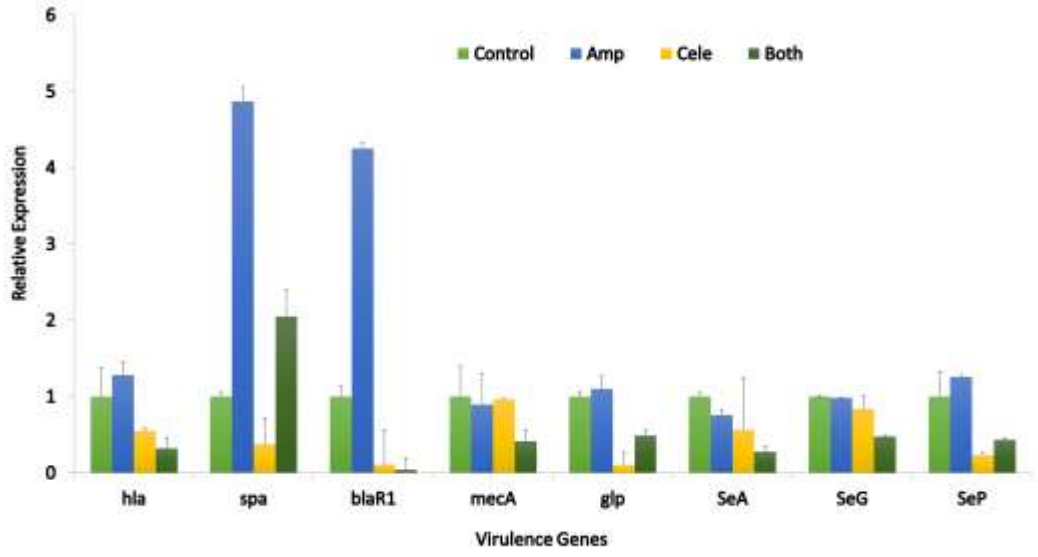


Figure 47: Relative fold change in the mRNA expression of some of the virulence genes

Objective 2: Study the changes in gene expression profile in ampicillin-celecoxib co-treatment to *S. aureus*

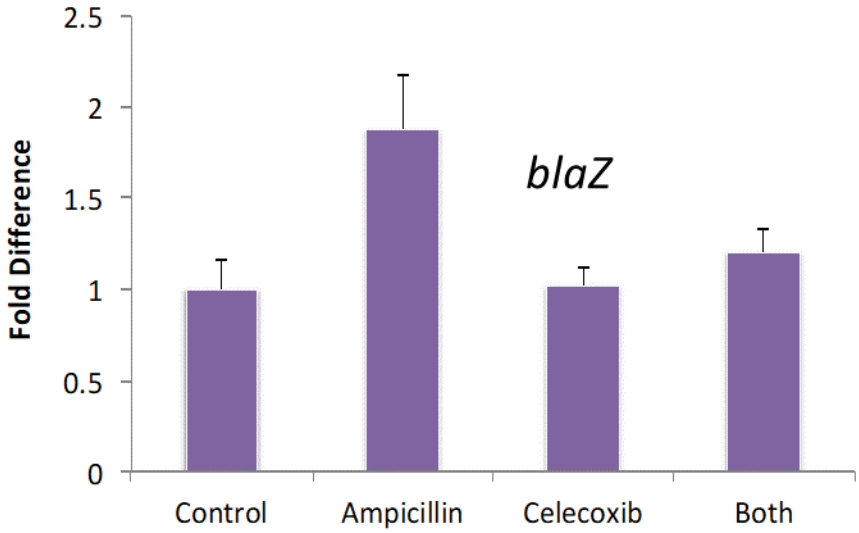


Figure 48: Relative fold change in the mRNA expression of *blaZ*

3.2.5. β – Lactamase activity assay using nitrocefin

Beta-lactamase enzyme can break the beta-lactam containing antibiotics like ampicillin and makes it ineffective. Microarray data and Real time PCR data clearly showed a significant decrease in Beta-lactamase gene (*blaZ*) expression. Therefore, to validate this further, we performed beta-lactamase activity assay. In presence of ampicillin, there was 6-fold increase of β-lactamase whereas in combinatorial treatment it was decreased to 4-fold (Figure 49). The combination treatment is effective in reducing the beta lactamase activity.

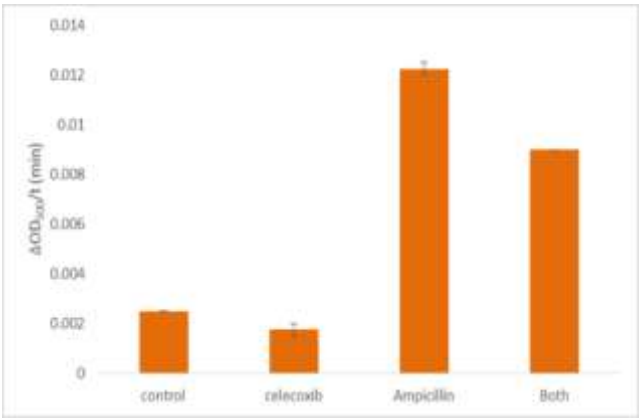


Figure 49: Beta-lactamase activity in various treatment conditions.

Objective 2: Study the changes in gene expression profile in ampicillin-celecoxib co-treatment to *S. aureus*

Table 11: Table showing the *blaZ* gene expression fold change as per microarray

Control	Cells+ celecoxib	Cells+ ampicillin	Cells+ ampicillin+ celecoxib
0.00	-0.05	1.25	0.71

Results from microarray (Table 11), quantitative real-time PCR analysis of *blaZ* gene (Figure 48) and beta-lactamase activity assay (Figure 49) have clearly shown the efficacy of combinatorial treatment with a significant decrease ($p<0.001$) in the gene expression and activity of the enzyme when compared to ampicillin alone treatment.

3.2.6. Western Blot

The microarray data was further validated by performing western blot to detect the enterotoxin H and hemolysin alpha proteins in different treatment conditions. Enterotoxin H and hemolysin-alpha levels were remarkably decreased in combination treatment (Figure 50A and 50B), which was evidenced by western blot.

A **B**

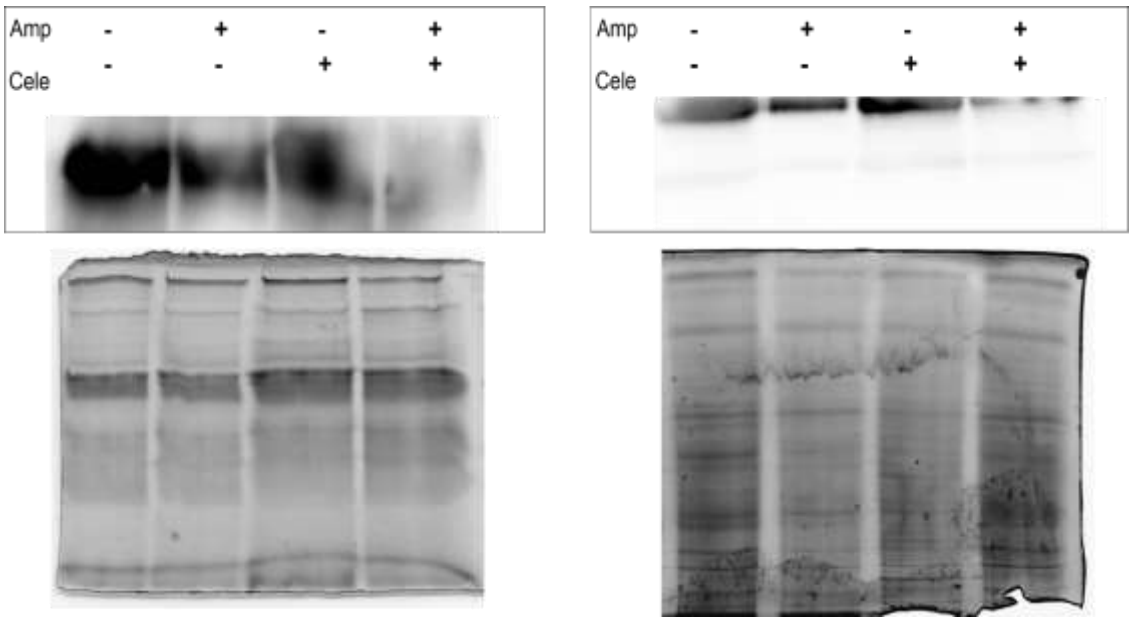


Figure 50: A) Western blot analysis of enterotoxin H. **B)** Immunoblot of α-hemolysin toxins in response to the treatments of celecoxib alone, ampicillin alone or both celecoxib and ampicillin. The Coomassie stained gels below the blots represent loading controls.

Objective 2: Study the changes in gene expression profile in ampicillin-celecoxib co-treatment to *S. aureus*

Microarray data showed that genes involved in lipid metabolism were also affected (Table 12), which may compromise the membrane lipid synthesis and may lead to increased membrane permeability. This is in line with the membrane permeability observed in bacterial ghosts and in flow cytometry.

Table 12: Table showing the expression of genes involved in fatty acid/lipid synthesis

Control	Celecoxib	Ampicillin	Both		
0.00	-0.56	1.13	-0.12	hmrB	acyl carrier protein
0.00	-0.45	0.69	-0.38	glpK	glycerol kinase
0.00	-0.09	1.81	0.93	fabD	malonyl CoA-acyl carrier protein transacylase
0.00	-0.23	0.69	0.21	fab	3-oxoacyl- synthase
0.00	0.00	1.48	1.12	FabH	3-oxoacyl-(acyl carrier protein) synthase III
0.00	-0.10	1.75	0.63	fabG1	3-oxoacyl-(acyl-carrier-protein) reductase
0.00	0.35	1.24	0.51	accC	acetyl-CoA carboxylase biotin carboxylase subunit
0.00	-0.16	1.26	0.16	-	acetyl-CoA carboxylase subunit beta
0.00	0.29	1.19	0.81	-	diacylglycerol glucosyltransferase
0.00	-0.14	1.38	0.27	-	fatty acid biosynthesis transcriptional regulator
0.00	0.08	0.67	-0.43	geh	glycerol ester hydrolase
0.00	0.05	0.63	0.04	tagD	glycerol-3-phosphate cytidylyltransferase
0.00	0.37	1.01	-0.24	-	HMG-CoA synthase
0.00	0.37	1.23	-0.36	-	HMG-CoA synthase, truncation
0.00	0.21	1.46	0.24	lip	triacylglycerol lipase precursor

Also, microarray data analysis showed that the genes necessary for the maintenance of membrane potential were differentially regulated in the combination treatment (Table 13) and it supports the flow cytometric analysis of membrane potential.

Table 13: Differential expression of genes involved in Membrane potential as per microarray data analysis

Control	Celecoxib	Ampicillin	Both		
0.00	0.37	1.20	-0.24	kdpA	potassium-transporting ATPase subunit A
0.00	0.36	5.26	-0.36	kdpB	potassium-transporting ATPase subunit B
0.00	0.00	3.82	-0.68	kdpC	potassium-transporting ATPase subunit C
0.00	0.34	1.83	1.40	mnhB	putative monovalent cation/H+ antiporter subunit B
0.00	0.11	1.88	1.19	mnhC	putative monovalent cation/H+ antiporter subunit C
0.00	0.20	1.96	1.07	mnhD	putative monovalent cation/H+ antiporter subunit D
0.00	0.17	1.98	0.76	mnhE	putative monovalent cation/H+ antiporter subunit E
0.00	0.03	1.61	0.65	mnhF	putative monovalent cation/H+ antiporter subunit F
0.00	-0.15	1.22	0.56	mnhG	putative monovalent cation/H+ antiporter subunit G
0.00	0.00	1.30	0.88	mnhA	monovalent cation/H+ antiporter subunit A

Objective 2: Study the changes in gene expression profile in ampicillin-celecoxib co-treatment to *S. aureus*

3.3 Discussion

To understand the mechanism of action of ampicillin and celecoxib combination on *S. aureus*, we have carried out global transcriptome expression analysis by microarray technology. The data analysis indicated that celecoxib alone treatment did not show much variation in the global transcription of *S. aureus* when compared to control. However, a significant increase in the gene expression, specifically the virulence genes, antibiotic resistance genes, two-component signal transduction genes etc was observed in ampicillin alone treated cells. However, all the genes upregulated due to ampicillin treatment, were down regulated in combinatorial treatment of celecoxib and ampicillin.

The process of infection caused by *S. aureus* is a complex process involving many proteins. Among them, the two classes of virulence factors (1) cell-wall associated proteins and (2) secreted proteins [143] are mainly involved in pathogenesis. Initially *S. aureus* uses cell-wall associated proteins to attach to host cells and enter into the cells. In later stages of infection, it secretes toxins that result in inflammation and necrosis of the tissue.

a) Capsular polysaccharides and fibronectin binding factors: The capsular polysaccharide proteins of *S. aureus* prevent phagocytosis of the bacteria and fibronectin binding proteins are required for promoting adherence of bacteria to fibronectin coated host molecules [144]. Targeting these proteins is a known strategy to combat *S. aureus* infections [145]. Furthermore, the clumping factor A (clfA) and elastin binding factor (ebpS) are also necessary for attachment of *S. aureus* to host cells [146]. Our data also suggested down regulation of these virulence genes in combinatorial treatment of ampicillin and celecoxib indicating the efficacy of combinatorial treatment.

b) Secreted proteins: *Staphylococcus aureus* secretes an arsenal of toxins including α-hemolysin (HLA), γ-hemolysin (HLG), leukocidin (Luk), panton-valentine leukocidin (PVL), toxic shock syndrome toxin (TSS-1), enterotoxins, staphylokinase and superantigens [147]. Surprisingly, all these virulence factors were down regulated in combinatorial treatment of celecoxib and ampicillin further strengthening our hypothesis that celecoxib sensitizes bacteria to antibiotic.

Antibiotic resistance genes: Among the different mechanisms of development of resistance to antibiotics, horizontal gene transfer of antibiotic-resistance genes,

enzymatic degradation of antibiotics and efflux of antibiotics are common in *S. aureus* [148]. Penicillin Binding Protein 2a (PB2a) is involved in resistance to methicillin while beta lactamase is responsible for degradation of penicillin-like antibiotics with β-lactam ring. Resistance to other antibiotics is conferred by horizontal gene transfer. In the current study, down regulation of all these resistance genes was observed with combinatorial treatment.

Two-component signal transduction: Two-component system of bacteria is a communication link between internal and external environment wherein the bacteria sense the external stimulus and translate it internally to modulate the gene expression. The two component system has histidine kinase (HK), sensor in the membrane, and response regulator (RR) in the cytoplasm which are often arranged in operons and thus co-transcribed. Two such important operons are accessory gene regulator (*agr*) and staphylococcal accessory regulator (*sar*) which generally regulate the production of all virulence factors through RNA III [149]. Our data presented here is in line with earlier studies where down regulation of *agr* and *sar* operons by combinatorial treatment was show to down regulate all the virulence genes. Our data was also further validated using real time PCR, Western blot analysis and activity assay.

In conclusion, the toxicogenomic analysis of *S. aureus* to combinatorial treatment of celecoxib and ampicillin indicate a significant down regulation in various virulence factors including, enterotoxins, two-component signal transduction systems and antibiotic resistance genes. In presence of celecoxib, there was an increased uptake of ampicillin by bacteria due to increased membrane permeability and cocrystal studies have indicated interaction between the two compounds, which might also affect the physical properties of the drugs and thereby increased permeation. This effect of celecoxib in combination cannot be defined by synergism since celecoxib alone does not show any effect on the bacterial growth. The combinatorial treatment of celecoxib and antibiotic can be a better treatment strategy to combat *S. aureus* infections.

Objective 3

Evaluate the efficacy of ampicillin-celecoxib cotreatment in isolates of *S. aureus* obtained from bubaline mastitis

Objective 3: Evaluate the efficacy of ampicillin-celecoxib cotreatment in isolates of *S. aureus* obtained from bubaline mastitis

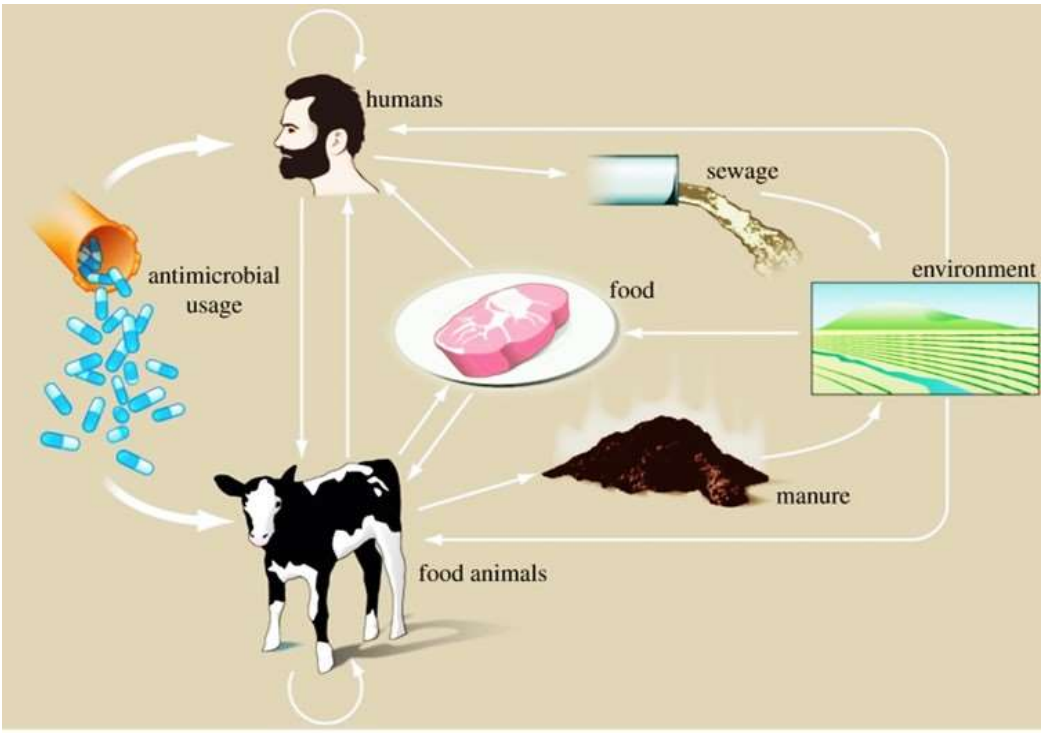
4.1 Introduction

Inflammation usually due to bacterial infection of the mammary gland (udder) in dairy cattle leads to mastitis causing a great economic loss globally. Improper use of antibiotics in livestock industry have led to the development of antibiotic resistance worldwide [150]. Nonetheless, the current treatment option for mastitis in cows and buffaloes is administration of antibiotics or corticosterone/anti-histamine that have their own limitations such as excretion of antibiotic residues in milk, which renders the milk for discard. Identification of complementary therapeutic formulation which helps in reducing the dosage (MIC) of antibiotic in combination with other agent, with fewer or no side effects is desirable to treat mastitis [151].

USA and China are the countries which use highest quantity of antibiotics for food production. As per FDA, in USA, 80 % of total antibiotics are used in agriculture. The quantity of antibiotics received by poultry and pigs is 10 times more than that of cows and sheep. Lack of laws in our country for controlling the use of antibiotics in farm animals is also indirectly responsible for AMR development. As discussed in the general introduction, antibiotics in sub therapeutic concentrations are used in farm animals as growth enhancers. But this continued use of antibiotics in farm animals will offer selection pressure for bacteria and hence susceptible bacteria will be eliminated and resistance bacteria will be selected and spread [152]. The studies on meat of farm animals showing the antibiotic residues in them, resulted from the over use of antibiotics. Most of the microorganisms live in human as well as animals. Wide spread AMR in farm animals was reported. Most of the drug resistant strains found in humans were also isolated from the animals. This shows the severity of the AMR. Many bacteria move from animals to humans (cause zoonotic diseases) through meat or by contact and hence care should be taken to control the development of AMR bacteria in animals [153] (Figure 51).

One Health is a “collaborative effort of multiple disciplines -working locally, nationally, and globally – to obtain optional health for people, animals and our environment” (Figure 52). Since the microbes can move among human, animals and environment, the drug resistance developed at any stage can affect the others. Six out of ten diseases are zoonotic, which represents the significance of one health approach.

Objective 3: Evaluate the efficacy of ampicillin-celecoxib cotreatment in isolates of *S. aureus* obtained from bubaline mastitis



Philos Trans R Soc Lond B Biol Sci. 2015 Jun 5; 370(1670): 20140083.

Figure 51: Transmission of drug resistance among humans, animals and environment

https://images.app.goo.gl/FovENzZrnvkoreZ49

Figure 52: One health concept

Since inflammation due to microbial infection of udder is the most significant factor for severity of mastitis [154], a combination therapy consisting of an anti-inflammatory drug and antibiotic might help in alleviating mastitis. We, therefore, tested the efficacy of celecoxib in combination with ampicillin on veterinary isolates of *S. aureus* isolated from milk of bubaline mastitis. We also tested the combination efficacy of meloxicam, a preferred NSAID for animals, in combination with ampicillin on same *S. aureus* isolates.

Objective 3: Evaluate the efficacy of ampicillin-celecoxib cotreatment in isolates of *S. aureus* obtained from bubaline mastitis

4.2 Materials and methods

4.2.1 Determination of MIC of ampicillin for clinical isolates of *S. aureus*

The minimum inhibitory concentration (MIC) of ampicillin for *S. aureus* clinical isolates was determined as described above. Clinical isolates were categorized into three categories, highly-resistant bacteria, intermediate-resistant bacteria and sensitive bacteria based on their MIC values for ampicillin.

4.2.2 Effect of ampicillin-celecoxib and meloxicam-celecoxib combination on veterinary *S. aureus* isolates

Ampicillin concentration for bacterial treatment for each group was fixed in such a way that there is no visual growth inhibition at that concentration.

Bacterial isolates of different resistance levels were incubated with celecoxib (25 µM) alone, meloxicam (25 µM) alone, or ampicillin (3 µg/mL or 0.3 µg/mL or 0.0155 µg/mL) alone or both (ampicillin-celecoxib and meloxicam-celecoxib**)** or neither (control) in 50 mL LB for 40 min at 37 ºC in shaker incubator. The number of colony forming units (CFUs) of every bacterial isolate was recorded.

4.2.3. Statistical analysis

One-way ANOVA (analysis of variance) was performed using Sigmaplot software version 12.3. $p<0.05$ was reported as significant.

Objective 3: Evaluate the efficacy of ampicillin-celecoxib cotreatment in isolates of *S. aureus* obtained from bubaline mastitis

Objective 3: Evaluate the efficacy of ampicillin-celecoxib cotreatment in isolates of *S. aureus* obtained from bubaline mastitis

4.3.1. Determination of MIC of ampicillin for *S. aureus* clinical isolates by broth micro dilution and classifying the isolates into different categories

A total of 25 isolates of *S. aureus* isolated from milk of bubaline mastitis were collected from NTR College of Veterinary sciences, Gannavaram, A.P., India and characterized by 16S RNA. Based on the MIC ampicillin, the isolates were categorized as highly resistant, intermediate resistant and sensitive bacteria (Table 14) and a concentration of ampicillin was reached for each category for further experiments.

Table 14: Categorization of veterinary isolates of *S. aureus* according to their resistance to ampicillin

Sample name	Ampicillin MIC (µg/ml)	Ampicillin used for combination assay (µg/ml)
Highly Resistant Bacteria		
KSP3, KSP4, KSP6, TVCC4, TVCC36	>16	3.0
G-162, G-173, U-152, MRSA	16	
Intermediate Resistant Bacteria		
GDV-2, GDV-3, G-177, G-167,	2	0.3
G-168, G-174, MSSA	1	
KSP7	0.5	
Sensitive Bacteria		
G-169, G-171	0.125	0.0155
GDV-1, TVCS3, GV15, G-170, U-151, U-153, U-155	0.0625	
TVCS2	0.03125	

Objective 3: Evaluate the efficacy of ampicillin-celecoxib cotreatment in isolates of *S. aureus* obtained from bubaline mastitis

4.3.2. Effect of ampicillin-celecoxib combination on veterinary *S. aureus* isolates

It was observed that all the highly resistant strains when treated with individual drug were not affected. When these highly resistant isolates were treated with a combination of ampicillin and celecoxib, showed a significant decrease in the colony forming units (CFU) (Figure 53).

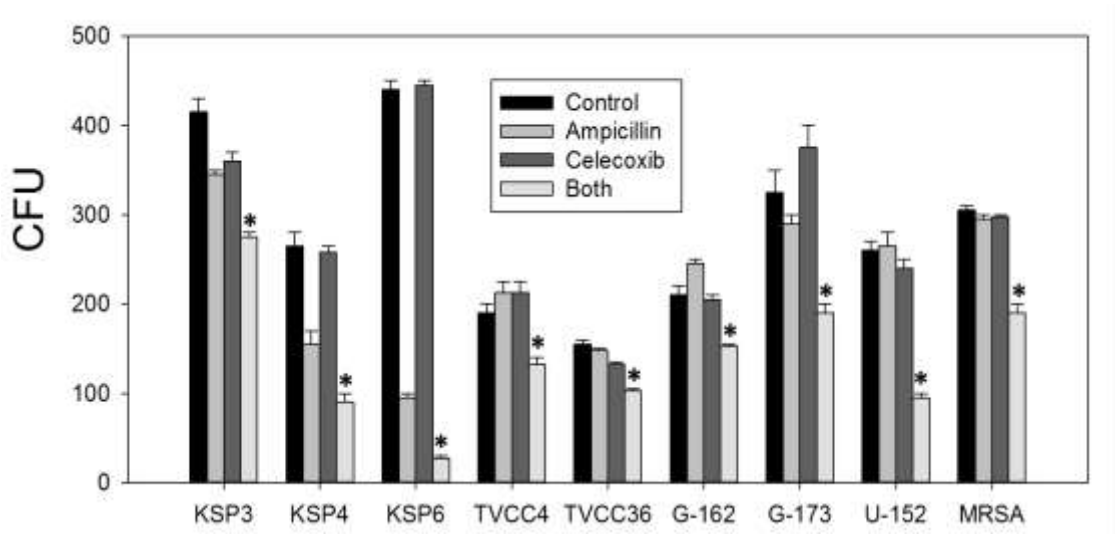


Figure 53: Effect of ampicillin, celecoxib and ampicillin-celecoxib combination on growth of highly resistant veterinary *S. aureus* isolates (* indicates the significant decrease in comparison to ampicillin alone treatment)

Similar results were observed with intermediate resistant (Figure 54) and sensitive strains (Figure 55) of clinical isolates of *S. aureus*.

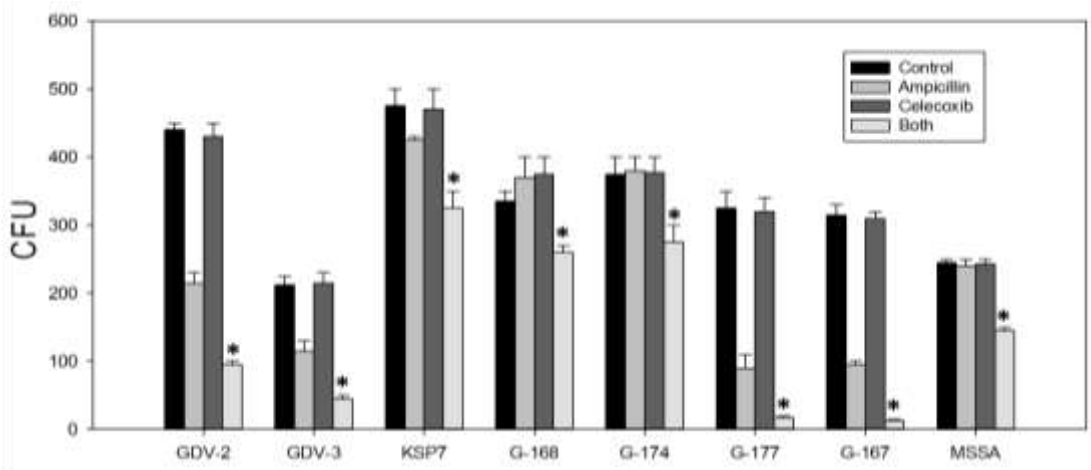


Figure 54: Effect of ampicillin, celecoxib and ampicillin-celecoxib on growth of intermediate resistant veterinary *S. aureus* isolates (* indicates the significant decrease in comparison to ampicillin alone treatment)

Objective 3: Evaluate the efficacy of ampicillin-celecoxib cotreatment in isolates of *S. aureus* obtained from bubaline mastitis

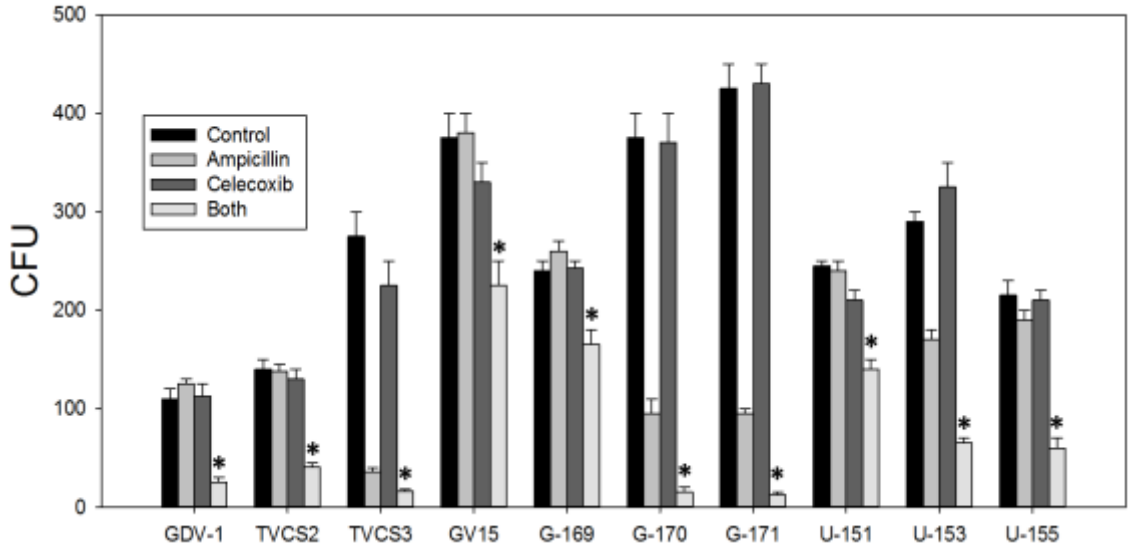


Figure 55: Effect of ampicillin, celecoxib and ampicillin-celecoxib on growth of sensitive veterinary *S. aureus* isolates (* indicates the significant decrease in comparison to ampicillin alone treatment)

Effect of ampicillin-meloxicam combination on veterinary *S. aureus* isolates

Meloxicam is preferred NSAID for veterinary animals. Ampicillin-meloxicam cotreatment also showed similar results and this combination is effective on all the three groups of veterinary isolates of *S. aureus* viz., highly resistant (Figure 56), intermediate resistant (Figure 57) and sensitive bacteria (Figure 58).

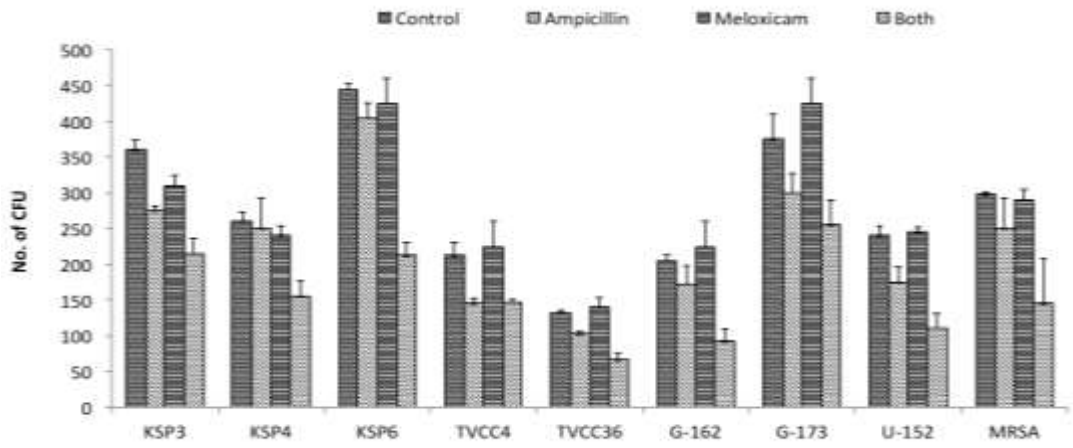


Figure 56: Effect of ampicillin, meloxicam and ampicillin-meloxicam combination on growth of highly resistant veterinary *S. aureus* isolates

Objective 3: Evaluate the efficacy of ampicillin-celecoxib cotreatment in isolates of *S. aureus* obtained from bubaline mastitis

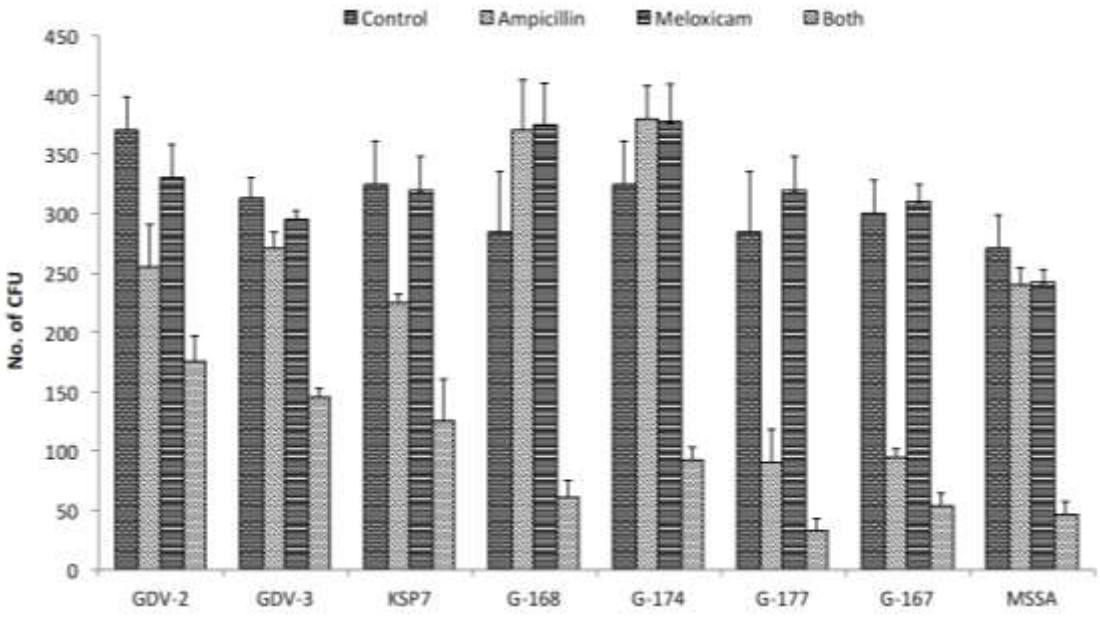


Figure 57: Effect of ampicillin, meloxicam and ampicillin-meloxicam combination on growth of intermediate resistant veterinary *S. aureus* isolates

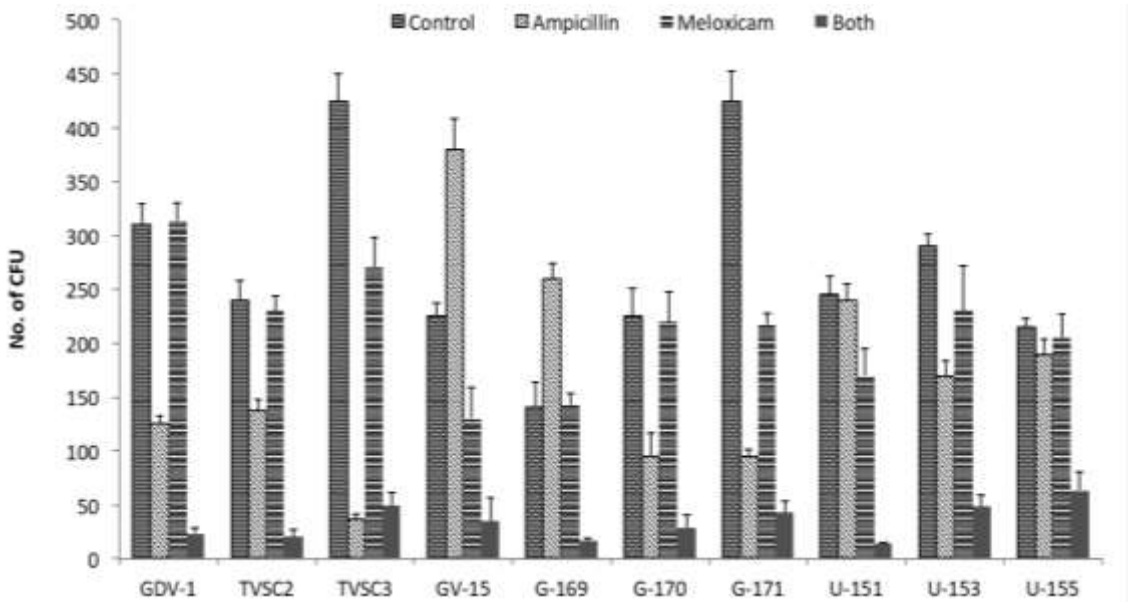


Figure 58: Effect of ampicillin, meloxicam and ampicillin-meloxicam combination on growth of sensitive veterinary *S. aureus* isolates

Objective 3: Evaluate the efficacy of ampicillin-celecoxib cotreatment in isolates of *S. aureus* obtained from bubaline mastitis

4.4 Discussion

Antimicrobial resistance of bacteria in animals pose a great threat to humans as these can cause zoonotic diseases in humans. Mastitis, mainly by *S. aureus* and *E. coli*, in cows and buffaloes cause high economic loss to dairy industry worldwide. Irrational use of antibiotics to control these infections is the main reason for emergence of antibiotic resistant pathogens. There is need for a better therapeutic formulation with least side effects. Previously, we have demonstrated that celecoxib, an anti-inflammatory drug, reduces the minimal inhibitory concentration (MIC) of the antibiotic in combination, in laboratory strains of *S. aureus*. Here, in current study we assessed the efficacy of the NSAIDs, celecoxib or meloxicam in combination with ampicillin on 25 *S. aureus* samples isolated from bubaline mastitis milk samples.

The results are in agreement with our earlier studies and clearly indicated the potential use of NSAIDs in combination with antibiotic to treat mastitis in buffaloes. Furthermore, the dose of antibiotic required in combinatorial treatment was half the MIC of antibiotic suggesting the remedy for overuse of antibiotic and probably lower antibiotic residues in the milk. Meloxicam in combination with ampicillin was very effective on resistant *S. aureus* strains.

NSAIDs, meloxicam and celecoxib, in combination with antibiotic can be potential therapeutic strategy to treat bubaline mastitis.

5. Conclusions

- ✓ NSAIDs are not inhibiting the *S. aureus* growth even at 100 µM concentration.
- ✓ Celecoxib is effective in inhibiting the bacterial growth in combination with other antibiotics.
- ✓ Celecoxib increased the ampicillin permeability into ghost membranes.
- ✓ Combinatorial treatment of ampicillin-celecoxib increased the membrane permeability and potential and thereby increased the entry of antibiotic into bacterial cells.
- ✓ Ampicillin-celecoxib co-crystals were developed and characterised by PXRD, DSC, FTIR which indicated weak interactions between the ampicillin and celecoxib.
- ✓ The logP value of ampicillin in co-crystal has increased from 1.16 to 2.08 indicating increased permeability into the cell.
- ✓ Celecoxib did not show significant variation in gene expression when compared with control.
- ✓ Ampicillin showed significant differential gene expression.
- ✓ Co-treatment of ampicillin and celecoxib resulted in reversal of gene expression compared to ampicillin alone treated cells.
- ✓ In co-treatment the expression of all virulence genes, antibiotic resistance genes were significantly down regulated when compared to ampicillin alone treated cells.
- ✓ There was no significant differential gene expression in efflux pumps indicating non-involvement of these in the beneficial effects of co-treatment.
- ✓ There was a significant down regulation of membrane permeability and potential genes which are in line with the flow cytometric results that indicate the potential effects of celecoxib in increased permeation of antibiotic.
- ✓ Combinatorial treatment of ampicillin-celecoxib was also effective on veterinary *S. aureus* isolated from bubaline mastitis milk.
- ✓ In combinatorial treatment the dose of antibiotic has been reduced to half the MIC.

6. Summary

Irrespective of how effective a new antibiotic is upon clinical introduction, there will be an invariable drug resistance by microbes, which limits the effectiveness of the new drug. Recent emergence of "superbugs," clinically resistant to several antibiotics, has posed a great problem in treating bacterial infections [155]. *S. aureus* is causing high mortality rate due to MDR [148]. MRSA infections are problematic in both community and health care settings. Chemotherapy involving combination of two or more antibiotics has been one of the therapeutic strategies for the treatment of MRSA infections [156]. Several mechanisms contribute to the intrinsic or acquired resistance for antibiotics in these bacteria. According to U.S. Food and Drug Administration (FDA), identification of non-traditional treatment strategies using combination of two or more drugs containing antibiotics targeting bacteria and another drug as potentiator of antibiotic activity would help in overcoming drug resistance [157].

Celecoxib, a nonsteroidal anti-inflammatory drug (NSAID), was shown to be involved in reversal of MDR -related cancers [158] and is approved for the treatment of Familial adenomatous polyposis (FAP) [159]. We have previously demonstrated that celecoxib inhibits growth of both Gram positive and negative bacteria in combination with antibiotics [118, 119]. Ampicillin, a penicillin class of antibiotic, is an essential medicine according to WHO. Although ampicillin is no more a preferred choice of antibiotic to treat *S. aureus* infections, in absence of new antibiotic, rescuing and repurposing old antibiotics will be an innovative economic strategy [160, 161]. With this idea, we have used ampicillin in combination with celecoxib to inhibit *S. aureus* and clearly demonstrated that celecoxib and ampicillin combination can limit the growth of both methicillin-sensitive and –resistant *S. aureus* [118, 119]. Recently, we demonstrated the efficacy of this combinatorial treatment *in vivo* in mouse cecum ligation and puncture model of sepsis and also on ESKAPE pathogens isolated from human patient samples [120]. Several others have also demonstrated the antibacterial effects of celecoxib [128, 162, 163]. We have elucidated the mechanism of action of the cotreatment in host cells. *In vitro* macrophages infected

S. aureus and *in vivo* polymicrobial sepsis murine bacterial infection models showed that celecoxib activates SIRT1 (member of sirtuin family) thus regulating inflammatory gene expression such as COX-2 (Cyclooxygenase-2), NO (nitric oxide), IL-6 (interleukin-6), MIP1α (macrophage inflammatory protein 1 alpha), IL-1β (interleukin-1 beta) via TLR2 (toll-like receptor 2), JNK (c-Jun N-terminal kinase) and NF-κB (nuclear factor kappa light chain enhancer of activated B cells) pathways and regulating ROS (reactive oxygen species) levels by increasing antioxidant enzyme levels there by providing conditions favourable for bacterial clearance by antibiotic. Celecoxib sensitizes intracellular *Staphylococcus aureus* to antibiotic there by limiting bacterial survival and infection. However, the mechanism of action of the combinatorial treatment in bacteria remains to be explored.

In this study, we aimed to investigate the molecular mechanism underlying the action of celecoxib in combination with ampicillin against *S. aureus* growth.

Development of alternate treatment strategies for drug resistant bacterial infections is the major responsibility of scientific community. Drug repurposing is one of the methods FDA has suggested for identification of drugs to treat MDR bacteria. We [118] and others [128] had previously shown that celecoxib, selectively inhibits COX-2, act as an antibacterial agent when given in combination with an antibiotic. In the present study we aimed at elucidating the mechanism of action of celecoxib on bacterial growth. To achieve this, first we have studied the growth kinetics of *S. aureus* ATCC29213 in presence or absence of ampicillin alone, celecoxib alone and a combination of both the drugs. As indicated in results, combinatorial treatment showed growth inhibition. Synergistic effect is defined as the joint action of drugs, that when taken together increases each other's effects. However, in the present study the synergistic definition does not hold good since celecoxib alone does not show any effect on the bacterial growth. Nevertheless, celecoxib in combination was enhancing ampicillin inhibitory effect. Growth inhibition of *S. aureus* by ampicillin was also shown to be increased in synergism when used in combination with other non-antibiotic compounds [129, 130].

To further understand the process, we have done global transcriptome expression analysis by microarray technology. The data analysis indicated that celecoxib alone treatment did not show much variation in the global transcription of *S. aureus* when

compared to control. However, a significant increase in the gene expression, specifically the virulence genes, antibiotic resistance genes, two-component signal transduction genes etc was observed in ampicillin alone treated cells. However, all the genes upregulated due to ampicillin treatment, were down regulated in combinatorial treatment of celecoxib and ampicillin.

The process of infection caused by *S. aureus* is a complex process involving many proteins. Among them, the two classes of virulence factors (1) cell-wall associated proteins and (2) secreted proteins [143] play an important role in the process of pathogenesis. Initially *S. aureus* uses cell-wall associated proteins to attach to host cells and enter into the cells. In later stages of infection, it secretes toxins that result in inflammation and necrosis of the tissue.

a) Capsular Polysaccharides and Fibronectin binding factors: The capsular polysaccharide proteins of *S. aureus* prevent phagocytosis of the bacteria and fibronectin binding proteins are required for promoting adherence of bacteria to fibronectin coated host molecules [144]. Targeting these proteins is a known strategy to combat *S. aureus* infections [145]. Furthermore the clumping factor A (clfA) and elastin binding factor (ebpS) necessary for attachment of *S. aureus* to host cells [146]. Our data also suggested down regulation of these virulence genes in combinatorial treatment of ampicillin and celecoxib indicating the efficacy of combinatorial treatment.

b) Secreted proteins: *Staphylococcus aureus* secretes an arsenal of toxins including α-hemolysin (HLA), γ-hemolysin (HLG), leukocidin (Luk), Panton-Valentine Leukocidin (PVL), Toxic Shock Syndrome toxin (TSS-1), enterotoxins, staphylokinase, superantigens etc [147]. Surprisingly, all these virulence factors have been down regulated in combinatorial treatment of celecoxib and ampicillin further strengthening our hypothesis that celecoxib sensitizes bacteria to antibiotic.

Antibiotic Resistance genes: Among the different mechanisms of development of resistance to antibiotics, horizontal gene transfer of antibiotic-resistance genes, enzymatic degradation of antibiotics and efflux of antibiotics are common in *S. aureus* [148]. Penicillin Binding Protein 2a (PB2a) is involved in resistance to methicillin while beta lactamase is responsible for degradation of penicillin-like antibiotics with β-lactam ring. Resistance to other antibiotics is conferred by

horizontal gene transfer. In current study, down regulation of all these resistance genes was achievable with combinatorial treatment.

Two-component signal transduction: Two-component system of bacteria is a communication link between internal and external environment wherein the bacteria senses the external stimulus and translates it internally to modulate the gene expression. The two component system has histidine kinase (HK), sensor in membrane, and response regulator (RR) in cytoplasm which are often arranged in operons and thus co-transcribed. Two such important operons are accessory gene regulator (*agr*) and staphylococcal accessory regulator (*sar*) which generally regulate the production of all virulence factors through RNA III [149]. Our data presented here is in line with earlier studies where down regulation of *agr* and *sar* operons was shown by combinatorial treatment had down regulated all the virulence genes. Our data was also further validated using real time PCR, Western blot analysis and activity assay.

Although the microarray data indicated promising role of celecoxib in sensitization of bacteria to ampicillin, we wanted to address the basic question does celecoxib enter the bacteria? With the ghost membrane drug uptake assay it was clear that indeed there was an increased uptake of ampicillin in presence of celecoxib (celecoxib is also entering into bacteria). Therefore, we next addressed the question whether the increased uptake/entry of ampicillin into bacteria in presence of celecoxib is due to the modulation of membrane potential and permeability? It is well established that bacteria attain resistance by altering their membrane permeability and potential to stop entry of the antibiotics and is very well known with ampicillin [131, 132]. Our flowcytometric results where we show increased membrane permeability and potential to combinatorial drug treatment are in line with the established results.

The bacterial ghost membrane assay and the microarray results were able to indicate probable mechanism of action of celecoxib as increased uptake of ampicillin by modulating membrane permeability and potential, it is not clear, however, as to how it is allowing increased uptake. To address this question we have carried out co-crystal studies. In recent years, a much focus has been in altering the physiochemical properties of a drug so as to improve its solubility, bioavailability, stability, permeability etc [133-136]. It was already established that the weak non-covalent interactions present in the amoxicillin-clavulanate co-crystals can alter the physicochemical properties of amoxicillin thus increasing its bioavailability. So, co-

crystals of celecoxib and ampicillin were prepared by co-grinding method followed by solvent evaporation at room temperature. The crystals were analysed by PXRD, FTIR and DSC which confirmed that celecoxib being a lipophilic compound and ampicillin hydrophilic, the weak electrostatic or hydrogen bonding interactions or van der Waals forces between the two drugs might be enhancing the entry or permeation of ampicillin. This was also evident from the increase in logP value of ampicillin from 1.16 to 2.08. Such an increase in the permeation and dissolution properties of a drug in cocrystals has been well documented [137-139].

In conclusion, the toxicogenomic analysis of *S. aureus* to combinatorial treatment of celecoxib and ampicillin indicate a significant down regulation in various virulence factors including, enterotoxins, two-component signal transduction systems and antibiotic resistance genes. In presence of celecoxib, there was an increased uptake of ampicillin by bacteria due to increased membrane permeability and cocrystal studies have indicated interaction between the two compounds, which might also affect the physical properties of the drugs and thereby increased permeation and bioavailability. This effect of celecoxib in combination cannot be defined by synergism since celecoxib alone does not show any effect on the bacterial growth. The combinatorial treatment of celecoxib and antibiotic can be a better treatment strategy to combat *S. aureus* infections.

The growth inhibitory efficacy of celecoxib and antibiotic at sub-MIC concentrations can

i) Decrease the overdose of antibiotic that is used to treat the infections
ii) The cost for the treatment can be decreased
iii) Side effects can be decreased
iv) Lower doses of antibiotic can reduce the selection pressure and hence the antibiotic resistance.

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PUBLICATIONS

1. **Gajapati Y N Varma**, Githavani Kummari, Pradip Paik, Arunasree M Kalle. Celecoxib potentiates antibiotic uptake by altering membrane potential and permeability in *Staphylococcus aureus*. J Antimicrob Chemother. 2019 Oct 5. pii: dkz391. doi: 10.1093/jac/dkz391. [Epub ahead of print] PubMed PMID: 31586409.
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Celecoxib potentiates antibiotic uptake by altering membrane potential and permeability in *Staphylococcus aureus*

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Background: We have shown previously that celecoxib enhances the antibacterial effect of antibiotics and has sensitized drug-resistant bacteria to antibiotics at low concentrations using *in vitro* and *in vivo* model systems and also using clinically isolated ESKAPE pathogens.

Objectives: To identify the mechanism of action of celecoxib in potentiating the effect of antibiotics on bacteria.

Methods: Toxicogenomic expression analysis of *Staphylococcus aureus* in the presence or absence of ampicillin, celecoxib or both was carried out by microarray followed by validation of microarray results by flow cytometry and real-time PCR analysis, cocrystal development and analysis.

Results: The RNA expression map clearly indicated a change in the global transcriptome of *S. aureus* in the presence of cells treated with ampicillin alone, which was similar to that of celecoxib-treated cells in co-treated cells. Several essential, non-essential and virulence genes such as α-haemolysin (HLA), enterotoxins and β-lactamase were differentially regulated in co-treated cells. Further detailed analysis of the expression data indicated that the ion transporters and enzymes of the lipid biosynthesis pathway were down-regulated in co-treated cells leading to decreased membrane permeability and membrane potential. Cocrystal studies using Powder-X-Ray Diffraction (PXRD) and differential scanning calorimetry (DSC) indicated interactions between celecoxib and ampicillin, which might help in the entry of antibiotics.

Conclusions: Although further studies are warranted, here we report that celecoxib alters membrane potential and permeability, specifically by affecting the Na^+/K^+ ion transporter, and thereby increases the uptake of ampicillin by *S. aureus*.

Introduction

Irrespective of how effective a new antibiotic is upon clinical introduction, there will invariably be drug resistance of microbes that limits the effectiveness of a new drug. Recent emergence of 'superbugs', clinically resistant to several antibiotics, has posed a great problem in treating bacterial infections.[1] One of the bacterial pathogens well known for its MDR and for causing a very high global mortality rate for all human infections is *Staphylococcus aureus*.[2] Infection caused by *S. aureus*, particularly MRSA strains, has been declared problematic in both community and healthcare settings. Chemotherapy involving combinations of two or more antibiotics has been one of the therapeutic strategies for the treatment of MRSA infections.[3] However, several mechanisms contribute to the intrinsic or acquired resistance to antibiotics in these bacteria. According to the FDA, identification of non-traditional treatment strategies using combinations of two or more drugs containing antibiotics targeting bacteria and another drug as a potentiator of antibiotic activity would help in overcoming drug resistance.[4]

Celecoxib, a non-steroidal anti-inflammatory drug (NSAID), was shown to be involved in reversal of MDR-related cancers[5] and is approved for the treatment of familial adenomatous polyposis (FAP).[6] We have previously demonstrated that celecoxib inhibits growth of both Gram-positive and -negative bacteria in combination with an antibiotic.[7,8] Ampicillin, an antibiotic of the penicillin class, is an essential medicine according to the WHO. Although ampicillin is no longer a preferred choice of antibiotic to treat *S. aureus*, in the absence of new antibiotics, rescuing and repurposing old antibiotics will be an innovative economic strategy.[9,10] With this idea, we have used ampicillin in combination with celecoxib to inhibit *S. aureus* and clearly demonstrated that the celecoxib and ampicillin combination can limit both methicillin-susceptible

S. aureus and MRSA.[7,8] Recently, we demonstrated the efficacy of this combinatorial treatment *in vivo* in a mouse caecum ligation and puncture model of sepsis and also using ESKAPE pathogens isolated from human patient samples.[11] Several others have also demonstrated the antibacterial effects of celecoxib.[12–14] This antibacterial effect, however, was not noted either with rofecoxib, a more potent COX-2 inhibitor than celecoxib, or other NSAIDs such as indomethacin and ibuprofen, suggesting it to be a unique pharmacological feature of celecoxib, probably because of its structure.[13]

In this study, we aimed to investigate the molecular mechanism underlying the action of celecoxib in combination with ampicillin against *S. aureus* growth. Global transcriptome analysis indicated that, in combination with antibiotic, celecoxib not only down-regulated all the virulence and essential genes for *S. aureus* infection and pathogenicity but also lowered its membrane potential and permeability, allowing increased uptake of the antibiotic.

Materials and methods

Bacterial strains and materials

Methicillin-susceptible *S. aureus* (ATCC 29213) was obtained from ATCC and maintained in tryptic soy broth (TSB). Celecoxib was obtained from Aurobindo Pharma Ltd, Hyderabad, India. A stock solution (10 nM) was prepared in DMSO and stored at −20°C.

Growth curves

S. aureus was taken from glycerol stock and grown overnight in TSB medium at 37°C, with shaking at 180 rpm. For growth curve analysis, 1% of overnight culture was inoculated into 50 mL of fresh TSB medium, with or without supplementation by ampicillin (3 μg/mL), celecoxib (10 μM) or both ampicillin and celecoxib, and incubated at 37°C, with shaking at 180 rpm. Cell growth was monitored spectrophotometrically and cfu were measured by plating the serially diluted culture every 15 min on agar plates.

Treatment with celecoxib and ampicillin

S. aureus was grown overnight in TSB medium. For treatment, 50 mL of TSB broth was inoculated with 1% overnight culture and allowed to grow until reaching the log phase (OD_{600} = 0.4). The cells were treated with celecoxib (10 μM) or ampicillin (3 μg/mL) and incubated for 40 min. For treatment of cells with both celecoxib and ampicillin, celecoxib was added after 20 min of incubation of cells with ampicillin. The untreated cells served as the control. After 4 h, the cells were harvested by centrifugation at 5000 rpm at 4°C for 5 min, washed with 25 mM Tris pH 7.5, snap frozen in liquid nitrogen and stored at −80°C.

RNA isolation

Total RNA was isolated using the TRIzol method (Life Technologies, USA) as per the manufacturer's instructions. The concentration and purity of the extracted RNA were evaluated using a NanoDrop 1000 Spectrophotometer (Thermo Scientific). The integrity of the RNA was analysed on a Bioanalyzer 2100 system (Agilent). RNA was considered to be of good quality based on the 260/280 values (NanoDrop), rRNA 23S/16S ratios and RNA integrity number (RIN) (Bioanalyzer).

Labelling, amplification and scanning

The samples were labelled using an Agilent Quick Amp Kit (Part number: 5190-0442) according to the manufacturer's protocol. Briefly, 500 ng of total RNA was reverse-transcribed using random hexamer primers. The cDNA thus obtained was converted into double-stranded cDNA and further converted into cRNA in the *in vitro* transcription step using T7 RNA polymerase enzyme; Cy3 dye was incorporated into the newly synthesized cRNA strands. The cRNA obtained was cleaned up using QIAGEN RNeasy columns (QIAGEN, Cat No: 74106). The concentration and amount of dye incorporated was determined using NanoDrop. Two micrograms (2000 ng) of Cy3-labelled cRNA samples was fragmented and hybridized on an Agilent Custom *S. aureus* 8×15k instrument designed by Genotypic Technology Private Limited (AMADID: 27491) using the Gene Expression Hybridization Kit (Part Number 5190-0404; Agilent) in Sure Hybridization Chambers (Agilent) at 65°C for 16 h. This gene chip included MW2, N315, COL and Mu50. The array covered 3613 genes, for which 15 150 probes were designed. Hybridized slides were washed using Agilent Gene Expression Wash Buffer (Part No: 5188-5327) and scanned at 5 μm resolution on a G2505C scanner (Agilent Technologies). Data extraction was done using Agilent Feature Extraction software, version 10.7.

Microarray data analysis

Feature-extracted data was analysed using GeneSpring GX version 11.5 software from Agilent. Normalization of the data was done in GeneSpring GX using the 75th percentile shift. Test samples were compared with control samples to obtain fold expression values. Genes that were up-regulated and down-regulated in the test samples compared with the control sample were identified. Differentially regulated genes were grouped using hierarchical clustering to identify significant gene expression patterns. Clustering analysis was performed using GeneSpring GX software using the average linkage rule with the Pearson uncentred distance metric. The microarray-related data was submitted to Gene Expression Omnibus with the accession number GSE56100.

RT–PCR

RT–PCR was performed to validate microarray data. Primers were designed using Primer3 software[15] for *spa, sei, sea, seg, sep* and *blaZ* to generate an amplicon size of 100–150 bp (Table 1). 16S rRNA served as the reference gene for normalization of the amount of RNA input and the efficiency of each cycle. cDNA was amplified using the Maxima SYBR Green/ROX qPCR Master Mix (Fermentas) on the 7500 Fast Real-Time PCR System (Applied Biosystems) with an initial incubation of 95°C for 10 min followed by 40 cycles of 15 s at 95°C, 30 s at 56°C and 30 s at 72°C. The relative expression or fold change in expression of genes in the test samples was analysed using the comparative Ct method.

Western blotting

Protein levels of enterotoxin H (SEH) and α-haemolysin (HLA) were analysed by western blotting. The growth conditions and treatments were the same as described above. The culture supernatants were collected by centrifugation at 5000 rpm and 4°C for 10 min. The protein in the culture supernatant was precipitated by adding four volumes of acetone, incubating overnight at 4°C and collecting by centrifugation for 30 min at 14 000 rpm and 4°C. The protein was air-dried, dissolved in PBS and the concentration was determined by Bradford assay. A total of 100 μg of protein was loaded onto 15% SDS polyacrylamide gel. After electrophoresis the proteins were transferred onto nitrocellulose membrane. The membrane was blocked with 5% non-fat dry milk in Tris-buffered saline with 0.01% Tween 20 (TBST) and probed with primary sheep polyclonal antibody to HLA (Abcam, ab15948) and rabbit polyclonal

Table 1. List of genes that are differentially expressed in *S. aureus* treated with ampicillin alone or a combination of ampicillin and celecoxib

S. No.	Gene name	Product	Fold change: ampicillin (mean±SE)	Fold change: both (mean±SE)	*P* value
1	—	DNA polymerase	2.01±0.65	(–) 0.32±0.00	<0.0001
2	—	ABC transporter	(–) 0.89±0.89	(–) 0.27±0.17	<0.05
3	*adhE*	bifunctional acetaldehyde-CoA/alcohol dehydrogenase	1.21±0.24	(–) 0.18±0.00	<0.05
4	*aroA*	3-phosphoshikimate 1-carboxyvinyltransferase	2.22±1.95	(–) 0.10±0.05	<0.001
5	*ddl*	D-alanyl-alanine synthetase A	1.70±0.23	1.42±0.00	<0.05
6	*binL*	DNA-invertase	1.63±1.91	(–) 0.32±0.03	<0.01
7	—	FtsK/SpoIIIE family protein	4.25±3.03	(–) 0.07±0.03	<0.0001
8	*pre*	plasmid recombination enzyme	1.67±0.45	(–) 0.23±0.13	<0.01
9	—	truncated Pre protein	1.96±2.46	(–) 0.10±0.08	<0.001
10	*rep*	replication protein Rep	1.11±0.10	(–) 0.96±0.31	<0.001
11	—	replication-associated protein	0.38±0.39	1.98±2.38	<0.05
12	—	staphylococcal tandem lipoprotein	0.11±0.70	2.51±3.05	<0.0001
13	*kdpB*	potassium-transporting ATPase subunit B	2.36±2.89	(–) 0.24±0.12	<0.0001
14	*kdpC*	potassium-transporting ATPase subunit C	1.89±1.93	(–) 0.47±0.21	<0.001
15	*agrD*	AgrD	1.81±2.32	(–) 0.25±0.17	<0.001
16	—	putative ATP/GTP-binding protein	2.32±2.77	(–) 0.38±0.40	<0.0001
17	*aacA*	*N*-acetyltransferase	1.86±1.00	(–) 0.58±0.26	<0.0001
18	—	pathogenicity island protein	2.72±1.10	(–) 0.31±0.05	<0.0001
19	*set16*	superantigen-like protein	0.42±1.84	2.86±3.41	<0.0001
20	*set26*	superantigen-like protein	1.73±2.21	(–) 0.22±0.16	<0.01
21	*mecR1*	methicillin-resistance MecR1 regulatory protein	1.70±2.19	(–) 0.23±0.15	<0.01
22	—	holin	1.62±1.15	(–) 0.76±0.11	<0.0001
23	—	phage repressor	0.11±0.75	(–) 1.44±0.22	<0.05
24	—	portal protein	2.04±2.63	(–) 0.03±0.14	<0.001
25	*int*	prophage L54a, integrase	3.74±2.93	(–) 0.66±0.22	<0.0001
26	—	prophage L54a, major tail protein, putative	2.75±3.34	(–) 0.27±0.10	<0.0001
27	—	prophage L54a, *N*-6-adenine-methyltransferase	2.16±1.75	(–) 0.62±0.52	<0.0001
28	—	IS*1272* transposase	1.64±2.12	(–) 0.17±0.13	<0.01
29	—	IS*3* family transposase	2.19±2.68	0.46±0.52	<0.01
30	*tnp*	transposase	1.61±1.53	(–) 0.15±0.43	<0.001
31	—	DNA-binding response regulator	(–) 0.57±0.66	1.97±2.34	<0.0001
32	—	staphylococcal tandem lipoprotein	0.11±0.70	2.51±3.05	<0.0001
33	*sek*	staphylococcal enterotoxin	0.16±0.60	3.50±1.12	<0.0001
34	*set12*	superantigen-like protein	(–) 1.05±0.51	0.02±0.43	<0.01

S. No., serial number.

antibody to SEH (Abcam, ab15902) diluted 1:1000 in TBST, incubated overnight at 4°C followed by a horseradish peroxidase-conjugated anti-sheep and anti-rabbit IgG. The proteins were visualized using a chemiluminescence detection kit and a Kodak imaging system.

Bacterial ghost membrane preparation

Bacterial membrane ghosts were prepared as described previously.[16] The ghost membranes were incubated with celecoxib (10 μM), ampicillin (3 μg/mL) or both the drugs in PBS. The entry of drugs into the membrane ghosts was monitored by spectrophotometry at wavelengths of 239 and 259 nm for ampicillin and celecoxib, respectively.

Flow cytometry analysis

The membrane potential of untreated and drug-treated *S. aureus* was determined using rhodamine 123 as described previously.[17] The membrane permeability was analysed using 7-aminoactinomycin D (7-AAD) as described earlier.[18]

Cocrystal development of ampicillin and celecoxib

Ampicillin and celecoxib cocrystals were prepared by co-grinding followed by solvent (methanol) evaporation at room temperature as described earlier.[19,20] Briefly, accurately weighed ampicillin and celecoxib in different molar ratios (1:1, 1:2, 1:3, 1:4, 2:1, 3:1 and 4:1) were co-ground together with a mortar and pestle for 30 min followed by dissolving the known amount of co-ground mixture in methanol. The solvent was slowly evaporated at room temperature and cocrystals, formed in 5 days, were characterized by powder-X-ray diffraction (PXRD) and differential scanning calorimetry (DSC) for stability. They were named AC01 (only celecoxib), AC10 (only ampicillin), AC11, AC12, AC13, AC14, AC21, AC31 and AC41.

Partition coefficient (logP) determination

The *n*-octanol–water partition coefficient of ampicillin and cocrystal AC21 was determined by the shake-flask method as described previously.[21] Briefly, equal volumes of *n*-octanol (10 mL) and water (10 mL) were taken

in a flask and mutually saturated on a thermostat shaker at 100 rpm at 25°C for 24 h. After saturation, the two phases were allowed to separate on standing at room temperature. A calibration curve was prepared for serial dilutions of ampicillin in water and *n*-octanol phases by measuring the absorbance at 259 nm on a UV-VIS spectrophotometer (Shimadzu). The concentration of ampicillin in the AC21 cocrystal in both the phases was determined using the calibration curve and logP was calculated as $\log_{10}$ of the ratio of the concentration of ampicillin in the *n*-octanol phase to the concentration of ampicillin in the water phase. The logP value was the average of a minimum of three replicates ± SD.

Statistical analysis

One-way ANOVA statistical analysis was performed using SigmaPlot software version 12.3. $P<0.05$ was reported as significant.

Results

Global gene expression changes on combinatorial treatment with ampicillin and celecoxib

Growth curve analysis of *S. aureus* ATCC 29213 in the presence or absence of ampicillin and/or celecoxib indicated that cells in the presence of celecoxib followed the same growth kinetics as untreated cells whereas ampicillin delayed the growth of cells. In cells treated with both ampicillin and celecoxib, there was a significant reduction in the absorbance, indicating the reduction in the growth of bacteria (Figure 1a).

To further understand the mechanism of growth inhibition by the combinatorial treatment of ampicillin and celecoxib, we analysed the global transcriptomics of exponentially growing *S. aureus* treated with ampicillin and/or celecoxib using Agilent Gene Expression Microarrays. For microarray analysis, to analyse the gene expression we added celecoxib to cells 20 min after ampicillin addition. Celecoxib treatment alone did not have any effect on the growth of the cells, as observed in the growth curve; however, in combination with ampicillin, the growth was inhibited. So, we wanted to see exactly what celecoxib was doing to cells in combination with ampicillin. The reason to add celecoxib after 20 min was to induce gene expression changes by ampicillin and then evaluate the effect of celecoxib. The addition of celecoxib to cells along with ampicillin or 20 min after ampicillin addition did not show any difference in the growth inhibition. The microarray data was normalized to untreated cells (control). The gene expression profiles of control cells and cells treated with celecoxib alone showed no significant difference. A huge change in the expression pattern of genes was observed in cells treated with ampicillin (Figure 1b), as expected. Most of the genes up-regulated upon ampicillin treatment were involved in antibiotic resistance, signal transduction, infection and pathogenesis, transposition and phage proteins etc., indicating a survival strategy of bacteria. Ampicillin not only inhibited cell wall synthesis in bacteria but also down-regulated the genes involved in metabolic processes of carbohydrates, amino acids, lipids, nucleic acids etc., along with other transporter genes.

The microarray data analysis of the duplicate sets revealed that a total of 1223 genes were differentially expressed in cells treated with celecoxib and ampicillin when compared with cells treated with ampicillin alone. Of these, 163 were essential genes according to the Database of Essential Genes (DEG) (Figure 1c) and the remaining 1060 were non-essential genes (Figure 1d). Although many genes were down-regulated in combinatorial treatment, the most significant effect was seen in genes responsible for pathogenesis, drug resistance and upstream signal transduction in combinatorial treatment when compared with ampicillin (Tables 1, 2 and 3). However, the data analysis of one set of treatments showed a significant change in expression of most of the virulence genes. Real-time PCR of a few virulence genes (Figure 2a) and western blot analysis of HLA (Figure 2b) and SEH (Figure 2c) further validated the microarray results.

Celecoxib potentiated entry of ampicillin into the bacterial membrane ghosts

We next determined the concentrations of celecoxib and ampicillin inside bacteria using empty bacterial membrane vesicles. The results clearly indicated increased absorbance for ampicillin in the presence of celecoxib in the pellet fraction, suggesting increased entry of ampicillin (Figure 3a). The membrane permeability of bacteria in the presence or absence of drugs was analysed by flow cytometry and the results clearly indicated decreased permeability in ampicillin-treated cells that was increased upon addition of celecoxib (Figure 3b). Furthermore, the microarray data analysis also indicated down-regulation of membrane permeability and membrane potential genes in the presence of celecoxib (Figure 3c and d).

Cocrystal studies indicated interaction between celecoxib and ampicillin

We also developed cocrystals with different proportions of ampicillin and celecoxib using a neat grinding method followed by slow solvent evaporation. The cocrystals were then used to determine the percentage growth inhibition of bacteria at various concentrations. The results clearly demonstrated increased inhibition of growth by cocrystals. However, the cocrystal of proportion AC21 (two parts ampicillin and one part celecoxib) showed more potency (Figure 4a and b). The PXRD analysis of the cocrystals showed that there were weak interactions between ampicillin and celecoxib, which is evidenced from the new peaks observed and the disappearance of existing peaks in drug mixture crystals when compared with crystals made from pure compounds (AC01 and AC10) (Figure 4c and d and Figure S1, available as Supplementary data at *JAC* Online). DSC analysis of cocrystals showed that the crystalline nature of celecoxib was preserved in all combinations, but there was a phase transition in ampicillin in combination with celecoxib in AC21 when compared with the amorphous form of the pure compound (Figure 4e) (Figure S2).

Further, to determine the effect of celecoxib on increasing the permeation of ampicillin in the cocrystal, we incubated the bacterial membrane ghosts with AC21 and a physical mixture (PM) of both the drugs (without crystallization) and measured the absorbance of ampicillin and celecoxib at 239 and 259 nm, respectively, in the pellet (membrane) and supernatant fraction. The cocrystal AC21 showed more absorbance at 239 nm, corresponding to ampicillin in the pellet fraction, suggesting increased entry of the ampicillin (Figure 4f) compared with the PM.

Next, we experimentally determined the logP value, indicating the permeation/solubility of the drug for the cocrystal AC21 and

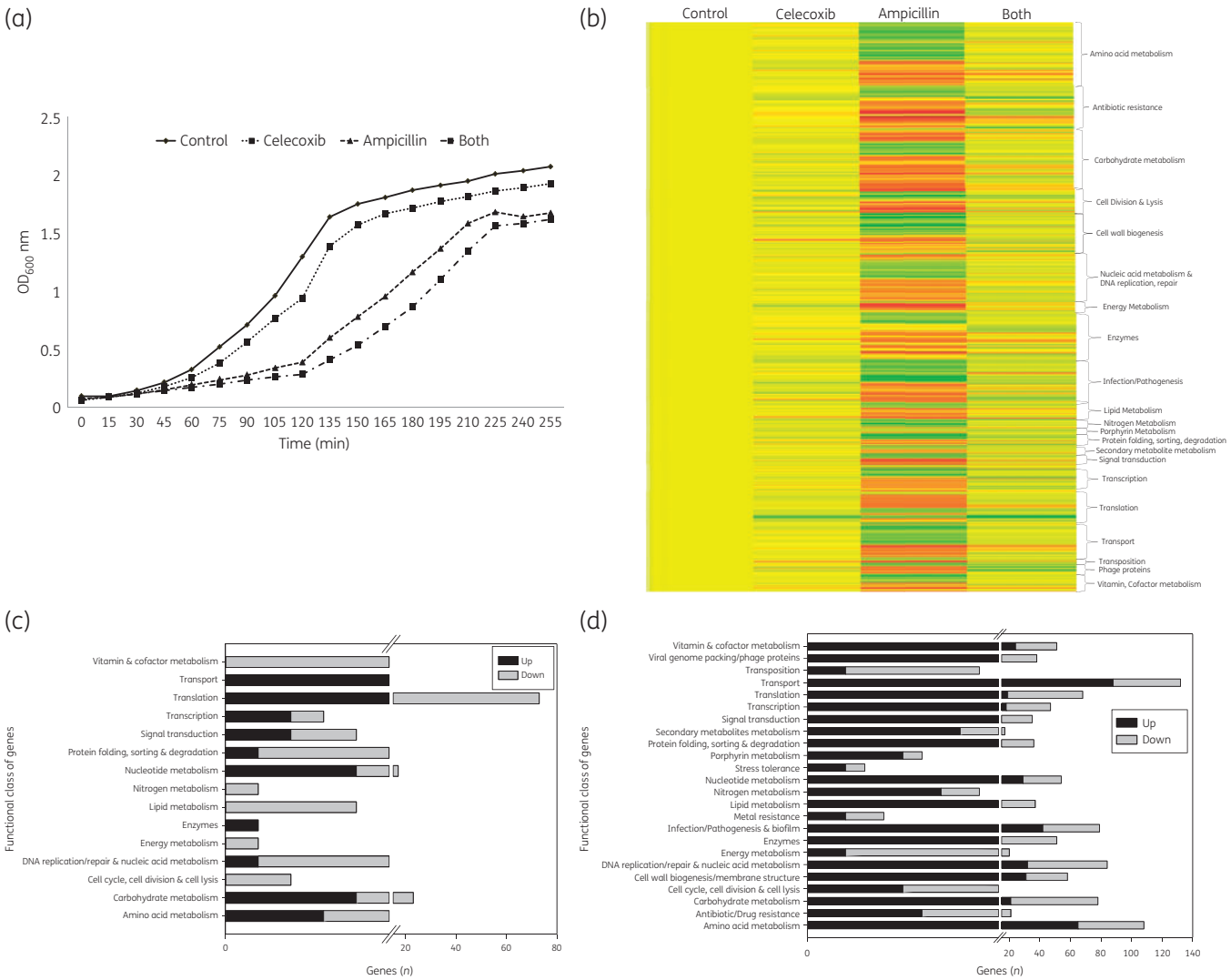


Figure 1. Growth and transcriptome analysis of *S. aureus* ATCC 29213 in different drug-treated conditions. (a) Representative graph showing the growth kinetics of *S. aureus* in the presence or absence of celecoxib (10 μM), ampicillin (3 μg/mL) or both celecoxib and ampicillin. (b) Colour map showing the differential gene expression of *S. aureus* treated with or without ampicillin, celecoxib or both 4 h after addition of drugs. Expression in control cells is represented by yellow colour, more than 2-fold increase by red and 2-fold reduction in expression compared with control is represented by green colour. (c) Graphs showing the differentially expressed essential genes (according to the functional class) in cells treated with the combination of celecoxib and ampicillin when compared with ampicillin alone. (d) Non-essential genes differentially expressed in cells treated with drugs alone or in combination. This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

ampicillin using the shake-flask method, along with prediction of logP using various pieces of software such as ALOGPS and Molinspiration by giving the simplified molecular-input line-entry system (SMILES) of both ampicillin and celecoxib together as structural input in ampicillin/celecoxib and celecoxib/ampicillin format since we do not have the structural information of the cocrystal. The experimental logP value was determined to be 2.08±0.089 for AC21 and 1.16±0.12 for ampicillin. ALOGPS predicted the logP of the cocrystal as 2.01 and Molinspiration predicted it to be 2.3. The logP value for ampicillin alone according to the PubChem database is 1.35, which is much lower than the AC21 value we obtained. The logP value indicates the solubility and permeation of a drug. A drug with logP between 1 and 3 shows moderate solubility and moderate permeation. An increase in the logP value of ampicillin from 1.35 to 2.08 in co-crystal AC21 clearly indicates increased permeation of ampicillin in the presence of celecoxib. This result is in line with our experimental permeability assay by flow cytometry using 7-AAD.

Discussion

Development of alternative treatment strategies for drug-resistant bacterial infections is a major responsibility of the scientific community. Drug repurposing is one of the methods the FDA has suggested for identification of drugs to treat MDR bacteria. Celecoxib, a selective COX-2 inhibitor, has been shown previously by us[7] and others[13] to be an antibacterial agent when given in combination with an antibiotic. In the present study we aimed to

Table 2. List of virulence factors down-regulated in combinatorial treatment with both celecoxib and ampicillin when compared with ampicillin treatment alone

Gene	Gene product	Fold difference	
		Ampicillin	both
Capsule polysaccharides and fibronectin-binding factors			
cap8B	capsular polysaccharide synthesis enzyme Cap8B	0.26	0
cap8E	capsular polysaccharide synthesis enzyme Cap8E	1.07	0.17
cap8F	capsular polysaccharide synthesis enzyme Cap8F	0.55	−0.41
cap8H	capsular polysaccharide synthesis enzyme Cap8H	1.22	−0.05
cap8I	capsular polysaccharide synthesis enzyme Cap8I	4.56	−0.17
cap8J	capsular polysaccharide synthesis enzyme Cap8J	4.71	−0.35
cap8K	capsular polysaccharide synthesis enzyme Cap8K	4.65	−0.15
ebpS	elastin-binding protein	1.8	0.36
clfA	fibrinogen-binding protein	0.45	−0.69
spa	staphylococcal protein A	0.97	−0.31
Secreted proteins			
—	HLA	0.25	−0.52
hlgA	HLG component A	0.74	0.67
hlgB	HLG component B	0.58	0.15
hlgC	HLG component C	1.02	0.57
lukS	PVL chain S precursor	1.01	0.81
tsst-1	toxic shock syndrome toxin-1	0.86	−0.32
seb	staphylococcal enterotoxin B	1.47	−0.37
sep	enterotoxin P	1.18	−0.21
seg2	staphylococcal enterotoxin SeG	0.78	−0.91
sek2	staphylococcal enterotoxin Sek	1.45	−0.55
—	SEH	1.33	−0.45
set16	superantigen-like protein	1.27	−0.55
set21	superantigen-like protein	0.8	−0.2
set26	superantigen-like protein	3.94	−0.39
sak	staphylokinase precursor	0.84	−0.38
Antibiotic resistance genes			
mecI	methicillin resistance regulatory protein	1.75	−0.29
mecA	PBP 2a	1.22	−0.65
mecR1	methicillin-resistance MecR1 regulatory protein	3.9	−0.39
blaZ	β-lactamase	1.25	0.71
bleO	bleomycin resistance protein	0.99	0.6
fmtC	oxacillin resistance-related FmtC protein	1.08	0.66
femA	factor essential for expression of methicillin resistance	0.83	0.12
Two-component system			
agrA	Agr protein A	1.44	0.19
agrD	Agr protein D	1.21	−0.37
agrD	AgrD protein	1.23	0.27
sarA	staphylococcal accessory regulator A	1.85	0.06
—	RNAIII-activating protein TRAP	1.09	0.84
—	staphylocoagulase precursor	0.83	−0.82

elucidate the mechanism of action of celecoxib on bacterial growth. To achieve this, first we studied the growth kinetics of *S. aureus* ATCC 29213 in the presence or absence of ampicillin alone, celecoxib alone and a combination of both the drugs. As indicated in the results, combinatorial treatment showed a delayed growth curve. Synergistic effect is defined as the joint action of drugs that, when taken together, increase each other's effects. However, in the present study the synergistic definition does not hold well since celecoxib alone does not show any effect on the bacterial growth. Nevertheless, celecoxib in combination was enhancing ampicillin's inhibitory effect. Growth inhibition of *S. aureus* by ampicillin was also shown to be increased in synergism when used in combination with other non-antibiotic compounds.[22,23]

Table 3. Real-time PCR data indicating $2^{-\Delta\Delta Ct}$ for a few important virulence genes listed in Tables 1 and 2 to validate microarray data

Gene	Gene product	Fold difference	
		ampicillin	both
spa	staphylococcal protein A	4.8	2.0
hla	HLA	0.78	0.50
sea	staphylococcal enterotoxin A	0.96	0.57
seg	staphylococcal enterotoxin G	0.98	0.17
sep	staphylococcal enterotoxin P	1.25	0.23
blaZ	β-lactamase	2.05	0.72

To further understand the process, we performed global transcriptome expression analysis by microarray technology. The data analysis indicated that celecoxib treatment alone did not show much variation in the global transcription of *S. aureus* when compared with the control. However, a significant increase in gene expression, specifically of the virulence genes, antibiotic resistance genes, two-component signal transduction genes etc., was observed in cells treated with ampicillin alone. However, all the genes up-regulated due to ampicillin treatment were down-regulated in combinatorial treatment with celecoxib and ampicillin.

The process of infection caused by *S. aureus* is complex, involving many proteins. Among them, the two classes of virulence

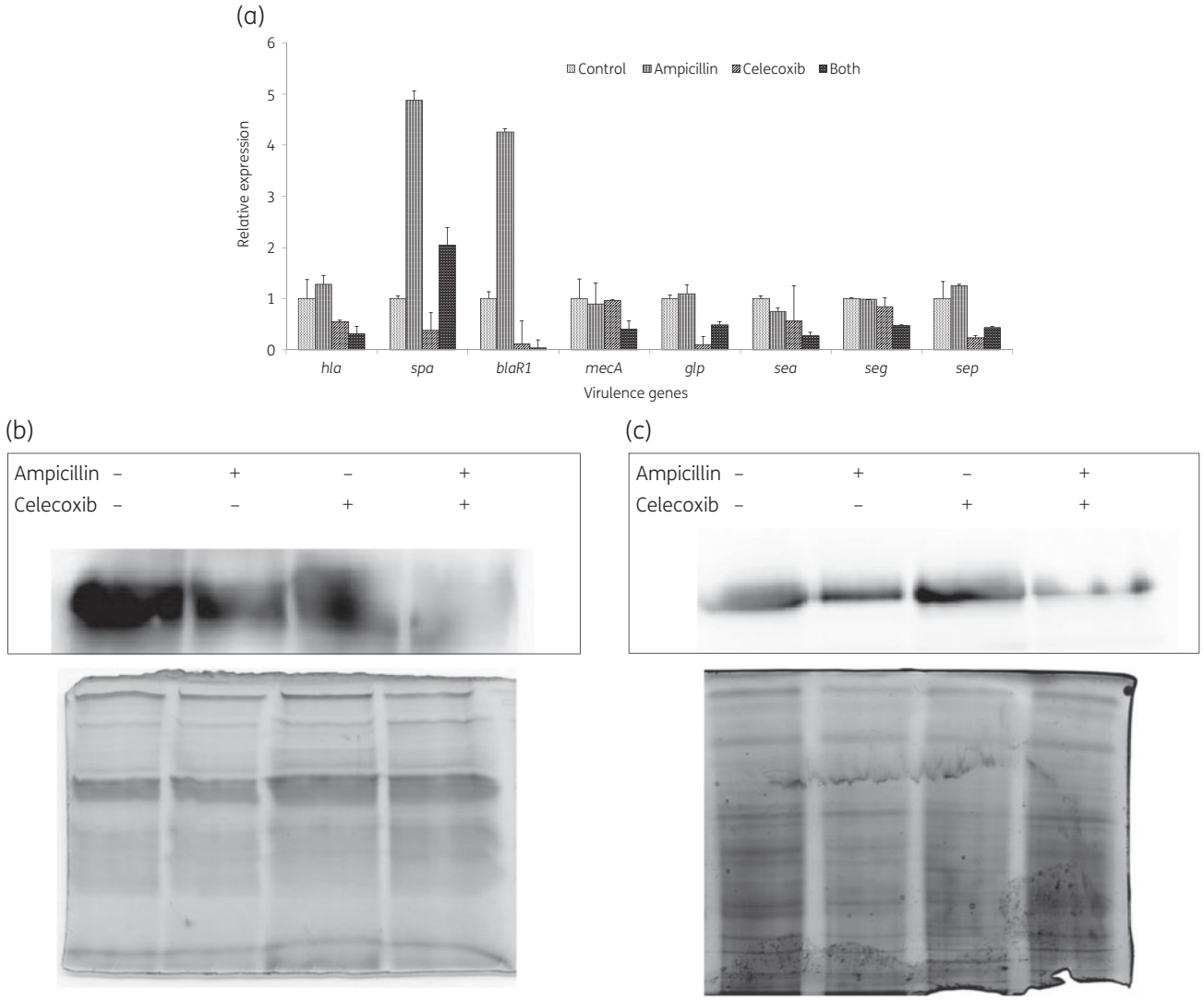
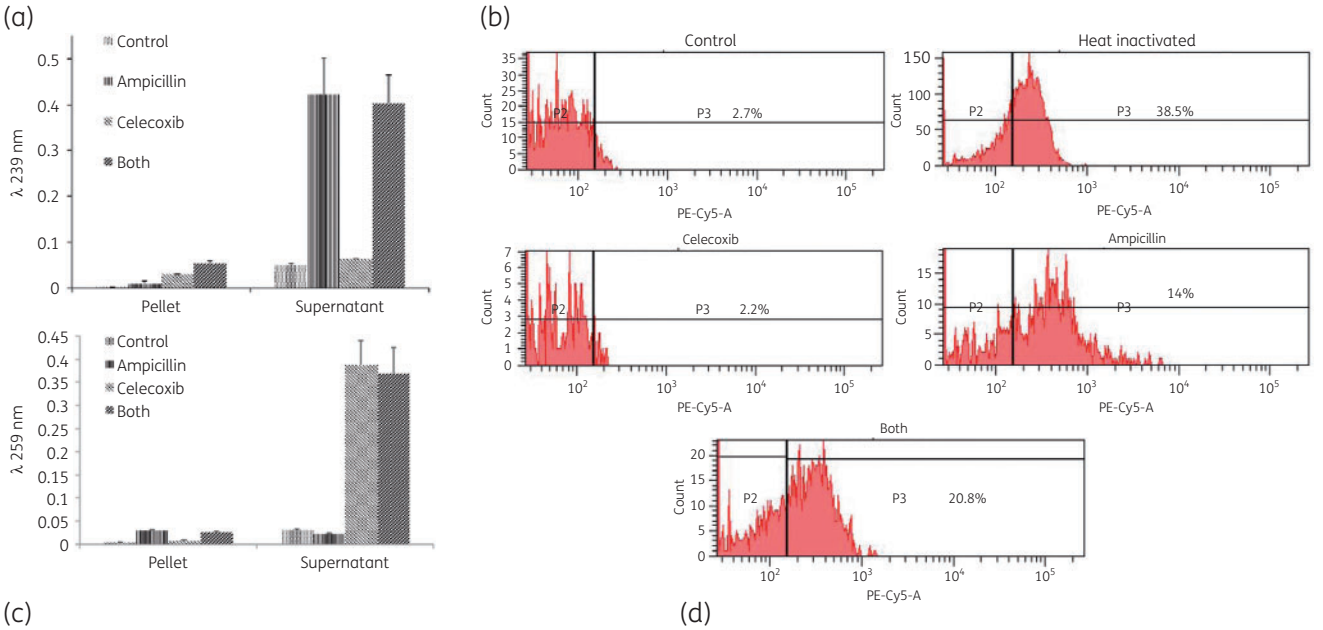


Figure 2. Validation of microarray data of a few genes. (a) Relative fold change in the mRNA expression of some of the virulence genes. (b) Western blot analysis of SEH. (c) Immunoblot of HLA toxins in response to the treatments of celecoxib (10 μM) alone, ampicillin (3 μg/mL) alone or both celecoxib and ampicillin. The Coomassie-stained gels below the blots represent loading controls.



(c)

Control	Celecoxib	Ampicillin	Both		
0.00	−0.56	1.13	−0.12	hmrB	acyl carrier protein
0.00	−0.45	0.69	−0.38	glpK	glycerol kinase
0.00	−0.09	1.81	0.93	fabD	malonyl CoA-acyl carrier protein transacylase
0.00	−0.23	0.69	0.21	fab	3-oxoacyl-synthase
0.00	0.00	1.48	1.12	FabH	3-oxoacyl-(acyl carrier protein) synthase III
0.00	−0.10	1.75	0.63	fabG1	3-oxoacyl-(acyl-carrier-protein) reductase
0.00	0.35	1.24	0.51	accC	acetyl-CoA carboxylase biotin carboxylase subunit
0.00	−0.16	1.26	0.16	–	acetyl-CoA carboxylase subunit beta
0.00	0.29	1.19	0.81	–	diacylglycerol glucosyltransferase
0.00	−0.14	1.38	0.27	–	fatty acid biosynthesis transcriptional regulator
0.00	0.08	0.67	−0.43	geh	glycerol ester hydrolase
0.00	0.05	0.63	0.04	tagD	glycerol-3 phosphate cytidylyltransferase
0.00	0.37	1.01	−0.24	–	HMG-CoA synthase
0.00	0.37	1.23	−0.36	–	HMG-CoA synthase,truncation
0.00	0.21	1.46	0.24	lip	triacylglycerol lipase precursor

(d)

Control	Celecoxib	Ampicillin	Both		
0.00	0.37	1.20	−0.24	kdpA	potassium-transporting ATPase subunit A
0.00	0.36	5.26	−0.36	kdpB	potassium-transporting ATPase subunit B
0.00	0.00	3.82	−0.68	kdpC	potassium-transporting ATPase subunit C
0.00	0.34	1.83	1.40	mnhB	putative monovalent cation/H+ antiporter subunit B
0.00	0.11	1.88	1.19	mnhC	putative monovalent cation/H+ antiporter subunit C
0.00	0.20	1.96	1.07	mnhD	putative monovalent cation/H+ antiporter subunit D
0.00	0.17	1.98	0.76	mnhE	putative monovalent cation/H+ antiporter subunit E
0.00	0.03	1.61	0.65	mnhF	putative monovalent cation/H+ antiporter subunit F
0.00	−0.15	1.22	0.56	mnhG	putative monovalent cation/H+ antiporter subunit G
0.00	0.00	1.30	0.88	mnhA	monovalent cation/H+ antiporter subunit A

Figure 3. Increased entry of ampicillin in the presence of celecoxib. (a) Top: increase in absorbance at 239 nm of ampicillin in the pellet fraction of bacterial ghost membranes in the presence of celecoxib. Bottom: absorbance of celecoxib at 259 nm. (b) Flow cytometric analysis of membrane permeability using 7-AAD. 'Count' indicates the number of bacteria. (c) Differential expression of genes involved in membrane permeability as per microarray data analysis. (d) Differential expression of genes involved in membrane potential as per microarray data analysis. This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

factors (cell-wall associated proteins and secreted proteins)[24] play an important role in the process of pathogenesis. Initially *S. aureus* uses cell wall-associated proteins to adhere to host cells and invade the cells. In later stages of infection, it secretes toxins that result in inflammation and necrosis of the tissue.

Capsular polysaccharides and fibronectin-binding factors

The capsular polysaccharide proteins of *S. aureus* prevent phagocytosis of the bacteria and fibronectin-binding proteins are required for promoting adherence of bacteria to fibronectin-coated host molecules.[25] Targeting these proteins is a known strategy to combat *S. aureus* infections.[26] Furthermore, clumping factor A (ClfA) and elastin-binding factor (EbpS) are necessary for attachment of *S. aureus* to host cells.[27] Our data also suggested down-regulation of these virulence genes in combinatorial treatment with ampicillin and celecoxib, indicating the efficacy of combinatorial treatment.

Secreted proteins

S. aureus secretes an arsenal of toxins including HLA, γ-haemolysin (HLG), leucocidin (Luk), Panton–Valentine leucocidin (PVL), toxic shock syndrome toxin (TSST-1), enterotoxins, staphylokinase, superantigens etc.[28] Surprisingly, all these virulence factors have been down-regulated in combinatorial treatment with celecoxib and ampicillin, further strengthening our hypothesis that celecoxib sensitizes bacteria to antibiotics.

Antibiotic resistance genes

Among the different mechanisms of development of resistance to antibiotics, horizontal gene transfer of antibiotic resistance genes, enzymatic degradation of antibiotics and efflux of antibiotics are common in *S. aureus*.[2] PBP 2a is involved in resistance to methicillin while β-lactamase is responsible for degradation of penicillin-like antibiotics with a β-lactam ring. Resistance to other antibiotics is conferred by horizontal gene transfer. In the present study, down-regulation of all these resistance genes was achievable with combinatorial treatment.

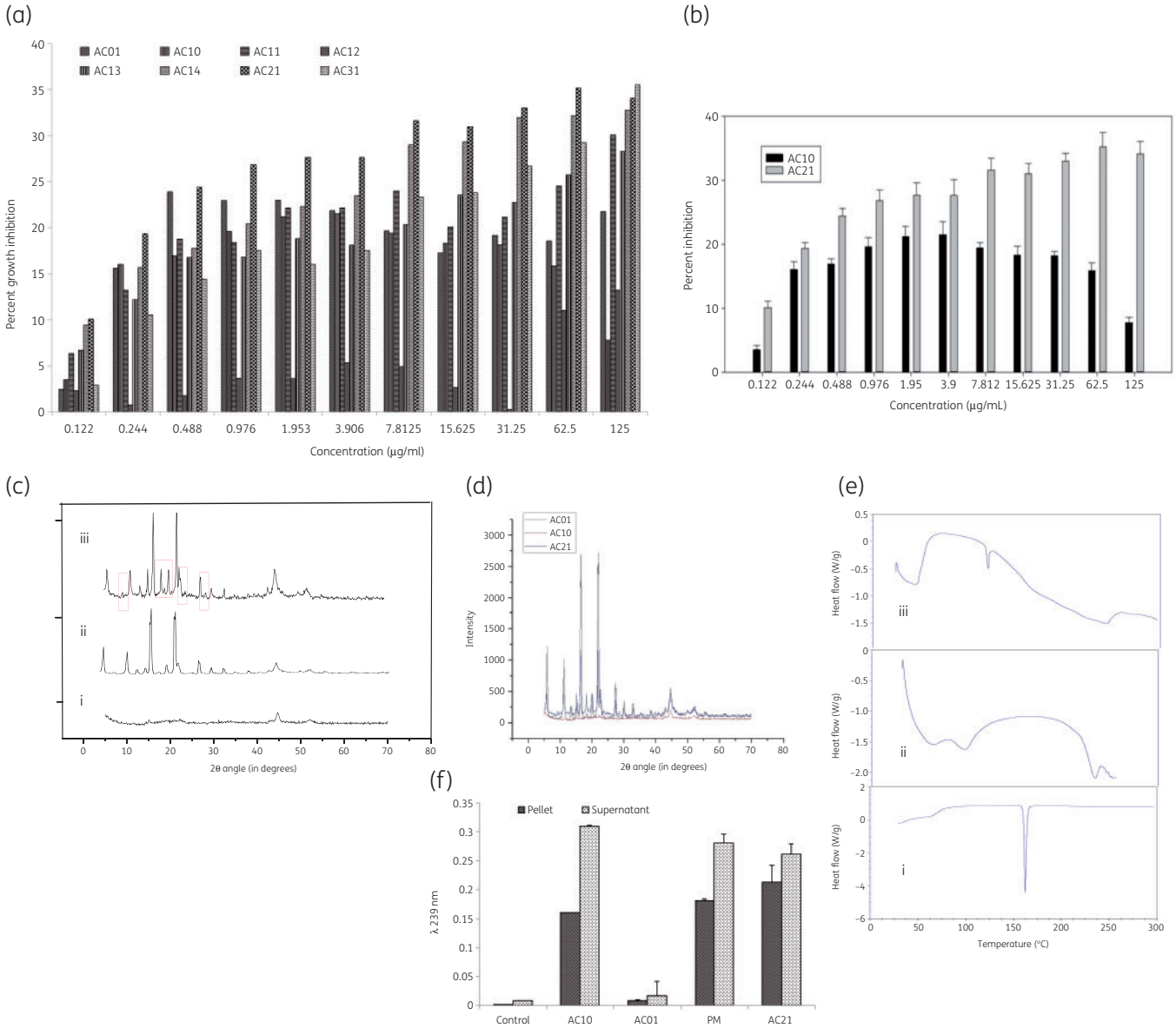


Figure 4. Cocrystal of ampicillin and celecoxib is effective in inhibiting *S. aureus* growth. (a) Cell viability of *S. aureus* in the presence of ampicillin and celecoxib cocrystals at various concentrations. (b) Graph showing efficacy of cocrystal AC21 against *S. aureus* when compared with ampicillin alone (AC10). (c) PXRD analysis of the crystals AC10 (ampicillin alone) (i), AC01 (celecoxib alone) (ii) and cocrystal AC21 (iii). (d) Merged chromatograms of the PXRD. (e) DSC analysis of the crystals AC10 (ampicillin alone) (i), AC01 (celecoxib alone) (ii) and cocrystal AC21 (iii). (f) Graph showing the absorbance of ampicillin at 239 nm in membrane pellets and supernatant fractions of membrane ghosts treated with AC10 (ampicillin), AC01 (celecoxib), AC21 and PM of ampicillin and celecoxib. This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

Two-component signal transduction

The bacterial two-component system is a communication bridge between the external and the internal environment wherein the bacteria sense the external stimulus and translate it internally to modulate gene expression. The two-component system consists of a membrane-associated sensor histidine kinase (HK) and a cytoplasmic response regulator (RR) that are often arranged in an operon and thus co-transcribed. Among the 16 known two-component systems in *S. aureus*, the two important operons[29] that generally regulate the production of all virulence factors through RNA III are the accessory gene regulator (*agr*) and the staphylococcal accessory regulator (*sar*).[30] Our data presented here are in agreement with previous studies showing that down-regulation of *agr* and *sar* operons by combinatorial treatment had down-regulated all the virulence genes. Our data were also further validated using real-time PCR, western blot analysis and activity assay.

Although the microarray data indicated a promising role of celecoxib in sensitization of bacteria to ampicillin, we wanted to address the basic question of whether celecoxib enters the bacteria. With the ghost membrane drug-uptake assay it was clear that indeed there was an increased uptake of ampicillin in the

presence of celecoxib. Therefore, we next addressed the question of whether the increased uptake/entry of ampicillin into bacteria in the presence of celecoxib is due to the modulation of membrane potential and permeability. It is well established that bacteria attain resistance by altering their membrane permeability and potential to stop entry of the antibiotics; this is very well known with ampicillin.[31,32] Our flow cytometry results and microarray data are in line with the established results.

The bacterial ghost membrane assay and the microarray results were able to indicate that the probable mechanism of action of celecoxib is increased uptake of ampicillin by modulating membrane permeability and potential; it is not clear, however, as to how it allows increased uptake. To address this question we carried out cocrystal studies. In recent years, much focus has been on altering the physicochemical properties of a drug so as to improve its bioavailability, stability, permeability etc.[33–36] Cocrystals of celecoxib and ampicillin were prepared by a co-grinding method followed by solvent evaporation at room temperature. The crystals were analysed by PXRD and DSC, which confirmed that the weak electrostatic or van der Waals forces or hydrogen bonding interactions between the two drugs (celecoxib being a lipophilic compound and ampicillin hydrophilic) might be enhancing the entry or permeation of ampicillin. This was also evident from the increase in logP value of ampicillin from 1.16 to 2.08. Such an increase in the permeation and dissolution properties of a drug in cocrystals has been well documented.[37–39]

In conclusion, the toxicogenomic analysis of *S. aureus* in combinatorial treatment of celecoxib and ampicillin indicate a significant down-regulation in various virulence factors including enterotoxins, two-component signal transduction systems and antibiotic resistance genes. In the presence of celecoxib, there was increased uptake of ampicillin by bacteria due to increased membrane permeability, and cocrystal studies have indicated interaction between the two compounds, which might also affect the physical properties of the drugs and thereby increase permeation and bioavailability. This effect of celecoxib in combination cannot be defined by synergism since celecoxib alone does not show any effect on the bacterial growth. The combinatorial treatment with celecoxib and antibiotic may be a better treatment strategy to combat *S. aureus* infections.

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Transparency declarations

None to declare.

Author contributions

G. Y. N. V. performed the experiments, analysed the results and drafted the manuscript. G. K. performed the real-time PCR analysis and western blots, P. P. helped in performing and analysis of the cocrystals by PXRD and DSC. A. M. K. conceptualized the study, analysed the results and reviewed the manuscript.

Supplementary data

Figures S1 and S2 are available as Supplementary data at *JAC* Online.

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ORIGINAL RESEARCH
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Celecoxib Enhances the Efficacy of Low-Dose Antibiotic Treatment against Polymicrobial Sepsis in Mice and Clinical Isolates of ESKAPE Pathogens

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Treatment of multidrug resistant bacterial infections has been a great challenge globally. Previous studies including our study have highlighted the use of celecoxib, a non-steroidal anti-inflammatory drug in combination with antibiotic has decreased the minimal inhibitory concentration to limit *Staphylococcus aureus* infection. However, the efficacy of this combinatorial treatment against various pathogenic bacteria is not determined. Therefore, we have evaluated the potential use of celecoxib in combination with low doses of antibiotic in limiting Gram-positive and Gram-negative bacteria *in vivo* in murine polymicrobial sepsis developed by cecum ligation and puncture (CLP) method and against clinically isolated human ESKAPE pathogens (*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa*, and *Enterobacter* species). The *in vivo* results clearly demonstrated a significant reduction in the bacterial load in different organs and in the inflammatory markers such as COX-2 and NF-κB *via* activation of SIRT1 in mice treated with imipenem, a choice of antibiotic for polymicrobial sepsis treatment. Combinatorial treatment of ampicillin and celecoxib was effective on clinical isolates of ESKAPE pathogens, 45% of tested clinical isolates showed more than 50% reduction in the colony forming units when compared to ampicillin alone. In conclusion, this non-traditional treatment strategy might be effective in clinic to reduce the dose of antibiotic to treat drug-resistant bacterial infections.

Keywords: antibiotic drug resistance, celecoxib, polymicrobial sepsis, ESKAPE pathogens, imipenem, ampicillin

INTRODUCTION

Multidrug resistance is an adaptation of bacteria for its survival (Sorlozano et al., 2014). Bacteria are evolving rapidly challenging the current antibiotic treatment strategies. The cost involved in the treatment of drug-resistant bacterial infections such as methicillin-resistant *Staphylococcus aureus* (MRSA) is a burden to both patient and the country (Filice et al., 2010). Furthermore, the cost and time involved in the development of new drug for the treatment of such "superbugs" is very huge which is soon followed by the development of resistance to the new antibiotic. In this context,

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Anti-inflammatory and Anti-proliferative Properties of *Chromolaena odorata* Leaf Extracts in Normal and Skin-Cancer Cell Lines

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School of Life Sciences, University of Hyderabad, Hyderabad, India

Leaf extracts of Chromolaena odorata *were evaluated for anti-inflammatory and anti-proliferative activities in normal and cancerous skin cell lines. Ethyl acetate extract was high in phenolic, flavonoid contents and exhibited antioxidant and free radical scavenging capacity. A dose-dependent decrease in LPS-induced TNF-α and IL-1β was observed in HaCaT cells treated with ethyl acetate extract. Hexane and ethyl acetate extracts showed cytotoxicity and arrest of A431 cells in sub-G1 phase.*

KEYWORDS *Anti-inflammatory, anti-proliferative*

INTRODUCTION

The inflammatory response orchestrates host defenses, but progressive destruction of the tissue would compromise the survival of the organism (15). Epidemiological evidence points to a connection between inflammation and a predisposition for the development of cancer (i.e., long-term inflammation leads to the development of dysplasia) (7).

Studies indicate a number of antioxidants like C-phycocyanin (17,23) are particularly effective as anti-inflammatory and anti-cancer agents. Skin gets exposed to various chemicals, allergens, irritants, pollutants, and even to ultra violet light. Some of these chronic exposures can nurture skin cancer. Conventionally, fresh leaves of *Chromolaena odorata* (Asteraceae), a weed of plantation crops and fields of southern Asia and western Africa,

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ANTI-PLAGIARISM CERTIFICATE

Understanding the growth inhibitory mechanism of action of celecoxib in combination with antibiotics in Staphylococcus aureus

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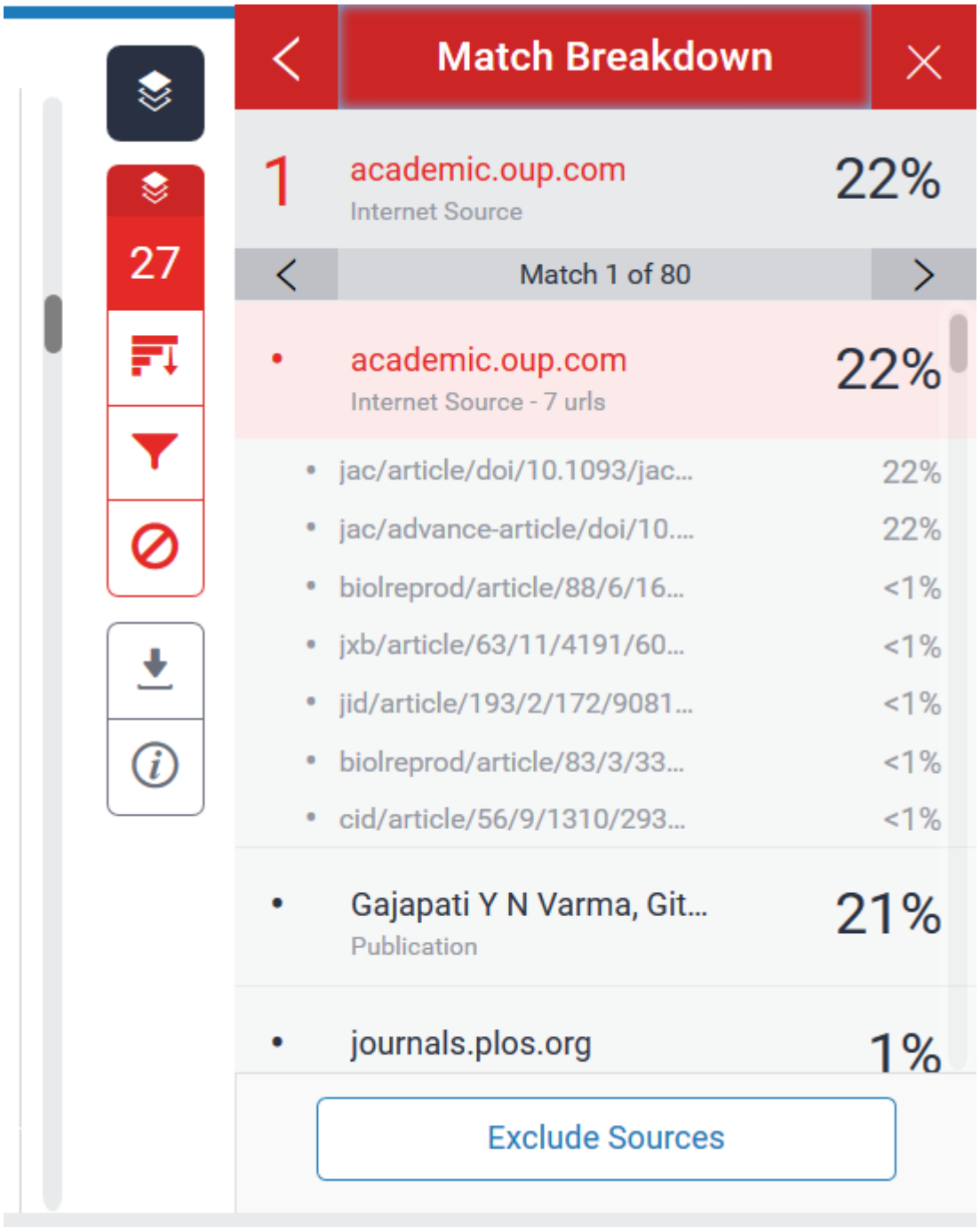
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