MICROBIOLOGICAL AND MOLECULAR DOCKING STUDIES OF BIOACTIVE COMPONENTS FROM MEDICINAL PLANTS AGAINST RESISTANT *S. aureus* ISOLATED FROM CLINICAL SAMPLES OF TERTIARY CARE HOSPITAL, PARSA, NEPAL

A Thesis

Submitted in partial fulfillment of the requirements for the

award of the degree of

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In

Microbiology

By

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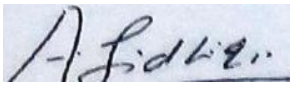
DECLARATION

I hereby declare that the work for the thesis entitled, **"Microbiological and Molecular Docking Studies of Bioactive Components from Medicinal plants against resistant *S. aureus* isolated from clinical samples of Tertiary care Hospital, Parsa, Nepal"** submitted to **School of Bioengineering and Biosciences,** Lovely Professional University, Phagwara for the award of degree of Doctor of Philosophy (Microbiology) is entirely my own work and has not been submitted in part or full for any other degree/diploma at this or any other University/Institution. All the ideas and references have been duly acknowledged.



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CERTIFICATION

It is hereby certified that the work for the thesis entitled **"Microbiological and Molecular Docking Studies of Bioactive Components from medicinal plants against resistant *S. aureus* isolated from clinical samples of Tertiary care Hospital, Parsa, Nepal"** submitted to S**chool of Bioengineering and Biosciences,** Lovely Professional University, Phagwara, for the award of degree of Doctor of Philosophy (Microbiology) was carried out in the Department of Microbiology by **Amrullah Shidiki** under my guidance and supervision. To the best of my knowledge, the present work is the result of his original investigation. No part of this thesis has ever been submitted for any other Degree/Diploma at this or any other University/Institution. The thesis is fit to be considered for the award of degree of Ph.D.

Dr. Ashish Vyas
Professor and Head
(SUPERVISOR)

Abstract

Antimicrobial agents are necessarily important in diminishing the infectious diseases globally. However, appearance and dissemination of multidrug resistant (MDR) in pathogenic bacteria especially methicillin resistant *S. aureus* (MRSA) and macrolide-lincosamide-streptogramin B (MLSB) resistant strains of *S. aureus* have become a significant public health threat as there are fewer, or even sometimes no, effective antimicrobial agents available for the infection caused by these pathogenic bacteria. In the light of evidence of rapid spread of these resistant clinical isolates globally, the demand to find new antimicrobial agents is of paramount importance. Thus, a vast number of medicinal plants have been recognized as valuable resources antimicrobial compounds as an alternative that can potentially be effective in the treatment of these problematic resistant strains of bacteria. Thus, the work of this study is to isolation of antibiotic resistant strains of *S. aureus* from clinical samples, evaluation of antimicrobial activity of medicinal plant extract against MRSA and MLSB resistant strains of *S. aureus*, phytochemical screening, LCMS analysis, molecular docking studies of compounds to penicillin binding protein 2a (PBP2a) from MRSA and erythromycin ribosomal methylase (ERM) from MLSB resistant *S. aureus* and evaluation of drug likeliness properties of studied compounds.

A total of 2000 patient samples (blood, pus, swabs, body fluids, sputum, urine and stool) were processed as per microbiological method. 867 (43.35%) samples were showed positive bacteria growth among which 465 (53.63%) were gram negative bacteria and 402 (46.36%) were gram positive bacteria. Among the gram negative bacteria, the most frequent isolates were *E.coli, Klebsiella spp., Proteus spp, Salmonela spp., Enterobacter spp., Pseudomonas spp., Acinetobacter spp., Citrobacter spp.* and *N. gonorrhoeae*. Similarly, among the gram positive bacteria, the mostly finding isolates were *S. aureus, Enterococcus spp., Streptococcus spp.* and *CoNS*. Out of total positive growth of bacteria, 310 (35.7%) were *S. aureus* isolates. The maximum numbers of *S. aureus* were isolated from blood sample. The isolated *S. aureus* were subjected for selection of methicillin resistant as per method of oxacillin disc diffusion method and MLSB resistant *S. aureus*

in two phenotype of either iMLSB or cMLSB strains through the application of double disc diffusion method (D-test). Out of total *S. aureus*, 242 (79.08%) MRSA, 64 (20.91%) MSSA, 204 (66.67%) ERY-R *S. aureus*, 132 (64.70%) iMLSB and 72 (32.29%) cMLSB were identified. The *S. aureus* and its antibiotic resistant strains were found maximum in male patients with adult age. The iMLSB strains were found more in MRSA and MSSA in comparison to cMLSB.

Despite relatively small area, Nepal has large diversity of flora with many endemic species. In nepali folk medicine plant materials have been used to treat various microbial diseases since ancient times. The goal of our research was to evaluate antimicrobial efficacy of leaves of five different medicinal plants species viz. *Syzygium cumini*, *Azadirachta indica*, *Moringa oleifera*, *Nyctanthes arbortristis* and *Tinospora cordifolia* which are commonly used in Nepali tradition medicine. Plant crude extracts were obtained with maceration method using five solvents: distilled water, 80% ethanol, methanol, acetone and hexane. Agar well diffusion assay was used for evaluation of antimicrobial properties of plant material at four different concentrations (25, 50, 100, 200 mg/ml) against two antibiotic resistant phenotypes: (iMLSB and cMLBS) of MRSA. Among five plants, only *Syzygium cumini*, *Azadirachta indica* and *Nyctanthes arbortristis* were showed antibacterial activity with formation of zone of inhibition to studied bacterial strains. Maximum zone of inhibition (25.36±0.15 & 27.30± 0.10) was obtained with aqueous extract of *Syzygium cumini* to iMLSB and cMLSB strains of MRSA. Amongst five studied plants, highest antibacterial activity was shown by *Syzygium cumini*. Our result also showed that solvent polarity greatly affects the phytochemical contents and extractive yields which is mainly increasing with increasing solvent polarity index and suddenly decreasing at very high polarity.

The aim of this work is to characterize the active constituents present in the aqueous extract of leaves of *Syzygium cumini* is attributed to its higher antibacterial effect. It was deemed necessary to confirm the identity of their active compounds through the application of LCMS analysis where the identification of active compounds was

established on the basis of molecular ion peaks and their fragmentation patterns. The LCMS analysis showed the presence of 20 (twenty) compounds in tested plant extract, including phenolic compounds: caffeic acid, 3-(3-hydroxy phenyl) propionic acid, xanthoxylin, ferulic acid, quinic acid, diferulic acid, methyl gallate and gallic acid, flavonoids: astragalin, catechin, butin, kaempferide, hydroxyflavan, taxifolin, isoquercetin and 3,5,7,4'-tetrahydroxy-6-(3-hydroxy-3-methylbutyl) flavones, coumarins: 6-O-feruloyl-D-glucose, organic acids: palmitic acid and punicic acid, terpenoids: cedrol.

This study focuses on analyzing the antibacterial potential of the leaves extract of *Syzygium cumini* and predicts its mechanism of action by *in silico* studies. The compounds were subjected protein-ligand docking study using *i*GEMDOCK. The phytochemicals reported from *Syzygium cumini* were also analyzed for ADMET properties using SwissADME and AdmetSAR tools. Among the analyzed twenty phytochemical compounds, diferulic acid and taxifolin demonstrated significant target protein inhibition potential against penicillin binding protein 2a (PBP2a) from MRSA strains and erythromycin ribosomal methylase (ERM) from MLSB resistant strains of *S. aureus* with a free binding energy of -102.37 kcal/mole and -103.12 kcal/mole, respectively when compared with the standard linezolid drug. All the twenty compounds except isoquercetin and astragalin were predicted to be suitable drug like molecules, with biocompatible physiochemical parameters.

S. aureus and its antibiotic resistant strains have gotten frighteningly resistant to many of common available antibiotics. The emergence of rate of antibiotic resistance strains is terrifying high. The emergence of antibiotic resistance highlights the value of prudent prescribing of antimicrobials and avoiding their irrational use. This study may help to suggest an alternative possible leading compound for development of new antimicrobial agent from plant sources against MRSA and MLSB resistant strains of *S. aureus*. The aqueous extract of leaves of *S. cumini* showed the highest antimicrobial activity and the phytoconstituents of same were identified by LC-MS analysis. Molecular docking of phytoconstituents of *S. cumini* showed that it was the safest and most potent inhibitors of

PBP2a and erm from MRSA and MLSB resistant strains of S. aureus respectively when compared with the standard linezolid drug. *In silico* ADMET predictions revealed that except isoquercetin and astragalin all other compounds had minimal toxic effects and had good absorption as well as solubility characteristics. These compounds may serve as potential lead compound for developing antimicrobial drugs.

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"All praise and glory to Almighty Allah who gave me strength, courage and patience to Carry out this work."

This thesis is the end of my journey in obtaining my Ph.D. Pursuing a Ph.D. project is a dual experience for me both painful and enjoyable. It's like climbing a high peak, step by step, with bitterness, hardships, frustration, encouragement and loyalty and with a lot of people's kind help. This is really a pleasant task to express my thanks and gratitude to all those who contributed in various ways to the success of this study. At the end of my thesis I would like to thank all those people who made this thesis possible and an unforgettable experience for me.

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(Amrullah Shidiki)

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CHAPTER-I

1.1 Introduction

The most cardinal factor in causing mortality and morbidity in the human population is Infectious Diseases (IDS). Infectious disease still remains to be the most prominent cause of reasons for health illness globally. In one of the studies conducted in 2001 by Global Burden of Disease Study (GBDS) has reported 56.2 million people died by all causes and one third populatin (26.1%) were due to infection causes(Michaud, 2009). The entire mankind was perturbed by deadly contagious diseases until the exploration of novel antimicrobial agents in the mid of twentieth centenary and the infectious diseases are still known as the biggest killer of all adolescents, children, and young people with accounts of more than 16 million deaths per year worldwide. Globally with especially in developing countries for every two death, one death is caused by infectious organisms in case of infectious disease (Deyno *et al*., 2017).

One of the prominent menaces to the world, health and novel anti-infectives is the emergence of antimicrobial resistance (Thabit *et al.,* 2015). As per Founou *et al.,* 2017, the survival of microorganisms in the presence of drugs otherwise would normally bactericidal and bacteriostatic leading to the emergence of AMR. AMR is the most deadly infection resulting in the death of approximately 700,000 people yearly and this will be increased to touch 10 million in the next three decades (O'Neill, 2018). Many bacterial species like *Enterococcus faecium*, *Pseudomonas aeruginosa, Staphylococcus aureus, Klebsiella pneumoneae, Enterobacter spp* were highlighted by the Infectious Disease Society of America. The above-mentioned pathogens are referred to as ESKPE pathogens and are known to show a high degree of resistance to a different class of antibiotics (Boucher *et al.,* 2009).

Among the three species of genus *Staphylococcus, S. aureus* is a medically important bacterial pathogen causing a wide range of diseases among both humans and animals (De Lencastre *et al.,* 2007). The different range of diseases caused by *S. aureus* is osteomyelitis, endocarditis, meningitis, pneumonia, toxin mediated food poisoning, skin

and ocular infections (Nickerson *et al.,* 2009; Bassetti *et al.,* 2014). The rise of antibiotic-resistant mutant serovars of *S. aureus* who exhibits resistance to antibiotics like methicillin are called methicillin-resistant *S. aureus* (MRSA) and macrolide-lincosamide-streptogramin B is called MLSB resistant *S. aureus*. Further, The MLSB resistant *S. aureus* are of two different phenotypes, one is inducible clindamycin resistant *S. aureus* (iMLSB) and constitutive clindamycin resistant *S. aureus* (cMLSB) (Reich *et al.,* 2016).

In both humans and animals, MRSA is the major cause of high mortality and morbidity among different antibiotic-resistant strains of *S. aureus* (VanEperen and Segreti, 2016). It mainly related to hospital-acquired infections and produced differently such as superficial, deep, and systemic infections (Mihu *et al.,* 2015). Due to the leading killer of MRSA among humans, among 12 deadliest drug resistant bacteria MRSA was official ranked one of them in January 2017 (WHO, 2017). The first incidence of MRSA was announced in the UK in 1961. The reason behind the emergence of MRSA is the carry of a novel *mecA* gene in its genome make up of *S. aureus* (Katayama *et al.,* 2000). The novel penicillin-binding protein (PBP2a or PBP2') made by *mecA* gene in MRSA caused inhibition of binding the affinity of penicillin and cephem group of antibiotics (Hartman and Tomasz 1984). The resistance produced by *mecA* gene is unlike to penicillinase mediated resistance, conferring the MRSA showed resistance to all antibiotics of the β-lactam group except ceftaroline and ceftobiprole (Chamber and Dele, 2009). It leads to the selection of another group of antibiotics mainly MLSB group of antibiotics. The antibiotics in macrolides include azithromycin, clarithromycin, and erythromycin; in lincosamides includes clindamycin and in streptogramin B includes quinupristin and dalfopristin. Among MLSB group of antibiotics, clindamycin is the most used antibiotic in patients caused by MRSA because of its outstanding pharmacokinetic properties of being excellent assemblage in abscess and tissue perforation (Bottega *et al.,* 2014). Guimarães *et al.,* 2010 have reported that different MLSB antibiotics are different chemically but they share the same mode of action by inhibiting protein synthesis. Antibiotics target bacterial 50S subunit ribosome. The disproportionate uses of these MLSB drugs have resulted in the development of *S. aureus* resistant strains. There are

three ways of resistance as observed in *S. aureus* viz. drug modification and efflux, modification of target site by methylation reaction, prevention of binding of antibiotic through mutation process (Yilmaz G *et al.,* 2007). The MLSB resistance can be inducible (iMLSB) and constitutive (cMLSB). The iMLSB is expressed in presence of strong inducers such as 14 member macrolides (erythromycin, clarithromycin, dirithromycin), 15 member macrolides (azithromycin) and 16 member macrolides (josamycin, spiramycin) for methylase synthesis. Strains of constitutive (cMLSB) are expressed in absence of inducers for methylase synthesis constitutively (Fiebelkorn *et al.,* 2003).

The inappropriate use of immunosuppressive drugs, antibiotics and in the condition of intravenous catheters, organ transplantation, and the ongoing epidemic of human immunodeficiency virus (HIV) infections are the key reasons for the emergence of multi drug resistant (MDR) bacteria (Selvamohan *et al.,* 2012). One report showed about 2.20 million patients were hospitalized with adverse drug reaction of synthetic drugs and 106,000 patients died in every year in USA (Joshi *et al.,* 2011). This emergence of MDR bacteria and adverse drug reaction encouraged to find out different sources of antibacterial compounds that can combat the problems associated with them (Selvamohan *et al.,* 2012). The sources of antibacterial compounds are microorganism, fungi, algae, symbiotic lichen, and mosses and medicinal plants (Hewage *et al.,* 1998). Among different sources, 80% of the world population depends on use of plant materials throughout the world in the treatment of different diseases (Pallant and Steenkamp *et al.,* 2008).

Globally 250-500 thousand plant species have been identified among which a few plants were reported for their antimicrobial effect against different microorganisms (Petrosyan *et al.,* 2015). The antimicrobial properties of plants are due to the presence of different secondary metabolites with key compounds include alkaloids, flavonoids, terpenoids, coumarins, quinones, steroids, etc. which cause inhibition of growth of microorganism through disrupting microbial membrane (eugenol, thymol, carvacrol), impairing cellular metabolism (cinnamaldehyde), inhibition of biofilm formation (geraniol, thymol,

carvacrol, etc), inhibition of capsule formation in bacteria (salicylic acid and their derivatives), controlling quorum sensing, inhibition of microbial toxin synthesis (dihydroisosteviol, RG tannins etc) (Upadhyay *et al.,* 2014).

The qualitative and quantitative analysis of secondary metabolites or bioactive compounds of medicinal plants depends on different extraction methods (Sasidharan *et al.,* 2011). The standardization and optimization of herbal products with bioactive compounds are affected through the selection of suitable extraction methods. The various extraction methods include maceration, percolation, infusion, decoction, and with more advanced supercritical fluid extractions (SFE), microwave-assisted solvent extraction (MASE), and ultrasound-assisted solvent extraction (UASE) (Wu *et al.,* 2011; Ollanketo *et al.,* 2002). The diversified chemical structures and physicochemical properties of plant compounds made great challenges for the analysis of herbal compounds. To solves such challenges, different types of advanced analytical tools have been used those include liquid chromatography and mass spectrometry (LCMS), nuclear magnetic resonance spectrometer (NMR), and evaporative light scattering detectors (ELSD). LC-MS is the gold standard and most used for qualitative and quantitative analysis of herbal products. The LC-MS operates at different analytical stages in which the sample first runs into the LC column. The different compounds from plant extract become separated on the basis of their different properties such as affinity to stationary phase (affinity chromatography), size (size exclusion chromatography), hydrophobicity (reversed-phase chromatography), and polarity (ion-exchange chromatography). The most important analytical basis in LCMS is the determination of retentiontime (RT) of compounds. The retention time (RT) defines as the time duration in minutes between the injection of sample and development of compound peak. The different compounds have their own specific retention time which helps for the identification of compounds. (Katajamaa and Oresic, 2005).

The prediction of the degree of interaction of different target proteins or nucleic acid molecule (DNA or RNA) with ligands is of great importance in Computer-Aided Drug Design (CADD) in molecular docking technique. Now a day, molecular docking is

widely used for screening of different ligand compounds from plants to combat inhibition of target essential proteins in microorganisms and further used in drug development. The molecular docking procedure is well represented in Figure 1. The result of molecular dockings is represented in bond energy values. After docking, the values of bond energy represent the energy required to form the highest affinity between the receptor protein and ligand compounds. Lower the bonding energy value, more stable the bond and the compound is a candidate of drug design (Somer, 2004).

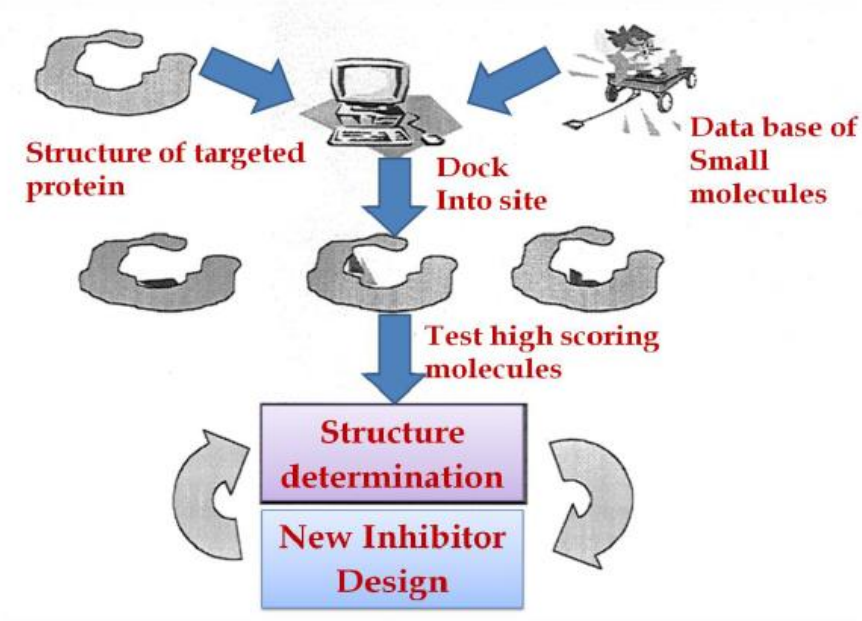


Figure 1.1: Schematic diagram outlining the docking procedure

The penicillin-binding proteins (PBPs) are the key enzymes for bacterial peptidoglycan synthesis and are the target site of action of a β-lactam group of antibiotics. The MRSA showed resistance against β-lactam antibiotics through the formation of novel PBP2a. The MLSB resistant *S. aureus* showed antibiotic resistance through the formation of erythromycin ribosome methylase (ERM) protein by dimethylation of 23S rRNA of 50S ribosomal subunit at adenine A2058 of N6 position in peptidyl transferase loop in domain V (Leclercq and Courvalin 1991). The pharmacokinetic profiles of studied compounds

are carried out through studies of their absorption, distribution, metabolism, excretion, and toxicity of potential compounds (Tsaioun *et al.,* 2016).

The present study is conducted to evaluate *in vitro* antibacterial activity of plant extracts against multidrug-resistant (MDR) bacteria especially for MRSA and MLSB resistant *S. aureus*. The selection of studied plants is due to its great biological and pharmacological properties used in the treatment of different types of diseases in Nepal. Plant extracts in five different solvents viz. aqueous, ethanol, methanol, acetone, and hexane were prepared as per method of maceration extraction technique.In our study, investigation of phytocompounds from the leaves of plants was carried out through the application of chemical methods, the antibacterial activity of plant extracts were evaluated to MLSB resistant *S. aureus* and MRSA with agar well diffusion method. Identification of active compounds in plant extracts were performed through the application of the LCMS method. Molecular docking was performed to find binding affinities between identified compounds of plant extract to two common target proteins, penicillin-binding protein 2a (PBP2a) (MRSA inhibitory target) and erythromycin ribosomal methylase (*erm* gene product) (MLSB SA inhibitory target) and finally, ADMET and drug likeliness properties of plant compounds were evaluated for drug safety (Beck and Geppert, 2014).

CHAPTER-II

2. Review of Literature

2.1 *S. aureus:*

2.1.1 Historical perspective:

S. aureus was the most frequent pathogen found in human beings since its evolution several million years ago. Sir Alexander Ogston and Louis Pasteur first described the Staphylococci as "Micrococci" from the case of furuncle and abscesses in 1880. Ogston has first coined the word *Staphylococcus* in 1883. Anton Rosenbach a German scientist first described the taxonomy of the genus *Staphylococcus* and classified it into two species on the basis of the formation of colored colonies viz. *Staphylococcus pyogenes* var. *aureus* (golden-colored colonies) and *Staphylococcus pyogenes* var. *albus* (white-colored colonies) (Licitra, 2013).

The genus *Staphylococcus* was placed in family Micrococcaceae with genus *Micrococcus.* The two genera are not closely related through the study of their molecular phylogenetic and chemical analysis. The two genera were different through the standard oxidative-fermentative (OF) test, the composition of the cell wall and comparison on oligonucleotide cataloging of 16S rRNA sequence-based on catalog of oligonucleotide array (Evans *et al.,* 1995).

2.1.2 Morphology:

Staphylococcus – [Greek; *Staphyle* - grape-like clusters; *aureus* – gold]*Staphylococci* are gram-positive cocci with diameter of 1μm, found in single, pairs and grape-like clusters because of its plane of division which occurs at right angles and cocci separate slowly. They are non-motile, non-spore former and usually un-capsulated but some strains contain capsule materials. It grows readily on basal media produced golden-yellow colonies on nutrient agar with round, opaque, and about 1 – 2mm in diameter. On sheep blood agar, it shows beta-hemolytic colonies (Brooks *et al.,* 2007).

2.1.3 Cultural, biochemical and physiological characteristics:

S. aureus is a facultative anaerobic bacterium. The optimum temperature for growth is 37 ºC, but it can grow between 8- 45ºC and the optimum pH is 7.2 (range 4.8 – 9.4). It is resistant to unfavorable conditions such as desiccation and high concentration of salt (7.5 -10% NaCl). They are catalase positive, oxidase negative, and produce urease, DNase, gelatinase, lipase, coagulase and carbonic acid. It ferments glucose, lactose, maltose, sucrose, xylose, glycerol, and mannitol to acid. A number of media can support the growth of *S. aureus*, including nutrient agar, blood agar, Baird Parker agar, egg yolk agar, casein agar, MacConkey agar, and mannitol salt agar. It produces golden yellow pigment on basal media and is pronounced after 24 hours at room temperature, or in media enriched with acetate or glycerol. On mannitol salt agar, *S. aureus* produces yellow-colored colonies due to the fermentation of mannitol. Few strains produce abundant exopolysaccharides, whereas most strains produce only a microcapsule (Durai *et al.*, 2012).

2.1.4 Virulence factors:

Staphylococcus aureus carries numerous virulence factors which help for the enhancement of pathogenicity. The virulence factors are of different types and are represented in Table 1.

Table 2.1: Virulence factors of *S. aureus*

S. No.	Virulence Factor	Name of Factors	References
1	Components linked with bacterial cell wall	Carbohydrate moieties associated as capsule	O'Riordan & Lee, 2004
		Teichoic acid	Brown *et al.*, 2012
		Peptidoglycan	Francius *et al.*, 2008
		Protein A	Peterson *et al.*, 1977
2	Toxins	α- toxin	Berube and Wardenburg, 2013

		β-toxin	Huseby *et al.,* 2007
		γ-toxin	Verdon *et al.,* 2009
		δ-toxin	Otto, 2014
		Exfoliative toxin	Bukowski *et al.,* 2010
		Toxic shock syndrome toxin (TSST)	Ross and Onderdonk, 2000)
3	Enzymes	Catalase	Mustafa, 2014
		Coagulase	Cheng *et al.,* 2010
		Staphylokinase	Jin *et al.,* 2004

2.1.5 Epidemiological aspects of *Staphylococcus aureus* with associated pathogenesis:

S.aureus is medically important because of its different virulence factors, adapt in different types of environments, and cause different types of life-threatening diseases (Lowy, 2003). It causes skin and soft tissues infections, pleuropulmonary infections, device-related infections and infective endocarditis (Tong *et al.,* 2015). *S. aureus* is the most common causes of endemic and epidemic nosocomial infections. A survey conducted by the National Nosocomial Infections Surveillance system in U.S. hospitals from 1955 to 1979 was accounted for 13% of isolates from patients with nosocomial infections were *S. aureus* (Eiff *et al.,* 2011). In the year 2008, the most frequently characterized microorganism from the patients of bacteremia was *S. aureus* after *Escherichia coli*.There has also been increasing in the prevalence of bacteremia due to *S. aureus* from 2002 to 2008 from one of the studies in Europe. It was recently reported that the second commonest cause of healthcare-associated infections in the United States was *S. aureus* after *Clostridium difficile* (Kobayashi *et al.,* 2015).

The normal flora of *S. aureus* in the nasal is the key factor for the increase in epidemiology and pathogenesis of infections. Three states nasal carrier has been reported in *S. aureus* viz. d: non-carriers, intermittent carriers and persistent carriers. Kluytmans *et al*., 1997 reported 20% of the individuals without harboring any organism are known as non-carrier followed by 60% of large population harboring different strains of *S. aureus* are known as intermittent carriers and finally, persistent carriers having carried one type of strain A with tentatively 20% of the individuals.

Infections caused by *S. aureus* are mainly in localized skin infections such as folliculitis, furuncle, carbuncles, impetigo, mastitis, and wound infections. Other infections are from deep to systemic infections like bacteremia and also major causes in bones, joints, lungs (Licitra, 2013).

2.2 Methicillin resistant *S. aureus* (MRSA):

2.2.1 Evolutionary significancein MRSA:

After the emergence of penicillin resistant bacteria, methicillin was developed in 1960 which is a penicillinase-resistant semisynthetic penicillin group of antibiotic solved the problem causing infections by penicillin-resistant *Staphylococcus aureus*. But after one year in 1961 and in 1968 British scientist and the scientists from USE has reported MRSA (Chambers and DeLeo, 2009)

2.2.2 Pathogenesis and epidemiology of MRSA:

The degree of pathogenicity of *S. aureus* is influenced by presence of virulence factors such as arginine catabolic mobile element (ACME), phenol soluble modules (PSMS), Panton-Valentine leucocidin (PVL), alpha hemolysin (alpha-toxin) and a regulatory locus referred to as *agr* (Tong*et al.*, 2005). The methicillin resistance gene (*mec A*) in MRSA produced a changed penicillin binding protein called PBP2a (Utsui&Yokota, 1985).

2.2.3 Classes of MRSA:

There are three classes of MRSA viz. healthcare-acquired MRSA (HA-MRSA), community-associated MRSA (CA-MRSA) and live associated MRSA (LA-MRSA)

- **HA-MRSA:**

HA-MRSA has been reported is most commonly found in America & East Asia in comparison to Europe. The more prevalence with the value of 70% was found in Vietnam, Sri Lanka, and South Korea; with less than 50% in Italy, Greece, and Portugal. The different clonal complexes (CCs) found in HA-MRSA, two CCs (CC5 and CC8) are frequently found. CC22 is obtained from Canada, Australia, and Indonesia, and clonal complex (CC45) found in the US and Europe (Cuny *et al.,* 2015).

- **CA- MRSA:**

Generally, it is identified in community origin. CA-MRSA is more commonly found in younger peoples than others. Several strains of CA-MRSA carry one gene (*PVL*) that encodes Panton-Valentine Leukocidin (PVL) toxin causes on leucocytes and subsequently tissue damage (Bukharie, 2010)

- **LA-MRSA:**

The LA-MRSA is mostly found in animals such as cattle and pigs. It is more prone to animal rearers and people who work in close contact with farm animals and who slaughter the animals. The clonal complex (CC398) is found in LA-MRSA commonly in Europe viz. Belgium, Austria and Denmark (Cuny*et al.,* 2015).

2.2.4 Studies of MRSA with prevalence and antibacterial effect of medicinal plants:

The prevalence of MRSA was 40% in antibiotic-resistant strains of bacteria (Gupta et *al.,* 2015). The maximum isolation of MRSA with the value of 68.7% was from pus samples out of 323various clinical samples (Debnath and Chikkaswamy 2015). The *S. aureus*, MRSA, and MSSA were found with the value of 104 (24.5%), 40(38.5%), and 64 (61.5%) among 424 clinical samples. The highest prevalence was observed in wound swabs (29.4%) whereas the least was observed in blood samples (6.7%) (Nsofor*et al.,*2016). The preponderance of MRSA is a high concern and has increased from 12% (1992) to 80.89% (1999) in India (Verma *et al.,* 2000). The disc diffusion test with cefoxitin is the superior method to another phenotypic method for MRSA detection. The result with cefoxitin was found to be in agreement with the findings of *mecA* gene detection by PCR (Anand *et al.,* 2009). The occurrence of *mec A* gene among MRSA

with the application of polymerase chain reaction compared to use of a conventional method (Tacconelli *et al.,* 2009). Ethanolic extract of five different plants in Australia was reported to show antimicrobial activity against clinical isolates of MRSA (Palombo & Semple, 2002). The combined effect of antimicrobial activity from essential oils of *Thymus schimper, Boswellia ogadensis,* and *Blepharis cuspidata* was shown resistant against MDR *E. coli, K. pneumoniae,* and MRSA (Gadisa *et al.,* 2009). The extract of *Inula helenium* was evaluated the bactericidal effect against 200 clinically significant Irish *Staphylococcus aureus isolates* with MRSA & MSSA (O'Shea *et al.,* 2009). The eight Libyan traditional medicinal plants were shown to potential to inhibit MRSA (Abouzeed *et al.,* 2013).

2.3 MLSB resistant *S. aureus*:

Antibiotics from the MLSB family are mostly used for infections caused by MRSA. Among MLSB antibiotics, clindamycin is perfectly applied to infections related to soft tissues and dermal applications. Clindamycin has been observed to show excellent tissue penetration, no need for renal dose adjustment, and good oral absorption (Nwokah and Abbey, 2016). The indiscriminate use of clindamycin has led to an increase in MLSB resistant *S. aureus* (Zaher, 2017).

2.3.1 Mechanism of resistant to MLSB antibiotics:

There are two mechanisms through which *Staphylococci* showed resistance against the use of different MLSB groups of antibiotics. The first mechanism involves macrolide efflux through a special design efflux pump encoded through *msr A* gene among *Staphylococci*. This energy dependant efflux pumps effectively throws out the entered macrolides from the bacterial cell before their target site binding on the ribosome. The resistance mechanism notably happened only with macrolides and azalides (eg. erythromycin, azithromycin) and group B streptogramins (eg quinupristins). The second mechanism of resistance developed through modification of target binding protein on the ribosome. All antibiotics of macrolides, lincosamides, and streptogramins B group follow this mode of resistance mechanism commonly referred to as "MLSB resistance". This resistance mechanism can be expressed in form of either constitutive or inducible.

Therefore, two phenotypic forms of MLSBcan are named either in form of constitutive (cMLSB) or inducible (iMLSB) (Seifi *et al.*, 2012).

Constitutive MLSB (cMLSB):

The cMLSBphenotypes always expressed erythromycin ribosome methylase (*erm*) gene product called erythromycin ribosome methylase transferase protein. The methylase is continuously producing through binding of a macrolide to upstream translational attenuator sequences of mRNA secondary structure. This modification can take place through duplications, deletions, mutations (Leclercq, 2000).

Inducible MLSB (iMLSB):

The iMLSB of *Staphylococci* are always producing an uncomplete mRNA which is inadequate to encode methylase protein. However, the methylase of *erm* gene product is produced in presence of macrolide in form of an inducer. The iMLSB strains are shown erythromycin-resistant (an inducer) and clindamycin sensitive in double-disk diffusion method (Juyal *et al.,* 2013). The characteristics of the phenotypes identified by the D-test are described in Table 2.2 (Abbas et *al.*, 2015).

Table 2.2: Characteristics of phenotypes identified by D-test

Phenotype	ERY result	CLI result	Characteristics
Inducible MLSB ($iMLS_B$)	**R**	**S**	D-like zone of inhibition
Constitutive MLS_B($cMLS_B$)	**R**	**R**	Growth around ERY and CLI discs
MS_B	**R**	**S**	Growth around ERY and clear zone of inhibition around CLI

R=Resistant; S=Sensitive; CLI=Clindamycin; ERY=Erythromycin

2.3.2 Earlier studies indicating MLSB resistant *S. aureus*:

Different studies have been conducted on MLSB resistant *S. aureus*. Spiliopoulou *et al.,* 2004 has reported the prevalence of iMLSB strains with higher 53 (3.5%) than cMLSB 106 (7.2%) and MSB 13 (0.9%) from different clinical samples. The prevalence of cMLSB and iMSLB phenotypes was higher among MRSA than MSSA (Gadepalli *et al.,* 2006). Among 366 *S. aureus*, 68 (18.6%) were inducible MLSB phenotype of *S. aureus*followed by 14 (3.8%) constitutive MLSB phenotypic strains and the 3 (0.8%) MSB phenotypic strains (Mallick *et al.,* 2009). The prevalence of iMLSB, cMLSB, and MSB phenotypes of strains of *S. aureus* was found with values of 7.94%, 14.6%, and 26.35% in 373 *S. aureus*. Juyal *et al.,* 2013 has been reported 19.4% iMLSB, 29% cMLSB, and 38.7% MS phenotypes of MLSB resistant strains of *S. aureus* among total isolates of MRSA.

2.4 Antibiotic resistance with mechanism of action:

Staphylococcus aureus has been shown resistance against several available antibiotics through following different types of resistance mechanism depicted in Table 2.3.

Table 2.3: Antibiotics; its mode of action and bacterial resistance mechanism

Antibacterial Agent	Mechanism of Action	Mechanism of Resistance	References
β-Lactams antibiotics (cephalosporins, monobactams, carbapenems, penicillins)	Cell wall inhibition	Drug inactivation by Penicillinase Altered PBP targets	Lowy, 2003
Glycopeptides (vancomycin, teicoplanin)	Binds to precursor of peptidogiycan	Modification of precursor	Lowy, 2003
Aminoglycosides (gentamicin,	Inhibiting protein synthesis (binds to	Drug-modifying enzymes	Brook *et al.*,

tobramycin, amikacin)	30S ribosomal subunit)	Methylation at ribosome binding site Active efflux	2007
Lincosamides (clindamycin)	Inhibiting protein synthesis (binds to 50S ribosomal subunit)	Methylation at ribosome binding site Enzymatic Modification	Juyal, 2013
Streptogramins (quinupristin, dalfopristin)	Inhibiting protein synthesis (binds to 50S ribosomal subunit)	Methylation at ribosome binding site Enzymatic Modification	Juyal, 2013
Quinolones (ciprofloxacin, levofloxacin, norfloxacin)	Inhibits DNA gyrase and DNA topoisomerase IV	Altered targets Active efflux Drug-modifying enzymes	Lowy, 2003
Tetracyclines (tetracycline, doxycycline, minocycline)	Binds on ribosomal subunits	Efflux pumps Ribosome protection proteins	Schnappinger and Hillen, 1996
Chloramphenicol	50S ribosomal subunit	Drug-modifying enzymes	Schnappinger and Hillen, 1996
Oxazolidinones (Linezolid)	50S ribosomal subunit	Mutation in 23 rRNA and ribosomal proteins	Long and Vester, 2012
Macrolides (erythromycin,	Inhibiting protein synthesis (binds to	Methylation at ribosome binding site	

clarithromycin azithromycin)	50S ribosomal subunit)	Active efflux Enzymatic Modification	Juyal, 2013

2.5 Plants with Medicinal Properties:

The different parts of plants are used for the maintenance and treatment of diseases in humankind for ages. Different plant extracts are used for traditional therapies as folk medicine against the treatment of different infections by 80% of the global population as reported by World Health Organization. Fifty percentages of available clinical drugs are made from source of natural product with plants origin. Alkaloids, flavonoids, tannins, saponins, carotenoids, terpenoids, polyphenols, pigments, enzymes, minerals and vitamins (K, E, C, and A) were few medically important active phytochemicals which carries different mode of action in the treatment of a different form of diseases (Madhuri and Pandey, 2009).

The mode of action of different phytochemicals against different forms of diseases is still unclear. In the present scenario, the availability of different modern and sophisticated techniques has made possible for identification and analysis of phytochemical studied from different medically important plant origin. Medicinal plants possess different activities such as immunomodulatory, antioxidant, and antibacterial. The antimicrobial properties are of great significance for the treatment of different diseases which proved this efficacy through conducted different scientific studies (Nascimento *et al.*, 2000).Herbal medicine has a pivotal and indispensable role in Nepali culture. Nepal is considered as source of exploring diverse group of medicinally important plants. The geographical form of Nepal, plane, hill and mountains inhabit more than 500 plant species (Kunwar and Bussmann, 2008).

2.5.1 Plants with antimicrobial activity:

New Antibiotics need to be worked upon because of the effect of antimicrobial resistance (AMR) on public health. Medicinal plants are still considered one of the major sources for finding different antimicrobial products widely used against antimicrobial-resistant strains of bacteria. Several activities viz. antimicrobials, anti-inflammatory, and antioxidant activities have been reported from phytochemical for treatment of microbial infections (both topical and systemic application). In view of these medical important activities, the following are the literature surveys are carried out to disclose the antimicrobial activities of several medical important plants.

2.5.1.1 Medicinal plants exhibiting antibacterial activity:

The antimicrobial activity of different medicinal plants has been reported to act against different pathogenic bacteria. Among medicinal plants, few with antibacterial activity have been shown in Table 2.4.

Table 2.4: Antimicrobial efficacy of medicinal plants against bacteria (Gram positive and Gram negative)

S. N	Plant Names	Family Name	Part Used	References
1.	*Acorus calamus*	Araceae	Rhizomes	Singh *et al.*, 2012
2.	*Adhatoda vasica*	Acanthaceae	Leaves	Manandhar 2002
3.	*Centella asiatica*	Umbelliferae	Whole plant	Singh *et al.*, 2012
4.	*Cinnamomum camphora*	Lauraceae	Leaves, seeds, bark	Singh *et al.*, 2012
5.	*Curculigo orchioides*	Amaryllidaceae	Rhizomes	Manandhar 2002
6.	*Curcuma longa*	Zingiberaceae	Rhizomes	Singh *et al.*, 2012
7.	*Cuscuta reflexa*	Cuscutaceae	Whole plant	Singh *et al.*, 2012
8.	*Cynodon dactylon*	Poaceae	Whole plant	Singh *et al.*, 2012)
9.	*Eupatorium adenophorum*	Compositae	Leaves	Manandhar 2002
10.	*Psidium guajava*	Myrtaceae	Leaves, bark	Manandhar 2002

2.5.1.2 Plants having medicinal properties against *Staphylococcus aureus:*

The potential antibacterial properties of traditional medicinal plants have been reviewed. Selected medicinal plants with antibacterial activity against *Staphylococcus aureus* was represented in Table 2.5.

Table 2.5: Medicinal plants with antibacterial effect against *S. aureus*

S. No	Plant Names	Family Name	Part Used	References
1	*Syzygium cumini*	Myrtaceae	Leaves	Chaudhary and Mukhopadhyay, 2012
2	*Psidium guajava*	Myrtaceae	Leaves	Chaudhary *et al.,* 2012
3	*Tinospora cordifolia*	Menispermaceae	Stem	Narayanan *et al.,* 2012
4	*Zingiber officinale*	Zingiberaceae	Rhizome	Karuppiah and Rajaram, 2012
5	*Moringa oleifera*	Moringaceae	Leaves	Abalak *et al.,* 2012
6	*Azadirachta indica*	Meliaceae	Leaves	Francine *et al.,* 2015
7	*Warburgia salutaris*	Canellaceae	Leaves	Rabe and Van-Staden, 2000
8	*Dacryodes edulis*	Buseraseae	Leaves	Fonkeng *et al.,* 2015
9	*Ocimum gratissimum*	Lamiaceae	Leaves	Fonkeng *et al.,* 2015
10	*Commelina Erecta*	Commelinaceae	Leaves	Fonkeng *et al*., 2015
11	*Spilanthes filicaulis*	Asteraceae	Leaves and stem	Fonkeng *et al.,* 2015
12	*Nyctanthes arbor tristis*	Oleaceae	Leaves	Dhinakaran *et al.,* 2017

2.5.1.3 Medicinal plants with antibacterial activity against resistance strains of *Staphylococcus aureus*:

Potential plants with medicinal properties have been used as therapeutic use for curing infectious diseases in many countries. This provides a rationale for imposing natural products for the treatment of MRSA infection. Literature survey for medicinal plants with antimicrobial activity against resistant strains of *Staphylococcus aureus* has been shown in Table 2.6

Table 2.6: Medicinal plants with antibacterial effect against resistant *S. aureus*

S. No.	Plant Names	Family Name	Part Used	References
1.	*Zanthoxylum tingoassuiba*	*Rutaceae*	Root	Costa *et al.*, 2017
2.	*Terminalia avicennioides*	*Combretaceae*	Stem, bark	Akinyemi *et al.*, 2005
3.	*Bridella ferruginea*	*Euphorbiaceae*	Leaves	Akinyemi *et al.*, 2005
4.	*Ageratum conyzoides*	*Compositae*	Leaves	Akinyemi *et al.*, 2005
5.	*Acalypha wilkesiana*	*Euphorbiaceae*	Stem, bark	Akinyemi *et al.*, 2005
6.	*Cistus salvifolius*	*Cistaceae*	Leaves	Abouzeed *et al.*, 2013
7.	*Salvia officinalis*	*Labiatae*	Leaves	Abouzeed *et al.*, 2013
8.	*Arbutus pavarii*	*Ericaceae*	Leaves	Abouzeed *et al.*, 2013
9	*Pistacia atantica*	*Anacardiaceae*	Leaves	Abouzeed *et al.*, 2013
11.	*Myrtus communis*	*Myrtaceae*	Leaves	Abouzeed *et al.*, 2013
12.	*Syzygium cumini*	*Myrtaceae*	Leaves	Jahan *et al.*, 2011
13.	*Moringa oleifera*	*Moringaceae*	Leaves	Kamath *et al.*, 2016
14.	*Azadirachta indica*	*Meliaceae*	Leaves	Quelemes *et al.*, 2015
15.	*Tinospora cordifolia*	*Menispermaceae*	Stem	Chakraborty *et al.*, 2014

2.5.2 Phytochemcical Extraction:

Based on the extraction principle, the desired natural products from the raw material are separated through sublimation followed by solvent extraction, distillation, and pressing. The solvent extraction is considered as a gold standard method than others for extraction of natural products. There are two classes of extraction method have been widely used for purpose of extraction of desired compounds from different solvent extracts. One class of extraction method is known as conventional extraction methods in which different techniques applied are maceration (Albuquerque *et al.,* 2017), percolation (Zhang et *al.,*

2014), decoction ((Li *et al.,* 2010), soxhlet extraction (Wei *et al.,* 2013) and reflux extraction (Wei *et al.,* 2013). The second class of extraction is named modern extraction methods those are hydro distillation and steam distillation (Verma *et al.,* 2016), enzyme assisted extraction (Liu *et al.,* 2016), pulsed electric field extraction (Bouras *et al.,* 2016), microwave-assisted extraction (Xiong *et al.,* 2016), ultrasound assisted extraction (Chemat *et al.,* 2017), Supercritical fluid extraction (Conde-Hernandez *et al*., 2017) and pressurized liquid extraction (Vergara-Salinas *et al.,* 2013).

2.5.3 Preliminary Qualitative Analysis of Phytochemicals:

In the traditional system of medicine, medicinal plants are rich source different types of phytochemicals. These phytochemicals are responsible for the different colors, flavors, and smells of plants. Several potential active compounds have therapeutic effects on the human body with specific physiological action (Karunyadevi *et al.,* 2009). The important industrially and medicinally important compounds viz. alkaloids, flavonoids, phenolic compounds, saponins, steroids, tannins, terpenoids can be worked out through phytochemical screening (Akindele and Adeyemi, 2007). The tests for qualitative identification of phytochemical constituents from plant sources are shown in Table 2.7.

Table 2.7: A brief review on Phytochemical screening in plants

S. No	Compounds	Tests	References
1.	Saponins	Foam height test	Banso and Adeyemo, 2006
2.	Terpenoids	Salkowski test Liebermann-Burchard test	Iqbal *et al.,* 2015
3.	Steroids	Liebermann-Burchard test	Iqbal *et al.,* 2015
4.	Tannins	Extract + water+5% Fecl3	Banso and Adeyemo, 2006
5.	Alkaloids	Dragendorff's test Mayer's test wagner's test	Iqbal *et al.,* 2015
6.	Carbohydrates	Molisch's Test	Yadav *et al.,* 2014

7.	Flavonoids	Shinoda test 10% NaoH test	Maria *et al.,* 2018
8.	Quinones and anthraquinones	Borntrager's test Ammonium hydroxide test Sulfuric acid test	Maria *et al.,* 2018
9.	Proteins	Xanthoproteic Test	Yadav *et al.,* 2014
10.	Coumarin	10% NH_4OH test	Bhandari *et al.,* 2017
11.	Glycosides	Borntrager's test Keller-Killiani test	Joshi *et al.,* 2013

2.5.4 Separation of phytochemicals:

The polarity of different compounds from plant extract sources is a great factor for the separation, identification, and characterization of bioactive compounds. Table 2.8 shows different methods of separation.

Table 2.8: Methods for separation of phytochemicals

S. No	Methods	References
1.	Adsoption Chromatography	Perry *et al.*,1972
2.	Partition Chromatography	McNair&Miller 1998
3.	Ion-exchange Chromatography	Coskun O, 2016
4.	Affinity Chromatography	Coskun O, 2016
5.	Size exclusion Chromatography	Coskun O, 2016
6.	Paper Chromatography	Coskun O, 2016
7.	Thin layer Chromatography	Coskun O, 2016
8.	Column Chromatography	Coskun O, 2016
9.	Gas Chromatography	Coskun O, 2016
10.	HPLC	Coskun O, 2016
11.	HPTLC	Attimarad *et al.,* 2011
12.	Optimum performance laminar chromatography	Barabde 2015

2.5.5 Identification of separated compounds:

Different range of techniques based on spectroscopy viz. liquid chromatography-mass spectroscopy, UV- visible spectroscopy, Nuclear Magnetic Resonance (NMR) spectroscopy and Infrared (IR) spectroscopy are applied for the analysis of different compounds from herbal products. The principle of spectroscopy is based on the use of electromagnetic radiation through compounds that absorbs some of the radiation. Measurement of this amount of radiation, a spectrum specific to certain bonds in compounds, can be produced. The spectra obtained are used for structural clarification (Altemimi *et al.,* 2017).

2.5.5.1 Ultra Violet (UV)-Visible Spectroscopy:

This method is widely applied for the identification of compounds in both pure and biological mixtures. The different phyto-compounds like polymer dyes, phenol complexes, anthocyanins, and tannins have the ability to bind iron sources and help for the identification of compounds. Different ranges of ultraviolet radiation from 280 nm to 520 nm have been used for the determination of different secondary compounds (Altemimi *et al.,* 2017). Following is a brief review about the use of UV-Visible Spectroscopy for the identification of containing different molecules in the field of plant source.

The presence of quercetin and curcumin in the herbal formulation was identified through use of UV-Visible Spectroscopy (Salunkhe and Patil, 2014). Renuka *et al.,* 2017 has been reported about phytoconstituents in *Caralluma nilagiriana* through use of UV–visible spectroscopy. The phytochemical analysis of *Bougainvillea glabra* was found through the application UV-Visible Spectroscopy (Neha and Jyoti, 2013).

2.5.5.2 Infrared Spectroscopy:

In infrared spectroscopy, some frequencies of infrared light are absorbed and the remaining frequencies will be transmitted through samples containing organic compounds and. This absorption causes some vibration changes in molecules and helps

for the identification of these molecules in compounds of the sample. Different molecules having their own characteristic vibration frequencies due to having different nature of bonding either in form of single, double binds. Thus, this spectroscopy helps for the identification of molecules in organic compounds with the formation of characteristic frequency absorption bands with different bonds (Altemimi *et al.,* 2017). A brief reviews carried out for the application of infrared spectroscopy for the estimation of different phytoconstituents found in different plant sources.

The total terpenoids contain different aromatic plants found in different the geographical region of Turkey through the application of near-infrared spectroscopy (NIRS) (Ercioglu *et al*., 2017). Shameel *et al.,* 1996 has been reported about the different number and different nature of compounds from seeds of *Pongamia pinnata* through the use of infrared spectroscopy. The thiophenes, steroids, and terpenoids from different parts (root, stem, and leaves) of *Tagetes patula* was determined by the use of infrared spectroscopy (Bano *et al.,* 2002).

2.5.5.3 Nuclear Magnetic Resonance Spectroscopy:

The principle of nuclear magnetic resonance spectroscopy (NMR) depends on differences in magnetic properties of nuclei of molecules. It is widely used for the analysis of compounds of different materials. Following are some reviews that have been done about the application of nuclear magnetic resonance spectroscopy for the determination of molecules contains in plant sources (Altemimi *et al.,* 2017).

Quantitative determination of saccharides, amino acids, and organic acids in potato (*Solanum tuberosum*) tuber filaments was found by use of proton nuclear magnetic resonance spectroscopy (Aisala *et al.,* 2016). The phytoconstituents of methanolic extracts of 10 Annonaceae species was investigated through the use of nuclear magnetic resonance spectroscopy (Alves *et al.,* 2016). Exarchou *et al.,* 2001 identified the rosmarinic acids and caffeic acid from the Lamiaceae family of plant through the use of NMR. The concentration of the three major phytoconstituents such as canadine, β-

hydrastine, berberine, and alkaloids were determined in root extract of goldenseal (*Hydrastis canadensis)* through the use of nuclear magnetic resonance spectroscopy (Le *et al.,* 2018).

2.5.5.4 Liquid Chromatography-Mass Spectroscopy (LCMS):

The techniques for screening of information of various biomolecules in multiple samples can be done by liquid chromatography with mass spectrometry (LCMS). LCMS is widely used in the field of metabolomics and proteomics research. A typical LCMS includes sample pre-treatment and extraction. The sample passes through LC the column where molecules become separated on basis of their charge and mass ratio. The most important factor in LCMS is the determination of retention time (RT) which measure time in minute taken for a compound to pass through chromatography or it is the time from injection to detection. The separated compounds were pass through the LCMS interface become ionized and these ions were measured with the help of a mass spectrometer on basis of their different m/z values. For ionization purposes, two types of ionization methods have been used in the LCMS method viz. atmospheric pressure chemical ionization (APCI) and Electrospray ionization (ESI). The use of different quadrupole affects the mass of compounds. Brief reviews were carried for the importance of the LCMS method in the estimation and identification of phytochemical constituents of plant sources (Katajamaa and Oresic, 2005). The metabolite profile of leaves of wild strawberry (*Fragaria vesca*) was analysed by use of LC-ESI/LTQ Orbitrap/MS (D'Urso*et al*., 2017). Sulaiman *et al.*, 2014 reported phenolic compounds from the leaf of three *Salacia* species by use of LCMS. The estimation of phenolic antioxidants from *Ipomoea mauritiana* was determined through the use of spectrophotometric and LC-MS analysis (Sulaiman*et al.,* 2014). The phenolic and flavonoids constituents of *Ephedra alata* growing plant in Palestine was analyzed through the application of HPLC/PDA and HPLC/MS (Al-Rimawi *et al.,* 2017).

2.5.6. Evaluation of antibacterial activity of plant extracts:

The evaluation of antimicrobial properties of herbal products can be used for epidemiology, treatment outcomes and drug discovery. In our reviews for finding antimicrobial efficacy of plant

sources, we focused on some antimicrobial testing methods for evaluation of *in vitro* investigation of plant extracts as depicted in Table 2.9.

Table 2.9: Antimicrobial susceptibility testing methods

Methods	**Types**	**References**
Diffusion	Disc diffusion, E-test, agar well diffusion, agar plug diffusion, cross streak, poisoned food	Konate *et al.,* 2012; Gupta *et al.,* 2015; Valgas *et al.,* 2007; Elleuch *et al.,* 2010; Lertcanawanichakul *et al.,* 2008; Kumar *et al.,* 2013
Thin-layer chromatography (TLC) bioautography	Agar overlay bioassay, direct bio autography, agar diffusion	Mehrabani *et al.,* 2013; Dewanjee *et al.,* 2015
Dilution method	Broth dilution, Agar dilution	Imhof *et al.,* 2003
Flow cytofluorometric method	NA	Tang and Stratton 2013
ATP bioluminescence assay	NA	Finger *et al.,* 2013
Time-kill test	NA	Clancy *et al.,* 2006

2.5.7 Molecular docking:

Molecular docking is a computer-based method used for the prediction of the preferred orientation of one compound to another after the formation of a stable complex. The scoring function is used to explain the degree of association or binding affinity between two molecules in molecular docking. It is widely used in rational drug designing (Kitchen *et al.,* 2004).

2.5.7.1 Theory of Molecular Docking:

There are two steps in the method of molecular docking (Meng*et al.,* 2011);

1. Evaluation conformations formed after binding the ligands on the active site of target protein molecules

2. The stable conformations formed are ranked through the application of the scoring function.

2.5.7.2 Molecular Docking and its Types

Molecular dockings are of three types:

a) **Rigid Docking**: In this docking type, the conformation of complex (receptor and ligand) does not change after docking. It is the very simplest docking type not required more calculations. It is mostly used for larger compounds such as protein-protein docking or protein-nucleic acid docking (Liu*et al.*, 2016).

b) **Semi flexible Docking:** In this docking type, the conformation of the receptor remains unchanged but the conformation of the ligand is become changed after docking. The changing nature of ligand in this docking can be used for various applications. This docking is useful for small macromolecules such as proteins, nucleic acid, and small ligand compounds (Jiang *et al.*, 2016).

c) **Flexible Docking:** In this type, the conformation of both receptor and ligand is readily changed after docking. This is a very complicated docking process. It is generally used to study the interaction between molecules accurately (Roy*et al.*, 2018).

2.5.7.3 Docking algorithms and programs:

The making of the algorithm of docking is related to the concept of 'lock and key, however, the precise algorithm with this concept like a key (ligand) and lock (receptor proteins) varies with different docking programs. Recent research has developed a new algorithm and this algorithm particular docking program should mandatorily strike the balance between speed and accuracy with scoring function and binding affinity. Researchers can select different docking programs as per their requirements. The number of docking programs has been researched by various researchers. Goodsell & Olson,

1990 and Jones *et al.,* 1997 has reported Autodock and Gold as the most popular and common docking programs. Table 2.10 represents the lists of molecular docking programs with their algorithms.

Table 2.10: Molecular docking programs with their own algorithms

Algorithms	Name	References
Lamarckian genetic algorithm	AUTODOCK4	Morris *et al.,* 2009
Shape matching	DOCK	Allen *et al.,* 2015
Shape matching	OEDOCKING	Kelley *et al.,* 2015
Ensemble-based	FLEKSY	Wagener *et al.,* 2012
Evolutionary optimization	SWISSDOCK	Grosdidier *et al.,* 2011
Genetic algorithms	GOLD	Jones *et al*., 1997
Hybrid	GLIDE	Friesner *et al.,* 2004
Local optimization	VINA	Trott and Olson, 2009
Hybrid	RDOCK	Ruiz-Carmona *et al.*,2014
Simulated annealing	LEDOCK	Unzue *et al.,* 2016)
Ant colony optimization	PLANTS	Korb *et al.,* 2009
Hybrid	HADDOCK	Dominguez *et al.,* 2003
Shape matching	SURFLEX-DOCK	Spitzer and Jain 2012
Hybrid	MOE	Vilar *et al*., 2008
Shape matching	FLEXX	Kramer *et al*., 1999
Shape matching	LIGANDFIT	Venkatachalam *et al.,* 2003
Hybrid	ICM	Neves *et al.,* 2012
Evolutionary	IGEMDOCK	Yang and Chen, 2004

2.5.7.4 Docking studies related to this work:

Review of literature on molecular docking suggest that there are different ligand compounds used from different natural sources against different used receptor proteins considered in different biological systems. Table 2.11shows lists of ligand molecules

used against receptor protein molecules used in the process of different molecular docking methods.

Table 2.11: Molecular docking studies of some ligands (compound) with target

S. No	Ligand Name	Target Protein	Tools	Reference
1.	3-methyl-2-(2-oxopropyl) furan, 3-cyclopentylpropionic acid, 4-methoxyphenyl ester	Tyrosine phosphatase (PDB ID: 3rof)	Autodock	Biswal *et al.*, 2020
2.	HMIC	(PDB ID:1XFF) Glucosamine-6-phosphate synthase	Autodock	Subbaiah *et al.*, 2017
3.	Apigenin, rosavin, quercetin, feruloyl glucoside, loliolide, luteolin 7-glucuronide, apigenin 7-glucuronide, D-mannitol, L-aspartic acid, luteoline	Penicillin Binding Protein 2A(PDB ID: 3vsl) DNA gyrase (PDB ID:3g7b)	Gold	Emran *et al.*, 2015
4.	syringin, betulin, ursolic acid, lupeol, and iridoid	DNA gyrase A (PDB ID: 6FM4)	Autodock	Swain *et al.*, 2017
5.	Glucoliquiritin apioside, Shinflavanone, Shinpterocarpin	Glucosamine 6 phos. synthase : 4VF5	ArgusLab 4.0.1	Ravikumar *et al.*, 2019
6.	Flavogallol, ursolic acid etc	Penicillin binding protein 2a (PDB ID: 3ZG5) Dihydrofolate reductase (DHFR) (PDB ID: 4FGG) Dihydropteroate synthase (PDB ID: 1AD1) Topoisomerase-IV (PDB ID: 2INR)	Autodock	Ravi *et al.*, 2020

2.5.8 ADMET selection:

The determination of pharmacokinetic properties of identified compounds was carried through studies of ADMET for evaluation of drug candidates. All these parameters have been tested through several online software programs (Van De Waterbeemd and Gifford, 2003). In the case of gout, the ADMET properties of plant-based compounds were analyzed to predicting their drug likeliness for developing potent inhibitors of inflammatory cytokines IL17A and IL18 (Hari, 2019). The ADMET properties of phytoconstituents of medicinal plant *Bridelia scandens* were used for the evaluation of drug candidates (Shivakumar *et al.,* 2018). The effectiveness of bioactive molecules against glucocerebrosidase was tested through the use of molecular docking and also evaluate the ADME profile of active molecules by use of Schrodinger software (Subramaniyan *et al.,* 2018).The prediction of ADMET profiling of ferulic acid through using pkCSM online tool for characterization of a palate receptor involved in the ADP signaling pathway (Ekowati *et al*., 2018).

CHAPTER-III

3.1 Hypothesis:

iMLSB, cMLSB and MRSA are antibiotic-resistant bacteria that cause life dangerous diseases in both hospitals acquired and community-acquired infections. This strain of *S. aureus* shows resistance to different classes of antibiotics including the best known last antibiotic vancomycin. Hence, there is need to search for new therapeutic agents through the evaluation of phytochemicals and validation through docking studies against MRSA and MLSB resistant strains of *S. aureus*.

CHAPTER-IV

4.1 Objectives:

The designed and defined objectives for our study are as follows:

- Isolation and Identification of *Staphylococcus aureus*.

- Selection of Methicillin-resistant *Staphylococcus aureus* (MRSA), macrolide-lincosamide-streptogramin B (MLSB) resistant *Staphylococcus aureus*.

- Evaluation of antibacterial efficacy of medicinal plants against studied resistant strains of *S.aureus*.

- Qualitative and quantitative analysis of Phytochemical contents of plant extracts

- Molecular docking studies of bioactive compounds from plant extract to penicillin-binding protein 2a of MRSA and erythromycin ribosomal methylase protein of MLSB resistant strains of *S. aureus*.

CHAPTER-V

5. Methods and materials:

5.1 Clinical sample collection and processing:

The present study is conducted at National Medical College and Teaching Hospital, a tertiary Care Hospital in Birgunj, Parsa district of Province-2 state. The samples of patients covering eight districts from Province-2 state have been taken in the present investigation. The names of the districts are Bara, Parsa, Sarlahi, Rautahat, Mahotri, Dhanusha, Sirha and Saptari. Clinical samples were collected from the patients suffering from bacteremia, sepsis, and urinary tract infection, upper and lower respiratory infection, neurologic, soft tissue infection, and gynecologic cases. Seven types of clinical specimens which were categorized as blood, pus, body fluids, urine, swab, stool, and sputum are selected. Samples are collected and processed as per the standard microbiological procedures of Winn 2006. The Samples are:

5.1.1 Blood:

10-20 ml of venous blood was collected from adult patients and 1-3 ml from children group of patients. The cap of the blood glass bottle is disinfected with 70% isopropyl alcohol. 50 ml of brain-heart infusion broth (BHI) media was inoculated with blood and incubated at 37 ºC. The media bottles were observed two times per day for the first 3 days for any bacterial growth till 7 days. The growth of bacteria is characterized through the following observations;

- A floccules deposit on top of the blood layer
- Uniform or subsurface turbidity
- Haemolysis
- Coagulation of the broth
- A surface pellicle
- Production of gas

After observation of visible growth, one loop full of broth with the help of an inoculating loop was subculture on blood agar, nutrient agar, and mannitol salt agar media and incubated at 37 ºC for 24 hours (Cheesbrough, 2000).

5.1.2 Pus:

A sterile swab stick was used for the collection of pus. The sample was collected from swabbing from superficial pus with help of swab sticks but the aspiration of pus collected with help of a syringe in deep wound condition. The collected pus samples were inoculated into blood agar, nutrient agar, and mannitol salt agar media. The plates were incubated at 37 ºC for 24 hours for bacterial growth (Cheesbrough, 2000).

5.1.3 Urine:

Wide-mouth screw-capped universal containers are used for the collection of midstream urine.

- The female patients were instructed to hold labia apart. The first portion of voided urine should be discarded and midstream urine is collected in a sterile container
- The male patients were instructed to retract the foreskin. The first portion of voided urine should be discarded and collect midstream urine in a sterile container.

The collected midstream urine sample was inoculated into cysteine lactose electrolyte deficient agar media (CLED), Blood agar, and MacConkey agar. The inoculated plates were incubated at 37°C for 24 hours for any bacterial growth (Cheesbrough, 2000).

5.1.4 Swabs:

The specimens included in this category were ear pus discharge, throat, and high vaginal swab. The ear pus discharged was collected by inserting sterile swab sticks with rotating the swab stick. In throat swab sampling, the tongue of the patient was pressed with help of a tongue depressor and scrubbed on the inflamed area on the back of the pharynx and tonsil with help of a sterile cotton swab. The high vaginal swab (HVS) specimens were collected by inserting a sterile swab stick into the upper part of the vagina and uniformly rotated before withdrawing. All the three specimens after collection on swab stick were inoculated into mannitol salt agar (MSA), blood agar (BA), nutrient agar (NA), and

chocolate agar (CA) and further incubated at 37°C for 48 hours for bacterial growth (Cheesbrough, 2000).

5.1.5 Sputum:

The patient was advised to rinse their mouth for removal of excess oral flora with water. The sputum was collected in the sterile container through deeply coughing of patients. One loop full of sputum was inoculated into blood agar, mannitol salt agar, and NA plates. The streaked plates at 37°C for 48 hours were incubated for bacterial growth (Cheesbrough, 2000).

5.1.6 Body fluids:

Different fluids from the patient's body are collected through different modes. It includes ascetic, synovial, pleural, and cerebrospinal fluids. The synovial fluid, pleural fluid, and ascitic fluid were collected through aspiration with a sterile syringe and collect 9ml of fluid into a screw-capped tube or bottle containing 1ml of sterile trisodium citrate. The cerebrospinal fluid (CSF) was collected from vertebral puncture at arachnoid space. CSF sample was collected from 4^{th} and 5^{th} lumbar puncture in a container with the quantity of 2-3ml. the collected fluids were inoculated on NA, MSA, and BA media. Inoculated media at 37°C for 48 hours were incubated. The media were inspected for bacterial growth (Cheesbrough, 2000).

5.1.7 Stool:

A spoonful of stool containing blood, pus, and mucus was collected in a wide mouth plastic container. The sample was processed into MSA, MA, and BA media and incubated in an incubator at 37°C for 48 hours. The media was observed for bacterial (Cheesbrough, 2000).

5.2 Examination of bacterial growth:

After incubation for 18-24 hrs, different colonial morphology produced by different bacteria was recorded and has been shown in Table 5.1. The colonies were identified by phenotypic criteria as per Marmion 1996; Bailey and Scott's, 2018.

a) Gram Staining- gram positive and gram negative organisms
b) Preliminary tests like Catalase, Coagulase and Oxidase were performed.

c) Colonies morphology on SIM, TSI, MRVP, Urease and Citrate agar media.

Table 5.1: Different colonies morphology of isolated bacteria

Isolated Bacteria	Colony Morphology
Staphylococcus aureus	Blood agar: 1=3 mm diameter,densely opaque and butyrous consistency, glistening, smooth, low convex,Creamy yellow colony surrounded by narrow zone of beta haemolysis Mannitol salt Agar and Nutrient agar: Golden yellow colony
Streptococcus spp.	Blood Agar: Creamy white small colony with 0.5- 1 mm diameter, semi-transparent, low convex, discrete colony with matt or glossy surface
Coagulase Negative Staphylococcus (CONS)	Blood agar: 1=3 mm diameter, densely opaque and butyrous consistency, glistening Creamy white colony, smooth,low convex
Klebsiella pneumoniae	MacConkey agar: Large mucoid pinkish red colony
Escherichia coli	MacConkey agar: Smooth, dry, flat pink red colony Eosin Methylene Blue agar: greenish metallic colony
Pseudomonas aeruginosa	MacConkey agar: Faint yellowish flat colony, sticky consistency Blood agar: Spreading and flat. Serrated edges, confluent growth
Enterobacter spp.	MacConkey agar: Large mucoid pinkish white colony
Proteus spp.	MacConkey agar: Faint yellow colony

5.3 *S. aureus* identification*:*

S. aureus is identified through colonies characterization as it produced opaque, golden yellow or cream-colored on nutrient agar, mannitol salt agar, and β hemolysis on the blood agar (Winn, 2006). *S. aureus* identification was made by application of gram staining, coagulase and catalase biochemical tests for confirmation.

5.3.1 Microscopy:

Gram stain: Gram's staining was performed as per the standard procedure to visualize the irregular plane of division for grape-like cocci arrangement in *S. aureus* morphology under microscope. A smear of colonies of *S. aureus* was first prepared on a clean glass slide in gram staining. The slide was air-dried and heat-fixed to prevent smear from washing off during staining. The primary stain crystal violet was put on smear for one minute. After 1min. it was distilled water used to wash the smear. The mordant Grams iodine was applied to wash smear for 1min. The slide was treated with a decolorizing agent like Acetone for 5 to 10 seconds. The slide was again washed with tap water. Counterstain (Safranine) was poured on the slide and let slide stand for 30 seconds. The final wash was done with tap water. The slide was blot dry and observed under 40X in the compound microscope (Duguid 1996).

5.3.2 Catalase test: The catalase test was performed to distinguish *Staphylococcus* from *Streptococcus* which is catalase-negative. Catalase is an enzyme that has the ability to breakdown hydrogen peroxide (H_2O_2) into H_2O and O_2.

$$\mathbf{2H_2O_2 \text{——} 2H_2O + O_2\uparrow}$$

Using sterile inoculating needle, loop full of bacterial pure culture was picked and put on a clean glass slide. Few drops of freshly prepared 3% H_2O_2 were added. The formation of bubbles indicates catalase-positive bacteria (Winn, 2006).

5.3.3 Coagulase test: This test is the specific and gold standard for the identification of *S. aureus* from coagulase-negative *Staphylococcus* (CONS). The principle of coagulase is the conversion of fibrinogen into fibrin to form clots within contact with human or rabbit plasma. There are two types of coagulase tests; tube coagulase and slide coagulase. In the procedure of slide coagulase test, on a clean glass slide, few drops of 0.9% NaCl (normal saline) were transferred. Bacterial colonies were taken from the culture plate with the help of inoculating loop and emulsified properly in normal saline. Finally, 2-5 drops of plasma were mixed in suspension on the slide and seen clotting reaction within 15 seconds (Baird, 2012).

5.4 Methicillin resistant *S. aureus* (MRSA) screening:

5.4.1 Oxacillin disk diffusion method:

All the isolated *S. aureus* was subjected to 1µg oxacillin discs. The suspension of *S. aureus* equivalent to 0.5 Mc Farland standards was prepared. Lawn culture of suspension of *S. aureus* was prepared on Muller Hinton agar (MHA) media. One oxacillin disc (1µg) was put on an MHA plate and incubated at 37°C for 24 hours. The sizes of the zone of inhibition (ZOI) in mm was recorded as per standard of CLSI, 2011 viz. resistant, ≤10 mm; intermediate, 11-12 mm and susceptible, ≥ 13.

5.5 Screening of Inducible and Constitutive clindamycin resistant *S. aureus* (iMLSB and cMLSB):

5.5.1 Screening of erythromycin resistant *S. aureus*;

All the isolated *S. aureus* was subjected to 15µg erythromycin discs. The suspension of *S. aureus* equivalent to 0.5 Mc Farland standards was prepared. Lawn culture of suspension of *S. aureus* was prepared on Muller Hinton agar (MHA) media. One erythromycin disc (15µg) was put on an MHA plate and incubated at 37°C for 24 hours. The sizes of the zone of inhibition (ZOI) in mm were recorded as per standard of CLSI, 2011 viz. resistant, ≤13mm.

5.5.2 D-test:

The erythromycin-resistant *S. aureus* was subjected to 15µg erythromycin and 2µg clindamycin in D-test. The suspension of erythromycin-resistant *S. aureus* equivalent to 0.5 Mc Farland standards was prepared. Lawn culture of suspension of erythromycin-resistant *S. aureus* was made on an MHA plate. Two antibiotic discs (clindamycin 2µg and erythromycin 15µg) were placed at a distance of 15mm apart from edge to edge on processed MHA plate and incubated at 37°C for 24 hours. The appearances of the size of ZOI were recorded and interpreted as per the standard of following Steward *et al*., 2005.

5.5.2.1Inducible MLSB phenotype (iMLSB): The zone size was interpreted as per Steward *et al*. 2005: zone size ≤13mm (erythromycin resistant) and zone size ≥21mm (sensitive to clindamycin) called iMLSB strains of *S. aureus* isolates.

5.5.2.2 Constitutive MLSB phenotype (cMLSB): The zone size was interpreted as per Steward *et al*. 2005: zone size ≤13mm (erythromycin resistant) and zone size ≤14mm (clindamycin resistant) are called cMLSB strains of *S. aureus*phenotypes.

5.6 Collection and extract preparation from plant leaves:

5.6.1 Collection of plant leaves:

The leaves of five plants, *Nyctanthes arbortristis*, *Azadiracta indica*, *Syzygium cumini*, *Tinospora cordifolia* and *Moringa oleifera* were collected in October 2017 from different locations in and around Bara (27° 01' 60.00" North latitude &85° 00' 0.00" East longitude) and Parsa, Birgunj (27°02'31.2106" North latitude and 084°52'27.1720" East longitude), province-2, Nepal. All the leaves were identified and confirmed in the Department of Dravya Gun, Nepal Ayurveda Medical College and Teaching Hospital, Birgunj, Nepal.Plant species, local name, and parts used are shown in Table 5.2.

Table 5.2: Medicinal plants

Medicinal plants	Common Name	Family	Parts used	Place of collection
Syzygium cumini	Jamun	Myrtaceae	Leaves	Parsa
Azadirachta indica	Neem	Meliaceae	Leaves	Bara
Moringa oleifera	Shajan	Moringaceae	Leaves	Parsa
Nyctanthes arbortristis	Ratrani	Oleaceae	Leaves	Parsa
Tinospora cordifolia	Giloy	Menispermaceae	Leaves	Parsa

5.6.2 Preparation of leaves powder:

The leaves were washed with running tap water properly and left for seven days under shade for drying. After shading, the leaves were dried under a hot air oven at 50°C for 4-6 hours for removing excess moisture. A fine powder of leaves was made by grinding into a grinder (Azwanida, 2015).

5.6.3 Extraction through maceration method:

The powdered leaves (50gm) were put in five different conical flasks with 300ml of solvents viz. water, 80% ethanol, methanol, acetone, and n-hexane for seven (7) days with intermittent shaking (Handa, *et al.,* 2008). The suspension was filtered by muslin cloth followed by Whatman filter paper No. 1. The obtained filtrate was subjected to a hot air oven at 40°C for evaporation. The weight of residues was recorded and stored at 4°C for further experimentation (Mohamed *et al*., 2010).

5.7 Phytochemicals screening:

The qualitative screening of phytoconstituents, alkaloids, flavonoids, phenolics, saponins, steroids, tannins, couramins, and quinones form solvent extracts were performed through following use of different chemical tests;

5.7.1 Alkaloids; Alkaloids was screened through Wagner's test. The test is based on the principle of potassium alkaloid complex formation. In this test, 3ml of plant extracts is mixed with Wagner reagent in a test tube. The reddish-brown pigment formation indicates alkaloids in tested plant extract (Surendra *et al.,* 2016).

5.7.2 Terpenoids; the test of terpenoids is performed by Salkowski test based on the principle of formation of bi-sulphonic acid of bi-cholestadiene. In this test, five ml of plant extract is mixed in 3ml conc. H_2SO_4 and 2ml chloroform. The reddish-brown pigment formation indicates the positivity of terpenoids in plant extracts (Bhandari *et al.,* 2017).

5.7.3 Tannins; It is detected through the Ferric chloride test which is based on the principle that phenolics react with iron salt. In this test, 5 ml of extract is treated with 1ml of 1% $FeCl_3$ solution. Green or blue-green or bluish-black formation showed tannins in plants (Al-Daihan *et al.,* 2013).

5.7.4 Saponins; The saponins detection is based on the principle of the presence of glycosides that have the ability to produce foam in water hydrolyzed in glucose and other compounds. In this test, distilled water of 2 ml put in a test tube containing 2ml of plant extract. The suspension was transfer into a graduated cylinder to make volume up to 20 ml and shaken for 15 minutes. The appearance of foam indicates the presence of saponins (Al-Daihan *et al.,* 2013).

5.7.5 Steroids; the Salkowski test is used for the detection of steroids based on the principle of formation of bi-sulphonic acid of bi-cholestadiene. An equal volume (10ml) of conc. H_2SO_4 and chloroform were mixed in a test tube containing 1ml of extract. The formation of two different colors viz. red at the upper layer and yellow in the lower layer of suspension indicates a positive test for steroids in extracts (Bhandari *et al.,* 2017).

5.7.6 Quinones; Screening of quinones is based on the principle of reduction of quinones to the corresponding arenediols. 1ml of plant extract is mixed with 1ml of Conc. H_2SO4 in a test tube. The formation of the red color showed the presence of quinones in plant extracts (Bhandari *et al.,* 2017).

5.7.7 Flavonoids; the flavonoids were tested by a mineral acid test based on the principle of formation of the colored complex. In this test, the plant extract is mixed with ammonia solution and Conc. H_2SO4. The result of yellow color indicates the positive presence of flavonoids (Surendra *et al.,* 2016).

5.7.8 Phenols; the presence of phenol is tested by ferric chloride test based on the principle of the formation of a complex between phenols and iron ion. In this test, 1ml of extracts taken in a test tube is mixed with few drops of 1% $FeCl_3$. The observation of green color indicates the positive presence of phenol (Surendra *et al.,* 2016).

5.7.9 Coumarins; for coumarin observation, alkaline reagent test based on the principle of the colored complex formed between alkaline solution and couramins present. In this test 2ml of plant extract is mixed with 3ml of 10% NaOH solution in a test tube. The observation of yellow color indicated the positive presence of coumarins in plant extract (Bhandari *et al.,* 2017).

5.8 Antibacterial activity of plant extracts:

5.8.1 Preparation of Plant extracts:

Plant extracts in four concentration viz. 25mg/ml, 50mg/ml, 100mg/ml, and 200mg/ml were prepared as per Bhandari et *al.* 2017. Two solvents one is water used for aqueous extract and the other is dimethyl sulfoxide (DMSO) used for the preparation of different four concentrations of plant extracts.

5.8.2 Preparation of bacterial inoculums:

3-5 isolated colonies of iMLSB and cMLSB shairing common MRSA was inoculated into 5ml of BHI broth for inoculums preparation. The BHI broth media was incubated at 37°C for 1 hour. After that, the turbidity of inoculum bacterial suspension was made equivalent to the 0.5 McFarland standards (Arora & Kaur, 2009).

5.8.3 Agar well diffusion method:

The bacterial suspension was made lawn culture on MHA media. Five wells with 8mm diameter in the media of each plate were bored with the help of a sterile cork borer. 0.1ml of plant extracts from five solvents with four concentrations were added in four wells. In the fifth well 0.1ml of DMSO solution was added as the negative control and 30µg linezolid disc was placed as the positive control. The processed MHA media plates were incubated at 37°C for 24 hours. The size of the zone of inhibition was measured in triplicate mode (Olurinola, 1996).

5.9Liquid Chromatography Mass Spectroscopy (LCMS):

Analyses were conducted at SAIF, CDRI, Lucknow, India. It was carried out using a Waters, XEVO-TQD#QCA1232 having column compartment, autosampler, binary pump, and vacuum degassed. For chromatography separation, a SUNFIRE C-18, 250 × 4.6, 5 µm was used. The column compartment was held at 95% B (acetonitrile), 1.5% C (CH_4 OH), 1% D (0.1% HCOOH) for 0–1 min,95–70% B (acetonitrile), 1.5% C (CH_4 OH), 6% D (0.1% HCOOH) for (1–6 min), 70–40% B (acetonitrile), 1.5% C (CH_4OH) , 6% D (0.1% HCOOH) for (6–12 min), 40% B (acetonitrile) , 1.5% C (CH_4OH), 6% D

(0.1% HCOOH) for (12–16 min), 40–20% B (acetonitrile) , 1.5% C (CH_4OH) , 6% D (0.1% HCOOH) for (16–20 min), 20% B (acetonitrile), 1.5% C (CH_4OH), 6% D (0.1% HCOOH) for (20–24 min) and re-equilibrated by 20–95% B (acetonitrile), 1.5% C (CH_4OH) , 6%D (0.1% HCOOH) for 24–26 min and finally held at 95% B (acetonitrile), 1.5% C (CH_4OH) and 6% D (0.1% HCOOH) between 26 and 30 min. The following parameters were used throughout the column: auto-injection volume (25 µl), column temperature (30°C), the maximum pressure (300 bar), and VWD (225nm). The column outlet was coupled to a XEVO-TQD detector with an electrospray ionization interface (ESI) ion sources. In MS both positive and negative ion at capillary voltage 3.5 kV was set and followed by 350°C temperature, desolvation gas flow rate to 950 L/Hr, collision energies for MS1 and MS2 was set at 3 and 20 respectively (Waters, 2009) .

5.10 Molecular docking:

MolecularDocking was performed for active phytocompounds (ligands) of finalized plant extract to PBP2a from MRSA and to ERM from MLSB resistant strains of *S. aureus* as per the method of *i*GEMDOCK V 2.1(Kai-Cheng *et al.* 2011).

5.10.1 Protein structure retrieval:

The information about the two targets studied proteins; PBP2a with PDB ID: 1mwt and ERM with PDB ID: 3j7z was collected from an online protein data bank (PDB).The detail structure of any proteins and nucleic acids is retrieved from PDB database with different four-word identification codes (Kai-Cheng *et al.* 2011).

5.10.1.1 Prediction of active site on target proteins:

The sequences amino acids on the active site of both target proteins (PBP2a & ERM) were identified through the use of CASTp (Computes Atlas for Surface Topography of proteins (Dundas *et al.,* 2016).

5.10.2 Ligands structure retrievals:

Twenty different types of compounds have been identified from the interpretation of LCMS data of leaves of *S. cumini*. These compounds were chosen for the ligands for purpose of docking studies. The information about the structure, molecular weight,

molecular formula of the ligands, and one control drug Linezolid was retrieved from the Pubchem database (Kim *et al.,* 2015).

5.10.3 Docking module:

5.10.3.1 *i*GEMDOCK:

The *i*GEMDOCK software was run to dock 20 different identified compounds to two target proteins, PBP2a and ERM. The following parameters were used in the docking method: the number of runs was set to 10; maximum interaction was 2000; population size was 200 and the energy threshold was set to 100. The values of bond energies are varied according to the electrostatic, Van der wall's (VdW) interactions and hydrogen bonding between proteins and ligands. The compounds showing the lowest score in negative was preferred compound in comparison to others (Kumar *et al.,* 2016).

5.11 Drug likeliness properties analysis:

5.11.1 Lipinski's rule of five:

The lipinski's rule or shortly rule of five (ROF) is widely used for the characterization of drug likeliness of compounds. It was done through the run of free available online software of Molinspiration. It provides information about logP, molecular weight, violation, and number of hydrogen bond donors and acceptors (Lipinski *et al.,* 2012).

5.11.2 ADME and Toxicity properties of bioactive compounds:

The pharmacokinetic properties of studied compounds were studied in term of absorption, digestion, metabolism, excretion, and toxicity (ADMET). Freely available online SwissADME software package (Kramer *et al.,* 2017) and admet SAR (Cheng *et al.,* 2012) were used.

CHAPTER-VI

6. Results and discussion

6.1 Introduction

The emergences of new strains of antibiotic-resistant microorganisms are a serious global issue. Among different strains of antibiotic-resistant bacteria, *S. aureus* in two different phenotypes; MRSA and MLSB resistant *S. aureus* are listed as superbug to cause great killer in patients caused by them. Thus, the investigation aims at studying the prevalence of the nature of *S. aureus* and their resistant forms; MRSA, iMLSB, and cMLSB among clinical samples. The limited numbers of antibiotics are available to treat infection caused by these superbugs so it encourages finding out the product of plants that shows antibacterial activity against these superbug. The plant product which showed the best antibacterial activity was further processed for molecular docking and its pharmacokinetic evaluation.

6.2 Clinical sample collection:

The clinical samples have been processed in National Medical College and Teaching Hospital, a Tertiary Care Hospital in Birgunj, Parsa, and Province-2. Patient samples covering eight districts from Province-2 state have been taken in the present investigation. The names of the districts are Bara, Parsa, Sarlahi, Rautahat, Mahotri, Dhanusha, Sirha and Saptari. Clinical samples were collected from patients in case of bacteremia, upper and lower respiratory infection, soft tissue infection, sepsis, neurologic, gynecologic, and urinary tract infection. Seven types of clinical specimens which were categorized as blood, pus, body fluids, urine, swab, stool, and sputum are selected. The samples included in this study are of 11-month duration i.e. from February 2017 to December 2017. The percentage distribution of different samples among 2000 clinical samples has been shown in Figure 6.1. The result shows the percentage distribution of samples with a higher number from urine specimens (636; 31.8%)

followed by blood (529; 26.45%), pus (488; 24.4%), sputum (136; 6.8%), body fluids (101; 5.05%), swabs (87; 4.35%) and stool (23; 1.15%) specimen.

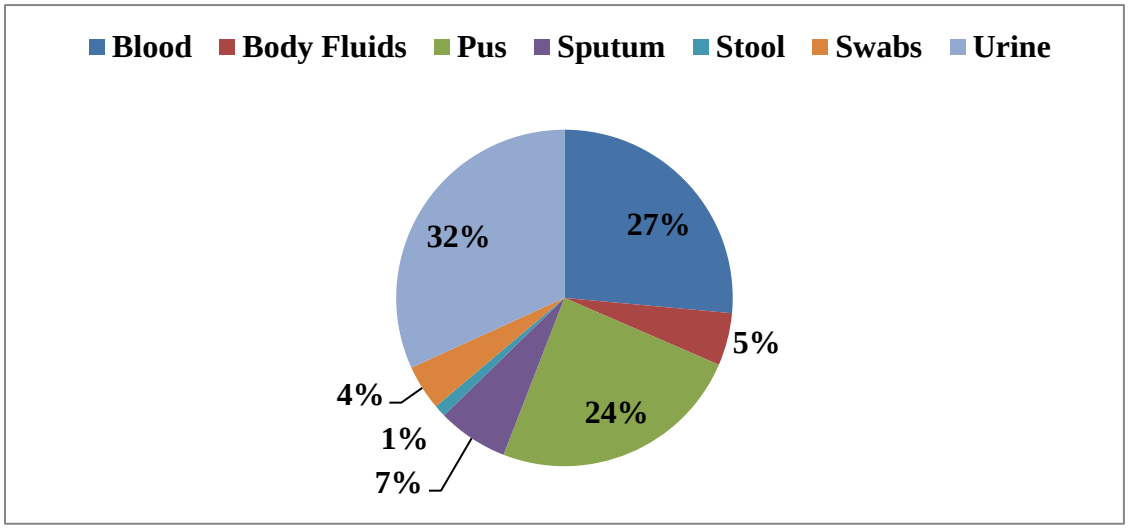


Figure 6.1: Percentage distribution of different clinical specimens

6.3 Bacterial growth:

Collected clinical samples showed 867 (43.35%) samples with positive bacterial growth after processing the samples. The percentage distribution of positive bacterial growth in different samples has been shown in Table 6.1. The result shows the percentage distribution of positive bacterial growth with a higher number in pus 285 (32.85%) followed by urine 266 (30.68%), blood 258 (29.75), sputum 43 (4.95%), body fluids 9 (1.03%), and swabs 6 (0.69%). In the characterization of gram reaction among positive bacterial growth, gram-negative bacteria were found higher in number 465 (53.63%) than gram positive bacteria 402 (46.36%). Table 6.1 and Figure 6.2 have been shown the sample-wise percentage distribution of gram-negative bacterial isolates. The result shows among gram-negative bacteria, the higher number is *E. coli* 181 (20.8%) followed by *Klebsiella spp.*152 (17.5%), *Pseudomonas spp.* 83 (9.5%), *Enterobacter spp.* 23 (2.6%), *Proteus spp.* 19 (2.1%), *N. gonorrhoeae* 3 (0.3%), *Acinetobacter spp.* 2 (0.2%), *Salmonellaspp.* 1 (0.1%) and *Citrobacterspp.* 1 (0.1%). The percentage distributions of different gram-positive bacteria in different samples have been shown in Table 6.1 and

Figure 6.3. The result shows *S. aureus* were found higher in number 310 (35.7 %) followed by coagulase-negative *Staphylococci* (CONS) 51 (5.8%), *Streptococcus spp.* 29 (3.3%) and *Enterococcus spp.* 12 (1.3%) among gram-positive bacteria. In our study, gram-negative bacterial isolates were found higher in comparison to gram-positive bacteria. This finding is well articulated that gram-negative bacteria are the important causative agent of nosocomial infections such as sepsis, pneumonia, and meningitis. It is predominately isolated among different clinical samples collected from patients. The result of the prevalence of both gram-positive and gram-negative bacteria was similar to others by Abebe *et al.*, 2019; Trojan *et al.*, 2016.

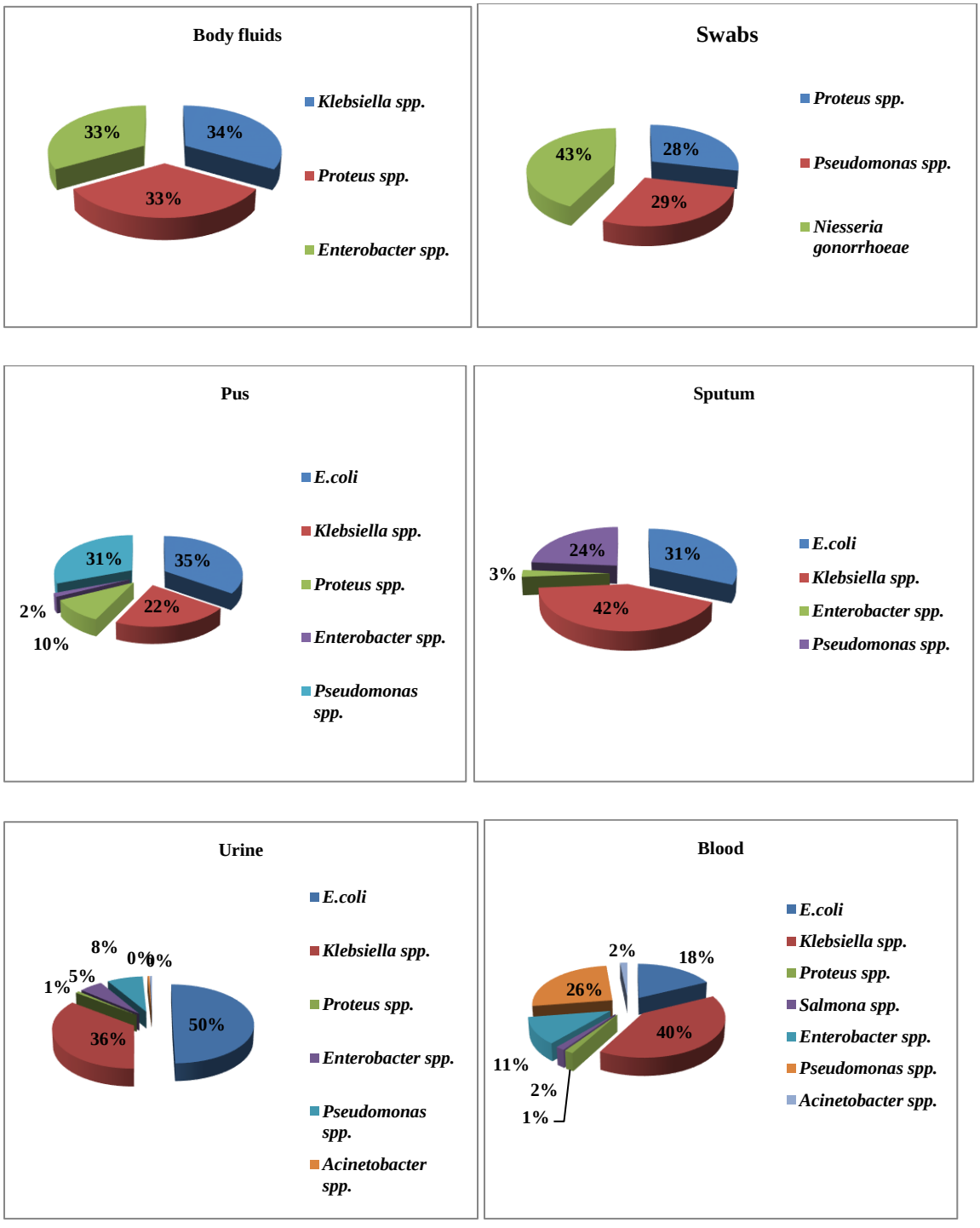


Figure 6.2: Percentage distribution of gram negative bacterial isolates among different samples

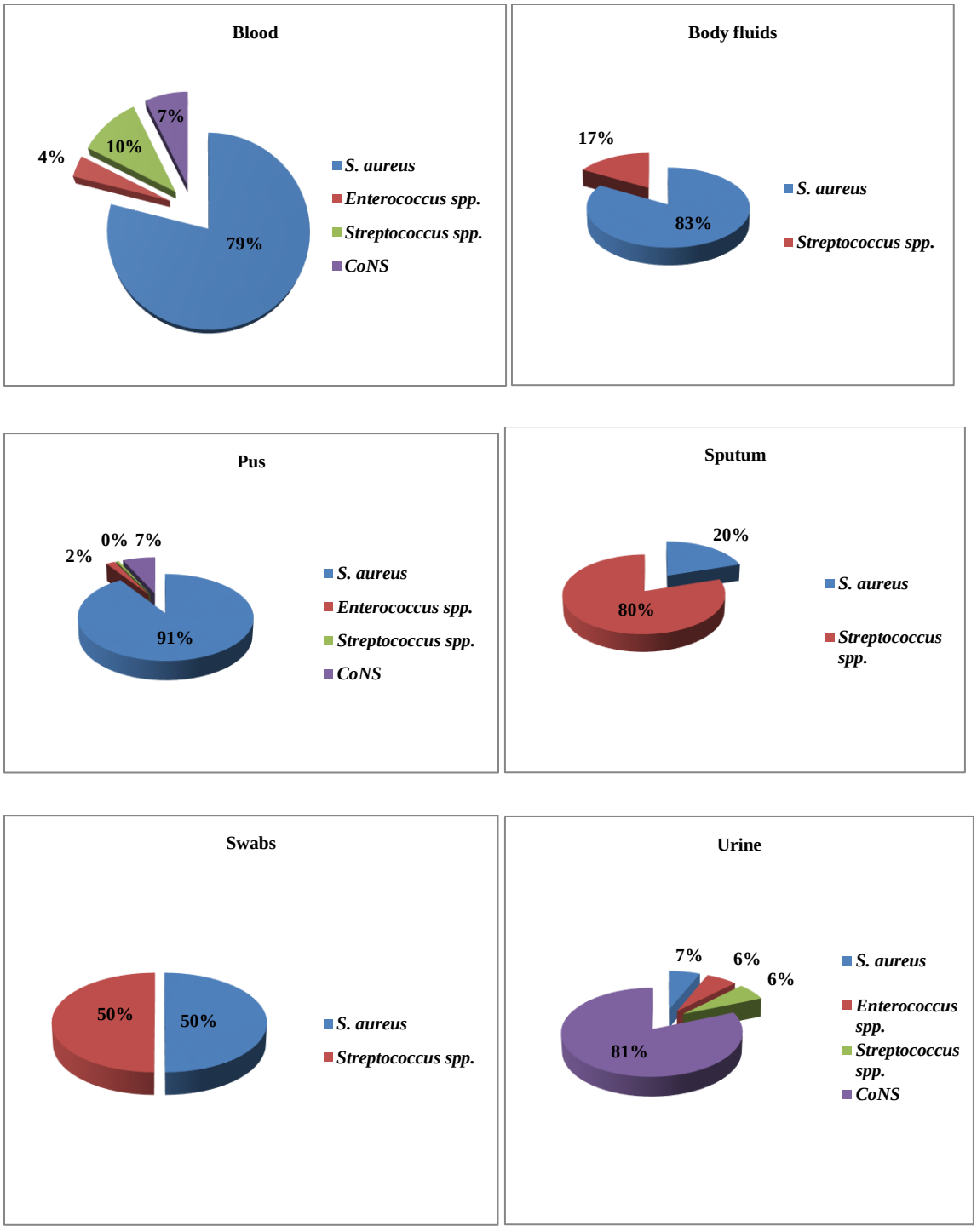


Figure 6.3: Percentage distribution of gram positive bacte

Table 6.1: Distribution of bacterial pathogens from different clinical samples

	Types of Samples							
Types of isolates	**Blood**	**Pus**	**Body Fluids**	**Sputum**	**Stool**	**Swab**	**Urine**	**Total (N %)**
E.coli	11(17.7%)	43(34.6%)	0	12(31.5%)	0	0	115(47.7%)	181(20.8%)
Klebsiella spp.	25(40.3%)	28(22.5%)	1(33.4%)	16(42.1%)	0	0	82(35.4%)	152(17.5%)
Proteus spp	1(1.6%)	13(10.4%)	1(33.4%)	0	0	2(28.5%)	2(0.8%)	19(2.1%)
Salmonela spp.	1(1.6%)	0	0	0	0	0	0	1(0.1%)
Enterobacter spp.	7(11.2%)	2(1.6%)	1(33.4%)	1(2.6%)	0	0	12(5.1%)	23(2.6%)
Pseudomonas spp.	16(25.8%)	38(30.6%)	0	9(23.6%)	0	2(28.5%)	18 (7.7%)	83(9.5%)
Acinetobacter spp.	1(1.6%)	0	0	0	0	0	1(0.4%)	2(0.2%)
Citrobacter spp.	0	0	0	0	0	0	1(0.4%)	1(0.1%)
N. gonorrhoeae	0	0	0	0	0	3(42.8%)	0	3(0.3%)
Total Gram negative bacteria	62(24%)	124(48%)	3(33.4%)	38(88.3%)	0	7(77.8%)	231(87.8%)	465(53.6%)
S. aureus	155(79%)	146(90.6%)	5(83.4%)	1(20%)	0	1(50%)	2(6.2%)	310(35.7%)
Enterococcus spp.	7(3.5%)	3(1.8%)	0	0	0	0	2(6.2%)	12(1.3%)

Streptococcus spp.	20(10.2%)	1(0.6%)	1(16.6%)	4(80%)	0	1(50%)	2(6.2%)	29(3.3%)
CoNS	14(7.1%)	11(6.8%)	0	0	0	0	26(81.2%)	51(5.8%)
Total Gram positive bacteria	196(75.1%)	161(56.4%)	6(66.7%)	5(11.6%)	0	2(22.2%)	32(12.1%)	402(46.3%)
Total	258(29.7%)	285(32.8%)	9(1%)	43(4.9%)	0	9(1%)	263(30.3%)	867(100%)

CoNS; Coagulase negative *Staphylococcus aureus*

6.4 Selection and identification of *S.aureus*:

6.4.1 Prevalence of *S. aureus* in clinical samples:

A total of 310 *S. aureus* was found to be present among clinical specimens. The percentage occurrence of *S. aureus* was 35.75% amongst all clinical specimens tested. The percentage occurrence of *S. aureus* was found to be highest in blood specimen 155 (50%) followed by pus 146 (47.09%), body fluids 5 (1.61%), urine 2 (0.65%), swab 1 (0.32%) and sputum 1(0.32%). *S. aureus* was not detected from stool specimens. The percentage occurrence of *S. aureus* in clinical specimens was shown in Figure 6.4. The rates of frequency of *S. aureus* from different clinical samples have shown a significant relationship with a value of P< 0.05. Our result about the rate of *S. aureus* isolates was in agreement with studies carried out by others (Dilnessa and Bitew 2016; Rajaduraipandi *et al.*, 2006; Gitau *et al*., 2018). In our result, the frequency of *S. aureus* isolates was more frequent in blood samples followed by pus. The finding of frequent isolates of *S. aureus* in blood samples was similar to other several studies carried out by Manishi *et al.*, 2005; Diekema *et al*., 1997and Schmitz *et al.* 1998. After blood sample, isolates of *S. aureus* was higher in the pus sample in comparison to others. These results were in agreement with other studies investigated by Gitau *et al*., 2018; Tiwari *et al*., 2009.

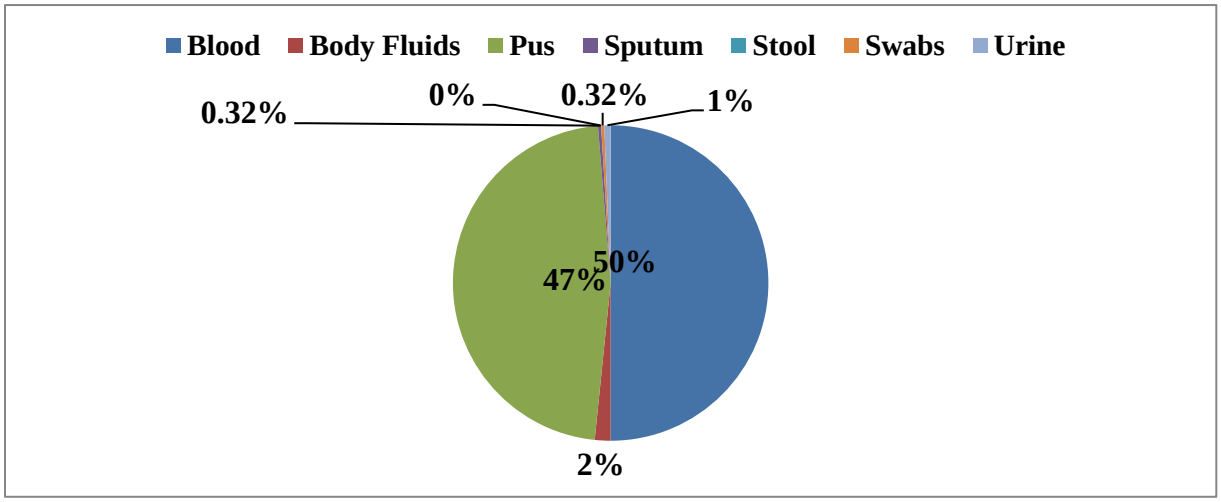


Figure 6.4: Sample wise percentage occurance of *S. aureus*

6.4.2 Gender wise and age wise occurance of *S. aureus*:

The selection of *S. aureus* was analyzed for the occurrence of organisms gender-wise and age-wise. The result has been shown in Figure 6.5 and 6.6. The result showed that males (51.61%) are more susceptible to *S. aureus* in comparison to female (48.38%). The data of age-wise occurrence of *S. aureus was* found higher among patients of 0-20 years of age 153(49.35%) followed by 21-40 years age 78 (25.16%), 41-60 years age 49 (15.80%), 61-80 years age 26 (8.38%) and >80 years age 4 (1.29%). The relationship between the rate of *S. aureus* isolates with gender was no significant with the value of P>0.05. But, the relationship between rates of *S. aureus* isolates with the age of patients was found significant with the value of P< 0.05. In our result, the frequent isolate of *S. aureus* was more in male patients than female. The reason for finding a high number of *S. aureus* in male patients might be due to visiting the maximum number of hospitals within cases of roadside and occupational injuries. Our results in gender-wise distribution of *S. aureus* agree to other studies carried out by Dilnessa *et al*., 2016; Deyno *et al.,* 2017; Geeta and Rama 2015 and Al-Zoubi *et al.*, 2015. On the basis of the different age group of patients, the result revealed that maximum numbers of isolates of *S. aureus* were found higher in age group 0-20 years (49.35%) followed by 21-40 years (25.16%). The 0-20 year's patient individuals are categorized into neonates, infants, children, and young peoples. The result of finding higher number isolates of *S. aureus* in age group 0-20 years is supported by other several studies of Chen *et al.*, 2017 and in >20-30 years aged with a value of 26.5% was supported by other studies by Deyno *et al.*, 2017.

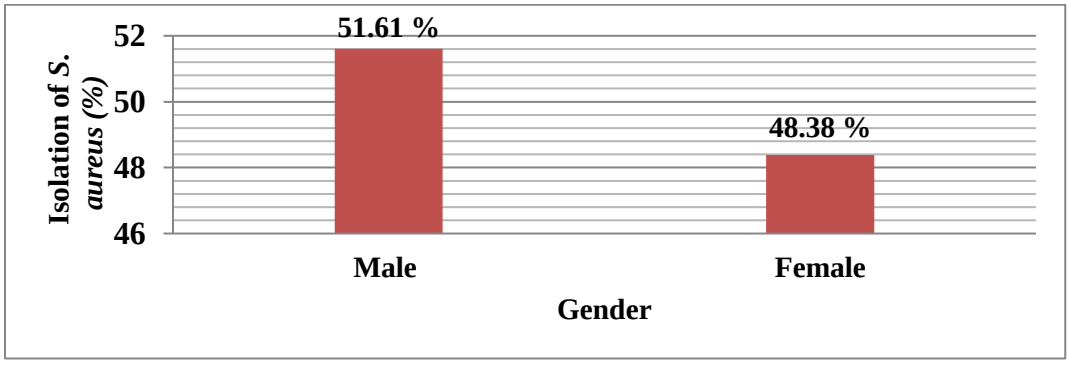


Figure 6.5: Percentage distribution of *S. aureus* with gender of patients

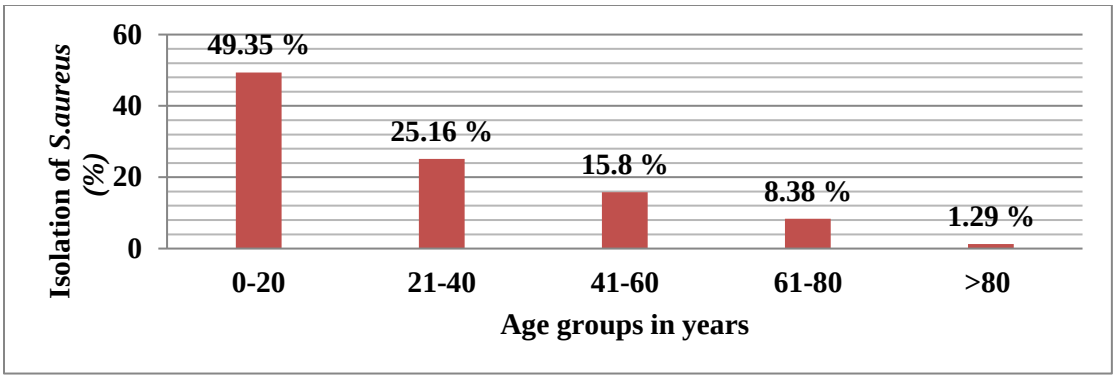


Figure 6.6: Age wise percentage occurance of *S. aureus*

6.5 Screening of MRSA and MSSA:

The emergence of MRSA in *Staphylococci* is made a serious problem in clinical practices for the treatment of patients. It showed resistance to all antibiotics of β-lactam and cephalosporin including vancomycin. The evaluation of the occurrence of MRSA and MSSA from isolated *S. aureus* strains was conducted in this study. The result has been shown in Figure 6.7 and it clearly depicts that the rate of isolation of MRSA was high with 242 (79.08%) in comparison to MSSA with 64 (20.91%). The result showed that MRSA is highly prevalent in comparison with MSSA in the studied *S. aureus* population. The prevalence of MRSA isolates is steadily increasing in the last few years and it lead to increased morbidity and mortality in both community and hospital peoples (Tong et *al.*, 2015). The increasing number of isolates of MRSA is due to some leading factors like lack of implementation of hospital control measure programs, insufficient implementation of prophylactic hygiene measures, indiscriminate use of different antibiotics and inadequate staff training. Related to our result, the diversified prevalence rate of isolates of MRSA was proved through several studies conducted by Baddour*et al.*, 2006; Blandino*et al.*, 2004.

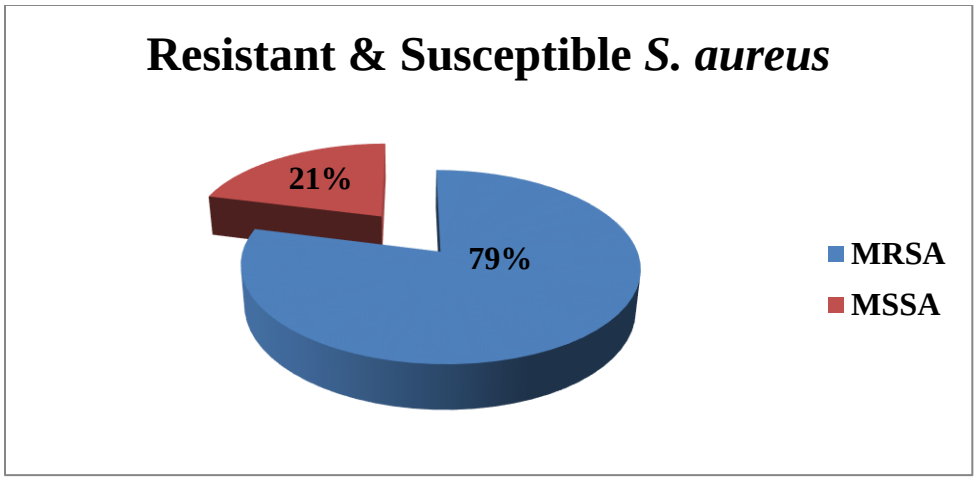


Figure 6.7: MRSA and MSSA among *S. aureus*

6.5.1 Percentage distribution of MRSA and MSSA in clinical samples

The number of MRSA was analyzed for their distributions in different clinical specimens were observed. The percentage distribution of MRSA and MSSA among specimens has been shown in Figure 6.8 and Table 6.2. The result clearly depicts the prevalence of MRSA was found to be higher in blood 135 (55.78%) followed by pus 101 (41.73%), body fluids 3 (1.23%), urine 1 (0.41%), sputum 1 (0.41%) and swabs 1 (0.41%). Similarly, MSSA was found to be higher in pus specimens 42 (65.62%) followed by blood 19 (29.68%), body fluids 2 (3.12%), and urine 1 (1.56%). The relationship between the rate of isolation of MRSA and MSSA among different specimens was showed significant relation with a value of $P< 0.05$. In our result, the isolates of MRSA was found maximum in a blood sample (55%) followed by pus (41%). The maximum isolates of MRSA in blood samples were in agreement with studies carried out by Ghahremani *et al.*, 2010. Pus is the second most important sample from which a higher number of MRSA isolates was found in comparison to other samples. Our result reports the finding of a higher number of MRSA from pus samples was in accordance with a study carried by Mohanasoundaram, 2011 showed that high prevalence of MRSA among pus 38% followed by swabs from different sites 25%, urine 17%, sputum7%, and blood 4%.

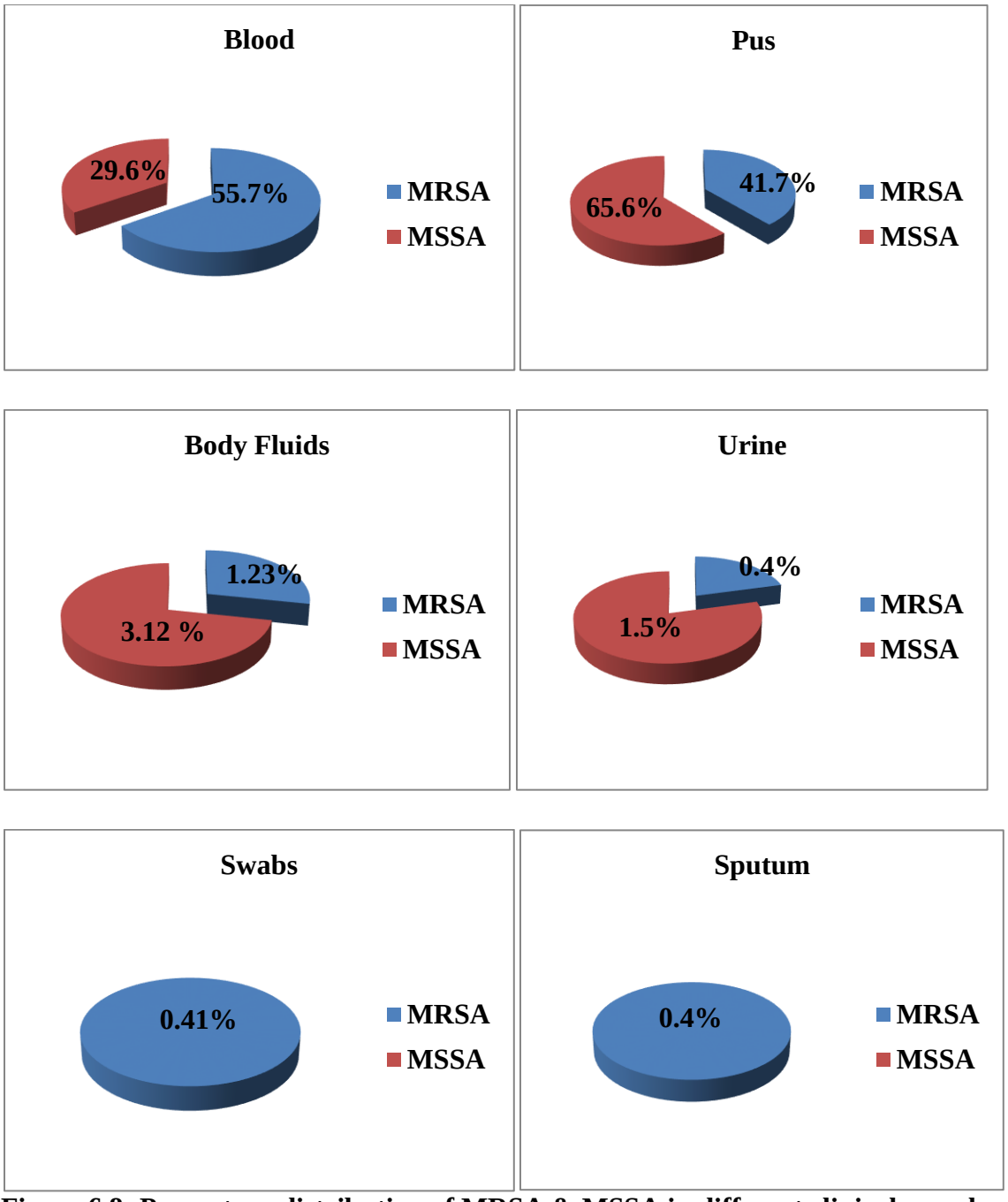


Figure 6.8: Percentage distribution of MRSA & MSSA in different clinical samples

Table 6.2: Percentage distribution of MRSA and MSSA among clinical samples

S. NO.	Clinical samples	MRSA (n=242)	MSSA (n=64)
1.	Blood	135 (55.78%)	19 (29.68%)
2.	Pus	101 (41.73%)	42 (65.62%)
3.	Body Fluids	3 (1.23%)	2 (3.12%)
4.	Urine	1 (0.41%)	1 (1.56%)
5.	Swabs	1 (0.41%)	00
6.	Sputum	1 (0.41%)	00
7.	Stool	00	00

6.5.2 Gender wise distribution MRSA and MSSA

The prevalence rate of MRSA and MSSA was studied in different gender. The data of frequency distribution of both MRSA and MSSA with genders has been shown in Figure 6.9. The data clearly shows that male individuals with 129 (53.30%) are more susceptible than the female with 113 (46.69%) for MRSA infections. Similarly for MSSA infection, the males 33 (51.56%) are slightly more susceptible in comparison with female 31 (48.43%). The relation between prevalence rate of MRSA with gender was found significant with the value of $P<0.05$. Instead, the relationship between prevalence rates of MSSA with gender was found no significant with the value of $P> 0.05$. The prevalence rate of isolation of MRSA and MSSA was found higher in male than the female which is in agreement with other studies of Khanal and Jha, 2010 in which isolation rate of MRSA is significantly higher in males (75%) than females (35%) reported. Kupfer *et al.,* 2010 was shown that a higher number of isolates of MRSA was found in males in comparision to females.

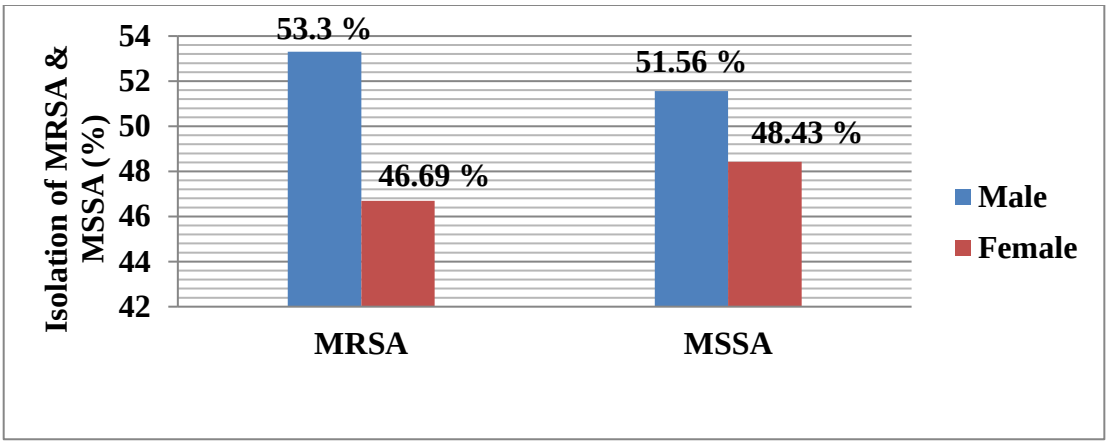


Figure 6.9: Percentage distribution of MRSA & MSSA with genders

6.5.3 Age wise occurance of MRSA and MSSA

The prevalence rate of MRSA and MSSA has been studied in different age groups. The prevalence rate of MRSA and MSSA with the age of patients has been shown in Table 6.3. The result clearly depict that MRSA is highly prevalent among the 0-20 age group with 138 (57.02%) followed by 21-40 age group with 80 (33.05%), 41-60 age group with 19 (7.85%), 61-80 age group with 04 (1.65%) and >80 age group with 01 (0.41%). Similarly among MSSA, the prevalence rate of MSSA was 29 (45.31%) in the age group of 0-20 years followed by 27 (42.18%) in the age of 21-40, 06 (9.37%) for the age of 41-60, and 02 (3.12%) in the age group of 61-80 years. The relationship between the prevalence rate of both MRSA and MSSA with the age of patients was found significant with the value of $P<0.05$. In our study, the number of isolation of MRSA and MSSA were found higher in the age group of 0-20 patients. This result is in accordance with several studies showed about the diversity of isolation of MRSA among clinical samples of different age groups viz. Jayakumar *et al.*, 2013; Al-Mayahie *et al.*, 2015.

Table 6.3: Percentage distribution of MRSA & MSSA among age of patients

S.NO.	Age in years	MRSA	MSSA
1.	0-20	138 (57.02%)	29 (45.31%)
2.	21-40	80 (33.05%	27 (42.18%)
3.	41-60	19 (7.85%)	06 (9.37%)
4.	61-80	04 (1.65%)	02 (3.12%)
5.	>80	01 (0.41%)	00

6.6 Screening of Erythromycin resistant *S. aureus*:

In gram-positive microorganisms including *S. aureus*, erythromycin has been widely used in place of cephalosporin and penicillin group of antibiotics treatment. However, the worldwide evolution of resistant strains against erythromycin has limited the use of this antibiotic. The present study focuses on analyzing the frequency distribution rate of ERY-R *S. aureus* and ERY-S *S. aureus* among total isolated *S. aureus* strains. The result has been shown in Figure 6.10. The result clearly depicts that the rate of occurrence for ERY-R *S. aureus* was high with 204 (66.67%) in comparison to ERY-S *S. aureus* with 102 (33.34%). This finding of a higher number of ERY-R *S. aureus* among total isolates of *S. aureus* is in comparison to studies conducted by Deotale *et al.*, 2010; Mokta *et al.*, 2015; Tiwari *et al.*, 2020.

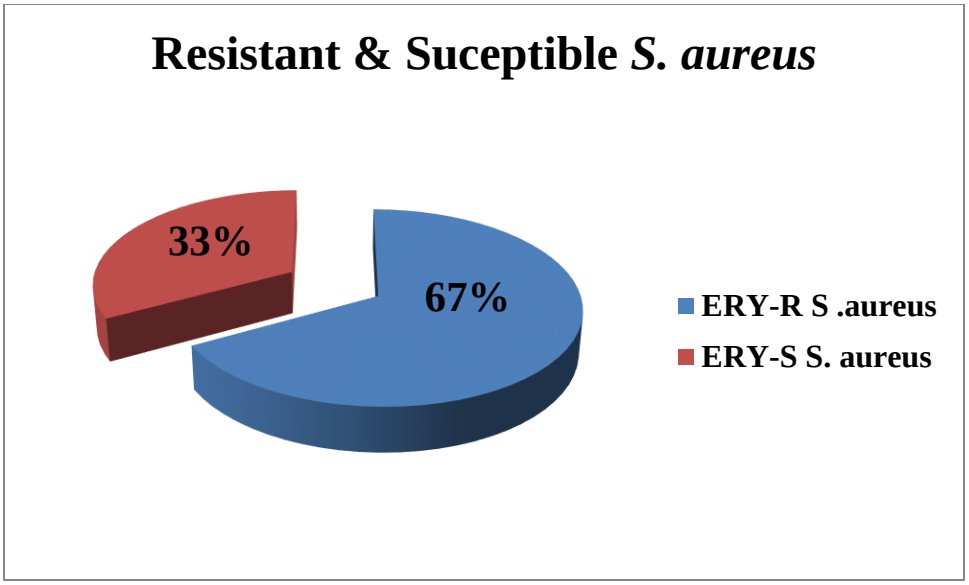


Figure 6.10: Percentage distribution of erythromycin resistant *S. aureus*

6.7 Selection of iMLSB and cMLSB:

6.7.1 Prevalence of iMLSB and cMLSB phenotypes among *S. aureus*:

The rationale of this research was to find out the percentage distribution of iMLSB and cMLSB resistant strains of *S. aureus.* The result has been shown in Figure 6.11. The result clearly shows that the occurrence of iMLSB strains of *S. aureus* was high with 132 (64.70%) in comparison to cMLSB strains of *S. aureus* with 72 (32.29%). The finding showed that iMLSB resistant *S. aureus* is highly prevalent in comparison with cMLSB resistant *S. aureus* in the studied population of *S. aureus*. These results agree to others investigators viz. Steward *et al*., 2005; Regha *et al*., 2016.

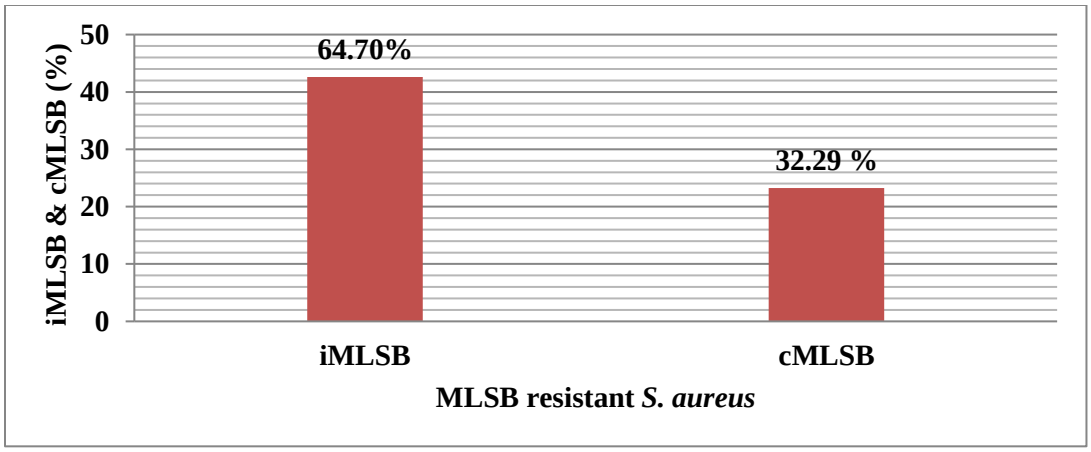


Figure 6.11: Percentage distribution of iMLSB and cMLSB among *S. aureus*

6.7.2 Percentage distribution of iMLSB and cMLSB among clinical specimens:

The percentage occurrence of iMLSB and cMLSB strains among specimens has been shown in Table 6.4. The result clearly shows that the occurrence of iMLSB strains was found to be higher in pus sample 76 (57.57%) followed by blood 54 (40.90%) and body fluids 02 (1.51%). Similarly, the cMLSB strains were found to be higher in blood sample 43 (59.72%) followed by pus 29 (40.27%). The relation between the prevalence of iMLSB and cMLSB strains in different clinical specimens was found significant with the value of $P<0.05$. Our results were in agreement with other studies by Manishi *et al.*, 2005; Diekema *et al.*, 1997.

Table 6.4: Percentage distribution of iMLSB and cMLSB

S. NO.	Clinical samples	iMLSB (n=132)	cMLSB (n=72)
1.	Blood	54 (40.90%)	43 (59.72%)
2.	Pus	76 (57.57%)	29 (40.27%)
3.	Body Fluids	02 (1.51%)	00
4.	Urine	00	00
5.	Swabs	00	00
6.	Sputum	00	00
7.	Stool	00	00

6.7.3 Gender wise percentage distribution of iMLSB and cMLSB strains:

The selection of strains of iMLSB and cMLSB strains of *S. aureus* were analyzed for the occurrence among total *S. aureus* in different gender. The result has been shown in Figure 6.12 and Table 6.5. The result showed that males 69 (52.27%) and 45 (62.5%) are more susceptible to iMLSB and cMLSB strains of *S. aureus* in comparison to female 63 (47.72%) and 31 (48.43%) respectively. The relation between prevalence of iMLSB and cMLSB strains of *S. aureus* with the gender of patients was found no significant with the value of P>0.05. The finding of a higher percentage of iMLSB and cMLSB in male than female is comparable to other studies conducted by Geeta and Rama, 2015; Al-Zoubi *et al.*, 2015.

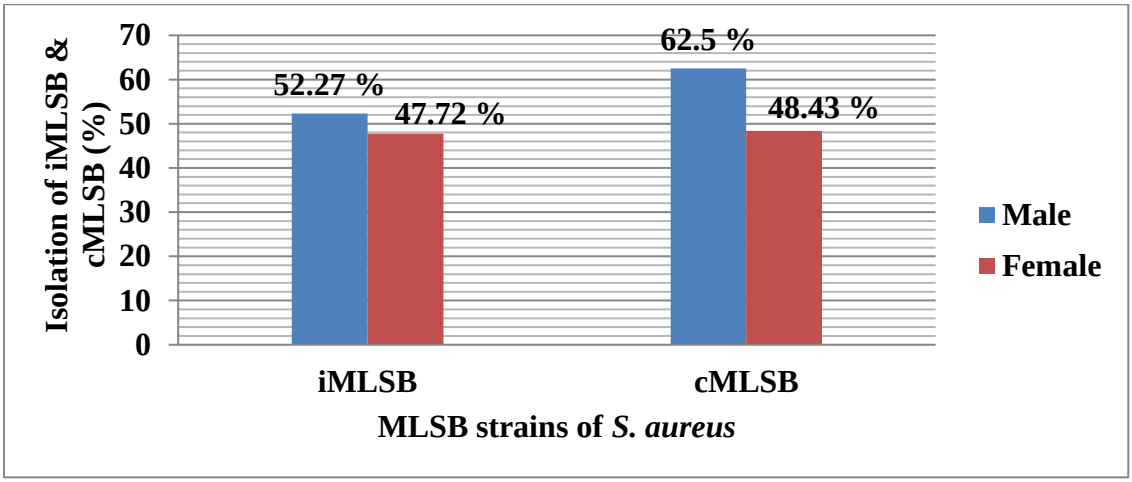


Figure 6.12: Percentage distribution of iMLSB and cMLSB strains among ERY-R *S. aureus*

6.7.4 Age wise distribution of iMLSB and cMLSB strains:

The selections of iMLSB and cMLSB strains were analyzed for the occurrence of bacteria in different age groups. The result has been shown in Table 6.5. The result of the age-wise distribution of iMLSB strains was found higher among patients of 0-20 age group with 73 (55.30%) followed by 21-40 age group with 47 (35.60%), 41-60 age group with 10 (7.57%) and 61-80 age group with 02 (1.52%). Similarly among cMLSB, the

prevalence rate of cMLSB strains were 38 (52.77%) in age group of 0-20 years followed by 23 (31.94%) in age of 21-40, 06 (9.37%) in age group 41-60, 03 (4.16%) in age group 61-80 and 01 (1.38%) in age group >80. The relation between prevalence of iMLSB strains of *S. aureus* with the age of patients was found significant with the value of P<0.05. Instead the relations between prevalence of cMLSB strains of *S. aureus* with age were found no significant with the value of P> 0.05. The results of our study are in agreement with others conducted by Al-Zoubi *et al.*, 2015.

Table 6.5: Prevalence distribution of iMLSB and cMLSB with age of patients

S.NO.	Age in years	iMLSB	cMLSB
1.	0-20	73 (55.30%)	38 (52.77%)
2.	21-40	47 (35.60%)	23 (31.94%)
3.	41-60	10 (7.57%)	07 (9.72%)
4.	61-80	02 (1.52%)	03 (4.16%)
5.	>80	00	01 (1.38%)

6.7.5 Selection of iMLSB and cMLSB strains among MRSA and MSSA strains

Among MRSA isolates, the selection rate of iMLSB strains of *S. aureus* was found to be 106 (43.80%) in comparison to cMLSB strains with 65 (26.89%). Similarly, among MSSA, the selection rate of iMLSB strains of *S. aureus* was found to be 26 (40.62%) in comparison to cMLSB strains with 7 (10.93%). The selection rate of iMLSB and cMLSB strains of *S. aureus* among both MRSA and MSSA has been shown in Figure 6.13. The relation between selection rate of iMLSB strains of *S. aureus* among both MRSA and MSSA was found significant with the value of P<0.05. Similarly, the relation between a selection of cMLSB strains of *S. aureus* in both MRSA and MSSA was found significant among MRSA with the value of P<0.05 and no significance among MSSA with the value of P>0.05. Both the inducible and constitutive clindamycin resistance phenotypes (iMLSB & cMLSB) were found significantly higher in MRSA (43.80% and 26.89%) in

comparison to MSSA (40.62% and 10.93%). These are results are supported by other studies carried by Gadepalli *et al*., 2006; Govindan *et al.,* 2014 and Mohapatra *et al*., 2009.

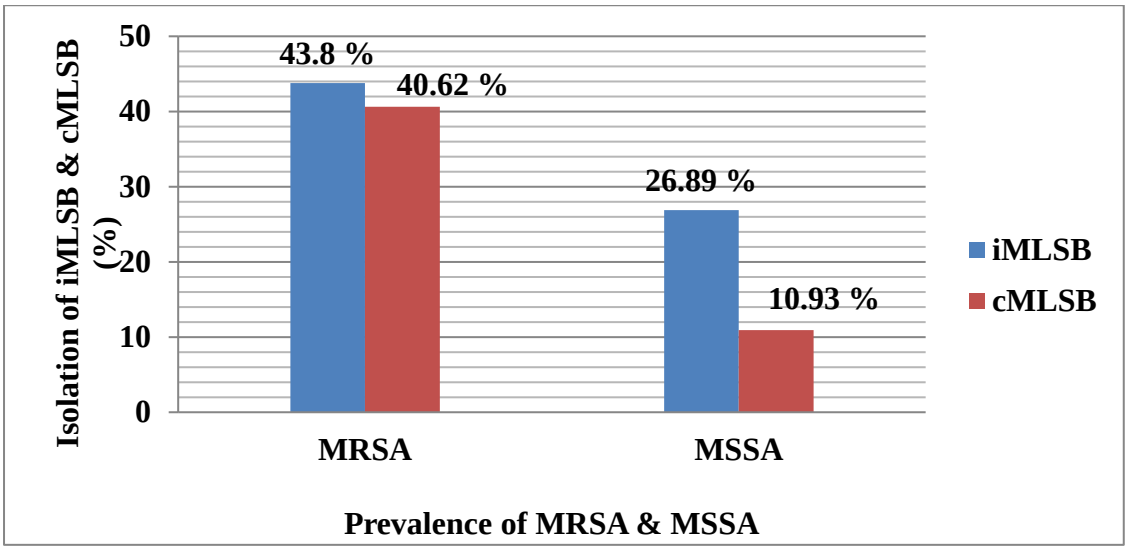


Figure 6.13: Percentage distribution of iMLSB & cMLSB in MRSA and MSSA

6.8 Preparation of coarse plant extract:

A large different number of plants with medicinal properties have been identified as the valuable source of natural antimicrobial products as an alternative for promotion of human health and potentially effective for the treatment of different diseases. Thus, in our present study, five different medicinal plants viz *Syzygium cumini*, *Azadirachta indica*, *Moringa oleifera*, *Nyctanthes arbortristis*and *Tinospora cordifolia* was used for secondary metabolite compounds characterization from natural sources. The extraction process involves various steps viz. grinding, milling, homogenization, and extraction for the study of bioactive compounds from the plants. The selection of method and solvents in extraction for bioactive compounds from the plant materials is an important step in phytochemical studies in the plant world. The present study uses five different types of solvents like distilled water and organic solvents (methanol, ethanol, acetone, and hexane) to extract phytoconstituents from mentioned plant leaves. The amount of crude

extract obtained by the maceration method was calculated and results were shown in Figure 6.14. The result clearly shows various extraction yields from different solvents. The yield (in grams) of water extraction was found to be in increasing order of *A. indica* (11.20±0.20) < *S. cumini* (13.6±0.2) < *N. arbortristis* (16.26±0.21) < *M. oleifera* (25.20±0.20) and *T. cordifolia* (37.90±0.10). In case of methanolic extracts, *A. indica* and *T. cordifolia* provided the least extraction yield with similar value (6.2±0.20), while *N. arbortristis* produced the highest yield (16.26±0.21). The extraction yield from methanol extraction was reported to be in increasing order of *A. indica* (6.2±0.20) and *T. cordifolia* (6.2±0.20) < *S. cumini* (11.2±0.1) < *M. oleifera* (12.23±0.25) < *N. arbortristis* (15.40±0.10). In 80% ethanolic extracts, *A. indica* resulted in less extraction yield in comparison to other plants. *N. arbortristis* produced the highest yield (20.43±0.15). The extraction yield from ethanol was found to be in increasing order of *A. indica* (8.23±0.66) < *T. cordifolia* (8.83±0.21) < *M. oleifera* (16.83±0.15) < *S. cumini* (18.1±0.1) < *N. arbortristis* (20.43±0.15). *A. indica* resulted in fewer yields (3.1±0.10) whereas *S. cumini* resulted in the highest yield (13.5±0.1) in organic solvent acetone. Acetone extracts yielded in increasing order of *A. indica* (3.1±0.10) < *T. cordifolia* (4.33±0.15) < *M. oleifera* (5.83±0.15) < *N. arbortristis* (8.63±0.15) < *S. cumini* (13.5±0.1). In hexane extracts, *S. cumini* resulted in fewer yields (0.3±0.05) in comparison to *N. arbortristis* which resulted the high yield (3.60±4.67). The yield from hexane extract was found to be in increasing order of *S. cumini* (0.3±0.05) < *A. indica* (0.76±0.05) < *T. cordifolia* (1.20±0.20) < *M. oleifera* (3.23±0.15) < *N. arbortristis* (3.60±4.67). The relationship between extraction yield of plant extracts with the use of different solvents was found to be significant with the value of $P<0.05$. The difference in extractive yields may be due to the presence of soluble extractive bio-compounds, variation in polarity of used solvents, structural differences of phytoactive compounds. These results are in agreement with other studies reported by Silva *et al.*, 2014; Felhi *et al.*, 2016a.

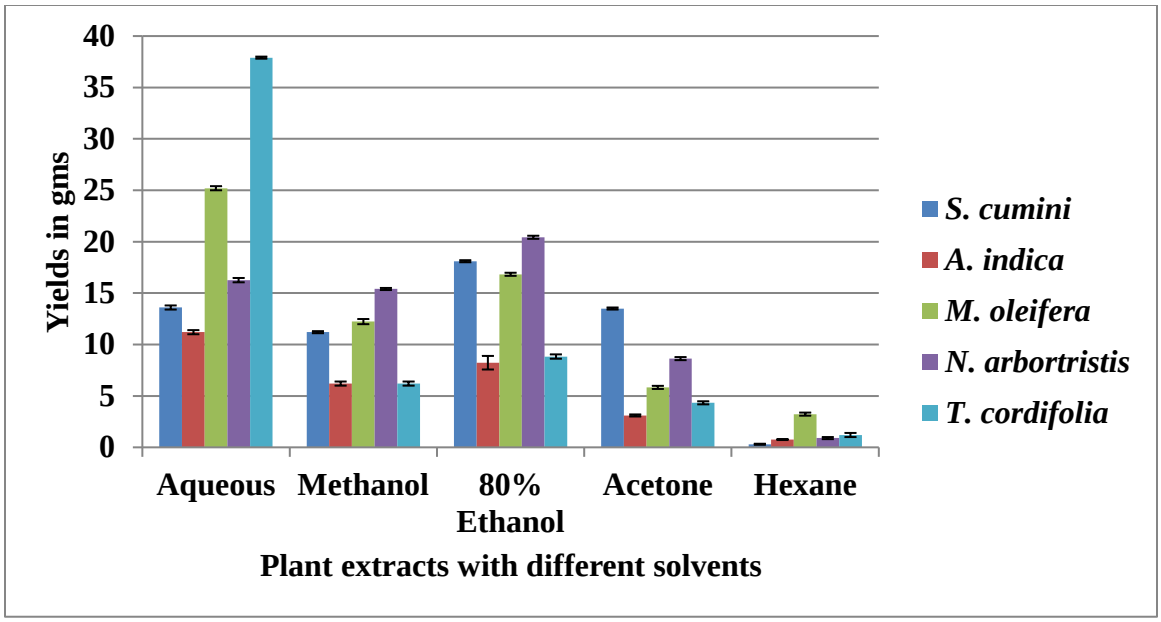


Figure 6.14: Extractive yields of plants obtained from five different solvents

6.9 Preliminary phytochemical screening:

Medicinal plants have been recognized as one of the biggest repository of a vast and diverse assortment of Phyto compounds used to treat human diseases. There is a wide diversity of compounds, especially secondary metabolites reported from plants and show a variety of antibacterial, antioxidant, anti-cancer, antitumor, analgesic, antiinflammatory, antiviral activities. Various phytochemical compounds like alkaloids, terpenoids, flavonoids, steroids, tannins, saponins, coumarins, and some other endogenous metabolites are present in plants. In the present investigation, phytochemical screening was carried for the presence or absence of compounds in form of alkaloids, flavonoids, tannins, steroids, quinones, coumarins, terpenoids, saponins, and phenols in the aqueous, methanol, ethanol, and acetone and hexane solvents for the preparation of plant extracts from five different plants. Table 6.6 shows the presence or absence of phytochemical secondary metabolites compounds.

6.9.1 *Syzygium cumini:*

The preliminary phytochemical screening of *S. cumini* in different solvents has been observed. Aqueous extract showed the positive appearance of steroids, tannins, terpenoids, flavonoids, phenols, and alkaloids. The coumarins, quinones, and saponins were found to be absent from the aqueous extract. Phytochemicals in ethanol extract observed a positive appearance of alkaloids, quinones, flavonoids, terpenoids, tannins, steroids, and saponins, whereas coumarins and phenols were absent. Methanol phytochemical extract revealed the appearance of tannins, steroids, phenols, alkaloids, flavonoids, terpenoids, quinones, and saponins, whereas coumarin is absent. Acetone phytochemical extract showed the presence of phenols, coumarins, steroids, tannins, terpenoids, flavonoids, quinines, and alkaloids whereas saponins are absent. Hexane extract showed the presence of coumarins and steroids, whereas alkaloids, phenols, flavonoids, terpenoids, tannins, quinones, and saponins are absent. The result has been shown in Table 6.6.

6.9.2 *Azadirachta indica:*

The preliminary phytochemical screening of *A. indica* in different solvents has been observed. Aqueous extract showed the positive appearance of steroids, coumarins, saponins, quinines, tannins, terpenoids, flavonoids, and alkaloids whereas phenols were found to be absent. Phytoconstituents in ethanol appeared the positive result of steroids, coumarins, saponins, flavonoids, and alkaloids whereas phenols, quinones, terpenoids, and tannins were found to be absent. Phytochemicals in methanol showed the presence of phenols, tannins, and steroids, whereas alkaloids, flavonoids, saponins, coumarins, quinines, and terpenoids are absent. Acetone phytochemical extract showed the presence of alkaloids, quinones, phenols, and terpenoids, whereas flavonoids, coumarins, tannins, steroids, and saponins were found to be absent. Hexane extract showed the presence of

quinones, terpenoids, tannins, and steroids, whereas alkaloids, coumarins, phenols, terpenoids, and saponins are absent. The results were represented in Table 6.6.

6.9.3 *Moringa oleifera:*

The preliminary phytochemical screening of *M. oleifera* in different solvents has been observed. Aqueous the extract showed all tested phytochemicals including tannins, terpenoids, quinones, phenols, steroids, coumarins, saponins, flavonoids, and alkaloids. Phytochemicals in ethanol appeared the positive result of tannins, terpenoids, quinones, steroids, coumarins, flavonoids, and alkaloids, whereas phenol and saponins were found absent. Phytoactive compounds in methanol observed for a positive result of flavonoids, coumarins, phenols, and alkaloids, whereas tannins, terpenoids, quinones, steroids, and saponins are absent. Acetone phytochemical extract showed the presence of alkaloids, phenols, quinones, and terpenoids, whereas tannins, steroids, coumarins, saponins, and flavonoids are found absent. Hexane phytochemical extract showed the absence of tested phytoconstituents; tannins, terpenoids, quinones, phenols, steroids, coumarins, saponins, flavonoids, and alkaloids. The results were shown in Table 6.6.

6.9.4 *Nyctanthes arbortristis:*

The preliminary phytochemical screenings of *N. arbortristis* in different solvents has been observed. Aqueous extract results in the positive appearance of tested saponins, tannins, and alkaloids, whereas terpenoids, coumarins, steroids, phenols, quinones, and flavonoids are found to be absent. Phytochemicals in ethanol expressed the positive appearance of the presence of tannins, steroids, coumarins, saponins, flavonoids, alkaloids, phenols, and quinones, whereas terpenoids are found to be absent. Phytochemicals in methanol indicated the positive result of tannins, terpenoids, coumarins, steroids, flavonoids, alkaloids, phenols, and quinones, whereas saponins were found to be absent. Acetone phytochemical extract indicated the positive result of terpenoids, coumarins, phenols, quinones, and flavonoids, whereas alkaloids, tannins, steroids, and saponins are found to be absent. Hexane phytochemical extract results in the

appearance of phenol, whereas steroids, saponins alkaloids, flavonoids, terpenoids, tannins, coumarins, and quinones were found to absent. The results were shown in Table 6.6.

6.9.5 *Tinospora cordifolia:*

The preliminary phytochemical screenings of *T. cordifolia* in different solvents has been observed. Aqueous extract indicated the positive result of quinones, coumarins, alkaloids, saponins, terpenoids, and steroids, whereas flavonoids, phenols, and tannins were found to be absent. Phytochemicals in ethanol result in the appearance of coumarins, quinones, steroids, saponins, alkaloids, tannins, and terpenoids, whereas flavonoids and phenols are found absent. Phytoactive compounds in methanol revealed the positive appearance of coumarins, alkaloids, and flavonoids, whereas phenols, quinones, terpenoids, steroids, saponins, and tannins are found to be absent. Acetone phytochemical extract expressed the positive result of coumarins, alkaloids, quinines, and terpenoids, whereas flavonoids, phenols, tannins, saponins, and steroids were found to be absent. Phytochemicals in hexane revealed the positive result of terpenoids, whereas tannins, quinones, alkaloids, coumarins, steroids, phenols, and flavonoids are found to be absent. These results were shown in Table 6.6.

Phytochemical screening in our study indicated that none of the plant extracts had all the studied phytochemicals. The presence of compounds in one plant extract and their absence in others indicates the variation of biosynthetic and physiological reactions of plant parts. The qualitative phytochemical screening is of great importance for the identification of either presence or absence of studied different phytochemicals and further may lead to their quantitative estimation (Saradhajyothi and Subbarao, 2011).
The screened different phytochemicals in our results have different medical, biological, and physiological importance. The screened alkaloids have been reported as important for the analgesics, antispasmodic, and antibacterial properties (Okwu and Okwu, 2004). The phenol compounds have been reported for their great importance for use in the inhibition of angiogenesis and cell proliferation in case of cancer, improvement of endothelial

function, cardiovascular protection, management of inflammation, anti-aging (Han *et al.*, 2007). The studied steroids have been reported for use as antibacterial and sex hormones compounds (Epand and Savage, 2007). The observed tannins in our result have been reported for its importance of different properties of antifungal, antibacterial, anti-yeast, and antiviral (Chung *et al.*, 1998). The screened terpenoids in result have been reported for their several medicinal values such as antibacterial, antiviral, antitumor, anti-inflammatory, ant malarial effects (Yang *et al.*, 2020). The quinones positive in our results have been reported for its great use in case of Parkinson's and cardiovascular diseases along with having powerful antioxidant properties (Pepe *et al.*, 2007). The screened flavonoids in our results have been studied in a number of investigation for it's widely used in cancers, cardiovascular disease, antibacterial, antiviral, and other age related disease (Kumar *et al.*, 2013). The positive result of the presence of coumarins in our results have been suggested for their use in different medicinal values such as anticoagulant, antioxidant, antimicrobial, anticancer, antidiabetic, analgesic, and anti-inflammatory agents (Kapoor, 2013). The screened saponins have been reported for its different medicinal use as an antibacterial, antifungal, anti-inflammatory, anticancer, and hemolytic factor (ElAziz *et al.*, 2019).

Table 6.6: Preliminary phytochemical screening of plant extracts with solvents

Plants	**Solvents**	**Phytochemical constituents**								
		Alk.	Cou.	Qui.	Phe.	Fla.	Ter.	Tan.	Ste.	Sap.
S. cumini	Aqueous	+	-	-	+	+	+	+	+	-
	80% ethanol	+	-	+	-	+	+	+	+	+
	Methanol	+	-	+	+	+	+	+	+	+
	Acetone	+	+	+	+	+	+	+	+	-
	Hexane	-	+	-	-	-	-	-	+	-
	Aqueous	+	+	+	-	+	+	+	+	+
	80% ethanol	+	+	-	-	+	-	-	+	+

A.indica	Methanol	-	-	-	+	-	-	+	+	-
	Acetone	+	-	+	+	-	+	-	-	-
	Hexane	-	-	+	-	-	+	+	+	-
M. oleifera	Aqueous	+	+	+	+	+	+	+	+	+
	80% ethanol	+	+	+	-	+	+	+	+	-
	Methanol	+	+	-	+	+	-	-	-	-
	Acetone	+	-	+	+	-	+	-	-	-
	Hexane	-	-	-	-	-	-	-	-	-
T. cordifolia	Aqueous	+	+	+	-	-	+	-	+	+
	80% ethanol	+	+	+	-	-	+	+	+	+
	Methanol	+	+	-	-	+	-	-	-	-
	Acetone	+	+	+	-	-	+	-	-	-
	Hexane	-	-	-	-	-	+	-	-	-
N. arbortristis	Aqueous	+	-	-	-	-	-	+	-	+
	80% ethanol	+	+	+	+	+	-	+	+	+
	Methanol	+	+	+	+	+	+	+	+	-
	Acetone	-	+	+	+	+	+	-	-	-
	Hexane	-	-	-	+	-	-	-	-	-

Alk., Alkaloids; Cou., Coumarins; Qui., Quinones; Phe., Phenols; Fla., Flavonoids; Ter., Terpenoids; Tan., Tannins; Ste., Steroids and Sap., Saponins. +: Present; -: Absent

6.10 *In vitro* antibacterial activity:

Globally, a vast number of different plants and their parts have been reported for its great role in the inhibition of microorganisms through carried a vast number of

phytoconstituents with antimicrobial properties. Thus, plants are the cheapest and safest alternative sources of different compounds with antimicrobial properties. This can further be utilized as the therapeutic curing and treatment of various deadly diseases caused by even drug-resistant strains of microorganisms. In light of these antimicrobial properties of medicinal plants, our study is undertaken to explore the antibacterial activity of five different medicinal plant leaves, *Syzygium cumini*, *Azadirachta indica*, *Moringa oleifera*, *Nyctanthes arbortristis*, and *Tinospora cordifolia* extracts with four different concentration (25, 50, 100 and 200 mg/ml) against iMLSB and cMLSB strains of *S. aureus* with common MRSA. Among five medicinal plants, only following three plants (*Syzygium cumini*, *Azadirachta indica*, and *Nyctanthes arbortristis*) showed antibacterial activity.

6.10.1 *Syzygium cumini:*

Among the five studied plants, our results showed that the antibacterial activity of *S. cumini* was the most effective one. The antibacterial evaluation was expressed through the measuring size of the zone of inhibition (ZOI) against both two stains of iMLSB and cMLSB with common MRSA. Different solvent extracts of *S. cumini* with four concentrations produced different sizes of the zone of inhibition. The size of the zone of inhibitions of five solvent extracts with four concentrations against iMLSB and cMLSB strains of MRSA have been shown in Figure 6.15 and Figure 6.16 respectively. Our results of aqueous extract, the size of the zone of inhibitions against iMLSB strain of *S. aureus* of MRSA was represented as in the order of 200 mg/ml (25.36±0.15) >100mg/ml (22.40 ± 0.01) >50mg/ml (19.26±0.20) >25mg/ml (17.23±0.20) and against cMLSB strains of *S. aureus* of MRSA, the order of size of the zone of inhibitions was 200 mg/ml (27.30±0.10) >100mg/ml (26.30±0.10) >50mg/ml (24.10±0.10) >25mg/ml (22.10±0.10). The results indicates the size of ZOI of methanolic extract towards iMLSB strains of MRSA were revealed in order of 200 mg/ml (20.36±0.15) >100mg/ml (18.20±0.20) > 50mg/ml (16.16±0.15) >25mg/ml (14.23±0.25) whereas the size of ZOI was represented in order of 200 mg/ml (25.26±0.25) >100 mg/ml (24.13±0.15) > 50 mg/ml (22.26±0.25)

> 25 mg/ml (20.36±0.15) towards cMLSB strains of MRSA. The results of size of ZOI appeared in 80% ethanolic extract against iMLSB strains of MRSA was depicted in the order of 200 mg/ml (22.23±0.15) >100mg/ml (20.23±0.15) > 50mg/ml (18.16±0.11) >25mg/ml (16.20± 0.10) whereas the ZOI sizes to cMLSB strains of MRSA was expressed in order of 200 mg/ml (17.30±0.20) >100 mg/ml (15.30±0.10) >50 mg/ml (13.30±0.10). The results of measured size of ZOI of acetone extract to iMLSB strains of MRSA revealed in order of 200 mg/ml (22.23±0.20) >100 mg/ml (20.23±0.15) > 50 mg/ml (17.16±0.05) > 25mg/ml (15.10±0.10) and against cMLSB strains of MRSA, the calculated ZOI size was represented in order of 200 mg/ml (27.34±0.15) >100 mg/ml (25.36±0.15) > 50 mg/ml (22.23±0.15) > 25 mg/ml (20.30±0.10). The study focused on the antibacterial activity of a variety of solvent extracts of *S. cumini*. The result of hexane extract has not produced any zone of inhibition against both studied strains of bacteria and ultimately this solvent extract of *S. cumini* showed a complete absence of antibacterial activity. The results of antibacterial efficacy of leaves of *S. cumini* were in comparison to other studies carried by Imran *et al.*, 2017; Ahmad and Beg, 2001.

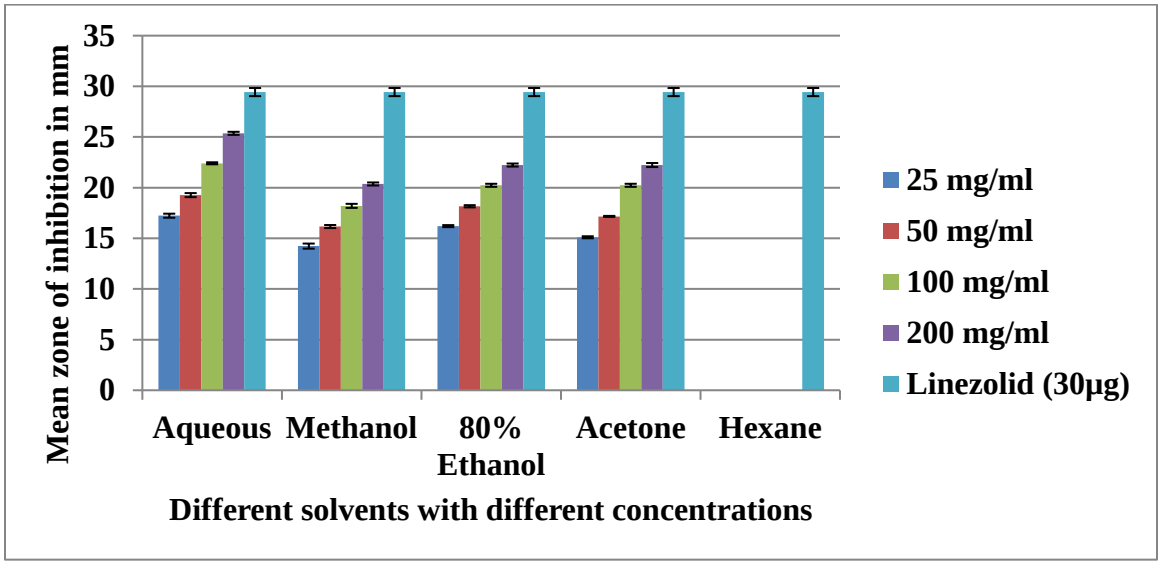


Figure 6.15: Antibacterial activity of leaves of *S. cumuni* against iMLSB strains of MRSA

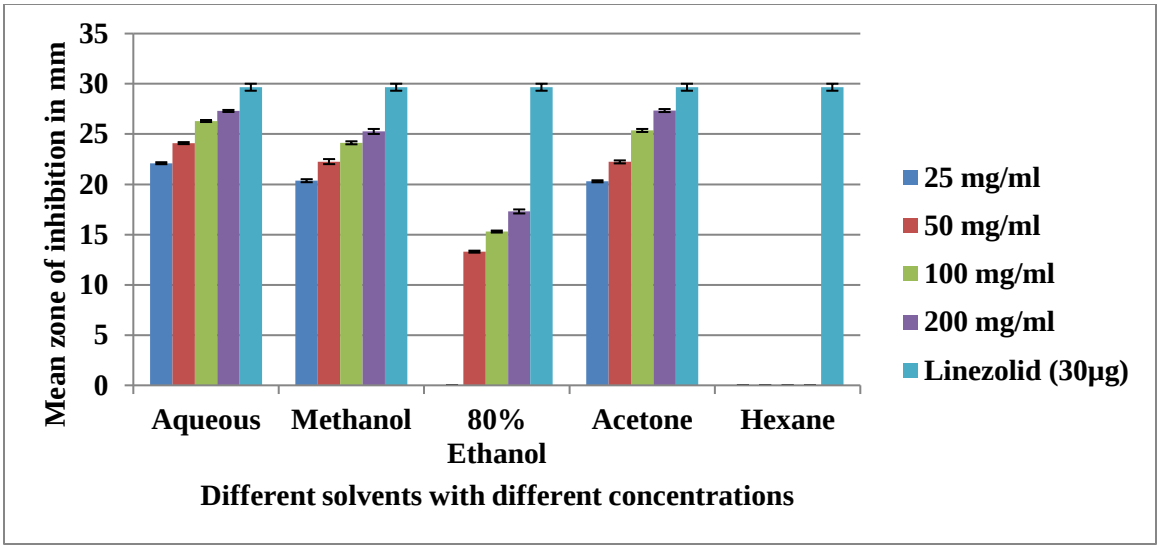


Figure 6.16: Antibacterial activity of leaves of *S. cumuni* against cMLSB strains of MRSA

6.10.2 *Azadirachta indica*

Azadirachta indica of the family Meliaceae is widely used for the treatment of different diseases. It is commonly found in Nepal, Bangladesh, Pakistan, and India. Numerous biological and pharmacological activities have been reported for their antibacterial, antifungal, antitumor, anti-inflammatory, antipyretic, and anti-arthritic activities. The antibacterial activity of leaves of *A. indica* from five solvents with studied four concentrations against iMLSB and cMLSB strains of MRSA has been shown in Figure 6.17 and Figure 6.18. Our results of antibacterial efficacy of five solvent extracts of *A. indica*shows that only two solvents (acetone and hexane) extract was exhibited the antibacterial activity. Our results of acetone extract of *A. indica*, the size of ZOI revealed in the order of 200 mg/ml (18.26±0.15) >100 mg/ml (16.16±0.15) > 50 mg/ml (15.20±0.20) whereas the size of ZOI against cMLSB strains of MRSA was measured in the order of 200 mg/ml (22.13±0.57) >100 mg/ml (20.13±0.15) > 50mg/ml (8.36±0.15). The results of hexane extract, only against cMLSB of MRSA showed the size of ZOI in order of 200 mg/ml (20.23±0.15) > 100 mg/ml (15.16±0.15) > 50mg/ml (9.3±0.20) > 25 mg/ml (5.2±0.20). Our result of antibacterial efficacy is in agreement with other studies

that have been reported from Neem carrying high concentration of azadirachtins, quercetin, and β-sitosterols (Subapriya and Nagini, 2005) & Maragathavalli *et al.*, 2005.

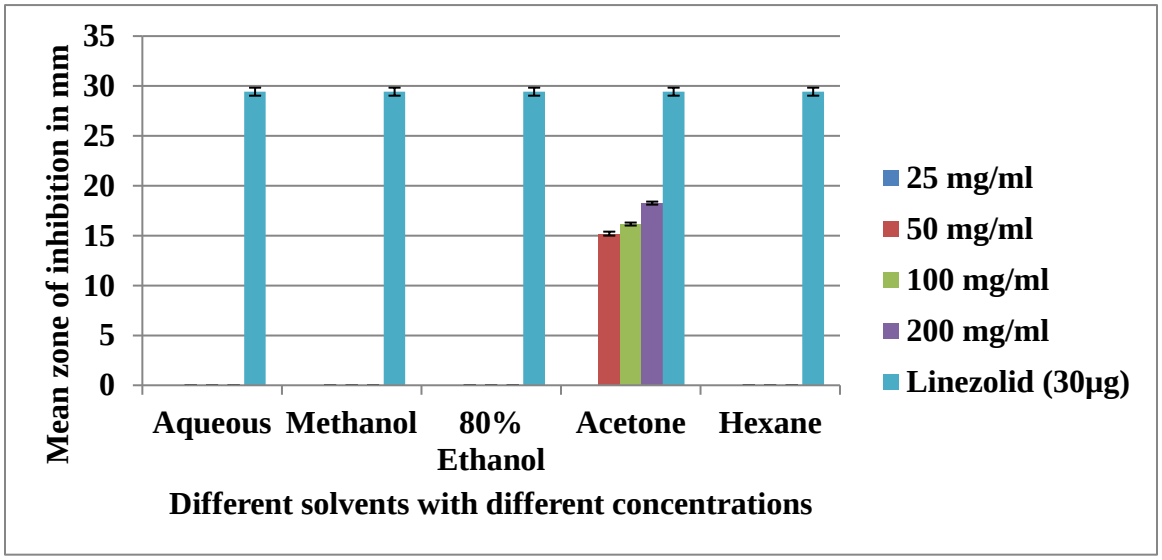


Figure 6.17: Antibacterial activity of *A. indica* against iMLSB strains of MRSA.

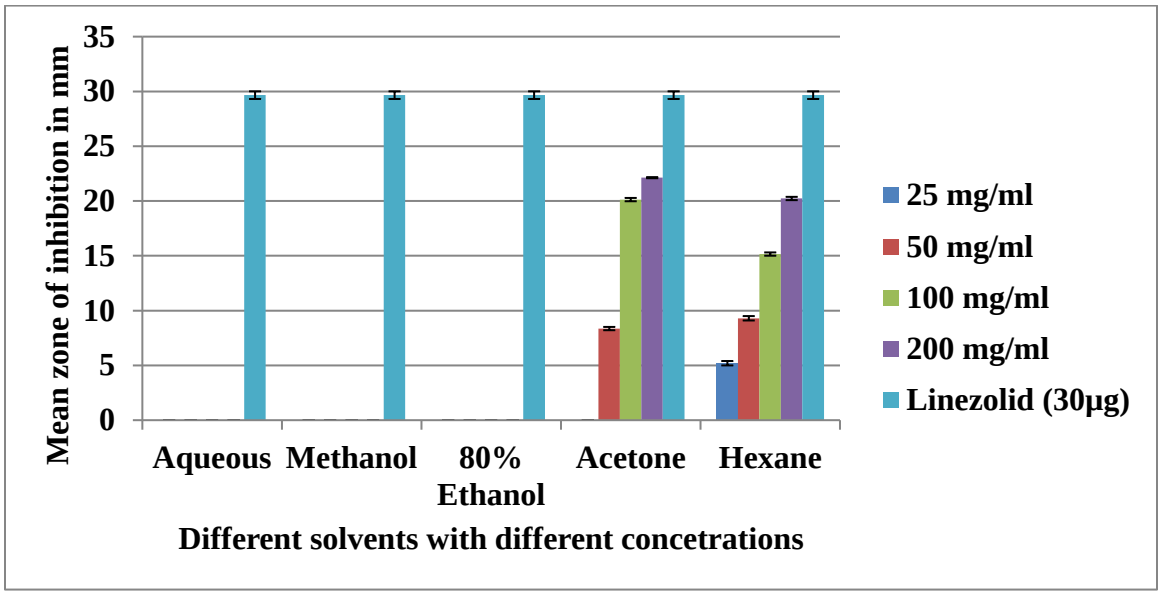


Figure 6.18: Antibacterial activity of *A. indica* against cMLSB strains of MRSA.

6.10.3 *Nyctanthes arbortristis*

In family of Oleaceae, *Nyctanthes arbortristis* is one of the most widely used medicinal plants in Asian countries. It is mostly found in both subtropical and tropical regions in the world. All the parts of the plant have been used for treatment and management of different diseases due to carried several pharmacological activities in form of antibacterial, antiviral, antifungal, antipyretic, anti leishmaniasis, antihistaminic, antimalarial, antioxidant, anti-inflammatory. The antibacterial activity of different solvent extracts with different concentrations against iMLSB and cMLSB strain of MRSA has been shown in Figure 6.19 and Figure 6.20. Antibacterial activity was observed in two solvent extracts out of five solvents. Aqueous, acetone, and hexane extracts of *N. arbortristis* did not show activity against studied resistant strains of *S. aureus*. The result showed that bacterial strains are resistant against these solvent extract with four different concentrations. Our results of measured size of ZOI of the methanolic extract against iMLSB strains of MRSA was represented in order of 200 mg/ml (15.33±0.10) >100 mg/ml (14.10±0.10) >50mg/ml (13.33±0.15) whereas the size of measured ZOI against cMLSB was 200mg/ml (14.20±0.20). the results of size of ZOI of 80% ethanol extract against iMLSB strains of MRSA was found to be in the order of 200 mg/ml (24.23±0.15) >100mg/ml (22.10±0.10) > 50mg/ml (17.23±0.15) > 25mg/ml (15.30±0.10) whereas the measured size of ZOI against cMLSB strains of MRSA was found to be in 200 mg/ml (18.13±0.15). The obtained result showed that leaf extracts have better zone of inhibition in methanol and ethanol solvents. Thus from the following results, it can be ascribed that the antibacterial property of leaf of *Nyctanthes arbortristis* is very potent and it is highly effective against preventing the growth of microorganisms. Our result of antimicrobial efficacy of solvent extract of *N. arbortristis* is in agreement with different studies of Parsad & Sushant, 2014 showed the antimicrobial property of *N. arbortristis*. Kumar& Yadav, 2013 showed the significant antibacterial activity of extracts of leaves of *N. arbortristis*.

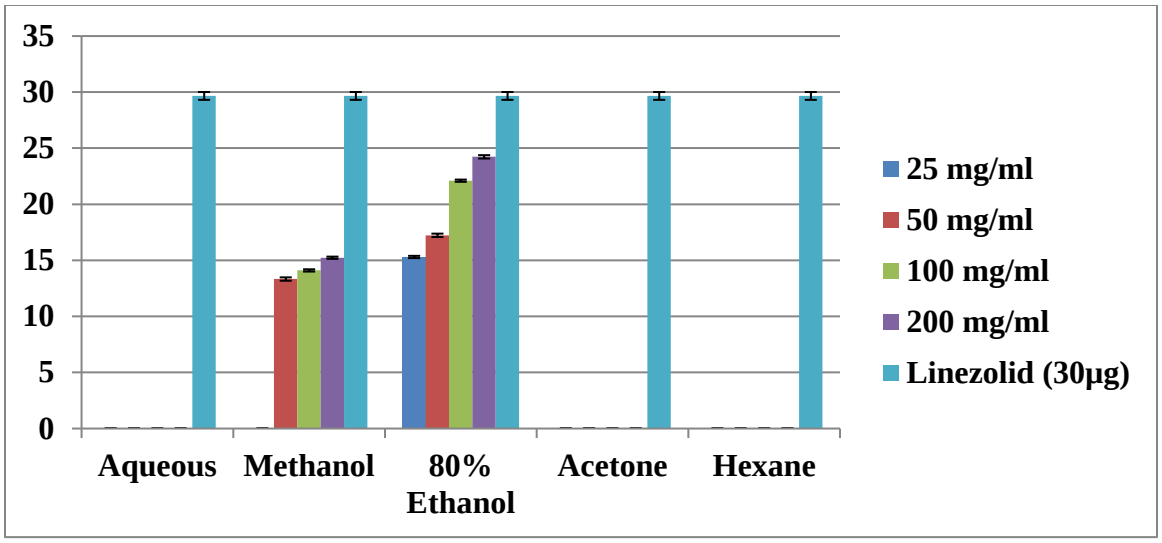


Figure 6.19: Antibacterial activity of *N. arbortristis*against iMLSB strains of MRSA.

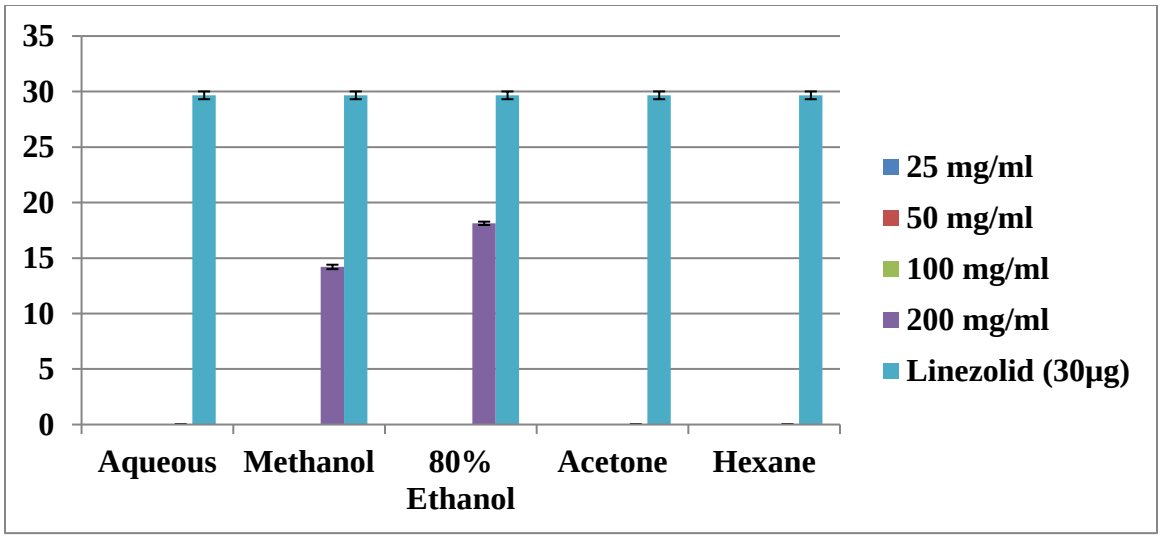


Figure 6.20: Antibacterial activity of *N. arbortristis*against cMLSB strains of MRSA

6.11 LCMS profiling of phytoconstituents in *S. cumini:*

LCMS has been greatly applied in the field of both proteomics and metabolomics research. This spectrometry is considered to highly advanced and analytical technology. LCMS is used for profiling of different compounds in samples with further structural elucidation. The different compounds in samples can be detected relatively or absolutely through analyzing spectral features from LCMS data. The compounds identification in LCMS chromatograph is carried out on the basis of mass-to-charge ration (m/z) and

retention time. In our study, profiling of phytoconstituents in aqueous extract of *S. cumin leaves* was carried out through positive and negative ionizationMS-ESI mode of LCMS method. The LCMS chromatograms in positive ionization mode are shown in Figure 6.21 and in Figure 6.22 for negative ionization mode. In comparison with the previous literature review, the bioactive compounds were characterized by their mass spectrum, retention time, and fragmentation profiles. The list of identified phytocompounds with their molecular weight, retention time, monoisotopic mass and chemical formula has been shown in Table 6.7 for positive ionization mode, in Table 6.8 for negative ionization mode, and Table 6.9 for both negative and positive ionization mode. Our results of interpretation of LCMS data was revealed about the identification of twenty different compounds comprising seven compounds in -ve ionization ($[M+H]^+$) form, ten compounds in +ve ionization ($[M-H]^-$) form and three compounds sharing both negative and positive ionization ($[M+H]^+/[M-H]^-$) forms.

The results of among seven identified compounds in negative mode comprising one coumarin viz. 6-O-feruloyl-D-glucose ($[M+H]^+$ion at m/z 355); one flavonoid viz. astragalin ($[M-H]^-$ ion at m/z 447) and five compounds were phenolic acid viz. quinic acid ($[M-H]^-$ ion at m/z 191), ferulic acid ($[M-H]^-$ ion at m/z 193), xanthoxylin ($[M-H]^-$ ion at m/z 195), 3-(3-hydroxy phenyl) propionic acid ($[M-H]^-$ ion at m/z 165) and caffeic acid ($[M-H]^-$ ion at m/z 179).

Our results of identified ten phytocompounds in positive ionization mode were represented as follows for their name and types. It comprising five flavonoids viz. taxifolin ($[M+H]^+$ion at m/z 305), hydroxyflavan ($[M+H]^+$ ion at m/z 227), kaempferide ($[M+H]^+$ ion at m/z 301), butin ($[M+H]^+$ ion at m/z 274) and catechin ($[M+H]^+$ ion at m/z 291); two organic acids viz. punicic acid ($[M+H]^+$ ion at m/z 279) and palmitic acid ($[M+H]^+$ ion at m/z 258); one terpenoid viz.cedrol ($[M+H]^+$ ion at m/z 223) and two phenolic compounds viz. methyl gallate ($[M+H]^+$ ion at m/z 185) and diferulic acid ($[M+H]^+$ the ion at m/z 387). Three identified compounds were sharing both used ionization mode in our results. These compounds comprising two flavonouds viz.

3,5,7,4'-tetrahydroxy-6-(3-hydroxy-3-methylbutyl) flavones ($[M+H]^+$ ion at m/z 373 and $[M-H]^-$ ion at m/z 371), isoquercetin ($[M+H]^+$ ion at m/z 465 and $[M-H]^-$ ion at m/z 463) and one phenolic compound viz. gallic acid ($[M+H]^+$ ion at m/z 171 and $[M-H]^-$ ion at m/z 169).

In the present investigation, the positive ESI mode was found to be highly significant than negative ESI mode for the detection of contents of diversified compounds from plant extract because of the formation of protonated molecules at low pH (Manners *et al.*, 2003). A similar observation was shown by Mari *et al.*, 2013 and Santos *et al*., 2013 for the significance of the positive ionization mode. It was observed that the different peaks were obtained at different retention times. In our results of the LC chromatogram, different compounds with their pharmacological were assigned on the basis of their peaks produced at different retention times (R_T) as below.

- The retention time (R_T) 1.67 min of the peak corresponded to caffeic acid which pharmacological and antimicrobial effects have been reported in other studies by Ramya *et al.*, 2012.
- The retention time (R_T) 2.46 min of the peak was corresponding to ferulic acid. It has been reported in other studies by Ramya *et al.*, 2012.It is widely used as in the role of antimicrobial, anticancer, anti-inflammatory, antioxidant against different diseases (Tee-ngam *et al.*, 2013).
- The retention time (R_T) 2.82 min of the peak was corresponding to the quinic acid compound which has been reported by Singh *et al*., 2018. It has been reported that quinic acid is used for the therapeutic curing of many diseases. It has been observed that it exhibit a broad spectrum of pharmacological activity in the role of anti-inflammatory, hepatic protection, antioxidant (Soh *et al.*, 2013).
- The retention time (R_T) 3.18 min of the peak was assigned to gallic acid which has been reported in several other studies one by Timbola *et al.*, 2002. A vast number of applications of gallic acid in different diseases are due to carried great pharmacological and biological activity as in the role of hepatic protection,

antimicrobial, anticancer, anti-inflammatory, antioxidant (Pandurangan *et al.,* 2015).

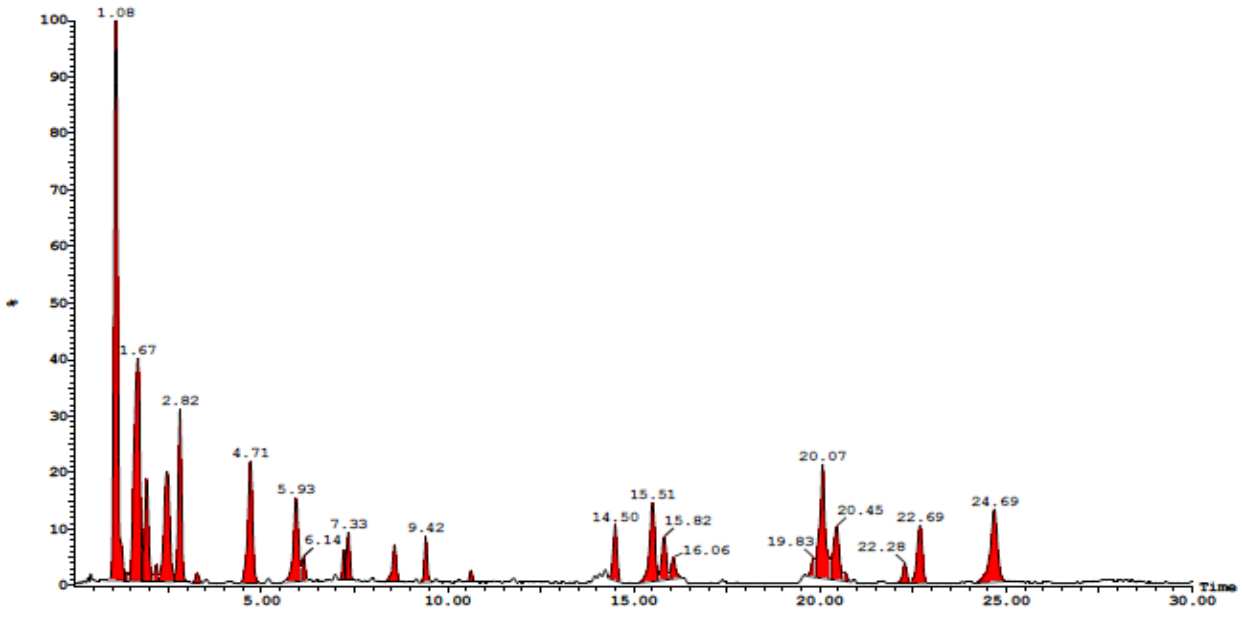
- The retention time (R_T) 3.27min of the peak was corresponding to the 4'-Hydroxyflavan. It has been reported in several studies one by Jagetia 2017. It is of great importance for use in the prevention of different diseases as its role in antibacterial effect (Wang *et al.,* 1998).
- The retention time (R_T) 1.74 min of the peak was assigned to the 3-(3-hydroxyphenyl) propionic acid (3-HPP). It has been reported in many other studies one by Soh *et al.,* 2013. It has wide use in health beneficial compounds as its role in anti-inflammatory, antiviral, antioxidant, antibacterial, anticancer (Rauf *et al.,* 2019).
- The retention time (R_T) 1.92 min of the peak was revealed to the presence of xanthoxylin. It has been previously reported in different investigations one by Chagas *et al.,* 2015. It has been proved for its great use as a phenolic compound in drug development industries for the management of different diseases (Wen *et al.,* 2009).
- Retention time (R_T) of value 7.93 has been observed which corresponds to the astragalin (kaempferol-3-O-β-D-glucoside). It has been previously reported in different studies one by Bijauliya *et al.,* 2017. It has been well known for its wide use in multiple medical importances as for its role in cardioprotection, neurological disorder protection, anticancer, antibacterial, anti-inflammatory, antioxidant (Kotani *et al.,* 2000).
- The retention time (R_T) 6.14 of the peak was corresponding to the diferulic acid. It has been reported in previous studies one by Ramya *et al.,* 2012. It has been proved for its great use in biological activities as in the role of antithrombotic, hepatic protection, vasodilator, anticarcinogenic, antiallergic, antibacterial, anti-inflammatory (Middleton *et al.,* 2000).
- The retention time (R_T) 8.58 min of peak revealed as the presence of methyl gallate (meGAL). It has been reported in different other studies one by Bijauliya*et*

al., 2017. It has been great important used in different biological activities as in role of lung injury protection, radical scavenging activity, ant platelet (Lim*et al.,* 2004).

- Retention time (R_T) of value 2.18 has been observed which correspond to catechin compound. It has been reported previously in different studies one by Chhikara *et al.,* 2018. It has been widely used in the role of suppression of cholesterol, anti-mutagenic, anti-inflammatory, antiallergic, anticarcinogenic, antidiabetic (Ozcan *et al.,* 2014).
- The retention time (R_T) 9.42 min of the peak was corresponding to the butin compound. It has been investigated in different studies one by Chhikara *et al.,* 2018. It has been very popular in the use of protection of DNA damage, radical scavenging activity, antioxidants (Zhang *et al.,* 2008).
- The three different retention times (R_T) 15.51, 15.82, and 16.06 min of peaks were assigned to the kaemferide. It has been reported in different studies one by Chhikara *et al.,* 2018. It has been widely used in the role of anti-allergic, analgesic, antimicrobial, neuroprotective, antidiabetic (Yang *et al.,* 2010).
- The retention time (R_T) 7.21min of the peak was corresponding to the isoquercetin compound. It has been reported in several studies one by Chhikara *et al.,* 2018. It has been widely used for the treatment of different diseases as its great role in different biological activities (Boots *et al.,* 2008).
- The retention time (R_T) 7.33 min of the peak was assigned to the 3, 5, 7, 4'-tetrahydroxy-6-(3-hydroxy-3-methylbutyl) flavones which have been reported in different studies one by Chhikara *et al.,* 2018. It has been widely used because of its great pharmacological properties as in the role of antiviral, anti-inflammatory, anticancer, radical scavenging (Russo *et al.,* 2012).
- The retention times (R_T) 20.07 and 20.45 min was corresponding to the taxifolin or dihydroquercetin compound which has been reported previously in different studies by Singh *et al.,* 2018. It has been widely using as in the role of antipyretic,

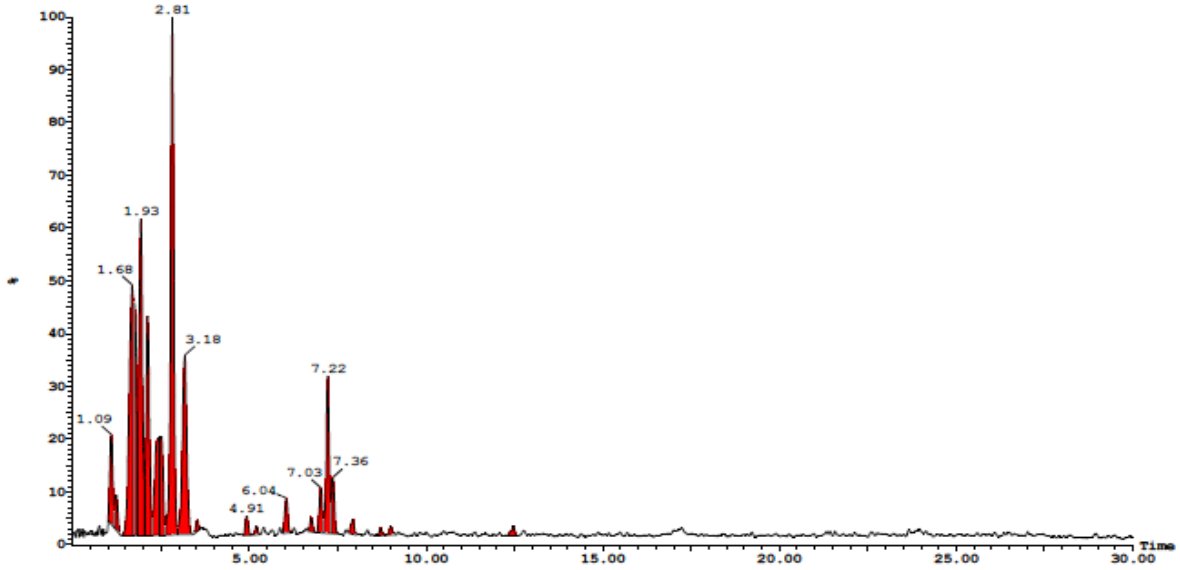
antioxidant, analgesic, anti-inflammatory, antibacterial, antifungal, anticancer (Asmi *et al.,* 2017).

- The retention time (R_T) 14.50 min of the peak was assigned to the cedrol compound which has been reported in different studies one by Satpute and Vanmare 2018. It has been reported for its pharmacological activities as in the role of sedative agents, anti-inflammatory, antibacterial, antifungal (Dayawansa *et al.,* 2003).
- The retention time (R_T) 10.63 min of the peak was assigned to the palmitic acid (PA) which has been reported in several studies by one Ayyanar and Subash-Babu 2012. It has been widely used as in the role of anticancer, antiatherosclerosis, neurogenerative protection (Carta *et al.,* 2017).
- The retention time (R_T) 22.28 min of the peak was corresponding to the punicic acid compound which has been reported in different studies one by Franzke *et al.,* 1982. It has been widely used as in the role of various metabolic ailments for the treatment of different diseases (Aruna *et al.,* 2016).
- The retention time (R_T) 3.52 min of the peak was corresponding to the presence of 6-O-feruloyl-D-glucose which has been reported in different studies one by Ramya *et al.,* 2012. It has been reported for its different roles in as antioxidant, probiotic effect, and antimicrobial properties (Ou and Sun 2014).



Time

Figure 6.21: LCMS chromatogram of *S. cumini* in positive mode showing peaks with identified compounds listed in Table 6.7



Time

Figure 6.22: LCMS chromatogram of *S. cumini* in negative mode showing peaks with identified compounds listed in Table 6.7

Table 6.7: Identification of active compounds in *S. cumini* leaves water extract by LCMS of in positive ionization mode

S. No	Rt (min)	Mol Wt	Monoisotropic mass	Chemical formula	compounds	References
1.	6.14	386	386.100168	$C_{20}H_{18}O_8$	Diferulic acid	Ramya *et al.*, 2012
2.	8.58	184	184.037173	$C_8H_8O_5$	Methylgallate	Ramya *et al.*, 2012
3.	2.18	290	290.079038	$C_{10}H_{12}O_4$	Cianidanol	Chhikara *et al.*, 2018
4.	9.42	273	272.068473	$C_{15}H_{12}O_5$	Butin	Ramya *et al.*, 2012
5.	15.82	300	300.063388	$C_{16}H_{12}O_6$	Kaempferide	Ramya *et al.* 2012
6.	3.27	226	226.09938	$C_{15}H_{14}O_2$	4'-Hydroxyflavan	Bijauliya *et al.*, 2017
7.	20.07	304	304.058303	$C_{15}H_{12}O_7$	Taxifolin	Singh *et al.*, 2018
8.	10.63	257	256.24023	$C_{16}H_{32}O_2$	Palmitic acid	Ayyanar and Subash-Babu 2012
9.	22.28	278	278.224580204	$C_{18}H_{30}O_2$	Punicic acid	Chagas *et al.*, 2015
10.	14.50	222	222.198365	$C_{15}H_{26}O$	Cedrol	Satpute and Vanmare, 2018

RT; Retention time, Mol. Wt.; Molecular weight

Table 6.8: Identification of active compounds in *S. cumini* leaves by LCMS of in negative ionization mode

S. No	Rt (min)	Mol. Wt	Monoisotropic mass	Chemical formula	Compounds	References
1.	1.67	180	180.042259	$C_9H_8O_4$	Caffeic acid	Ramya *et al.*, 2012
2.	1.74	166	166.062994	$C_9H_{10}O_3$	3(3Hydroxyphenyl) Propanoic Acid	Singh *et al.*, 2018
3.	1.92	196	196.073559	$C_{10}H_{12}O_4$	Xanthoxylin	Chagas *et al.*, 2015
4.	2.50	194	194.057909	$C_{10}H_{10}O_4$	Ferulic acid	Chhikara *et al.*, 2018
5.	2.82	192	192.063388	$C_7H_{12}O_6$	Quinic acid	Singh *et al.*, 2018
6.	7.93	448	448.100561	$C_{21}H_{20}O_{11}$	Astragalin	Bijauliya *et al., 2017*
7.	3.52	356	356.1107	$C_{16}H_{20}O_9$	6-O-Feruloyl-D-glucose	Ramya *et al.*, 2012

RT; Retention time, Mol. Wt.; Molecular weight

Table 6.9: Identification of active compounds in *S. cumini* leaves by LCMS of in positive and negative ionization mode

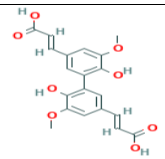
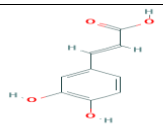
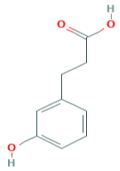
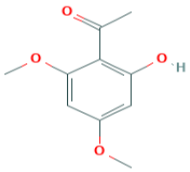
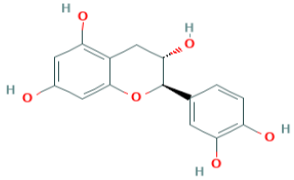
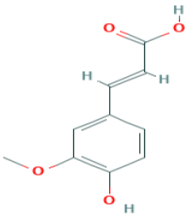
S. No	Rt (min)	Mol. Wt	Monoisotropic mass	Chemical formula	Compounds	References
1.	3.18	170	170.021523	$C_7H_6O_5$	Gallic acid	Timbola *et al.*, 2002
2.	7.21	464	464.095476	$C_{21}H_{20}O_{12}$	Isoquercetin	Jagetia, 2017
3.	7.33	372	372.120903	$C_{20}H_{20}O_7$	3,5,7,4'-Tetrahydroxy-6-(3-hydroxy-3-methylbutyl)flavone	Chagas *et al.*, 2015

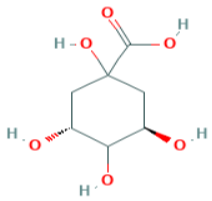
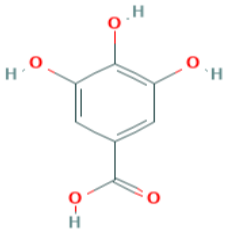
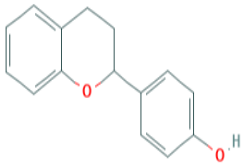
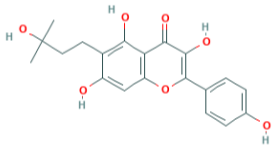
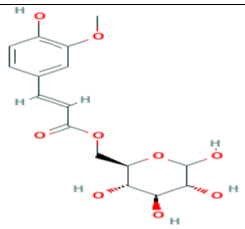
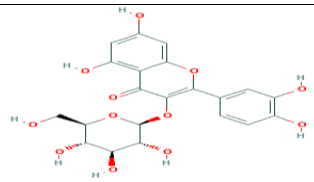
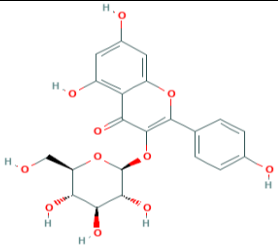
RT; Retention time, Mol. Wt.; Molecular weight

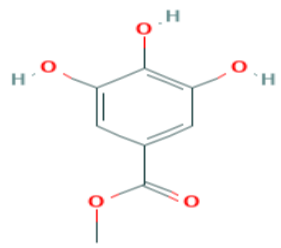
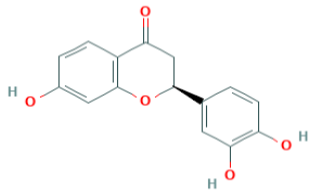
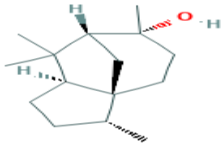
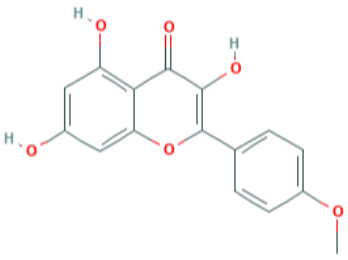
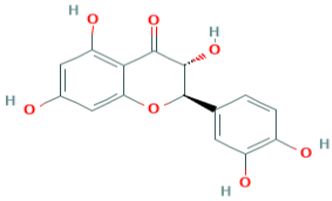
6.12 Molecular docking analysis

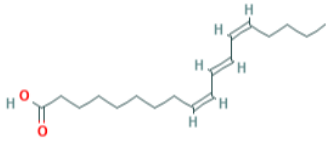
Diseases caused by MRSA and MLSB resistant strains of *S. aureus* are very difficult to be cured due to limited options of different synthetic drugs. In this context, our attempt to investigates novel compounds from different plant sources causing inhibition of MRSA and MLSB strains through targeting its proteins viz. PBP2a and ERM respectively. The interaction of identified compounds from LCMS was subjected to molecular docking analysis to made lists of compound ranks on basis of scoring. The least score in negative indicates the highest interaction and best compound for inhibition of target two studied proteins. In our study of *In Silico* method or molecular required molecular formula, PubChem identification and chemical structure of twenty analyzed compounds have been shown in Table 6.10. The structure of PBP2a and ERM proteins were located PDB data bank. The PBP2a is a homodimer protein with two chains (A & B) and ERM is having a single chain (A). The chain A participates in docking has been shown in Figure 6.22 as for A (PBP2a) and B (ERM).

Table 6.10: Lists of ligands with PubChem ID and structure

S. No.	Name of compound	Pub Chem ID	Mol. formula	Mol. Structure
1.	Diferulic acid	5281770	$C_{20}H_{18}O_8$	
2.	Caffeic acid	689043	$C_9H_8O_4$	
3.	3-(3-Hydroxyphenyl) Propanoic Acid	91	$C_9H_{10}O_3$	
4.	Xanthoxylin	66654	$C_{10}H_{12}O_4$	
5.	Cianidanol	9064	$C_{15}H_{14}O_6$	
6.	Ferulic acid	445858	$C_{10}H_{10}O_4$	

7.	Quinic acid	6508	$C_7H_{12}O_6$	
8.	Gallic acid	370	$C_7H_6O_5$	
9.	4'-Hydroxyflavan	20452436	$C_{15}H_{14}O_2$	
10.	3,5,7,4'-Tetrahydroxy-6-(3-hydroxy-3-methylbutyl)flavone	44259047	$C_{20}H_{20}O_7$	
11.	6-O-Feruloyl-D-glucose	11725795	$C_{16}H_{20}O_9$	
12.	Isoquercetin	5280804	$C_{21}H_{20}O_{12}$	
13.	Astragalin	5282102	$C_{21}H_{20}O_{11}$	

14.	Methylgallate	7428	$C_8H_8O_5$	
15.	Butin	92775	$C_{15}H_{12}O_5$	
16.	Palmitic acid	985	$C_{16}H_{32}O_2$	
17.	Cedrol	65575	$C_{15}H_{26}O$	
18.	Kaempferide	5281666	$C_{16}H_{12}O_6$	
19.	Taxifolin	439533	$C_{15}H_{12}O_7$	

20.	Punicic acid	5281126	$C_{18}H_{30}O_2$	

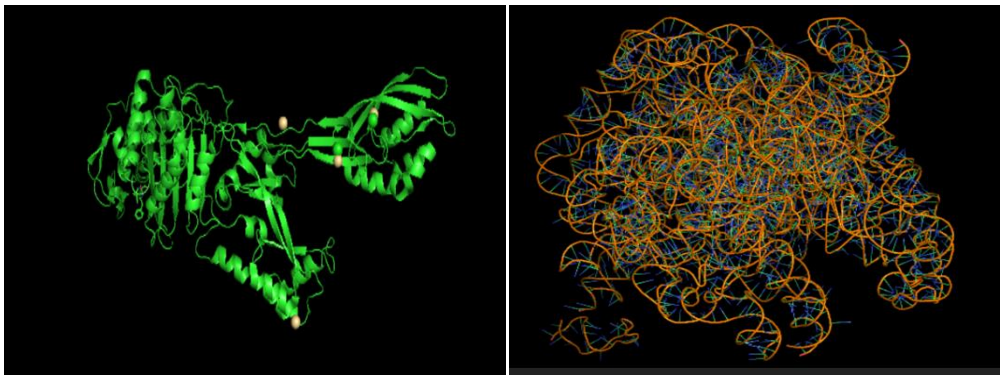


Figure 6.23: Chain A structure of A) PBP2a and B) ERM viewed using PyMol

6.12.1 Determination of active site in target proteins:

The sequences of amino acid determination on target proteins in molecular docking are of great importance as it making interact with analyzed compounds. The sequences in the pocket of PBP2a and ERM were determined as per the method of CASTp. The amino acid sequences results are depicted in Figure 6.23 for PBP2a and Figure 6.24 for ERM.

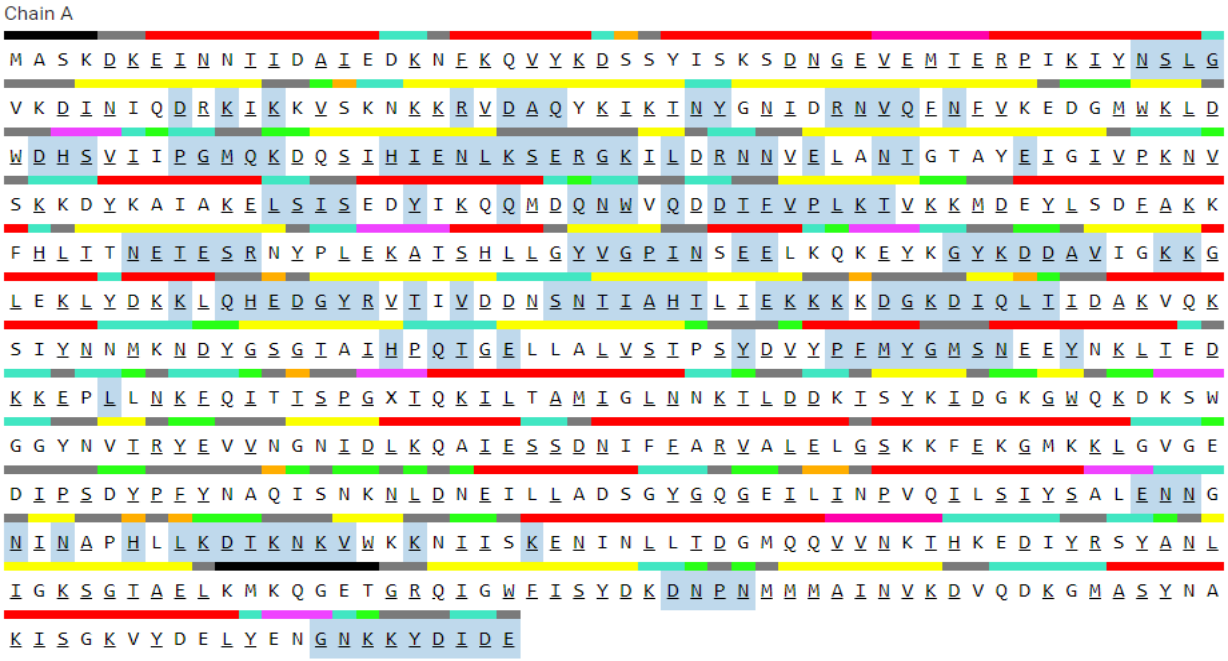


Figure 6.24: Amino acid sequencing in chain A of PBP2a with letters highlighted in blue colored indicates sequence of amino acids in active site

Figure 6.25: Amino acid sequencing in chain A of ERM with letters highlighted in blue indicates sequence of amino acids in active site

6.12.2 Analysis of docking simulation:

The degree of interaction or binding affinity of studied target proteins to analyzed compounds is the key factor in molecular docking analysis. The binding affinity is always expressed in scoring form. Less the score more will be the binding affinity and it is the drug of the candidate. The binding scores of twenty compounds to PBP2a and ERM proteins have been shown in Table 6.11 and Table 6.12 respectively. Our results showed that the values of binding scores were found in a range of -65.14 to 102.37 kcal/mole to PBP2a and -64.42 to -104.4kcal/mole to ERM as per method of *i*GEMDOCK. All the

score values were compared with scores (-102.95 kcal/mole for PBP2a and -102.61 kcal/mole for ERM) obtained from docking of analyzed compounds to linezolid as one of the standard drugs used for inhibition of MRSA and MLSB strains.

6.12.2.1 Penicillin binding protein 2a, PBA2a (PDB ID: 1mwt):

The results of docking of studied compounds to PBP2a were revealed that diferulic acid, phenol compound depicted the lowest binding energy score with the value of -102.37 kcal/mole. The score of binding energy with the value of -103 kcal /mol was found by linezolid to PBP2a. The interaction of linezolid to PBP2a was shown in Figure 6.24 A. Diferulic acid made binding to PBP2a through van der walls interaction with involvement of specific sequence of amino acids viz. Thr600, Met641, Tyr446, Asn464, His583, Gly599, Gln521 and Glu602 (Figure 6.25 B). Diferulic acid possesses great medicinal values as in the role of antimicrobial properties against fungal and bacterial organisms (Jeong *et al.,* 2000). Isoquercetin is the second last compound that produced the binding energy score with the value of 102.2 kcal/mol through the involvement of hydrogen bond with Ser403 (Figure 6.24 C). Isoquercetin is a flavonoid compound that carried a role of antimicrobial properties (Rauha *et al.,* 2000). The compound taxifolin was produced a binding score with the value of -99 kcal/mol (Figure 6.24 D). The taxifolin has been reported for its great role in the inhibition of a large spectrum of bacteria including *Staphylococcus aureus* (Kuspradini *et al.,* 2009). The compounds, cianidanol, 3,5,7,4'-tetrahydroxy-6(3-hydroxy-3-methylbutyl) flavones, kaempferide, butin and astragalin showed binding energies in range of -90 kcal/mol to -97.5 kcal/mol. The docking interactions were shown in Figure 6.24 from E to I. The other compounds such as 6-O-feruloyl D-glucose, punicic acid, caffeic acid, 4'Hydroxyflavan, ferulic acid, quinic acid, cedrol, 3-(3-hydroxyphenyl) propanoic acid, palmitic acid, xanthoxylin, methyl gallate, and gallic acid resulted in the binding energies with the values of -84 kcal/mol, -79.6 kcal/mol, -73.5 kcal/mol, -73.3 kcal/mol, -71.6 kcal/mol, -69.6 kcal/mol, -68.7 kcal/mol, -68.5 kcal/mol, -68 kcal/mol, -67.2 kcal/mol, -66.3 kcal/mol and 65.1 kcal/mo respectively. The interaction of these compounds to PBP2a has been shown in Figure 6.24 from J to U. The broad-spectrum antimicrobial properties of these compounds and

has been proved by several studies by Ou and Zheng, 2014; Shabbir *et al.*, 2017; Espíndola *et al.*, 2019; Mikell *et al.*, 2015; Zduńska *et al.*, 2018; Inbathamizh and Padmini, 2013; Bhatia *et al.*, 2008; Qian *et al.*, 2017; Kumar *et al*., 2017; Kahkeshani *et al.*, 2019.

Table 6.11: Molecular docking analysis of plant compounds with PBP2a

Target	**Ligands**	**Binding energy (Kcal/mol)**	**Binding Pocket residue (Aº)**	**VWD**	**H bond**	**Elec**	**Av.co pair**
PBP2a (PDB ID: 1mwt)	Linezolid (control)	-103	H-S-SER-403, H-S-GLN-521, H-M-GLY-599, H-M-THR-600, H-S-THR-600, V-S-TYR-446, V-M-THR-600, V-S-THR-600, V-S-MET-641	-79.4	-23.5	0	25.4
	Diferulic acid	-102.4	V-S-TYR-446, V-S-ASN-464, V-S-HIS-583, V-M-GLY-599, V-M-THR-600,V-S-THR-600, V-S-GLN-521, V-S-GLU-602, V-S-MET-641	-85.3	-17.1	0	25.5
	Isoquercitin	-102.2	H-S-SER-403, V-S-TYR-446, V-S-THR-600	-74.3	-28	0	25

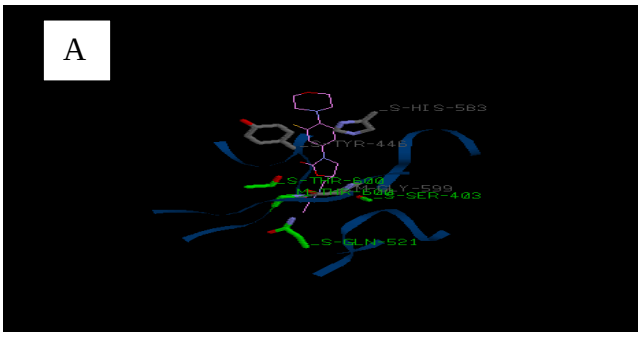
	Taxifolin	-99	H-S-SER-403, H-S-SER-462, H-S-HIS-583, H-S-SER-598, H-M-THR-600, H-S-THR-600, V-S-TYR-446, V-M-GLY-599, V-M-THR-600, V-S-THR-600, V-S-MET-641	-81	-17.1	0	26
	Cianidanol	-97.5	V-S-TYR-446, V-S-ASN-464, V-S-HIS-583, V-M-GLY-599, V-M-THR-600,V-S-THR-600, V-S-MET-641	-76	-21.5	0	26.3
	3,5,7,4'-tetrahydroxy-6 (3-hydroxy-3-methylbutyl) flavone	-97	H-S-GLN-521, H-M-ALA-642, H-M-SER-643, H-S-SER-643, V-S-TYR-446, V-S-ASN-464, V-S-HIS-583, V-M-GLY-599, V-S-THR-600, V-S-MET-641	-82.3	-14.8	0	24.5
	Kaempferide	-95.8	H-S-SER-403, V-S-TYR-446, V-S-THR-600	-80.7	-15	0	26.1
	Butin	-90.2	H-S-ASN-464, H-S-GLN-521, H-M-ALA-642, H-M-	-70.3	-19.1	0	26.7

			SER-643, V-S-TYR-446, V-S-ASN-464, V-S-HIS-583, V-M-THR-600, V-S-THR-600, V-S-MET-641, V-S-ASN-464, V-S-GLN-521, V-S-HIS-583, V-S-GLU-602, V-S-MET-641				
	Astragalin	-90	H-S-ASN-464, V-S-TYR-446, V-M-THR-600, V-S-THR-600, V-S-MET-641	-75	-15	0	21.5
	6-O Feruloylglucose	-84	H-S-ASN-464, V-S-TYR-446, V-M-THR-600, V-S-THR-600, V-S-MET-641	-60.8	-23.1	0	20.8
	Punicic acid	-79.6	H-S-SER-403, V-S-TYR-446, V-S-THR-600	-77.1	2.5	0	29.2
	Caffeic acid	-73.5	H-S-SER-403, H-S-SER-462, H-M-THR-600, H-M-ALA-642, H-M-SER-643, H-S-SER-643, V-S-TYR-446, V-M-GLY-599, V-S-MET-641	-51.1	-22.5	0	28.7

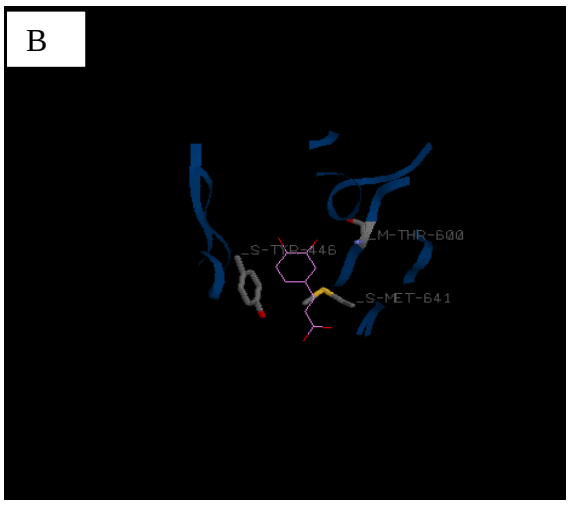
	4'Hydroxyflavan	-73.3	H-S-ASN-464, V-S-TYR-446, V-M-THR-600, V-S-THR-600, V-S-MET-641	-70.8	-2.5	0	25.3
	Ferulic acid	-71.6	H-M-TYR-446, H-S-ASN-464, H-S-HIS-583, H-S-SER-598, V-S-TYR-446, V-S-SER-462, V-M-THR-600	-49.3	-22.4	0	27.6
	Quinic acid	-69.6	H-S-SER-403, H-S-SER-462, H-S-HIS-583, H-S-SER-598, H-M-THR-600, H-S-THR-600, V-S-TYR-446, V-M-GLY-599, V-M-THR-600, V-S-THR-600, V-S-MET-641	-45.9	-23.7	0	27.7
	Cedrol	-68.7	V-S-TYR-446, V-S-ASN-464, V-S-HIS-583, V-M-GLY-599, V-M-THR-600,V-S-THR-600, V-S-MET-641	-62.7	-6	0	25.8
	3-(3-hydroxyphenyl) propanoic acid	-68.5	H-S-SER-403, H-S-SER-462, H-M-THR-600, H-M-ALA-642, H-M-SER-643, H-S-	-48.1	-20.4	0	27.5

			SER-643, V-S-TYR-446, V-S-HIS-583, V-M-GLY-599, V-S-MET-641				
	Palmitic acid	-68	H-S-SER-403, V-S-TYR-446, V-S-THR-600	-48.2	-19.8	0	23.5
	Xanthoxylin	-67.2	H-S-SER-403, H-S-SER-462, H-S-HIS-583, H-S-SER-598, H-M-THR-600, H-S-THR-600, V-S-TYR-446, V-M-GLY-599, V-M-THR-600, V-S-THR-600, V-S-MET-641	-52	-15.2	0	28.6
	Methyl gallate	-66.3	H-S-SER-403, V-S-TYR-446, V-S-THR-600	-49.3	-17	0	28.3
	Gallic acid	-65.1	H-M-TYR-446, H-S-ASN-464, H-S-HIS-583, H-S-SER-598, V-S-TYR-446, V-S-SER-462, V-M-THR-600	-42.3	-22.7	0	26.5

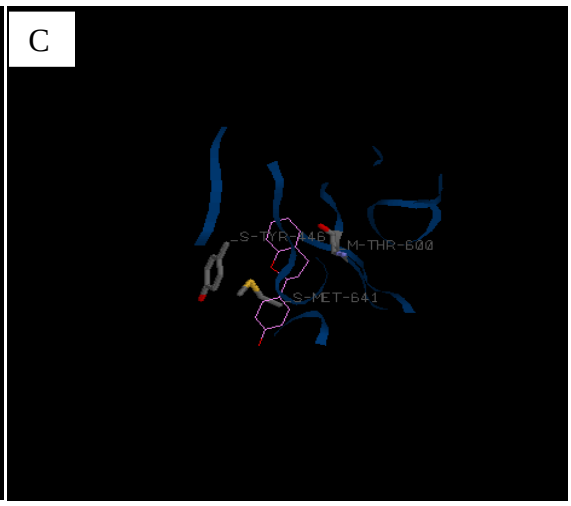
VDW: Van der Walls force, H-bond: hydrogen bond, Elec: Electrostatic interaction, Av. Co: Average cone pai



Linezolid docked with PBP2a

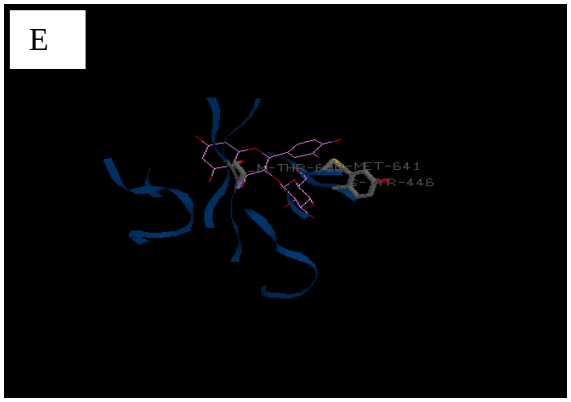


Diferulic acid docked with PBP2a

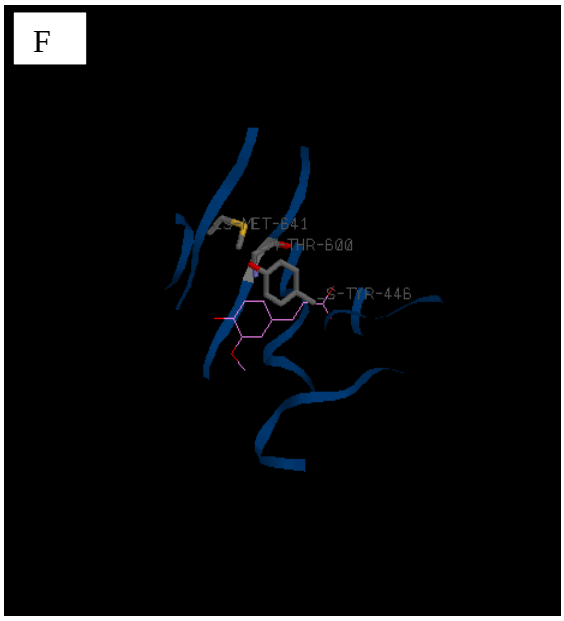


Isoquercetin docked withPBP2

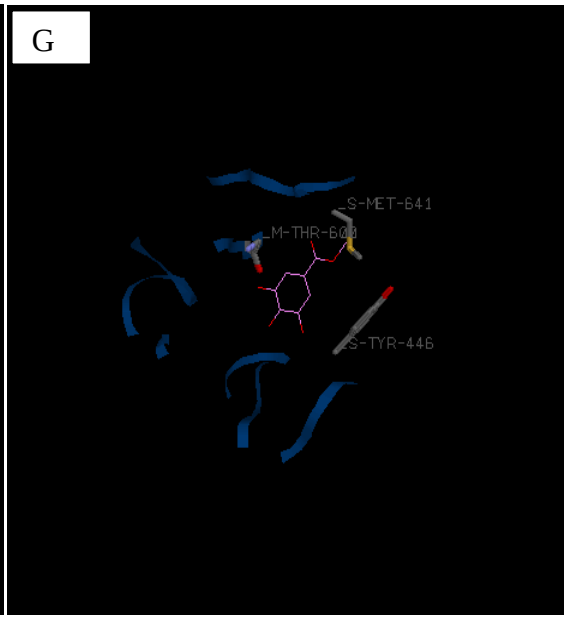
Taxifolin docked with PBP2a



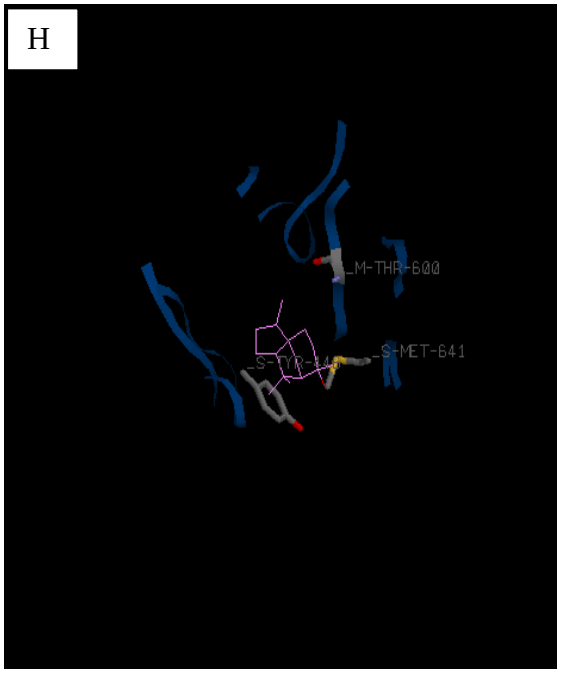
Cianidanol docked with PBP2a



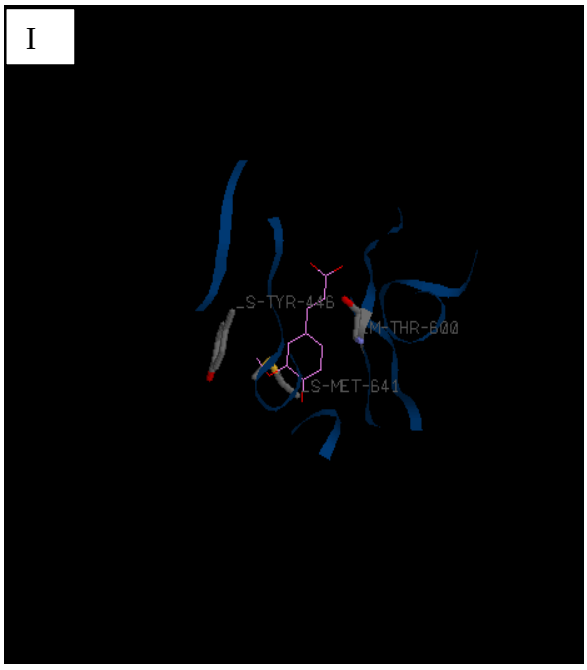
THMF docked with PBP2a



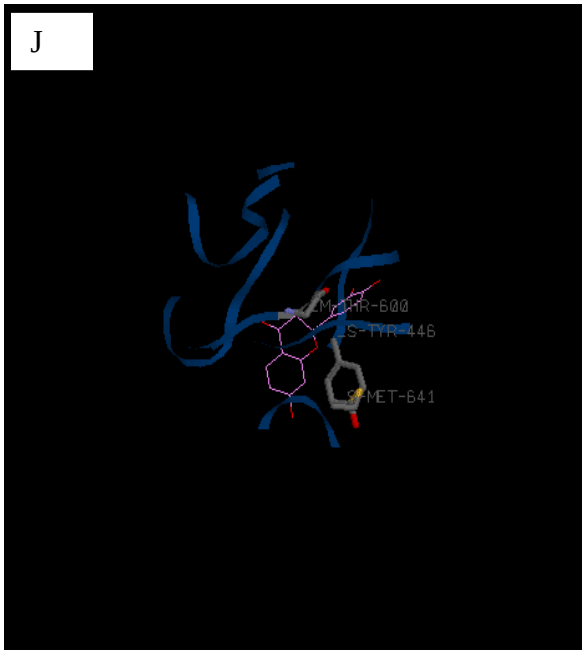
Kaempferide docked with PBP2a



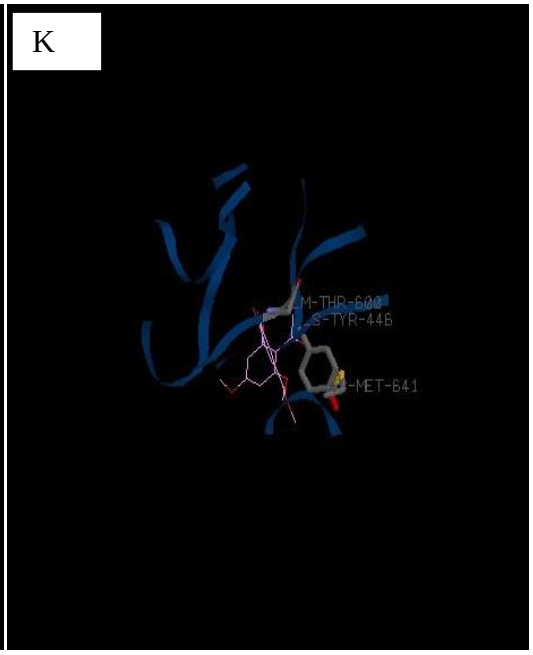
Butin docked with PBP2a



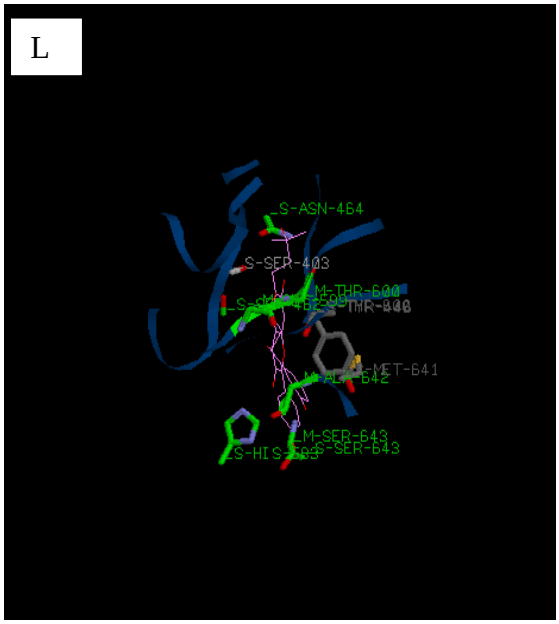
Astragallin docked with PBP2a



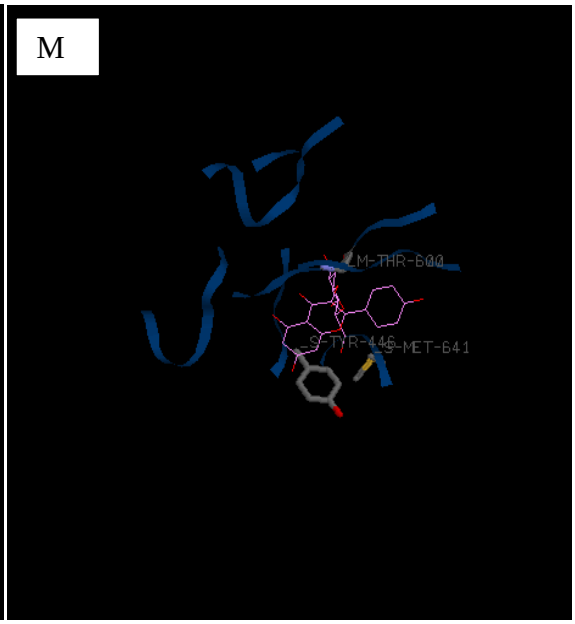
6-O-feruloylglucose docked with PBP2a



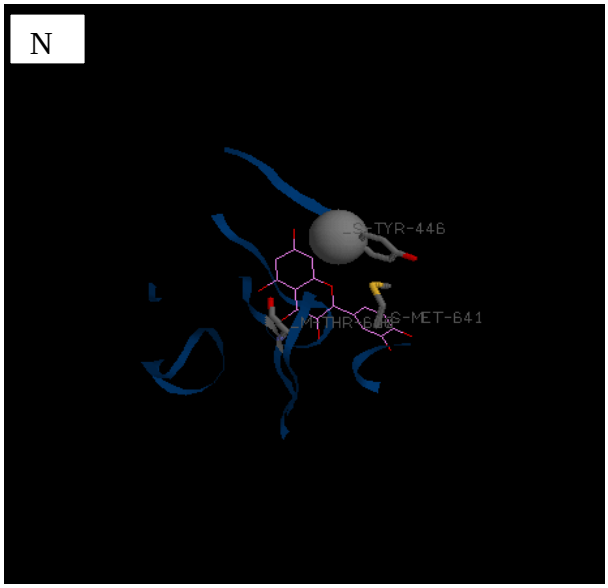
Punicic acid docked with PBP2a



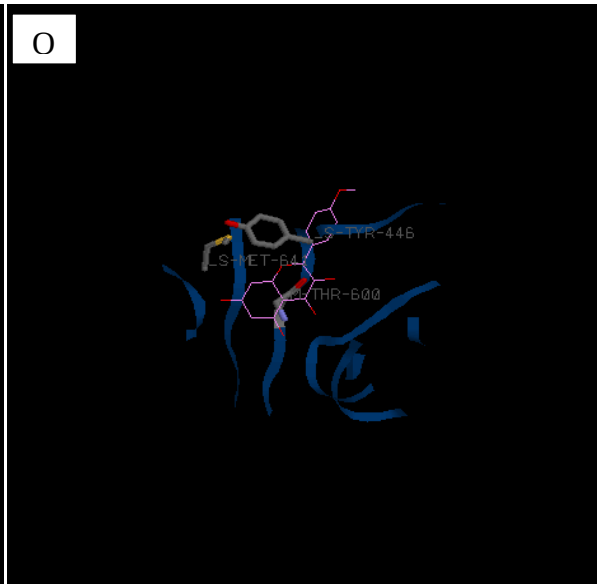
Caffeic acid docked with PBP2a



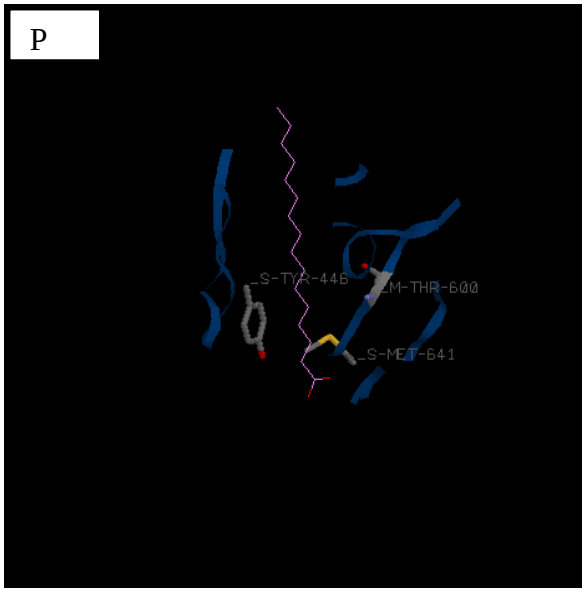
4'Hydroxyflavan docked with PBP2a



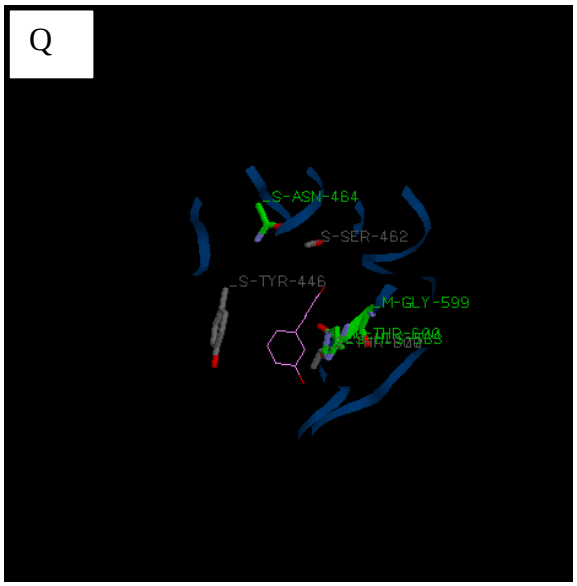
Ferulic acid docked with PBP2a



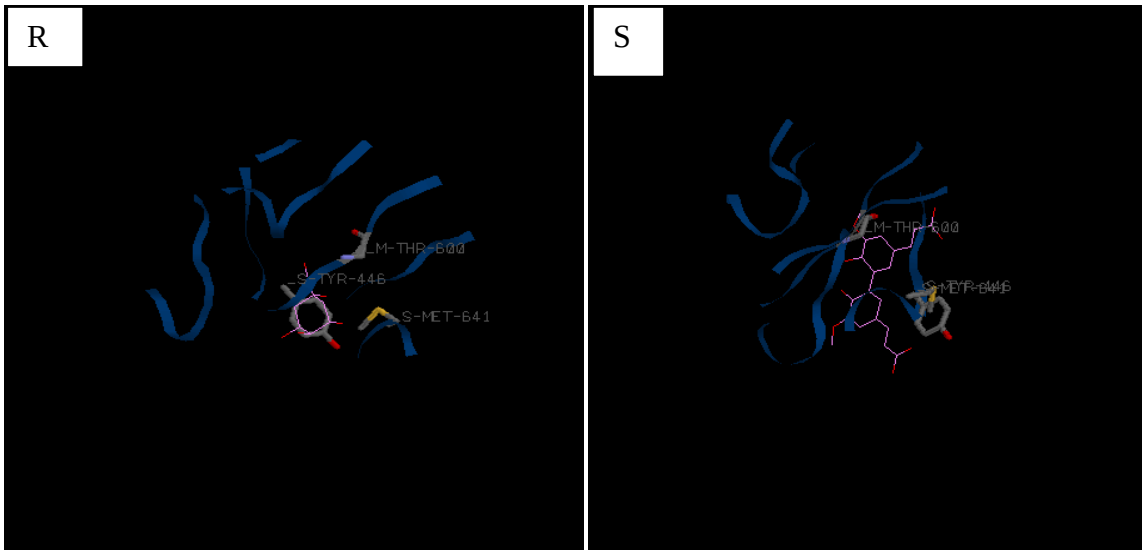
Quinic acid docked with PBP2a



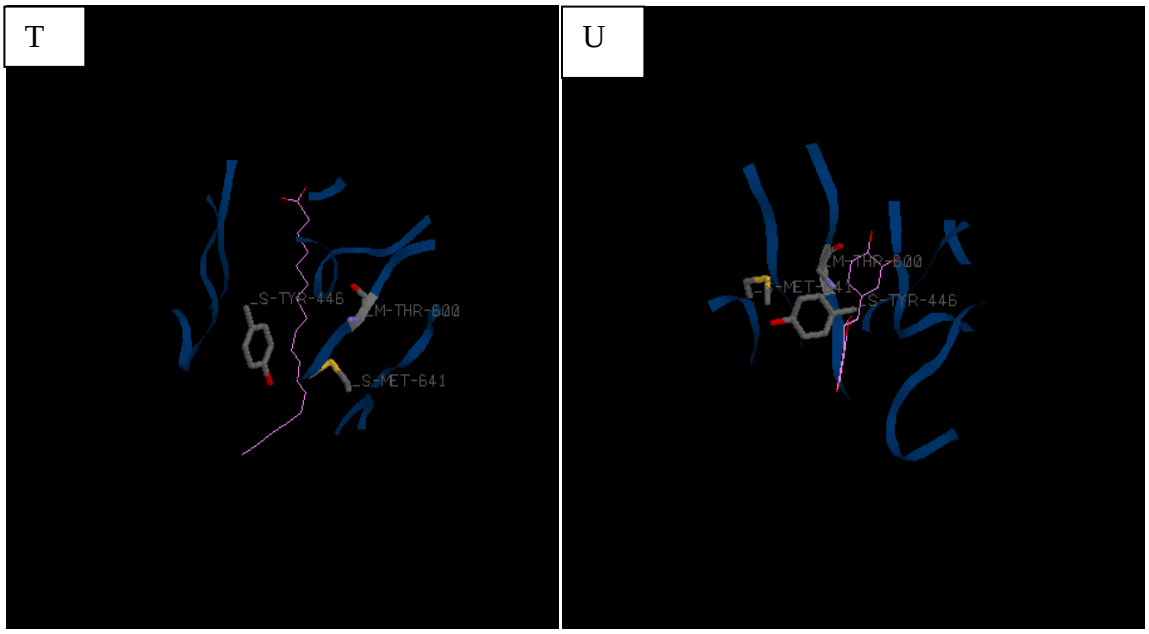
Cedrol docked with PBP2a



3-(3-hydroxy phenyl) propionic acid docked with PBP2a



Palmitic acid docked with PBP2a **Xanthoxylin docked with PBP2a**



Methylgallate docked with PBP2a **Gallic acid docked with PBP2a**

Figure 6.26: Docking pose of plant compounds with PBP2a

6.12.2.2 Erythromycin ribosomal methylase, *erm* (PDB ID: 3j7z):

Astragalin exhibited the least score binding energy with the value of -104.4 kcal/mol forming hydrogen bonds with Ser403, Thr444, Lys430, Tyr446, Ser462, Asn464, Gln521, His583, and Ser643 on the ERM protein among tested compounds (Fig. 6.27 A). The second compound which showed the score of binding energy with the value of -103.2 kcal/mol is taxifolin through making hydrogen bonds at Ser403, Tyr446, Ser462, Asn464, His583, Ser598, Gly599, Thr600, Ala642, and Ser643 amino acids on the Erm protein (Fig.6.27 B). Diferulic acid and isoquercetin showed a binding affinity with the values of -102.4 kcal/mol and -102.1 kcal/mol with Erm (Fig. 6.27 C-D). Cianidadole, 3,5,7,4'-tetrahydroxy-6 (3-hydroxy-3-methylbutyl) flavones, 6-O-feruloy-Dlglucose, Kaemferide, butin, and punicic acid produced the binding energy with the values of -97.6 kcal/mol, 97.1 kcal/mol, -96.2 kcal/mol, -92.6 kcal/mol, -89.4 kcal/mol, -79.6 kcal/mol with ERM (Fig. 6.27 E-J). The compounds viz. caffeic acid, 4'Hydroxyflavan, ferulic acid, quinic acid, cedrol, 3-(3-Hydroxyphenyl) propanoic acid, palmitic acid, xanthoxylin, methyl gallate, and gallic acid showed binding energy with the value of -73.5 kcal/mol, -73.3 kcal/mol, -71.6 kcal/mol, -69.6 kcal/mol, -68.7 kcal/mol, 68.5 kcal/mol, -68 kcal/mol, 67.2 kcal/mol, -66.3 kcal/mol and -64.4 kcal/mol respectively with ERM (Fig. 6.27 K-T). Thus, our result revealed that only two compounds, astragalin and taxifolin in comparison to other compounds produced the least binding energy than the standard drug, linezolid, which result in the binding energy of -102.6 kcal/mol with ERM (Fig. 6.27 U).

Table 6.12: Molecular docking analysis of plant compounds with ERM

Target	Ligands	Binding energy (Kcal/mol)	Binding Pocket residue (Aº)	VWD	H bond	Elec	Aver con pair
ERM (3j7z)	Linezolide (control)	-102.6	H-S-SER-403,H-S-GLN-521, H-M-GLY-599, H-M-THR-600, V-S-TYR-446, V-M-THR-600, V-S-THR-600, V-S-MET-641	-79.7	-23	0	25.3
	Astragalin	-104.4	H-M-SER-403, H-S-SER-403, H-S-LYS-430, H-M-THR-444, H-M-TYR-446, H-S-SER-462, H-S-ASN-464, H-S-TYR-519, H-S-GLN-521, H-S-HIS-583, H-S-SER-643, V-S-TYR-446, V-S-HIS-583, V-S-MET-641	-85.3	-17.1	0	25.5
	Taxifolin	-103.2	H-S-SER-403, H-M-TYR-446, H-S-SER-462, H-S-ASN-464, H-S-HIS-583, H-S-SER-	-63	-40	0	25.7

			598, H-M-GLY-599, H-M-THR-600, H-S-THR-600, H-M-ALA-642, H-M-SER-643, H-S-SER-643, V-M-THR-444, V-S-TYR-446, V-S-THR-600, V-S-MET-641				
	Diferulic acid	-102.4	H-S-SER-403, H-M-THR-600, H-M-ALA-642, H-M-SER-643, H-S-SER-643, V-S-TYR-446, V-S-ASN-464, V-S-HIS-583, V-M-GLY-599, V-M-THR-600, V-S-THR-600, V-S-MET-641	-85.1	-17.1	0	25.5
	Isoquercetin	-102.1	H-M-SER-403, H-S-SER-403, H-M-TYR-446, H-S-ASN-464, H-S-GLN-521, H-S-HIS-583, H-S-SER-598, H-M-THR-600, H-S-THR-600, H-M-GLU-602, V-M-TYR-446, V-S-TYR-446, V-M-SER-	-74	-28.1	0	24.1

			461, V-M-SER-462, V-S-SER-462				
	Cianidanol	-97.6	H-S-SER-403, H-M-THR-600, H-M-ALA-642, H-M-SER-643, H-S-SER-643, V-S-TYR-446, V-S-ASN-464, V-S-HIS-583, V-M-GLY-599, V-M-THR-600, V-S-THR-600, V-S-MET-641	-76	-21.7	0	26.4
	3,5,7,4'-tetrahydroxy-6 (3-hydroxy-3-methylbutyl)flavone	-97.1	H-M-SER-403, H-S-SER-403, H-S-LYS-430, H-M-THR-444, H-M-TYR-446, H-S-SER-462, H-S-ASN-464, H-S-TYR-519, H-S-GLN-521, H-S-HIS-583, H-S-SER-643, V-S-TYR-446, V-S-HIS-583, V-S-MET-641	-82.3	-14.7	0	24.7
	6- O feruloyl-D-glucose	-96.2	H-M-SER-403, H-S-SER-403, H-S-LYS-430, H-M-THR-444, H-M-TYR-446, H-S-	-59	-37.2	0	24.7

			SER-462, H-S-ASN-464, H-S-TYR-519, H-S-GLN-521, H-S-HIS-583, H-S-SER-643, V-S-TYR-446, V-S-HIS-583, V-S-MET-641				
	Kaempferide	-92.6	H-M-SER-403, H-S-SER-403, H-M-TYR-446, H-S-ASN-464, H-S-GLN-521, H-S-HIS-583, H-S-SER-598, H-M-THR-600, H-S-THR-600, H-M-GLU-602, V-M-TYR-446, V-S-TYR-446, V-M-SER-461, V-M-SER-462, V-S-SER-462	-75	-15	0	21.5
	Butin	-89.4	H-S-SER-403, H-M-THR-600, H-M-ALA-642, H-M-SER-643, H-S-SER-643, V-S-TYR-446, V-S-ASN-464, V-S-HIS-583, V-M-GLY-599, V-M-THR-600, V-S-THR-	-66.7	-22.6	0	25.7

			600, V-S-MET-641				
	Punicic acid	-79.6	H-S-SER-403, H-M-TYR-446, H-S-SER-462, H-S-ASN-464, H-S-HIS-583, H-S-SER-598, H-M-GLY-599, H-M-THR-600, H-S-THR-600, H-M-ALA-642, H-M-SER-643, H-S-SER-643, V-M-THR-444, V-S-TYR-446, V-S-THR-600, V-S-MET-641	-77.1	2.5	0	29.2
	Caffeic acid	-73.5	H-S-SER-403, H-M-THR-600, H-M-ALA-642, H-M-SER-643, H-S-SER-643, V-S-TYR-446, V-S-ASN-464, V-S-HIS-583, V-M-GLY-599, V-M-THR-600, V-S-THR-600, V-S-MET-641	-51.1	-22.5	0	28.7
	4'hydroxyflavan	-73.3	H-M-SER-403, H-S-SER-403, H-S-LYS-430, H-M-THR-444, H-M-TYR-446, H-S-	-70.8	-2.5	0	25.3

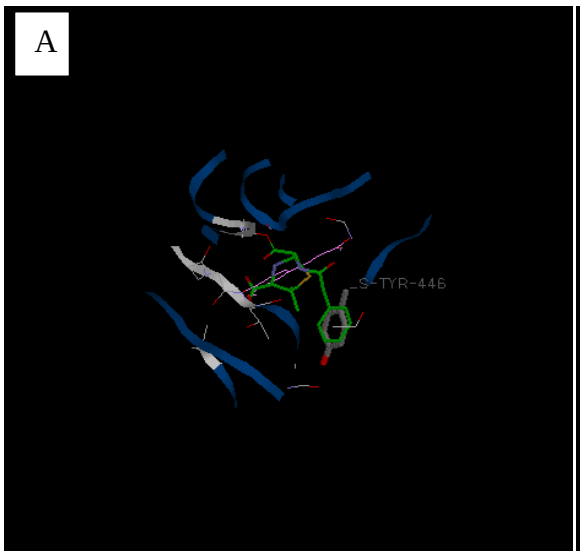
			SER-462, H-S-ASN-464, H-S-TYR-519, H-S-GLN-521, H-S-HIS-583, H-S-SER-643, V-S-TYR-446, V-S-HIS-583, V-S-MET-641				
	Ferulic acid	-71.6	H-M-SER-403, H-S-SER-403, H-M-TYR-446, H-S-ASN-464, H-S-GLN-521, H-S-HIS-583, H-S-SER-598, H-M-THR-600, H-S-THR-600, H-M-GLU-602, V-M-TYR-446, V-S-TYR-446, V-M-SER-461, V-M-SER-462, V-S-SER-462	-49.3	-22.5	0	28
	Quinic acid	-69.6	H-S-SER-403, H-M-TYR-446, H-S-SER-462, H-S-ASN-464, H-S-HIS-583, H-S-SER-598, H-M-GLY-599, H-M-THR-600, H-S-THR-600, H-M-ALA-642, H-M-SER-643,	-46.1	-23.5	0	27.6

			H-S-SER-643, V-M-THR-444, V-S-TYR-446, V-S-THR-600, V-S-MET-641				
	Cedrol	-68.7	V-S-TYR-446, V-S-ASN-464, V-S-HIS-583, V-M-GLY-599, V-M-THR-600,V-S-THR-600, V-S-MET-641	-62.7	-6	0	25.4
	3-(3-Hydroxyphenyl) propanoic acid	-68.5	H-M-SER-403, H-S-SER-403, H-S-LYS-430, H-M-THR-444, H-M-TYR-446, H-S-SER-462, H-S-ASN-464, H-S-TYR-519, H-S-GLN-521, H-S-HIS-583, H-S-SER-643, V-S-TYR-446, V-S-HIS-583, V-S-MET-641	-48.1	-20.4	0	27.5
	Palmitic acid	-68	H-S-SER-403, H-M-TYR-446, H-S-SER-462, H-S-ASN-464, H-S-HIS-583, H-S-SER-598, H-M-GLY-599, H-M-THR-600, H-S-THR-600, H-	-48.2	-19.8	0	23.3

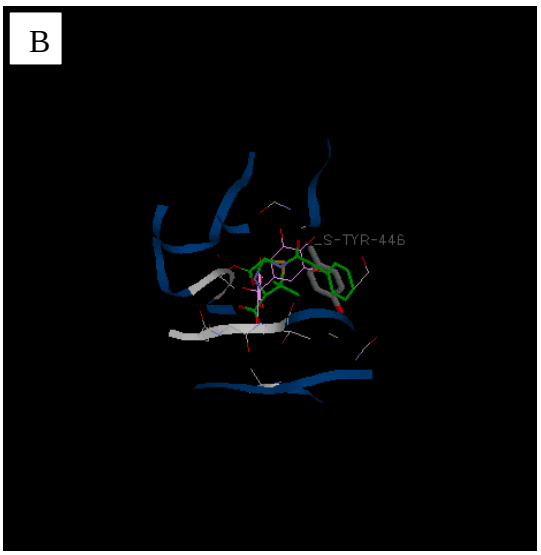
			M-ALA-642, H-M-SER-643, H-S-SER-643, V-M-THR-444, V-S-TYR-446, V-S-THR-600, V-S-MET-641				
	Xanthoxylin	-67.2	H-S-SER-403, H-M-TYR-446, H-S-SER-462, H-S-ASN-464, H-S-HIS-583, H-S-SER-598, H-M-GLY-599, H-M-THR-600, H-S-THR-600, H-M-ALA-642, H-M-SER-643, H-S-SER-643, V-M-THR-444, V-S-TYR-446, V-S-THR-600, V-S-MET-641	-52	-15.2	0	29
	Methyl gallate	-66.3	H-S-SER-403, H-M-TYR-446, H-S-SER-462, H-S-ASN-464, H-S-HIS-583, H-S-SER-598, H-M-GLY-599, H-M-THR-600, H-S-THR-600, H-M-ALA-642, H-M-SER-643, H-S-SER-643, V-M-THR-	-49.3	-17	0	28.2

			444, V-S-TYR-446, V-S-THR-600, V-S-MET-641				
	Gallic acid	-64.4	H-M-SER-403, H-S-SER-403, H-M-TYR-446, H-S-ASN-464, H-S-GLN-521, H-S-HIS-583, H-S-SER-598, H-M-THR-600, H-S-THR-600, H-M-GLU-602, V-M-TYR-446, V-S-TYR-446, V-M-SER-461, V-M-SER-462, V-S-SER-462	-44.7	-19.7	0	27.7

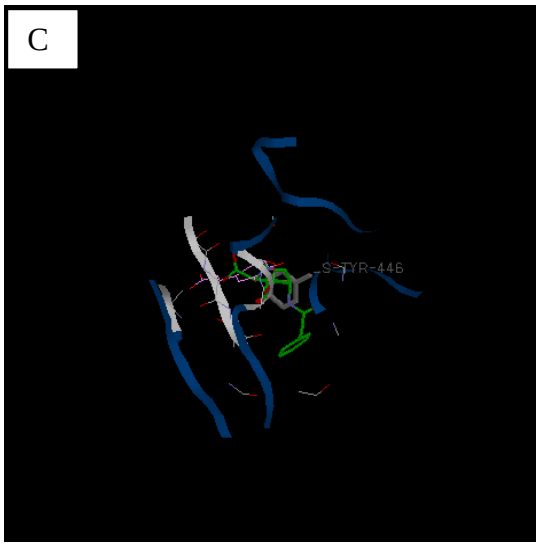
VDW: Van der Walls force, H-bond: hydrogen bond, Elec: Electrostatic, Av. Co: Average cone pai



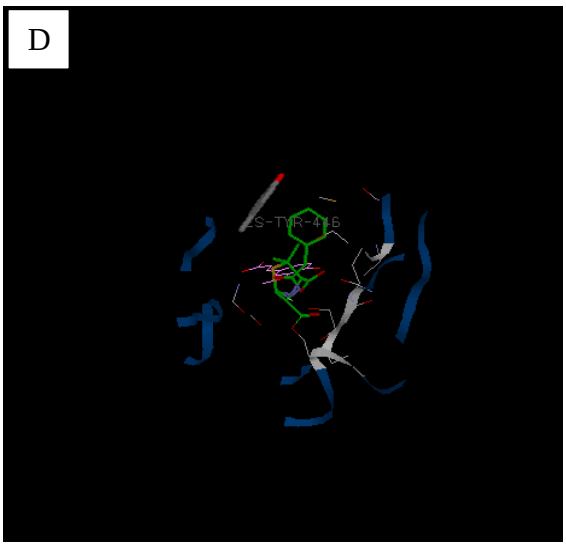
Astragalin docked with ERM



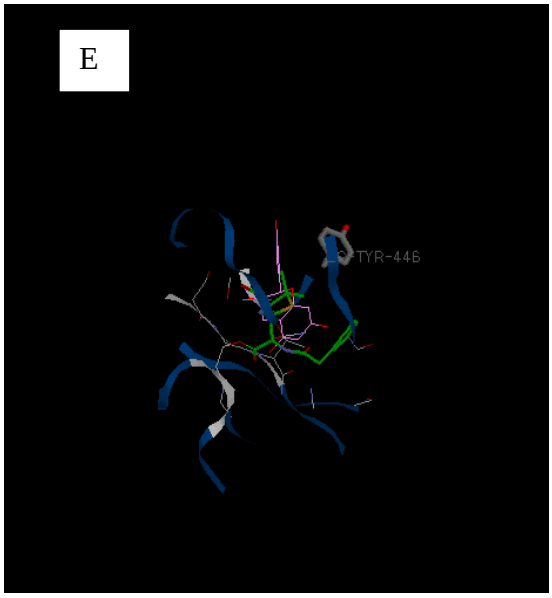
Taxifolin docked with ERM

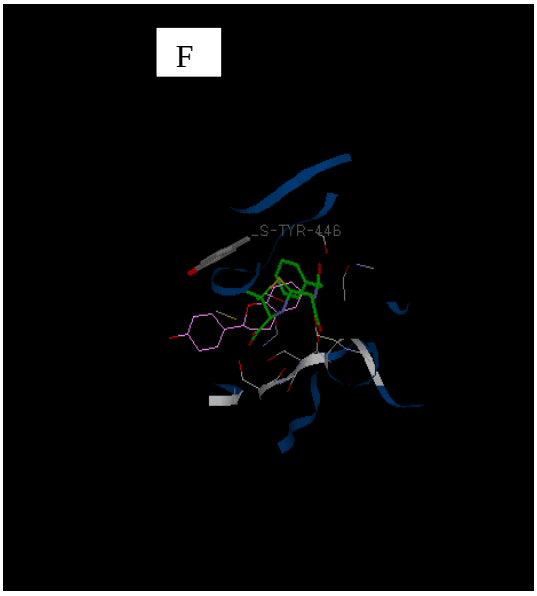


Diferulic acid dicked with ERM

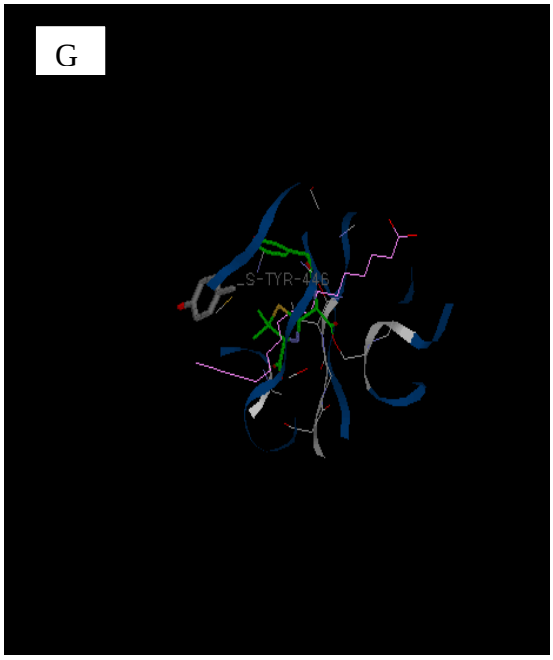


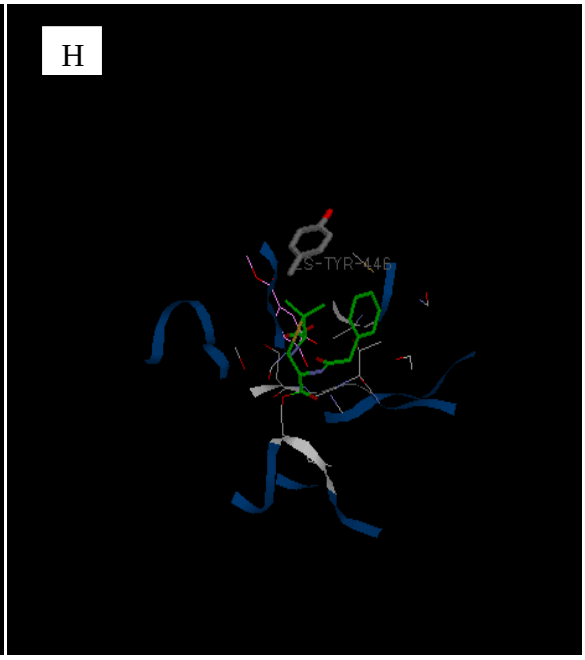
Isoquercetin docked with ERM



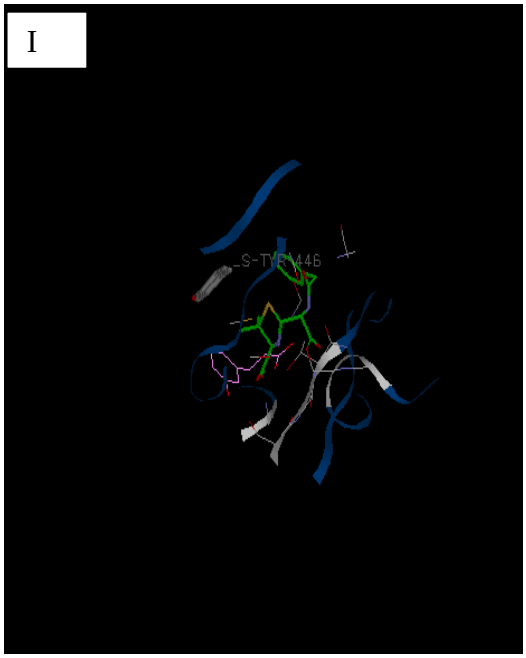


Cianidanol docked with ERM **THF docked with ERM**



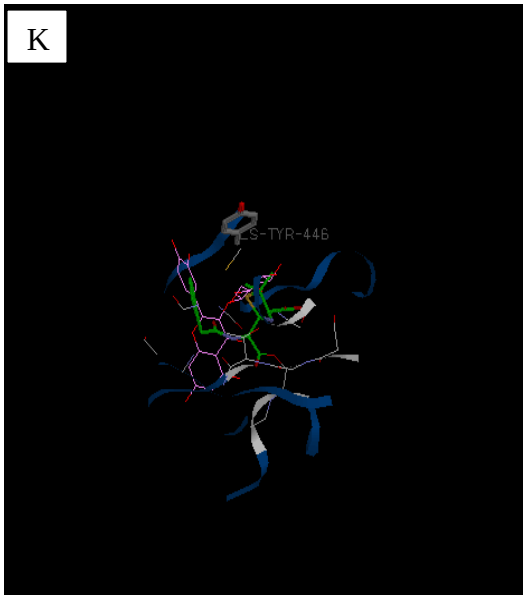


6-O-feruloyl-D-glucose docked with ERM **Kaempferide docked with ERM**

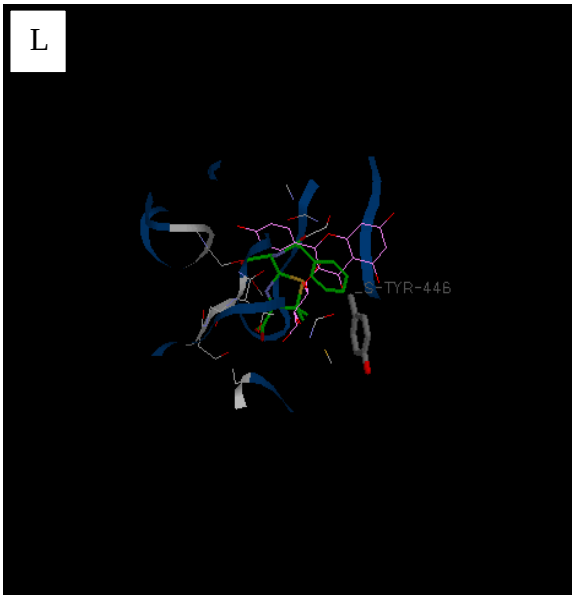


Butin docked with ERM

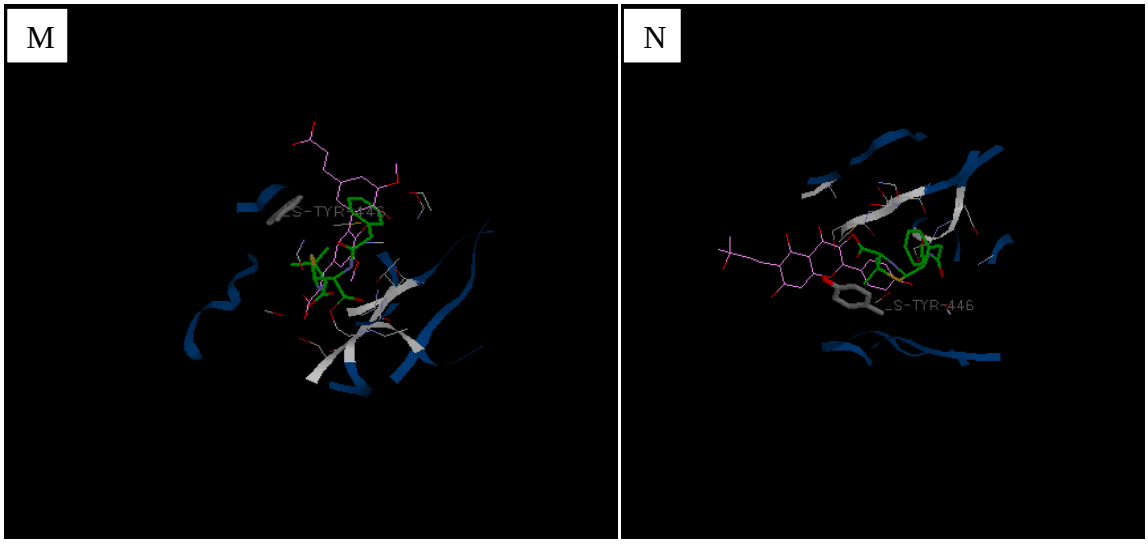
Punicic acid docked with ERM



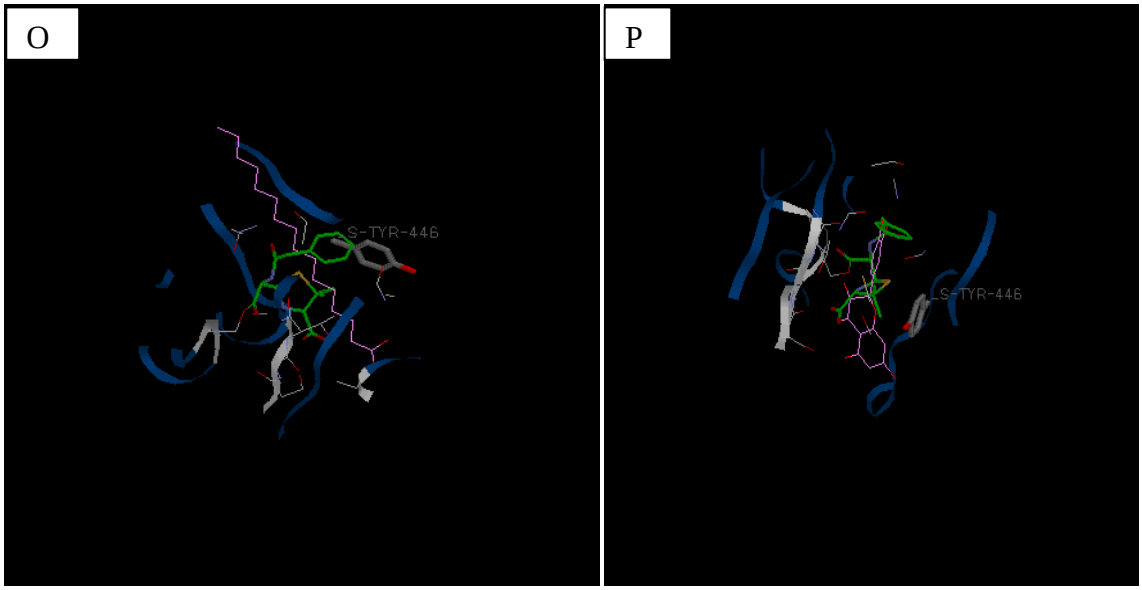
Caffeic acid docked with ERM



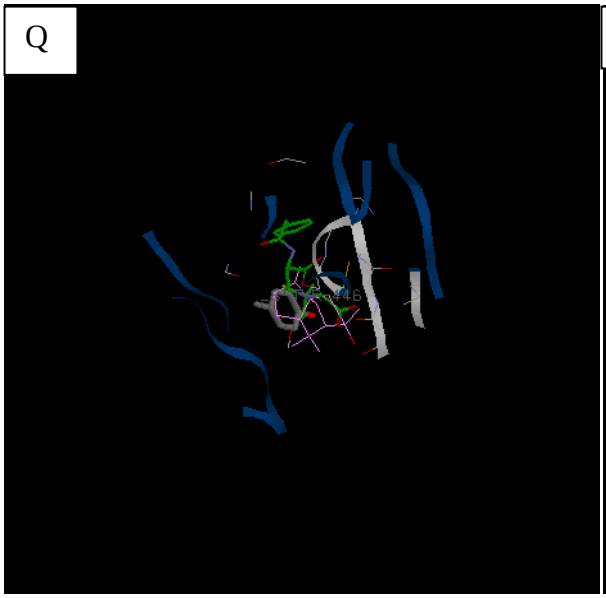
4' hydroxyflavan docked with ERM



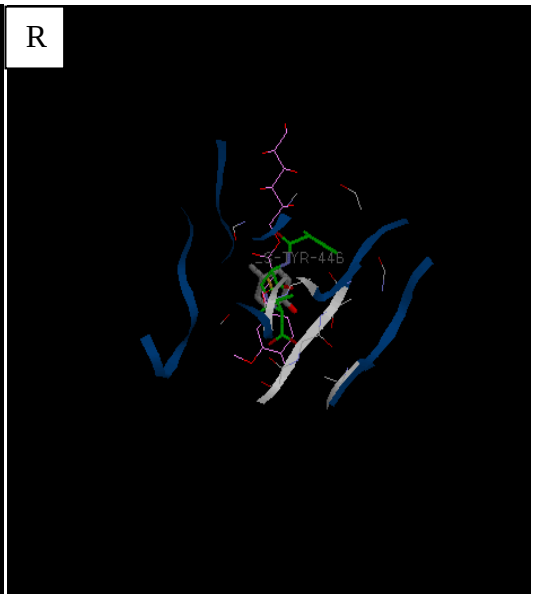
Ferulic acid docked with ERM **Quinic acid docked with ERM**



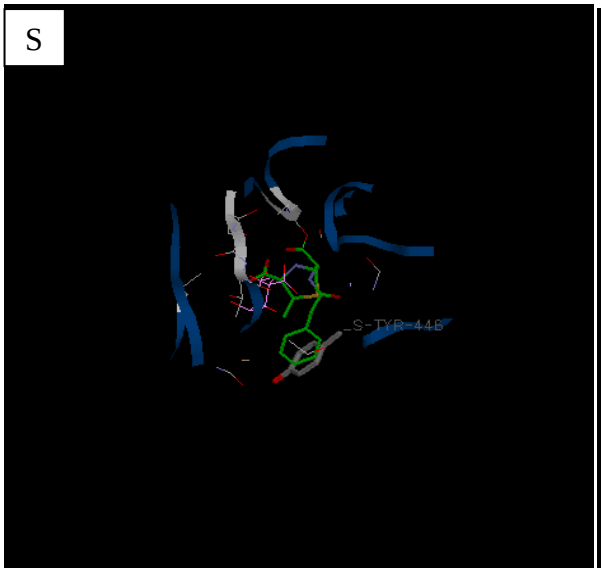
Cedrol docked with ERM **3-(3-hydroxyphenyl) propionic acid docked with ERM**



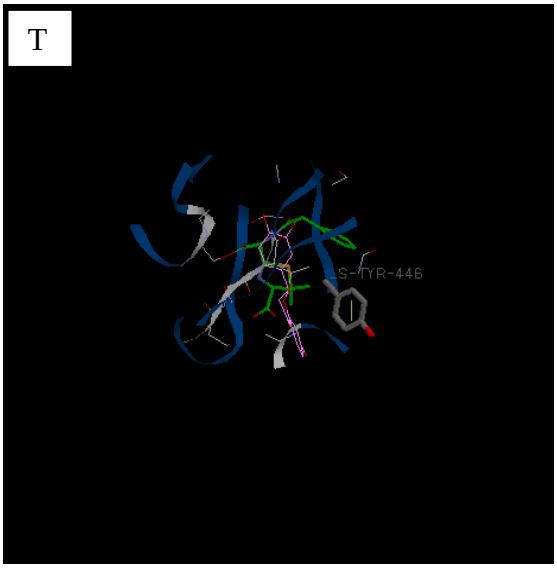
Palmitic acid docked with ERM



Xanthoxylin docked with ERM



Methygallate docked with ERM



Gallic acid docked with ERM

Linezolid docked with ERM

Figure 6.27: Docking pose of plant compounds with ERM

6.13 Drug likeliness properties of active compounds:

6.13.1 Lipinski's rule of five

Lipinski's rule of five (RO5) is the most widely accepted and used method for the evaluation of drug efficacy of different compounds from different sources. It is a computer-based technique in which molinspiration software has been used. In this software for Lipinski's rule of five, there are some properties of compounds that have been characterized viz. lipophilicity in terms of topological polar surface area (TPSA), log P, no of hydrogen bond acceptors, no of hydrogen bond donors, molecular weight and number of violations have been tested to determine and to evaluate that either compound is following or not following the RO5. The profile of drug-likeness properties of tested twenty different compounds in Lipinski's rule of five has been shown in Table 6.12. Our results showed that the molecular weight (MW) of studied compounds were in the range of 170-464. The values of molecular weight are in the reference values of molecular weight in RO5 i.e. ≤500. The hydrogen bond acceptor (H_a) and hydrogen bond donor (H_d) of compounds have been calculated and relates to the values of <10 (H_a) and <5 (H_d) in Lipinski's rule of five (Baell *et al.*, 2013). Our results showed that the twenty tested

compounds were in having <10 hydrogen bond acceptor (H_a) within range of 1-8. For hydrogen bond donor (H_d), all the compounds were found in reference value of <5 except cianidanol (6), gallic acid (5), quinic acid (6),3,5,7,4'-tetrahydroxy-6-(3-hydroxy-3-methylbutyl) flavones (7), 6-O-feruloyl-D-glucose (9), isoquercetin (12), astragalin (11), methygallate (5), butin (5), kaempferide (6), taxifolin (7). The characterization of lipophilicity of compounds is of great importance for its ability to cross or not the biomembrane of cells. This was tested by application of RO5 through the determination of values of log P and topological polar surface area (TPSA). The compounds with values of log P (<5) and TPSA (<140Aº) are the ability to cross the lipid membrane in their actions. Ourresultshowedthat all the twenty studied compounds are in the reference value log P<5 and therefore able to penetrate the cell membrane made by the lipid layer. Molecular polar surface area (PSA) is a descriptor showing the correlation with passive molecular transport through membranes, which permits for the prediction of human intestinal absorption, Caco-2 monolayers permeability, and blood-brain barrier penetration. The TPSA approach leads to the PSA values calculation with high-throughput calculation along with a fast estimation of related transport properties (Ertl *et al*. 2000). Our results showed that the TPSA values of only eighteen compounds except isoquercetin and astragalin are in the range of 20.23Aº-133.52Aº which meets the value of ≤140Aº and good in bioavailability. Thus, eighteen compounds out of twenty cleared the Lipinski's rule of five. Two compounds (isoquercetin and astragalin) are not to clear Lipinski's rule of five as showing two violations in comparison to others compounds.

Table 6.13: Lipinski properties of plant compounds analyzed using molinspiration

Name of compound & ID	Log P	MW	H_a (nOHNH)	H_d (nON)	TPSA	No. violations
Lipinski Rule of Five	<5	<500	<10	<5	<140Aº	
Diferulic acid (5281770)	2.67	386.36	8	4	133.52	0
Caffeic acid (689043)	0.94	180.16	4	3	77.75	0
Xanthoxylin (66654)	1.79	196.70	1	4	55.77	0
Cianidanol (9064)	1.37	290.27	5	6	110.37	0
Ferulic acid (445858)	1.25	194.19	2	4	66.77	0
Quinic acid (6508)	-2.33	192.14	5	6	118.21	0
Gallic acid (370)	0.59	170.12	4	5	97.08	0
4'-Hydroxyflavan (20452436)	3.61	226.28	1	2	29.46	0
3,5,7,4'-Tetrahydroxy-6-(3-hydroxy-3-methylbutyl) flavone (44259047)	3.44	372.27	5	7	131.35	0
6-O-Feruloyl-D-glucose (11725795)	-0.46	356.33	5	9	145.91	0
Isoquercetin (5280804)	-0.36	464.38	8	12	210.50	2
Astragalin (5282102)	0.12	448.38	7	11	190.28	2
Methylgallate (7428)	0.85	184.15	3	5	86.99	0

Butin (92775)	1.71	272.26	3	5	86.99	0
Palmitic acid (985)	7.06	256.43	1	2	37.30	1
Cedrol (65575)	3.77	222.37	1	1	20.23	0
Kaempferide (5281666)	2.71	300.27	3	6	100.13	0
Taxifolin (439533)	0.71	304.25	5	7	127.44	0
Punicic acid (5281126)	6.60	278.44	1	2	37.30	1
3-(3-Hydroxyphenyl) propanoic acid (91)	1.38	166.18	2	3	57.53	0

MW: Molecular weight; H_a: TPSA Topological Polar Surface Area: Hydrogen acceptor; H_d: Hydrogen donor

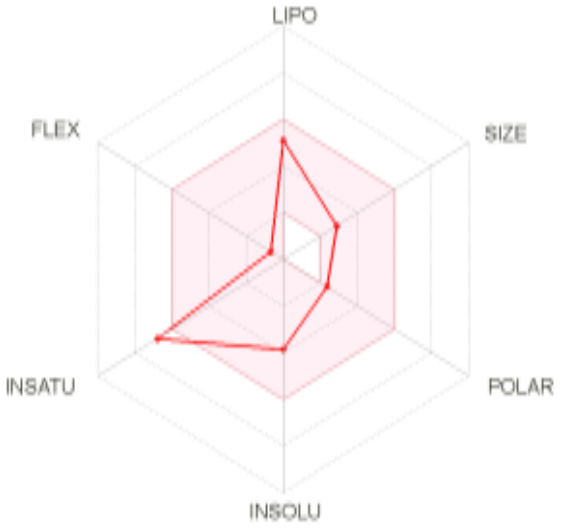
6.14 ADME-Toxicity (ADMET) prediction:

in Silico ADMET analysis is becoming popular in early drug discovery. In our study, we focused on the result of ADMET prediction of plant compounds as per the model of admetSAR. In the result of admetSAR, the first section introduces a prediction model of absorption properties of compounds in form of HIA, Caco-2 Permeability, P-glycoprotein Substrate (P-gps), P-glycoprotein Inhibitor and Renal Organic Cation Transporter inhibitor (ROCTi) and BBB. The second section concerns about prediction model of the metabolism of studied compounds to cytochromes (CYPS) are a superfamily of constitutive and inducible enzymes for the oxidative metabolism of various xenobiotics and bioactive endogenous compounds. Three forms of CYPS, CYP Inhibitory Promiscuity (CYPPRO), CYP substrates (CYP450 2C9, CYP450 2D6, CYP450 3A4) and CYP inhibitors (CYP450 1A2, CYP450 2C9, CYP450 2D6, CYP450 2C19 and CYP450 3A4) were studied. The third and final

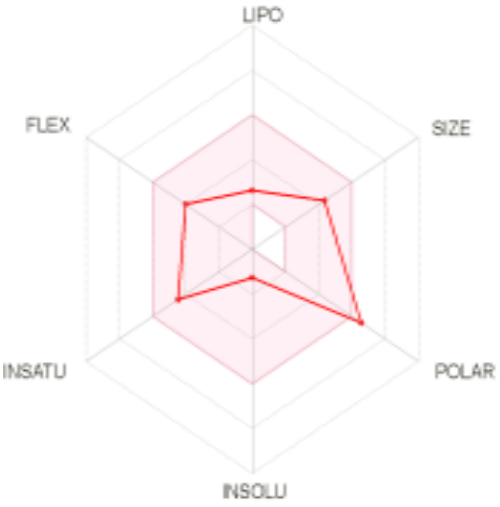
section relates to the prediction model of toxicity of compounds in the form of human ether-a-go-go-related gene inhibition (hERG), carcinogenicity, AMES Toxicity (Ames), Acute Oral Toxicity (AO), and LD_{50}.

6.14.1 Absorption:

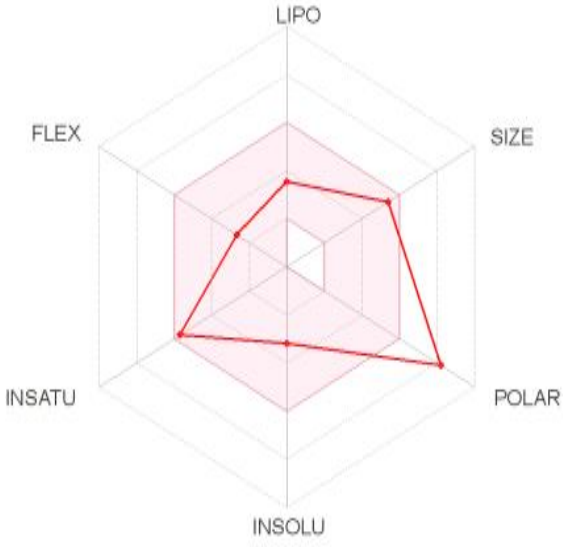
The property absorption of compounds is the key role for its function in drug development. This absorption process is very complex and depends on the nature of different physicochemical properties of compounds viz. size, polarity, solubility, saturation, lipophilicity, and flexibility (Daina and Zoete, 2016). The physicochemical properties affecting the absorption of studied twenty compounds were analyzed through the application of a radar plot as shown in Figure 6.27. The pink area of the plot defines the "oral drug-like" limits for the six properties, which are in the range of XLOGP3 − 0.7 to 5.0; molecular weight 150 to 500 g/mol; TPSA 20 to 130; log *S* < 6; sp3 >0.25 and number of rotatable bonds 0-9. The calculated values of studied compounds being analyzed are displayed as a red pentagon, which should fall within the pink area. The physical properties of tested compounds are of great importance on the influence of absorption has been shown in Figure 6.28 in form of a bioavailability radar plot. Our results revealed the analysis of radar plot that among twenty studied compounds, only four compounds [cedrol, quinic acid, xanthoxylin and 3,5,7,4'-tetrahydroxy-6-(3-hydroxy-3-methylbutyl)flavone] fulfilled the radar plot criteria as the red pentagon lies within the pink area. The eleven compounds viz. 4'-hydroxyflavan, butin, caffeic acid, cianidanol, ferulic acid, diferulic acid, gallic acid, kaempferide, methylgallate, 3-(3-Hydroxyphenyl)propanoic acid and taxifolin showed more instauration properties because the red-colored pentagon was left the pink-colored area and tilt to the instauration factor of radar plot. The three compounds (6-O-feruloyl-D-glucose, astragalin, and isoquercetin) were showed more polarity as the pentagon red-colored left the pink-colored region and pointed more to the polar properties of the radar plot. Similarly, two compounds (palmitic acid and punicic acid) were carried more lipophilic and flexibility properties of radar plot.



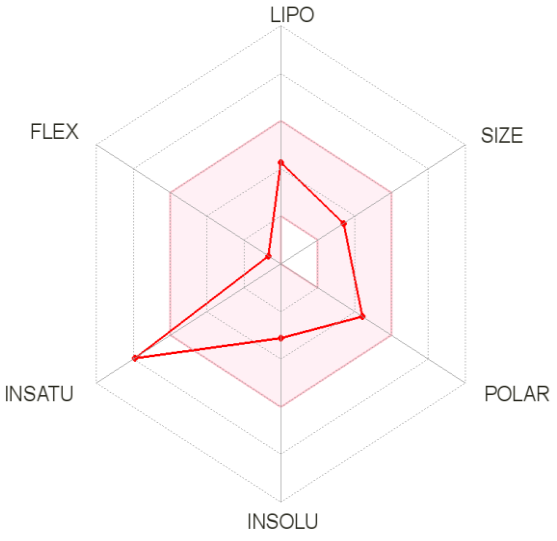
4'-hydroxyflavan



6-O-feruloyl-D-glucose

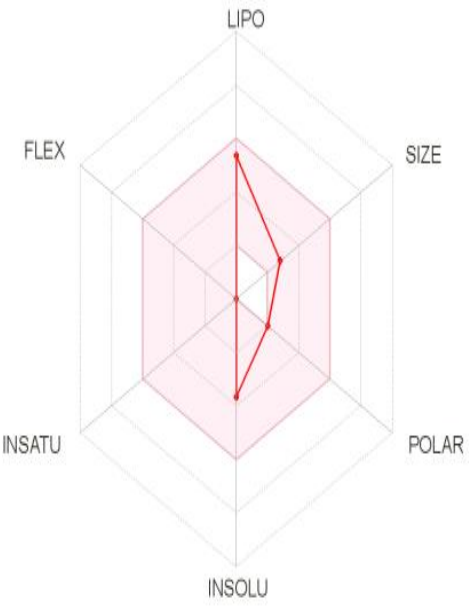


Astragalin

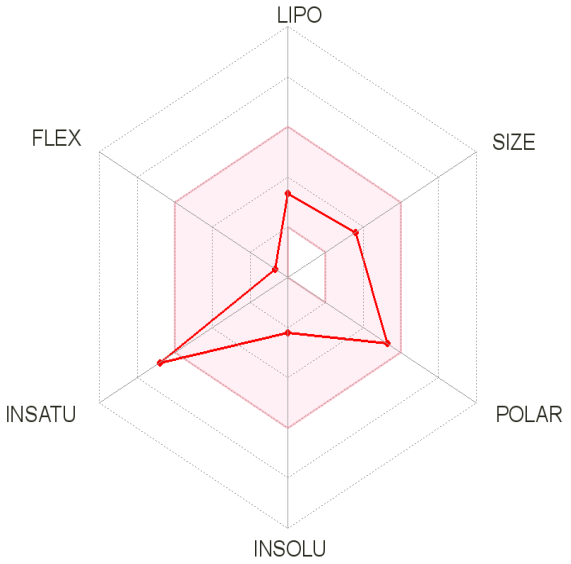


Butin

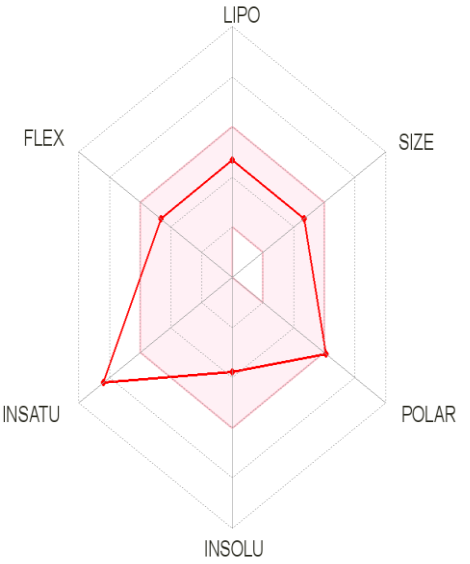
Caffeic acid



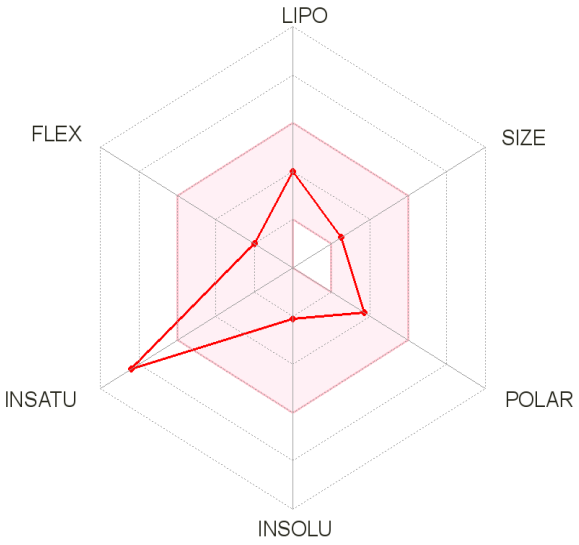
Cedrol



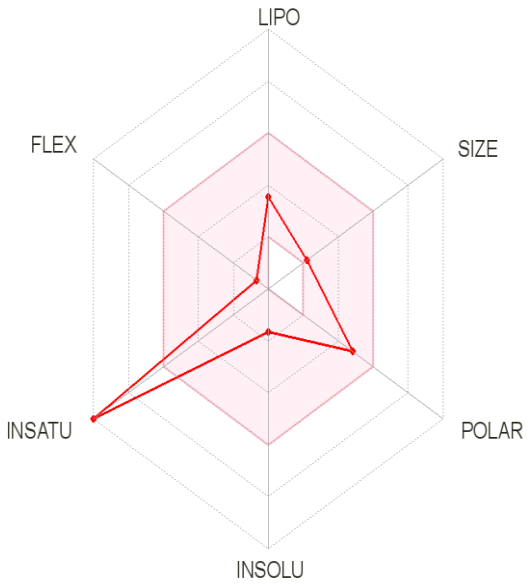
Cianidanol



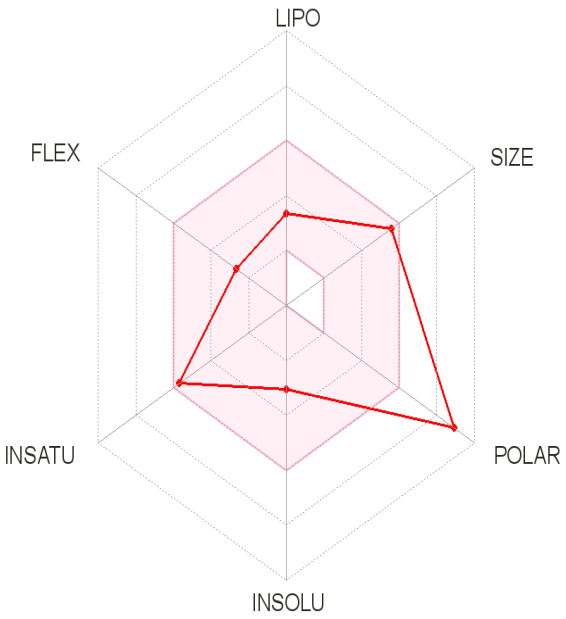
Diferulic acid



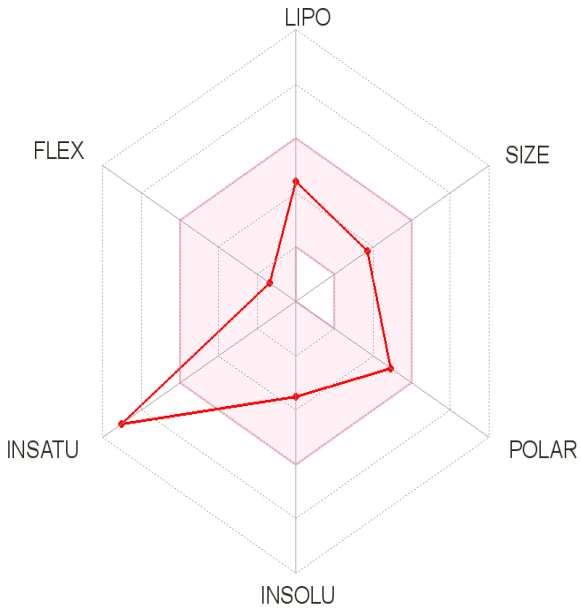
Ferulic acid



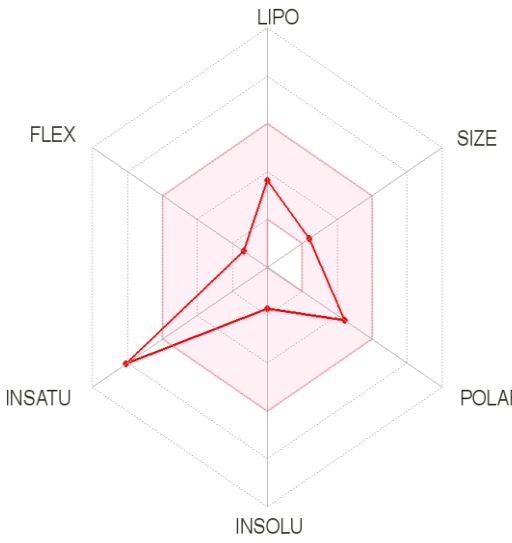
Gallic acid



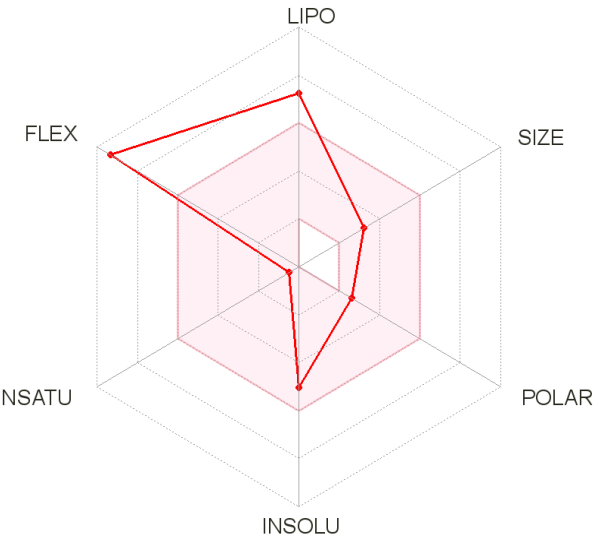
Isoquercetin



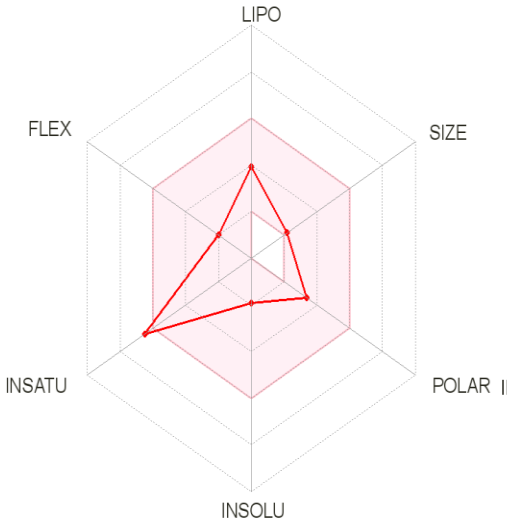
Kaempferide



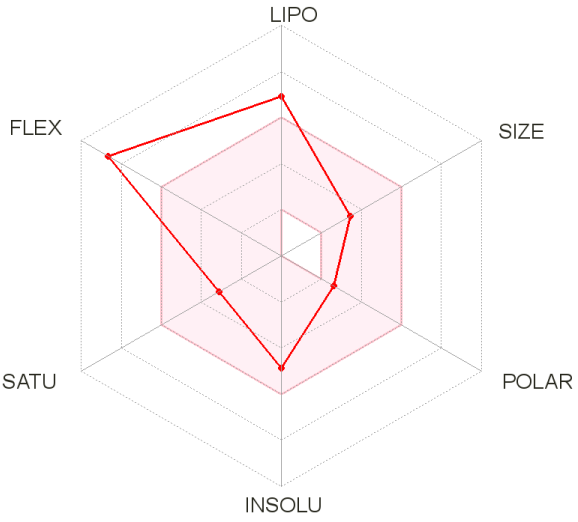
Methylgallate



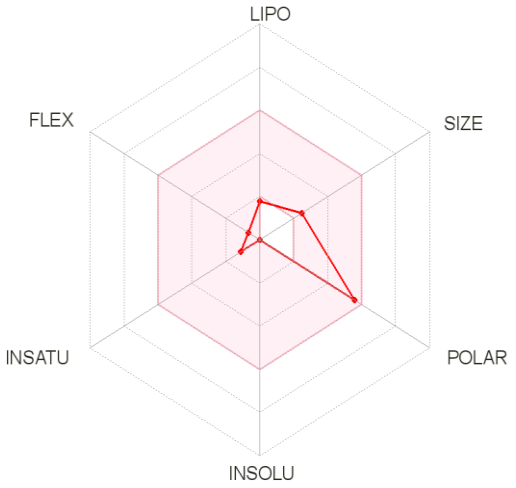
Palmitic acid



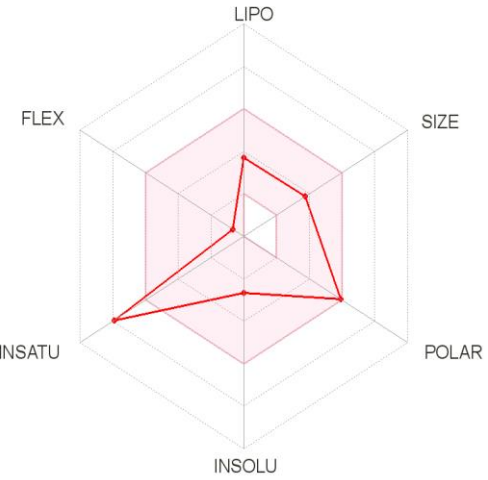
3-(3-hydroxyphenyl) propanoic acid



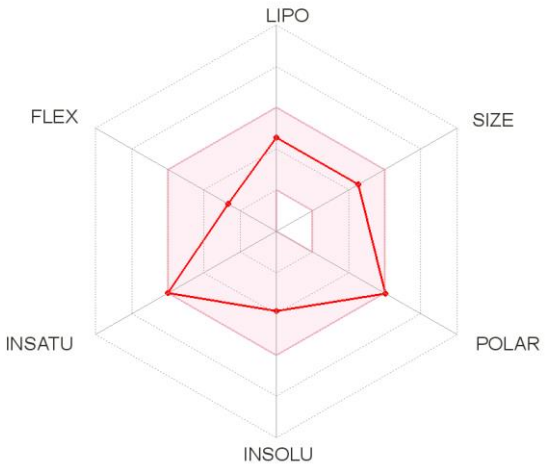
Punicic acid



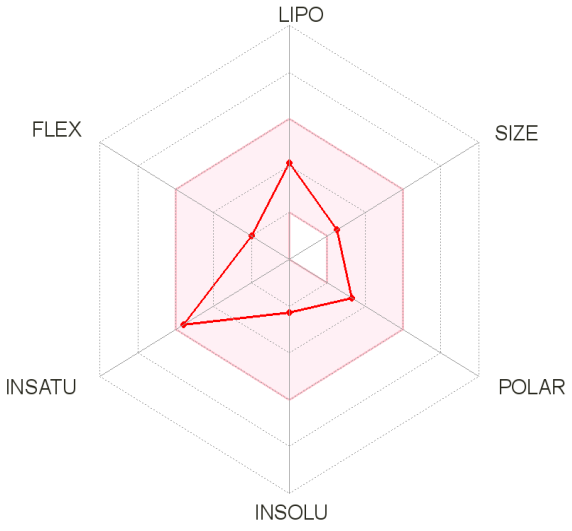
Quinic acid



Taxifolin



3, 5, 7, 4'-Tetrahydroxy-6-(3-hydroxy-3-methylbutyl) flavone



Xanthoxylin

Figure 6.28: The Bioavailability Radar plot of studied compounds.

6.14.1.1 HIA and BBB penetration:

The absorption of compounds in drugs through the intestine is the key properties in functioning after oral administration. HIA of the compound is usually expressed as HAI= D_{blood} /D_{oral}. D_{blood} is the dose or quantity of compound reached to the portal vein and D_{oral} is the dose of the orally administered compound. The blood-brain barrier (BBB) is made by the brain endothelium. It is highly selective for the passage of water and lipid-soluble compounds. The HIA and BBB penetration properties of studied compounds were analyzed through BOILLED-Egg model as per in SwissADME online software. This model is used for rapid analysis to predict the passive HIA and BBB asses of molecules. The HIA and BBB penetration properties of studied compounds have been shown as in BOILLED-Egg model in Figure 6.29. The BOILLED-Egg (brain or intestinal estimated permeation predicted model) is an instinctive graphical plot of the functions of lipophilicity and apparently polarity as described by WLOGP (atomistic octanol-water partition coefficient) and TPSA (topological polar surface area) respectively. The results of run BOILLED-Egg model revealed that compounds that are located in the yellow region (yolk) exhibits a high level of probability for BBB penetration whereas those represented in the white region exhibit the tendency of passive absorption through the GI tract. Among twenty analyzed compounds in BOILLED-Egg model, our results showed that nine compounds viz. caffeic acid (molecule 5), butin (molecule 4), kaempferide (molecule 12), cianidanol (molecule 7), gallic acid (molecule 10), methylgallate (molecule 13), taxifolin (molecule 18), diferulic acid (molecule 8) and 3,5,7,4' tetra hydroxyl-6 (3-hydroxy-3-methylbutyl) flavon (molecule 19) were foretell to have good gastrointestinal (GI) absorption. Similarly, seven compounds viz. 4' hydroxyflavan (molecule 1), cedrol (molecule 6), palmatic acid (molecule 14), punicic acid (molecule 16), ferulic acid (molecule 9), 3-(3-hydroxyphenyl) propanoic acid (molecule 15) and xanthoxylin (molecule 20) were predicted to have good blood brain barrier penetration. The three compounds viz. 6-O-feruloyl-D-glucose (molecule 2), astragalin (molecule 3) and quinic acid (molecule 17) were found outside of the white portion of the model and predicted for its poor absorption through the GI tract. The one compound, isoquercetin

was found to be not followed this model as it is neither absorbed through the GI tract nor penetrate the BBB.

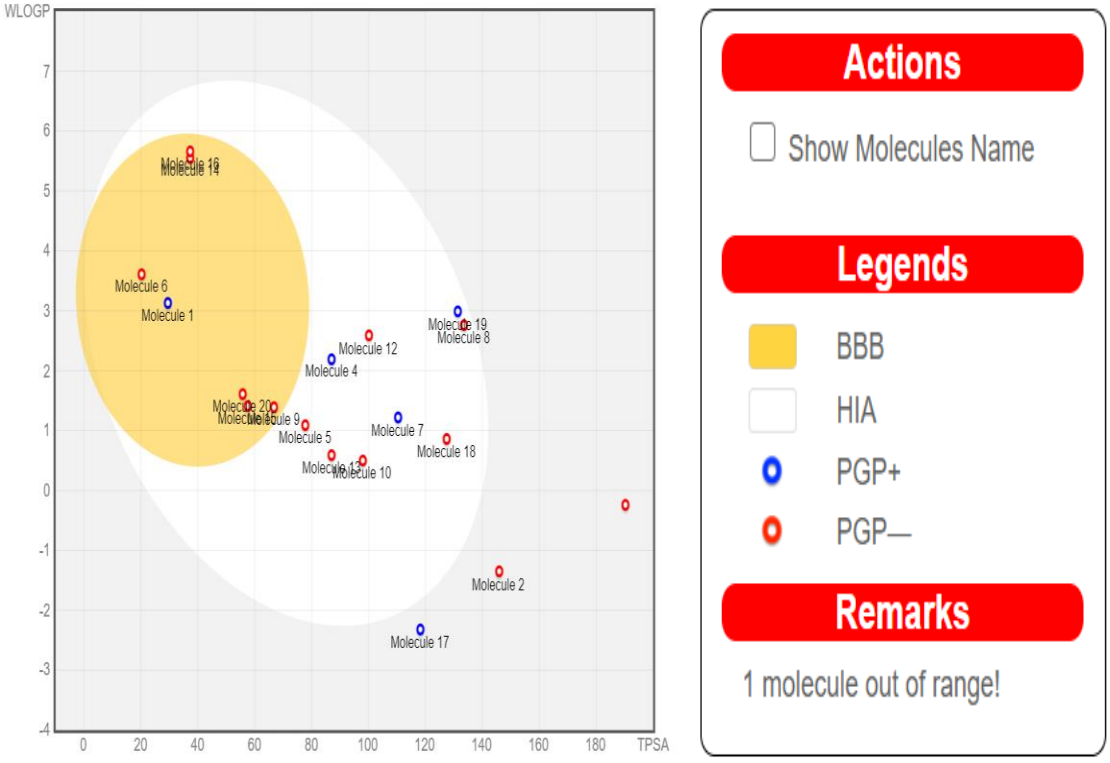


Figure 6.29: BOILLED-EGG graph depicting gastrointestinal absorption and brain penetration of compounds

6.14.1.2 Caco-2 permeability prediction:

The monolayer of human intestinal epithelial cells originates from human colorectal carcinoma cells (Caco-2). This method is used for checking the uptake efficiency of compounds and is the most effective *in vitro* gold standard for assessing into the body through expressing by cell permeability, P_{app} cm/s. The P_{app} has been effective for the uptake efficiency of drugs containing compounds. The *in vitro* or experimental method is cost-effective and time-consuming thus computation method is used as per the method of admetSAR for analysis of Caco-2 permeability prediction of compounds. The Caco-2 permeability of studied compounds has been shown in Figure 6.30. The predicted value of P_{app} is >0.90. Our results revealed that the values of P_{app} of studied compounds were in

the range of from -0.8 to 1.5. The six compounds viz. 4'hydroxyflavan (1.5), cedrol (1.5), palmitic acid (1.3), 3-(3-hydroxyphenyl) propanoic acid (1.1), punicic acid (1.4), and xanthoxylin (1.4) showed the value of P_{app} was greater than 0.9 and it can be assumed that the compounds have a high Caco-2 permeability in comparison to others has P_{app}<0.9 cm/s.

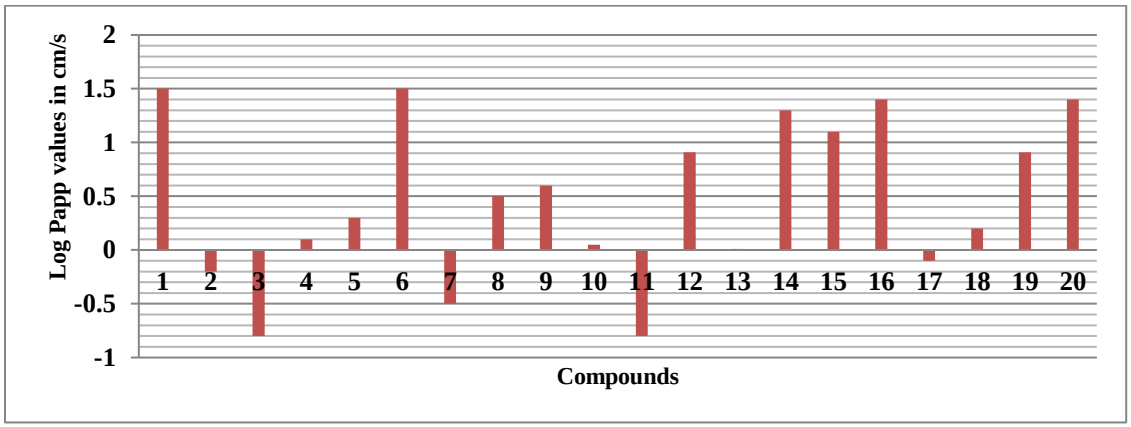


Figure 6.30: Cacco permeability properties of potential compounds

6.14.2 Distribution:

The P-gp plays a key role in determining the distribution property of ADMET analysis of many compounds. P-gp is an essential efflux transporter protein found mainly on the normal cells of the intestine, brain, liver, pancreas, adrenal gland. The function of P-gp is the prevention of accumulation of exogenous substances in cells by transporting functionally and structurally unrelated compounds from the interior of cells into the extracellular space. The compounds those are substrates of P-gp having low BBB permeability, low intestinal absorption and face the increased risk of metabolism in intestinal cells (Cuminns *et al.,* 2003). The P-gp modeling compounds are affecting the pharmacokinetic profiles of drugs which show that the drugs are either substrate or inhibitors of P-gp (Sugano *et al.*, 2010 & Szacaks *et al.*, 2006). The properties of tested compounds in the role of either substrate or inhibitor of P-gp as per the method of AdmetSAR have been shown in Figure 6.31 and in Table 6.14. Our results showed that out of twenty compounds, only nine compounds (Cianidanol, 3, 5, 7, 4'-tetrahydroxy-6-

(3-hydroxy-3-methylbutyl) flavones, 6-O-feruloyl-D-glucose, isoquercetin, astragalin, butin, cedrol, kaempferide and taxifolin) behave as the substrate to P-gp transporter protein while three compounds (diferulic acid, 3, 5, 7, 4'-tetrahydroxy-6-(3-hydroxy-3-methylbutyl) flavones and kaempferide) are the inhibitor of P-gp efflux protein.

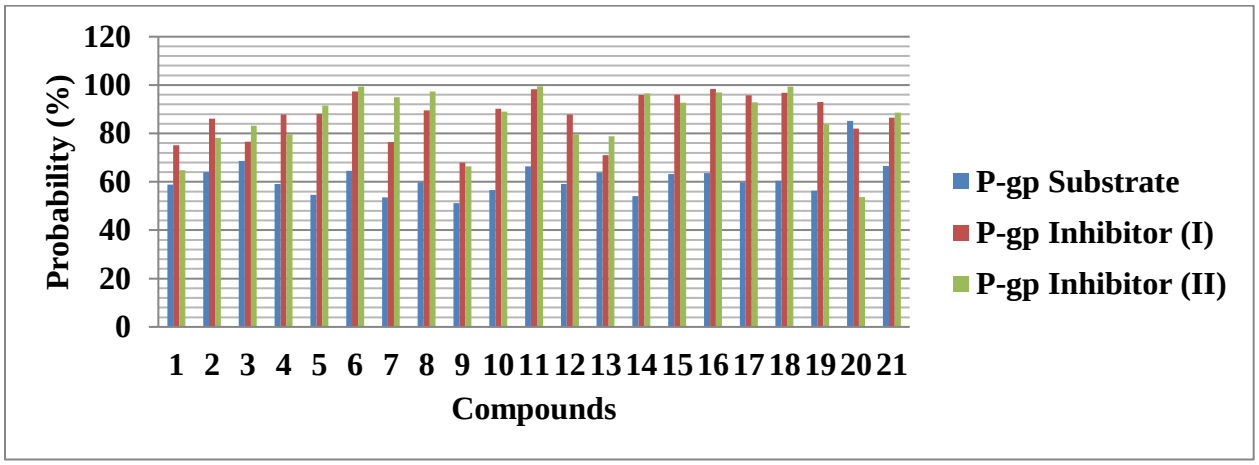


Figure 6.31: Percentage of compounds to be substrate/non substrate and inhibitor/non inhibitor of P-gp. The predictions were performed with AdmetSAR. 1: linezolid, 2: 4'-Hydroxyflavan, 3: 6-O-feruloyl-D-glucose, 4: Astragalin, 5: Butin, 6: Caffeic acid, 7: Cedrol, 8: Cianidanol, 9:Diferulic acid, 10: Ferulic acid, 11: Gallic acid, 12: Isoquercetin, 13: Kaempferide, 14: Methylgallate, 15: Palmitic acid, 16: 3-(3-Hydroxyphenyl) propanoic acid, 17: Punicic acid, 18: Quinic acid, 19:Taxifolin, 20: 3,5,7,4'-Tetrahydroxy-6-(3-hydroxy-3-methylbutyl) flavones, 21: Xanthoxylin

Table 6.14: Compounds predicted to be substrate/non substrate and inhibitor/non inhibitor of P-gp with AdmetSAR

Compounds	P-gps	P-gpi (I)	P-gpi (II)
Diferulic acid	NS	NI	I
Caffeic acid	NS	NI	NI
Xanthoxylin	NS	NI	NI
Cianidanol	S	NI	NI

Ferulic acid	NS	NI	NI
Quinic acid	NS	NI	NI
Gallic acid	NS	NI	NI
4'-Hydroxyflavan	NS	NI	NI
3,5,7,4'-Tetrahydroxy-6-(3-hydroxy-3-methylbutyl)flavone	S	NI	I
6-O-Feruloyl-D-glucose	S	NI	NI
Isoquercetin	S	NI	NI
Astragalin	S	NI	NI
Methylgallate	NS	NI	NI
Butin	S	NI	NI
Palmitic acid	NS	NI	NI
Cedrol	S	NI	NI
Kaempferide	S	NI	I
Taxifolin	S	NI	NI
Punicic acid	NS	NI	NI
3-(3-Hydroxyphenyl) propanoic acid	NS	NI	NI

P-gps: P-glycoprotein Substrate, P-gpi: P-glycoprotein Inhibitor, NS:Non-substrate, S: Substrate, NI: Non-Inhibitor; I: Inhibitors.

6.14.3 Metabolism:

Metabolism of most drugs and xenobiotic compounds is therefore of particular importance in clinical pharmacology for predicting the fate of drugs in patients. The cytochromes450 (CYPs) is the major enzyme family capable of metabolizing or oxidative biotransformation of most different drugs and lipophilic xenobiotics located in the liver and intestine. There are five different isomers of cytochromes450 (CYP 3A4, 2D6, 1A2, 2C9, and 2C19) that have been identified which are responsible for the metabolism of 75% of drug metabolism through oxidation. The profile of properties of inhibitor or non-inhibitor of compounds to CYP450 enzymes in comparison to linezolid drug in probability percentage was shown in Figure 6.32. The result showed that all the tested

compounds were non-inhibitor of CYP4501A2 isoenzyme except for six compounds viz. 4'-Hydroxyflavan, butin, palmitic acid, kaempferide, taxifolin and punicic acid. Out of twenty compounds, four compounds (diferulic acid, 4'-Hydroxyflavan, butin, and kaempferide) were the inhibitors of CYP450 2C9 isoenzyme. The twenty studied compounds have shown the non inhibitor properties to CYP450 2D6 isoenzyme. In the case of CYP450 2C19 isoenzyme, only three compounds viz. diferulic acid, 4'-Hydroxyflavan and kaempferide were the inhibitors among total tested compounds. Out of twenty tested compounds, three compounds (3, 5, 7, 4'-Tetrahydroxy-6-(3-hydroxy-3-methylbutyl) flavones, kaempferide, and taxifolin were the inhibitors of CYP450 3A4 isoenzyme.

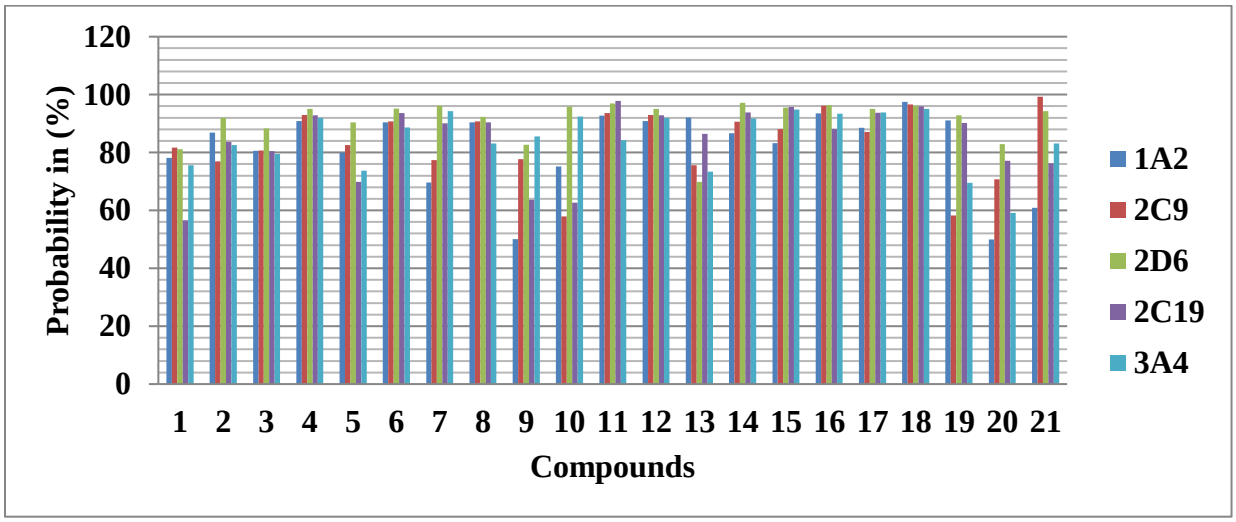


Figure 6.32: CYP inhibitor/noninhibitor with respect to various isomers of CYP450 (1A2, 2C9, 2D6, 2C19, 3A4). 1: linezolid, 2: 4'-Hydroxyflavan, 3: 6-O-Feruloyl-D-glucose, 4: Astragalin, 5: Butin, 6: Caffeic acid, 7: Cedrol, 8: Cianidanol, 9:Diferulic acid, 10: Ferulic acid, 11: Gallic acid, 12: Isoquercetin, 13: Kaempferide, 14: Methylgallate, 15: Palmitic acid, 16: 3-(3-Hydroxyphenyl) propanoic acid, 17: Punicic acid, 18: Quinic acid,19: Taxifolin, 20:3,5,7,4'-Tetrahydroxy-6-(3-hydroxy-3-methylbutyl) flavones, 21: Xanthoxylin.

The profile of properties of substrate or non substrate of compounds to isoenzymes of CYP450 (2C9, 2D6 and 3A4) in comparison to linezolid drug in probability percentage was shown in Figure 6.33. Our results revealed that twenty all tested compounds were not substrate to two isoenzymes (2C9 and 2D6) while all compounds except two compounds (cedrol and 3,5,7,4'-tetrahydroxy-6-(3-hydroxy-3-methylbutyl) flavones were also not the substrate to 3A4 isomer of enzyme CYP450.

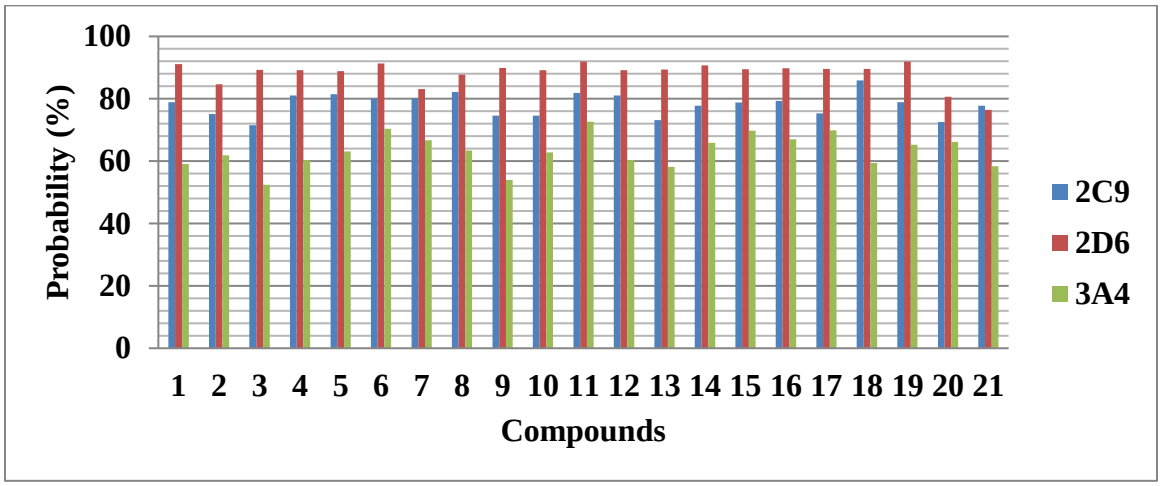


Figure 6.33: CYP substrate/non substrate with respect to various isomers of CYP450 (2C9, 2D6 and 3A4). 1: linezolid, 2: 4'-Hydroxyflavan, 3: 6-O-Feruloyl-D-glucose, 4: Astragalin, 5: Butin, 6: Caffeic acid, 7: Cedrol, 8: Cianidanol, 9: Diferulic acid, 10: Ferulic acid, 11: Gallic acid, 12: Isoquercetin, 13: Kaempferide, 14: Methylgallate, 15: Palmitic acid, 16: 3-(3-Hydroxyphenyl) propanoic acid, 17: Punicic acid, 18: Quinic acid,19: Taxifolin, 20: 3,5,7,4'-Tetrahydroxy-6-(3-hydroxy-3-methylbutyl) flavones, 21: Xanthoxylin

6.14.4 Excretion:

The excretion properties of drugs in ADMET studies is of great important for maintaining of concentration of compounds in patients through movement of exogenous and endogenous into and from the cells. The excretion of compounds are carried by different carrier proteins called solute carriers (SLCs) located on epithelial cells

especially on liver and kidney. The SLC proteins are of different types belonging ATP binding cassette (ABC) family. Among the solute carrier proteins, the excretion properties of tested compounds were analyzed through organic cation transporters (OCTs) proteins. The inhibitors of renal organic cationic transport decreases OCT-dependent renal clearance of a victim (substrate) drug and in fact decrease the efficacy of lead compounds (Ciarimboli, 2008). The profile of inhibitor/noninhibitor of renal organic cation transporter of compounds has been shown in Figure 6.34. The present result revealed that all twenty studied compounds were the non-inhibitor to the renal organic transporter proteins as similar to the control drug linezolid.

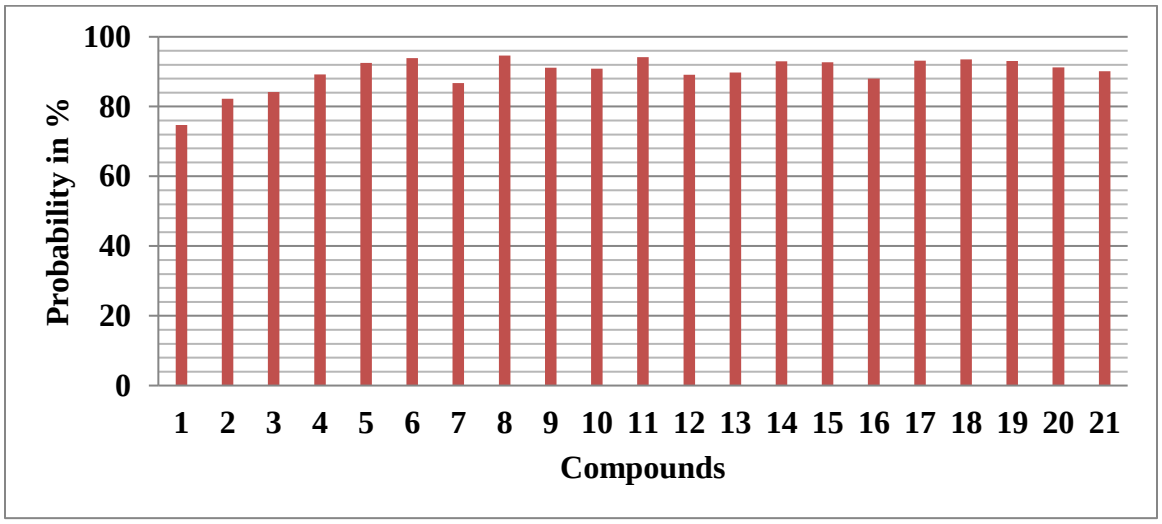


Figure 6.34: Percentage of compounds to be inhibitor/non inhibitor of ROC transporter protein. ROC: Renal organic cation, 1: linezolid, 2: 4'-Hydroxyflavan, 3: 6-O-Feruloyl-D-glucose, 4: Astragalin, 5: Butin, 6: Caffeic acid, 7: Cedrol, 8: Cianidanol, 9: Diferulic acid, 10: Ferulic acid, 11: Gallic acid, 12: Isoquercetin, 13: Kaempferide, 14: Methylgallate, 15: Palmitic acid, 16: 3-(3-Hydroxyphenyl) propanoic acid, 17: Punicic acid, 18: Quinic acid, 19: Taxifolin, 20: 3,5,7,4'-Tetrahydroxy-6-(3-hydroxy-3-methylbutyl) flavones, 21: Xanthoxylin

6.14.5 Toxicity:

The use of different natural products from sources of plants, animals, and marine origin as the revitalizers, health supplements, and agents for prevention of diseases are rising now a day. Diversified medicinal plants have different pharmacological activities could

show toxic effects when used internally without scientific validation. It is pertinent to assessing the toxic profile of different compounds used for the development of drugs. The result of AdmetSAR analysis to predict the toxicity properties of compounds with different criteria such as hERG, carcinogenic, ames toxicity and acute oral toxicity has been shown in Table 6.14.

The hERG potassium channel mediates an important function in maintaining cardiac activity and normal cardiac rhythm. Any changes or inhibition of hERG causes the disorder of cardiac repolarization and risk of sudden death due to dysfunction of cardiac function. Therefore, it is essential to analyze inhibitor or non-inhibitor properties of studied compounds to hERG as shown in Table 6.14. Our results revealed that all the tested compounds were showed non-inhibitor properties to hERG gene product and therefore these are safe for use in the drug development process.

The Ames test is important to studied compounds in order to understand the risks of their mutagenic properties. The mutagenic effect of the compound leads to cause cancer in patients and it could also further causes unknown defects in later generations. Thus, the Ames test or mutagenicity of compounds is an essential step before releasing drugs in commercial use. From Table 6.14, our results regarding the Ames test revealed that all twenty compounds were Ames test negative except isoquercetin and astragalin that indicate that both the compounds are mutagenic and therefore may act as carcinogens (Pires et *al*., 2015).

Any physical or chemical agents having the capacity to cause cancer in subjects are known as carcinogens. Thus, carcinogenic properties of studied compounds were analyzed in order to find which one is either carcinogenic or non-carcinogenic. Our results revealed that the tested twenty compounds were non-carcinogenic similar to the control drug linezolid as shown in Table 6.15.

The acute oral toxicity of compounds is analyzed by LD_{50}. LD_{50} defined as the amount of material required to cause mortality of 50% of a group of test animals. Depending on

LD_{50}, compounds are categorized into four groups as per the standard of WHO, 2010. The compounds with LD_{50} values ≤50mg/kg are placed in category I followed by compounds having the LD_{50} values ≥50mg/kg but ≤500mg/kg (category II), ≥500mg/kg but ≤5000mg/kg (category III), ≥5000mg/kg (category IV). The compounds in categories I and II are the toxic compounds and compounds in categories III and IV are considered as non-toxic compounds. The toxic or non-toxic properties of studied compounds through LD_{50} values in form of different categorization I, II, III, and IV have been shown in Table 6.15. Our results revealed that all the twenty tested compounds were found in category III and IV with non-toxic properties except two butin and taxifolin found in category II with toxic properties.

Table 6.15: hERG, AMES Toxicity, acute oral toxicity and Carcinogenicity profile of potential compounds

Compounds	hERG	AMES Toxicity	Carcinogens	Acute oral toxicity
Linezolid	WI	NT	NC	(III)
Diferulic acid	WI	NT	NC	(III)
Caffeic acid	WI	NT	NC	(IV)
Xanthoxylin	WI	NT	NC	(III)
Cianidanol	WI	NT	NC	(IV)
Ferulic acid	WI	NT	NC	(IV)
Quinic acid	WI	NT	NC	(III)
Gallic acid	WI	NT	NC	(III)
4'-Hydroxyflavan	WI	NT	NC	(III)
3,5,7,4'-Tetrahydroxy-6-(3-hydroxy-3-methylbutyl) flavone	WI	NT	NC	(III)
6-O-Feruloyl-D-glucose	WI	NT	NC	(III)
Isoquercetin	WI	T	NC	(III)
Astragalin	WI	T	NC	(III)

Methylgallate	WI	NT	NC	(III)
Butin	WI	NT	NC	(II)
Palmitic acid	WI	NT	NC	(IV)
Cedrol	WI	NT	NC	(III)
Kaempferide	WI	NT	NC	(III)
Taxifolin	WI	NT	NC	(II)
Punicic acid	WI	NT	NC	(IV)
3-(3-Hydroxyphenyl) propanoic acid	WI	NT	NC	(III)

WI: Weak inhibitor, NT: Non toxic, NC: Non-carcinogens, NI: non inhibitor, T: toxic

CHAPTER VII

7 Summary and conclusions:

7.1 Summary

The present studyafter observation of results it is concluded that 43.35% of total clinical samples were showed positive bacterial growth with higher isolates of Gram negative bacteria (53.63%) than Gram positive bacteria (46.36%). It is also concluded that 35.75% of total infections were caused by *Staphylococcus aureus* in the study area. This signifies the high rate of infection caused alone by *Staphylococcus aureus* than other bacteria. The investigation declared the infectious value of *S. aureus* and its antibiotic resistant strains.

310 *S. aureus* were isolated from seven different clinical samples. Most of the cases were male 51.61% than female 48.38%. The frequent isolation of *S. aureus*was from blood and pus samples among adult aged patients.

Resistant to methicillin was detected by using oxacillin 10µg/disc. We observed an increase in MRSA isolates with 79.08% than MSSA isolates with 20.91% among *S. aureus*. Majority of MRSA were found from blood (55.78%) followed by pus 101 (41.73%), body fluids 3 (1.23%), urine 1 (0.41%), sputum 1 (0.41%) and swabs 1 (0.41%). The majority of MSSA were found maximum in higher in pus specimens 42 (65.62%) followed by blood 19 (29.68%), body fluids 2 (3.12%) and urine 1 (1.56%). Most of patients were male 129 (53.30%) and 33 (51.56%) for MRSA and MSSA respectively than female 113 (46.69%) and 31 (48.43%). MRSA is highly prevalent among 0-20 age group with 138 (57.02%) followed by 21-40 age group with 80 (33.05%), 41-60 age group with 19 (7.85%), 61-80 age group with 04 (1.65%) and >80 age group with 01 (0.41%) than MSSA was 29 (45.31%) in age group of 0-20 years followed by 27 (42.18%) in age of 21-40, 06 (9.37%) for the age of 41-60 and 02 (3.12%) in age of 61-80 years.

The phenotypes of *Staphylococcus aureus* were examined for presence of either inducible or constitutive resistance. 204 (66.67%) *S. aureus* were found to be resistant to erythromycin. Erythromycin resistant *Staphylococcus aureus* (ERY-R *S. aureus*) were subjected to D- test, it was observed that 132 (64.70%) are of iMLSB phenotype and 72 (32.29%) of cMLSB phenotype. The occurrence of iMLSB strains were found to be higher in pus sample 76 (57.57%) followed by blood 54 (40.90%) and body fluids 02 (1.51%) than cMLSB strains were found to be higher in blood sample 43 (59.72%) followed by pus 29 (40.27%). The males 69 (52.27%) and 45 (62.5%) are more susceptible to iMLSB and cMLSB strains of *S. aureus* in comparison to female 63 (47.72%) and 31 (48.43%) respectively.The iMLSB and cMLSB strainswere found higher among patients of 0-20 age group with 73 (55.30%) and 38 (52.77%) respectively.

iMLSB strains of *S. aureus* were found higher 106 (43.80%) among MRSA in compassion to MSSA 26 (40.62%). cMLSB isolates were also predominant among methicillin resistant 65 (26.89%) when compared to methicillin sensitive isolates 7 (10.93%).

Five medicinal plants leave *Syzygium cumini*, *Azadirachta indica*, *Moringa oleifera*, *Nyctanthes arbortristis* and *Tinospora cordifolia* were selected for quantification of yields, phytochemical screening, antimicrobial screening, liquid chromatography mass spectroscopy analysis and molecular docking studies. Aqueous, ethanol, methanol, acetone and hexane plant extracts assessed. The yield of tested plants were found higher in aqueous solvent 37.90±0.10 in *T. cordifolia* followed by ethanol 20.43±0.15 in *N. arbortristis* and methanol 15.40±0.10 in *N. arbortristis*. Minimum yield was observed in acetone and hexane plant extracts.

The leaves of studied five medicinal plants posses numerous bioactive secondary metabolites with diverse therapeutically interesting pharmacological properties. The preliminary phytochemical screening revealed that the plant extract containalkaloids, terpenoids, flavonoids, steroids, phenols, tannins, quinones, saponins and coumarins.

The five different medicinal plants, *Syzygium cumini*, *Azadirachta indica*, *Moringa oleifera*, *Nyctanthes arbortristis*and *Tinospora cordifolia* extracts with four different concentration (25, 50, 100 and 200 mg/ml) were evaluated for antibacterial activity against iMLSB and cMLSB of *S. aureus* with sharing common MRSA. Among five medicinal plants, only following three plants (*Syzygium cumini*, *Azadirachta indica* and *Nyctanthes arbortristis*) showed antibacterial activity

In our study, the profiling of presence of phytoconstituent contains in aqueous leaves extract of of *S. cumini*was carried out through positive and negative ionization modes of LCMS method. Twenty different compounds (diferulic acid, caffeic acid, 3-(3-hydroxyphenyl) propanoic acid, xanthoxylin, cianidanol, ferulic acid, quinic acid, gallic acid, 4'-hydroxyflavan, 5,7,4'-tetrahydroxy-6-(3-hydroxy-3-methylbutyl) flavones, 6-O-feruloyl-D-glucose, isoquercetin, astragalin, methylgallate, butin, palmitic acid, cedrol, kaempferide, taxifolin and punicic acid) comprising phenolics, flavonoids, terpenoids, coumarins and organic acids were identified through the interpretation of LCMS data obtained.

In our investigation, twenty different phytoconstituents were found from LCMS analysis of leaves of *S. cumini*. The compounds were subjected to molecular docking to two target proteins, one is PBP2a considering from MRSA and other is ERM from MLSB resistant strains of *S. aureus*. The results of molecular docking showed that diferulic acid has lower binding score with value of -102.37 kcal/ mole in compression to other compounds to protein PBP2a while taxifolin has the lowest binding energy with value of -103.12 kcal/mole to ERM protein in comparison to others compounds.

The pharmacokinetic prediction was evaluated through ADMET properties of potential compounds. All the tested compounds were passed the ADMET test except isoquercetin and astragalin.

7.2 CONCLUSION:

Over all, the results of *in vitro*and *in silico* study highlight the potentialantibacterial activity of active compounds of medicinal plants and PBP2a and ERM binding activity of compounds against resistant strains of *S.aureus.* Hence these two compounds (Diferulic acid and taxifolin) are promising compounds for developing potent inhibitors against PBP2a and ERM active proteins for inhibition of MRSA and MLSB resistant strains of *Staphylococcus aureus*.

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