CAPITAL UNIVERSITY OF SCIENCE AND TECHNOLOGY, ISLAMABAD

Identification of Anti-Quorum Sensing Potential of *Reynoutria japonica* with Reference to *Staphylococcus aureus*

by

Maliha Fatima

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Every challenging work needs self-efforts as well as the guidance of elders. I dedicate this thesis to my mother whose affection makes me able to get such success and to my teachers whose encouragement has always been my source of inspiration.

CERTIFICATE OF APPROVAL

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Maliha Fatima

(MBS203042)

THESIS EXAMINING COMMITTEE

S. No.	Examiner	Name	Organization
(a)	External Examiner	Dr. Muhammad Imran	QAU, Islamabad
(b)	Internal Examiner	Dr. Tariq Khan	CUST, Islamabad
(c)	Supervisor	Dr. Arshia Amin Butt	CUST, Islamabad

Dr. Arshia Amin Butt
Thesis Supervisor
March, 2022

Dr. Sahar Fazal
Head
Dept. of Bioinformatics & Biosciences
March, 2022

Dr. Muhammad Abdul Qadir
Dean
Faculty of Health & Life Sciences
March, 2022

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(Maliha Fatima)

Abstract

It is estimated that by the end of 2025, 226 million people worldwide will be affected with infectious diseases. Although many treatments for these bacterial infections are available in the form of antibiotics but excessive and indiscriminate use of antibiotics to treat them has led to the emergence of multiple drug resistant strains. The global rise of anti-microbial resistance combined with the rapid rate of microbial evolution and the slower development of novel antibiotics focuses the urgent need of development of innovative therapeutics and new strategies to fight emerging infections. One of these strategies is to disturb quorum sensing system which is cell density dependent communication mechanism and responsible for virulence and biofilm formation in multidrug resistant strains of bacteria such as *Staphylococcus aureus*. Efforts to disrupt biofilms have enabled the identification of natural bioactive molecules produced by different plant species. One of these is *Reynoutria japonica* which is Japanese medicinal herb. Its root contains certain bioactive compounds with a wide spectrum of pharmacological effects and has been used for treatment of inflammation, jaundice and many other diseases. The motive of the present research was to discover potential antibacterial compounds from *Reynoutria japonica*. Ten bioactive compounds from this plant i.e. 2-Methoxy-6acetyl- 7- methyljuglone, emodin, emodin 8- o -b glucoside, polydatin, resveratrol, physcion, citreorosein, quercetin, hyperoside and coumarin were taken as ligands and docked with accessory gene regulator protein A, B, C and signal transduction protein TRAP. The 3D structure of the target proteins and the ligands was taken as the input for docking. The best ligand was selected on the basis of docking score ADMET properties and lipinski rule. By considering all these parameters resveratrol was seen obeying all drug-like properties with docking score -8.9 against accessory gene regulator protein C. It followed lipinski rule and toxicity and other ADME values are also in tolerable range as compared to other ligands. To check further effectiveness of resveratrol it was compared with commercially available antibiotic drug penicillin. A comparison of all drug-like characteristics showed that resveratrol is much better in many aspects over penicillin. Penicillin showed docking score - 6.7 while resveratrol has - 8.9, other pharmacokinetic

properties of resveratrol are also good than penicillin. So it is concluded here that resveratrol can prove itself as drug candidate in future antibiotic therapeutics.

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Abbreviations

ADMET:- absorption distribution metabolism excretion and toxicity

Agr:- accessory gene regulator

AHL :- autoinducer homoserine lactones

AI-2:- autoinducer 2

AI :- aliphatic Index

AIP:- autoinducer peptides

BBB:- blood brain barrier

CADD:- computer-Aided Drug Designing

CB Dock:- cavity-detection guided Blind Docking

CNS:- central Nervous System

CYP2D6:- cytochrome P450 2D6

EPS:- extracellular polymeric substance

FDA:- food and Drug Administration

GRAVY:- grand average of hydropathicity

HBA:- hydrogen Bond Acceptor

HBD:- hydrogen Bond Donor

hERG:- human Ether-a-go-go-Related Gene

II:- instability Index

KEGG:- kyoto Encyclopedia of genes and genomes

MDR:- multiple drug resistance

MRSA:- methicillin-resistant staphylococcus aureus

MW:- molecular weight

NR:- total number of negatively charged residues (Asp + Glu)

PSM:- phenol soluble modulins

PDB:- protein data bank

PR:- total number of positively charged residues (Asp + Glu)

QS:- quorum sensing

QQ:- quorum quenching

VDss:- volume of Distribution at steady state

Chapter 1

Introduction

Bacteria interact with each other through signals. As number of bacteria increases signal threshold concentration is reached which results in change in gene regulation of bacteria. This type of communication system which is based upon signalling and bacterial density is called quorum sensing [1]. The quorum sensing system operates through the release of special signals termed as autoinducers, whose accumulation depends upon cell density. Quorum sensing regulate bacteria's important behaviors e.g. attachment to surface, biofilm formation, bioluminescence, secretion of different type of chemicals, motility, virulence and pathogenicity [2].

Quorum sensing is also required for biofilm formation. A biofilm is well organized form of bacterial population. Biofilm protects bacteria from host defense systems, unsuitable environmental conditions and antibiotics approach. The main characteristic of biofilm forming bacteria is the release of extracellular polymeric substances. These polymers have sufficient amounts of polysaccharides along with protein and extracellular DNA which help in formation of matrix in which bacterial cells are embedded [3].

Quorum sensing process is dependent upon generation, release and detection of signals. Different bacterial species produce different type of signals. N-homoserine lactones (AHLs), furanosyl borates (AI-2) and autoinducer peptides (AIP) are

among some of the quorum sensing signals. Among most well studied signals are

- Autoinducer lactones, which are produced by more than 70 gram-negative bacterial species and diffuse across the cell membrane to bind to regulatory proteins within the cell, and
- Peptide-based quorum sensing systems in gram-positive bacteria, which work through membrane-bound receptor histidine kinases [4].

The upregulation of virulence and pathogenicity causing factors through quorum sensing is major cause of life-threatening diseases. *Staphylococcus aureus* is among those harmful microorganisms which exhibit complex quorum sensing systems. *Staphylococcus aureus* are gram-positive anaerobic facultative cocci. It is highly resistant to heat, high salt concentrations and high osmotic pressure. It shows catalase activity but no oxidase activity. The coagulase activity of *Staphylococcus aureus* distinguishes it from other *Staphylococcal* species [5].

Staphylococcus aureus is a commensal and opportunistic pathogen. It is linked to variety of infections specially related to skin, bones, respiratory structures and endovascular system. Food poisoning creating toxins i.e. Enterotoxin A and B and toxic shock syndrome are also caused by *Staphylococcus aureus*. Using quorum sensing strategy it is also seen to involve in series of diseases including skin diseases, cystic fibrosis, pneumonia, meningitis and several other [6].

A number of antibiotics had been tried in past as shown in figure 2.4 such as penicillin, vancomycin, linezolid, methicillin etc. but it always showed resistance against them. Methicillin resistance is frequently found in *Staphylococcus aureus* frequently called MRSA. Resistance is obtained by the integration of resistance gene mecA in the *Staphylococcus aureus* genome. This resistance gene is found on chromosome mec [7]. Historically, MRSA infections were only hospital-acquired but soon it was found that they spread through different sources. In 1999, several community-acquired MRSA infections were reported among children and adults.

The quorum sensing system in *Staphylococcus aureus* is peptide based and depends

upon mainly on autoinducer peptide. Agr and RAP/TRAP systems are main components of this type of quorum sensing. The Agr quorum sensing system is a global regulatory system which consists of two primary transcripts, RNAII and RNAIII. Transcription of RNAII depends on the activation of its promoter P2 [8]. The RNAII locus includes four genes that are co-transcribed, AgrA, AgrB, AgrC and AgrD [9]. Along with Agr another communication system found in *Staphylococcus aureus* is RAP/TRAP system. The luxS gene is also found in *Staphylococcus aureus*. Its transcript is necessary for the production of autoinducer-2. Recent studies depict that AI-2 system is controversial till date [10].

There are many ways to treat bacterial infections, among them one is the use of antibiotics. But repeated and overuse of antibiotics has made bacteria resistant resulting in emergence of multiple drug resistant strains. It created urge to look for alternative methods. Soon it was observed that control over virulence factors and biofilm formation can be made possible by controlling quorum sensing mechanism. Disrupting the quorum system mechanism is a novel and promising technique for bacterial infectious disease prevention. The mechanism causing the suppression of quorum sensing communication system is termed as quorum quenching [11].

Quorum quenching interferes with bacterial communication, controlling group behaviors such as the production of virulence factors. Quorum Quenching is now considered to be effective in the competitive inhibition and splitting of signaling molecules [12]. In *Staphylococcus aureus* it can be used for inhibiting the activation of the Agr quorum sensing system, thus leading towards decrease in virulence and biofilm formation. Quorum sensing can be inhibited at different levels of the Agr and RAP/TRAP systems. It can be blocked at the level of the signal, at the signal receptors or at the level of signal transduction. Quorum quenching is performed by quorum sensing inhibitors which can be obtained from different sources e.g. natural, synthetic or antibody based [13]. Quorum sensing inhibitors are likely to be found in legumes and traditional medicinal plants [14]. The Japanese knotweed *Reynoutria japonica* is a medicinal plant that is member of Polygonaceae family [15]. It contains a large number of secondary metabolites e.g. naphthoquinones, anthraquinones, flavonoids, stilbenes, coumarins, lignins

and phenolic compounds. These metabolites showed potential anti-bacterial activity against many pathogens. These metabolites are also useful against certain other type of diseases including inflammatory, viral and different infectious diseases [16].

A number of pharmaceutical applications are concerned with these bioactive compounds. 2-Methoxy-6-acetyl-7-methyljuglone is one of these belongs to quinones. It is proved effective in treatment of diabetes, tumors and different bacterial, viral and fungal infections. There are certain other bioactive compounds which could act as potential inhibitors for quorum sensing e.g. emodin, emodin 8-o-b glucoside, polydatin, resveratrol, physcion, citreorosein, quercetin, hyperoside and coumarin [17]. These chemical compounds act as ligands and can be docked with *Staphylococcus aureus* quorum sensing proteins e.g. Agr system proteins, accessory gene regulator protein A, B, C and target of RNAIII activating protein (TRAP) to check interactions between ligand and target proteins which will be useful in future drug designing purpose [18].

Docking is an insilco method for determining the correct structure of a ligand within the target binding site and estimating the strength of a bond between a ligand and a target protein using a special scoring function. The input for docking is the three-dimensional structure of the target proteins and ligands [19]. It is recognized that these novel small molecular compounds have important properties, such as a high interaction between target binding to target proteins, also proper absorption, distribution, metabolism, excretion and toxicity (ADMET) to help in target lead selection [20].

Molecular docking is process of molecular determination of target protein and its ligands. It also focuses on achieving the system's minimum independent energy, which includes properly aligned proteins and ligands. Small ligands, protein peptides, protein proteins, and protein nucleotides can all be performed in the molecular docking of proteins. Algorithm, receptor flexibility, and ligand flexibility are some of the docking mechanisms. Most frequent software used for docking are Auto Dock vina, Auto Dock, CB Dock and ICM etc. [21]. In insilico approach docking is main step to determine the binding affinity of certain compound against

target proteins. In this case number of ligands are taken because of their potential role in controlling certain diseases. These bioactive compounds showed positive results against resistant strains of *Helicobacter pylori* infections [78]. Therefore it is hypothesized that they could be effective against infectious *Staphylococcus aureus* strains as it has notorious record worldwide in spread of infections. In-silico method could be helpful in determination of bioactive compounds as best anti-bacterial agent on the basis of binding affinity.

1.1 Problem Statement

Bacterial infections specially with methicillin resistant *Staphylococcus aureus* are difficult to treat due the high resistance profile of *Staphylococcus aureus* and their ability to form biofilms. Probably it is estimated that by the end of 2025, 226 million people worldwide will be affected with infectious diseases. Despite of lot of antibacterial treatments we are not able to treat diseases because of inertness of pathogens.

1.2 Aim of the Study

The aim of this study is to better understand the quorum sensing process of *Styphylococcus aureus* as well as the identification of novel drug targets from *Reynoutria japonica* against this pathogen that may lead to the discovery of novel therapeutics for the active treatment of bacterial infections in human.

1.3 Objectives

The study entails following objectives:

1. To identify various bioactive compounds of *Reynoutria japonica* as potential inhibitors of target proteins.

2. To analyze the binding conformation between targeted proteins and other inhibitors as standard anti-quorum sensing potential.

3. To identify the lead compound as anti-bacterial drug candidate.

1.4 Scope

In low-income countries, people prefer traditional medicines over modern medicine, thanks to low cost and lesser side effects. Major issues with these traditional medicines are limited bioavailability, quantity and validity. This research is an attempt to determine novel antibacterial compounds of *Reynoutria japonica* to degrade the signals for quorum sensing, if successful will be instrumental to fight multi-drug resistance and combating nosocomial infections associated with biofilm bacteria.

Chapter 2

Review of Literature

2.1 Quorum Sensing

For hundreds of years, bacteria were thought to be alone and silent. But recently it has been discovered that they can perform their actions at the population level by creating, perceiving, and responding to small signal molecules [22] [23]. This mechanism of communication through signal molecules when specific threshold concentration is reached and living in the form of societies is known as quorum sensing. It is important for bacteria in my aspects as quorum sensing mechanisms are involved in regulation of bacterial behaviors. For example

- metabolism including antibiotic synthesis,
- pollutant biodegradation and bioenergy production etc [24].

Based on quorum sensing control in microbial pathogens, innovative diagnostic approaches and antibiotic drugs have been developed. However, the fundamental significance of quorum sensing is in the biofilm development process. Antibiotic resistance is caused primarily by biofilms, which are ordered structures and are responsible for number of important biological problems especially related to human health e.g. the formation of dental plaque is due to complex biofilms [25].

2.1.1 Quorum Sensing Link with Biofilms

A biofilm is an organized bacterial community. Exopolysaccharides, proteins and extracellular DNA are found in bacteria trapped in a biopolymer matrix. Biofilms produced by gram-positive bacteria like *Staphylococcus aureus* and gram-negative bacteria like *Pseudomonas aeruginosa* are extremely difficult to disseminate.
Biofilm is formed when biofilm-producing bacteria attach to solid surfaces in an aqueous environment and develop a network of extracellular polymeric substances, adopting a multicellular lifestyle. Biofilms pose serious problems in terms of infection prevention and treatment. To disturb biofilms, drug design and Insilco approaches can be designed to measure anti-microbial medication effectiveness .
Within a biofilm, bacteria are protected against the host's survival mechanisms, hostile environments and antibiotics. The process of formation of biofilm completes in four phases [25]. The dynamic adhesion of planktonic cells to a surface is first phase of biofilm formation. Van der Waal's forces, as well as hydrophobic and ionic interactions, play a key role in attachment. *Staphylococcus aureus* can bind to human tissues by expressing microbial surface components that sense sticky matrix molecules [26]. Attachment is followed by irreversible binding to surface and bacteria proliferate to form microcolonies. Microcolonies are obtained by intercellular adhesion. This is accomplished through the production of polysaccharide intercellular adhesin and protein A and protein G in case of *Staphylococcus aureus* and fibrinogen binding protein in methicillin resistant *Staphylococcus aureus* isolates. Maturation is the third phase of biofilm formation. In this stage bacteria continue to multiply to form a mature biofilm. Water channels are formed within the biofilm for the transfer of nutrients to each layer of the biofilm [27].

2.1.2 Types of Quorum Sensing

Alteration in gene expression through quorum sensing depends upon signal molecules termed as autoinducers which are of different types. Autoinducer homoserine lactones, which are made by most gram-negative bacterial species, and autoinducer

peptides which are found in gram-positive bacteria's quorum sensing mechanism, are the most widespread autoinducers [28].

2.1.2.1 AHL Based Quorum Sensing

This type of quorum sensing mechanism is present in gram-negative bacteria. Gene expression in AHL dependent systems is regulated by single synthase-regulator complex. The signal molecules are located in and around the cells and are produced constitutively at smaller amounts by the synthase gene [29]. Signal molecules adheres to its receptor at high cell concentration and triggers the transcriptional activator which then binds to the DNA thus stimulating the expression of genes regulated by the quorum sensing system. More than one autoinducers can be found in a single species, for example there are two homoserine lactones in *Pseudomonas aeruginosa* [30].

2.1.2.2 Peptide Based Quorum Sensing

Gram-positive bacteria use autoinducer peptides for quorum sensing. These oligopeptides work by directing changes in gene expression through a two- component signaling cascade that includes membrane-bounded sensory histidine kinase receptors and cytoplasmic transcriptional factors. Different species have their own systems as shown in table 2.1 for regulation of gene expression e.g. Agr system (Accessory gene regulator) is responsible for Quorum sensing in *Staphylococcus aureus* [31].

TABLE 2.1: Quorum sensing mechanism of different bacterial species [32].

Bacterial Species	**Quorum Signals**	**Quorum System**
Staphylococcus aureus	AIP, AI-2	Agr system, RAP/TRAP
Pseudomonas aeruginosa	AHL, PQS	LasI/R, Rh1I/R, LuxI/R
Vibrio fischeri	AI-1	LuxS/AI-2 system
Agrobacterium tumefacians	3-oxo-C8-HSL	TraR
Bacillus subtlis	ComX, CSF, PhrA	ComP/ComA, RAP proteins
Streptococcus pneumoniae	CSPs	ComD/ComE

Table 2.1 continued from previous page

Bacterial Species	Quorum Signals	Quorum System
Vibrio harveyi	HAI-1, CAI-1, AI-2	LuxLM, LuxN, LuxP, LuxQ

TABLE 2.2: Quorum sensing benefits of different bacterial species [32].

Bacterial Species	Group-Derived Benefits
Staphylococcus aureus	Biofilm formation, virulence factors
Pseudomonas aeruginosa	Multiple extracellular enzymes, Biofilm formation
Vibrio fischeri	Bioluminescence
Agrobacterium tumefacians	Ti plasmid , conjugation
Bacillus subtlis	Competence, sporulation, biofilm formation
Streptococcus pneumoniae	Competence, virulence, biofilm formation
Vibrio harveyi	Bioluminescence emission, symbiosis

2.1.3 Quorum Sensing Mechanism in *Staphylococcus aureus*

Staphylococcus aureus is gram-positive bacteria. It causes a variety of diseases ranging from minor skin rashes to more serious conditions including foodborne diseases, pneumonia and sepsis etc. It has a notorious record worldwide toward disease spread and its infections spread equally among infants and adults [32] [33]. The serious issue concerned with *Staphylococcus aureus* is that a number of antibiotics were tried to treat its infections but it always showed resistance against these drugs. Due to its resistivity it is now considered as a superbug. Methicillin-resistant *Staphylococcus aureus* is one of its dangerous form. It is a widespread superbug that can infect humans varying from normal skin infections to life-threatening infections in different parts of body. It can travel to any part of the body in less than 72 hours after it has taken control. It is also extremely resistant to almost all antibiotics. It has been reported that 80,461 severe MRSA infections and 11,285 MRSA-related deaths occur each year [34].

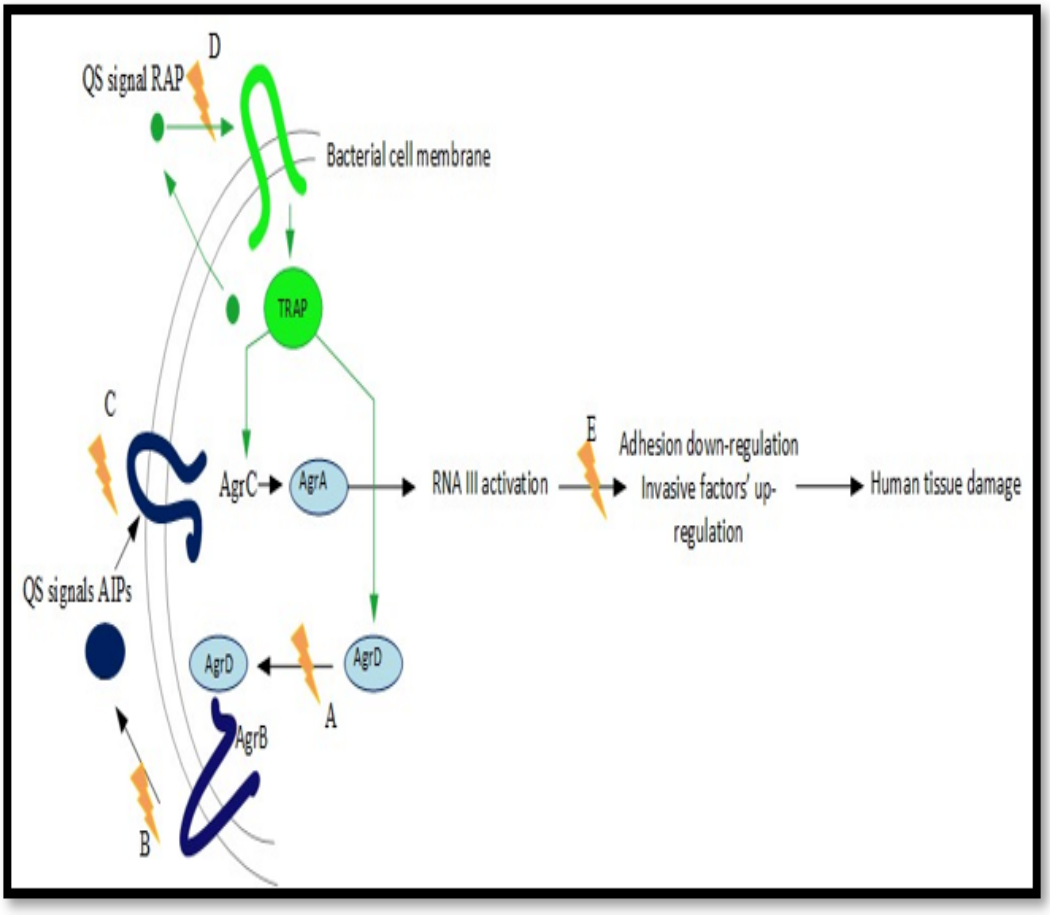


FIGURE 2.1: The link between quorum sensing and virulence in *Staphylococcus aureus [35].*

Antibiotic trials against MRSA was unsuccessful due to its resistant nature. Vancomycin is the standard treatment for infection, but the first case of vancomycin resistance was already reported in 1996 in Japan [35] [36]. This created urge among researchers to trial over alternate ways to treat MRSA related infections. One of these alternates is to disturb signaling mechanism in *Staphylococcus aureus*. According to figure 2.1, in *Staphylococcus aureus* quorum sensing mechanism operates through following systems.

2.1.3.1 Agr System

The Agr quorum sensing system in *Staphylococcus aureus* is formed by two primary transcripts, RNAII and RNAIII which are responsible for the expression of virulence factors, toxins such as haemolysins, phenol-soluble modulins, enterotoxins,

and enzymes such as proteases and lipases, as well as the formation of biofilms [37]. Transcription of RNAII depends on the activation of its promoter P2. The RNAII locus includes four genes that are co-transcribed i.e. AgrA, AgrB, AgrC and AgrD and synthesize and secretes autoinducing cyclic thiolactone peptides [38].

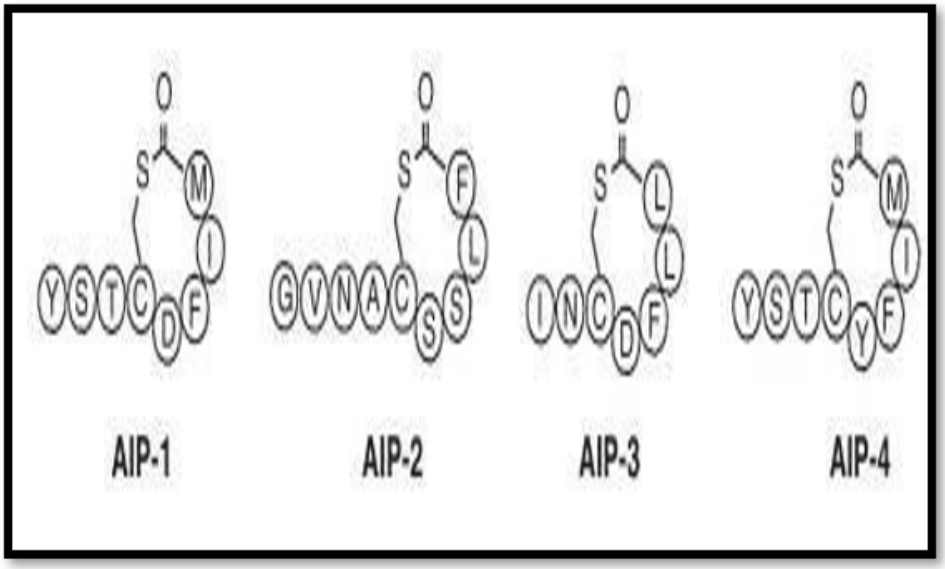


FIGURE 2.2: Chemical structure of the autoinducing peptides in *Staphylococcus aureus* quorum sensing [41].

Signals in *Staphylococcus aureus* are produced by AgrB and AgrD. AgrC is involved in detection of signals. These peptide-based signals attach with AgrC and another compound histidine kinase and activates them which then activate AgrA. The regulation of virulence factors depends mainly on activation of AgrA. The production of autoinducer peptides varies from species to species. Mainly there are four types of AIP as shown in figure 2.2. The four types of AIP exist as a result of sequence variation and have the following amino acid sequences: YSTCDFIM for AIP type I, GVNACSSLF for type II, INCDFLL for type III and YSTCYFIM for type IV. Each AIP is able to bind to its corresponding sensor kinase AgrC [39] [40].

2.1.3.2 RAP/TRAP System

The other signalling mechanism observed in *Staphylococcus aureus* is RAP/TRAP system. Here autoinducer is RNAIII-activating peptide (RAP) and it has a target

protein called target of RAP or TRAP [42] [43]. This target protein is the actual protein in this system which cause upregulation of virulence factors as shown in figure 2.3. In *Staphylococcus aureus*, it is also involved in the production of biofilms. This system is particularly active during initial stage of growth while Agr system active primarily during late stages of growth [44].

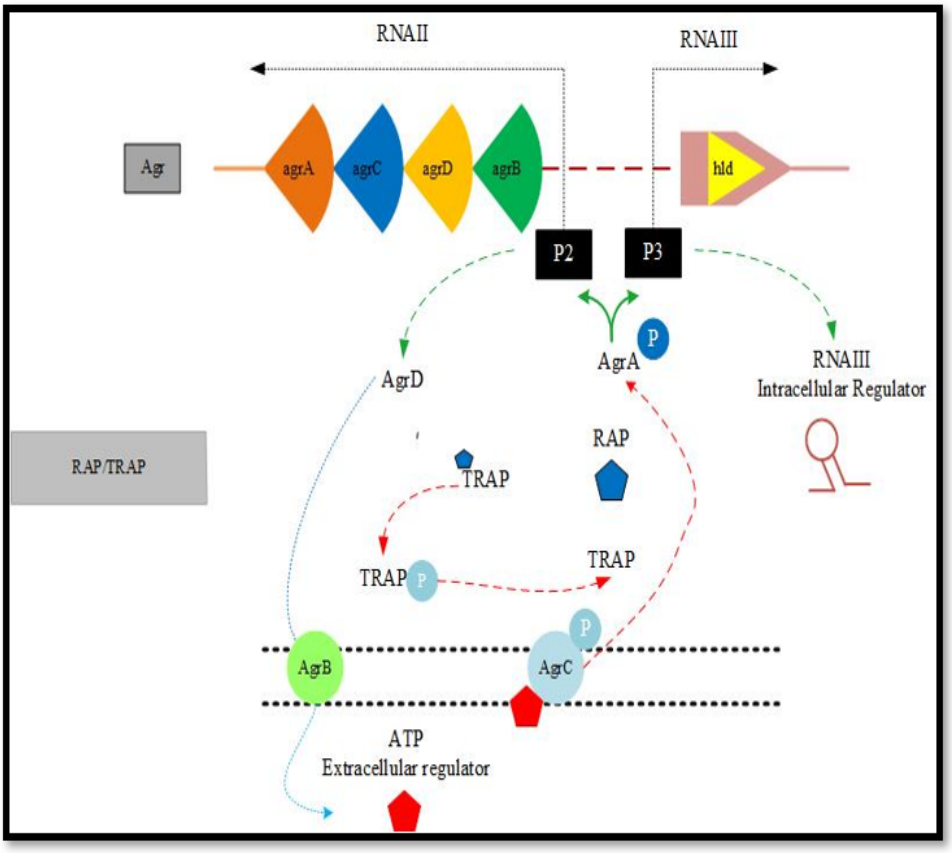


FIGURE 2.3: Quorum sensing regulation in *Staphylococcus aureus* [45].

2.1.3.3 Other Quorum Sensing Systems in *Staphylococcus aureus*

Staphylococcus aureus carries a luxS gene. Its transcript contributes in the production of autoinducer-2. However, the mechanism is unknown and researches reveal that it can be found in both gram-positive and gram-negative bacteria. Other regulatory mechanisms in *Staphylococcus aureus*, such as the staphylococcal accessory regulator (SarA), the *Staphylococcus aureus* exoprotein (Sae) operon, and the staphylococcal alternative sigma factor B (SigB), also affect virulence factor synthesis and biofilm formation [46].

2.1.4 Target Proteins of Quorum Sensing System

Agr and RAP/TRAP system consist of proteins which are responsible for establishment of infections. These proteins are described as under.

2.1.4.1 Accessory Gene Regulator Protein B and D

Accessory gene regulator protein B is an integral membrane-bound peptidase that is required for the synthesis of the autoinducing peptide, a quorum sensing system signal molecule involved in the regulation of virulence factor gene expression. It also plays a role in the proteolytic processing of AgrD, which is AIP ribosomal peptide precursor [47].

2.1.4.2 Accessory Gene Regulation Protein C

After the production of autoinducer peptide it is sensed by AgrC outside the cell. AgrC is the membrane-bound histidine kinase sensor. The binding of AIP to the AgrC receptor leads to histidine autophosphorylation [48].

2.1.4.3 Accessory Gene Regulator Protein A

It is essential for a number of secreted proteins expression at a high level once they have reached the post-exponential phase. AgrB interacts with AgrC in the membrane to activate AgrA, which upregulates transcription from both promoter P2 and promoter P3, increasing the response and triggering the production of a novel effector RNAIII [49].

2.1.4.4 TRAP

A key regulator of *staphylococcal* pathogenicity is the signal transduction protein TRAP. Phosphorylated TRAP activates the Agr system which results in RNAIII

production and the expression of many virulence factors. It upregulates most toxins and genes thought to be involved in biofilm formation [50].

2.1.5 Quorum Sensing Induced Worries

Quorum sensing based biofilms formed by infectious bacteria are big cause of spreading human infections. Quorum sensing system involves upregulation of motility, virulence and pathogenic factors which step towards the formation of biofilms which further cause disease. Different bacterial species are major source of different infections e.g. *Pseudomonas* species decline health of cystic fibrosis patients, *Clostridium botulinum* cause botulism, *Vibrio cholera* cause cholera etc. Fishery department is under-threat due to some disease causing bacteria *Aeromonas hydrophila, Aeromonas salmonicida, Vibrio anguillarum, Vibrio harveyietc* which express their pathogenicity through quorum sensing systems [51]. There are certain other areas like drinking water sources, treatment plants, buildings, agriculture etc. which are affected by biofilms produced by *Escherichia* and *Rhizobium* species [52]. The main contributors of infections are biofilms because they are quite resistant towards antibiotics. Also bacteria are provided with safety along with nutrients inside biofilms so they don't hesitate to cause infections [50] [48].

2.2 Antibiotic Usage and Resistance

The 20th century was good with relation to health aspect as it was the era of antibiotic discovery. Enormous medication was done by using antibiotics. But as the excess of everything is bad and due to evolutionary nature of bacteria they got resistant to those antibiotics as shown in figure 2.4. The results were quite surprising after a century because the drugs were ineffective e.g. to treat MRSA linezolid and daptomycin were used but bacteria showed resistance against them. Vancomycin was used for *Staphylococcus aureus* but soon bacteria became resistant

to it [53]. About 80% hospital strains of *staphylococci* are penicillin resistant. This is due to production of beta-lactamase which produce lactame ring in penicillin and cephalosporins. Also, there are multiple side effects of using these drugs [54].

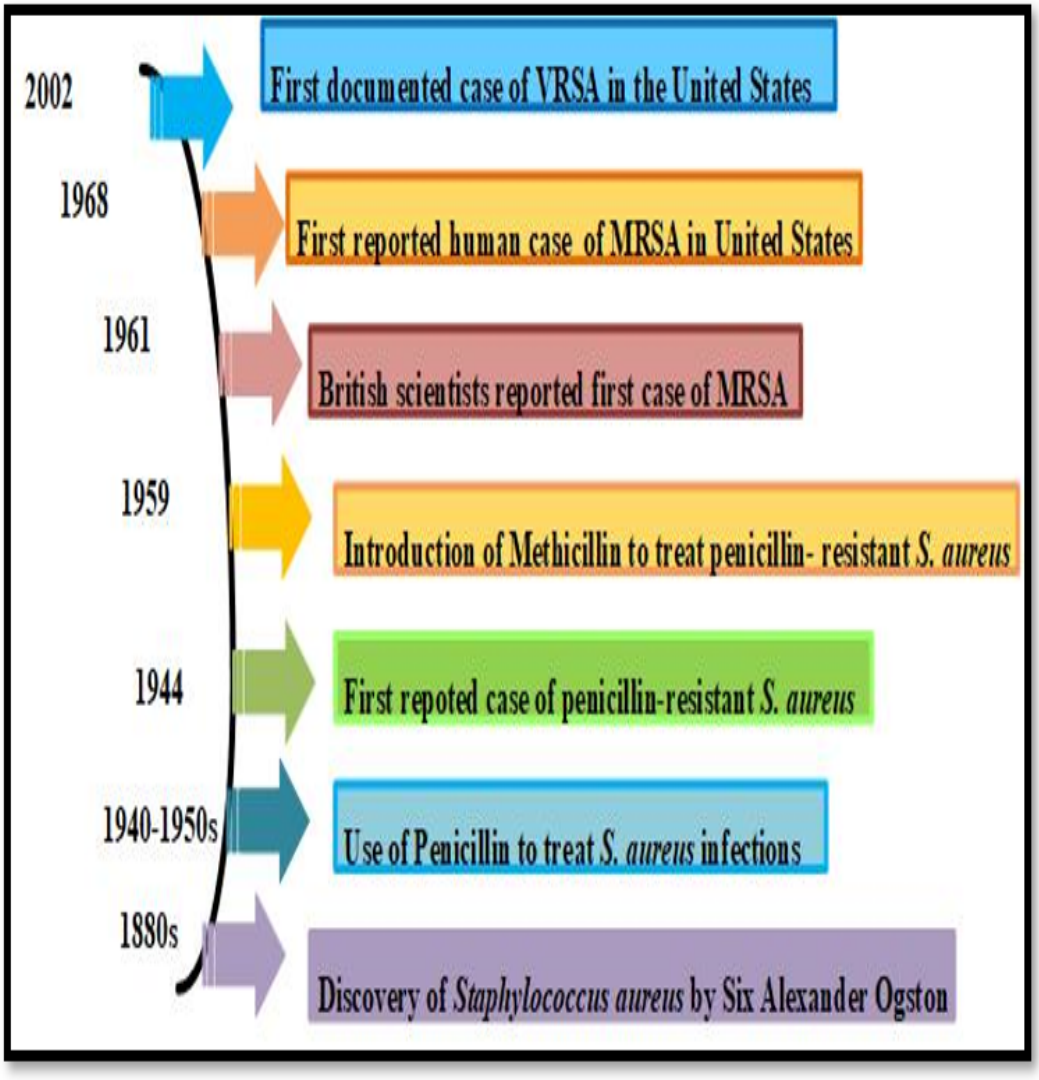


FIGURE 2.4: Timeline of resistance in *Staphylococcus aureus* [55].

Alternative techniques to fight these multidrug-resistant bacteria are required. It is the need of the hour because approximately 33000 people die each year as a result of bacterial infections. It is therefore mandatory to search for effective medication development proposals. Inhibition of quorum sensing is yet to be best proposed suggestion in this regard. This will protect ourselves from repeated antibiotic usage and their harmful side effects. Some antibiotics along with their mechanism of action and side effects are shown in table 2.3 [56].

TABLE 2.3: Antibiotics mode of action and their side effects [56].

Antibiotics	**Mechanism of action**	**Target bacteria**	**Side Effect**
Penicillin	Inhibits cell wall synthesis	Gram +ve bacteria	Hypersensitivity
Ampicillin	Inhibits cell wall synthesis	Broad spectrum	Gastrointestinal problems, seizures
Bacitracin	Inhibits cell wall synthesis	Gram +ve bacteria	Nephrotoxicity, Hypersensivity
Cephalosporin	Inhibits cell wall synthesis	Gram +ve bacteria	Hypersensivity
Tetracyclin	Inhibits protein synthesis	Broad spectrum	Tooth discoloration, Photosensivity
Streptomycin	Inhibits protein synthesis	Gram -ve bacteria, tuberculosis	Clumsiness, itching
Sulpha drug	Inhibits cell metabolism	Bacterial meningitis, urinary tract infection	Allergies, Liver and kidney injury
Rifampicin	Inhibits RNA synthesis	Gram +ve and Gram -ve bacteria	Bloody diarrhea, Stomach pain
Quinolones	Inhibits DNA synthesis	Urinary tract infections	Anxiety, Insomnia
Clindamycin	Inhibits protein	Gram +ve	Diarrhea,
Aminoglycoside	Inhibits protein synthesis	Gram -ve bacteria	Damage to kidney, Neurotoxicity

Table 2.3 continued from previous page

Antibiotics	Mechanism of action	Target bacteria	Side Effect
Chloramphenicol	Inhibits protein synthesis	Broad spectrum	Bone marrow suppression
Erythromycin	Inhibits protein synthesis	Broad spectrum	Mild gastrointestinal disturbance

2.3 Quorum Quenching

While the excessive use of antibiotics resulted in a large resistance problem and inefficiency in *Staphylococcus aureus* infection treatments, biofilms themselves are already very resistant to antibiotic therapy. To increase biofilm susceptibility towards antibiotics and to reduce virulence factors, quorum sensing inhibition is presented as an alternative to combat bacterial infections. Quorum sensing can be blocked at the level of the signal, at the signal receptors or at the level of signal transduction. Bacteria within biofilms multiply and cause most of the infectious diseases. These biofilms could be destroyed by using bioactive compounds [57].

2.3.1 General Strategies to Block Quorum Sensing

Quorum quenching interrupts bacterial communication and prevents group actions such as the expression of disease-causing factors. Quorum quenching can be done by many ways and it is now considered to be one of best strategies in signal inhibition and destruction [58].

2.3.1.1 Inhibition of Signaling Molecules

Signals are main components of quorum sensing. By controlling enzymes which take part in signal production, the signal synthesis can be blocked and in this

way quorum sensing can be controlled e.g. enoyl-ACP reductase is involved in formation of AHL signals. Triclosan is seen effective to reduce action of this enzyme [59].

2.3.1.2 Competitive Inhibition

In this strategy some structures similar to signals are selected. These similar structures can bind with receptors by process of competition and thus can block the binding of signals with receptor proteins e.g. halogenated furanones have structure similarity with AHLs and can be used for this purpose [60].

2.3.1.3 Degradation of Signal Molecules

For quorum sensing a large number of signals is required. This number can be reduced by using special enzymes which can degrade signals resulting in their lesser production. For this purpose, MacQ is used in gram-negative bacteria [61].

2.3.2 Mechanism of Quorum Quenching

As various types of signals exist in different bacterial species so the mechanism of quorum quenching is also different according to type of bacteria.

2.3.2.1 AHL Based Quorum Quenching

The quorum quenching of AHLs has received the greatest attention because they are involved in regulating disease causing factors. Either of these methods can be used to suppress or block AHL-mediated quorum sensing system [62].

1. Disturbing the production of AHL signals ,
2. Interrupting with the signal transmission,
3. Antagonizing the AHL receptors.

2.3.2.2 AIP Based Quorum Quenching

Due to cause of widespread diseases and resistant nature, *Staphylococcus aureus* has always remained a reference organism with respect to AIP based quorum sensing. A lot of research has done on this system to disturb its signalling system. Many ways to quench quorum sensing in *Staphylococcus aureus* are discussed under [63].

2.3.2.3 Quorum Quenching in *Staphylococcus aureus*

Quorum sensing can be inhibited at different levels of the Agr and RAP/TRAP systems. Monoclonal antibody was administered against the signaling molecule AIP to *Staphylococcus aureus* strains with Agr type IV. This resulted in decreased virulence factor expression. Co-administration of a *Staphylococcus aureus* inoculum and the anti-AIP antibody to mice protected the host against fatal infection. At the AgrC receptor level, different AIP types cause an AgrC receptor twist in the opposite direction. This prevents phosphorylation and hinders quorum sensing [64]. The signal transduction pathway can be blocked by the small molecule inhibitor savirin. Its target is the DNA-binding response regulator AgrA. A functional AgrA is crucial for the activation of P2 and P3. Savirin binding to AgrA leads to a blockage of the transduction pathway and consequently leads to quorum sensing inhibition. This results in decreased virulence gene expression [65]. Researches show that Apolipoprotein B is helpful in inhibition of virulence factor produced by Agr system in methicillin resistant strains of *Staphylococcus aureus*. Non-cognate AIPs can be used for this purpose. The autoinducers in *Staphylococcus aureus* bind always to their cognate receptors but when they bind to non-cognate receptor it will lead naturally in reduction of quorum sensing [66]. RNAIII-inhibiting peptides can also inhibit the entire quorum sensing process. The heptapeptide RIP has the following amino acid sequence: YSPXTNF. The amino acid sequence of RIP is similar to that of RAP which acts as an agonist for TRAP while RIP acts as an antagonist. RAP activates TRAP which regulates virulence factors. So, RIP can suppress TRAP phosphorylation thus

helping to control signaling mechanism. The non-peptide homologue of RIP is hamamelitannin. It is polyphenol found in nature and belongs to the tannin family. Hamamelitannin is extracted from the *Hamamelis virginiana* shrub's bark. It is able to inhibit and compete with RAP for RNAIII production, like RIP. It is also able to inhibit bacterial attachment and virulence. It acts as an antagonist by inhibiting its phosphorylation and serves as a quorum sensing inhibitor [64]. Different compounds having quorum quenching ability are shown in table 2.4.

TABLE 2.4: Quorum sensing and biofilm controlling compounds for *Staphylococcus aureus*

Compound	**Target**	**Mechanism of Action**	**Reference**
Non-cognate AIPs	Cognate AIP	Stabilize an inhibitory receptor conformation	[65]
Apolipoprotein B	AIP	Sequesters AIP1 inhibiting switch to invasive phenotype	[66]
RIP and hamamelitannin	RNA111, biofilms	Inhibits TRAP phosphorylation and Agr expression	[67]

2.3.2.4 AI-2 Based Quorum Quenching

AI-2 based quorum sensing can be blocked by using halogenated furanones which produce changes in LuxS gene which synthesize AI-2. Along this cinnamaldehyde analogs and fatty acids also proved effective to suppress AI-2 signalling pathway [67].

2.3.3 Quorum Sensing Inhibitors and their Sources

Quorum sensing inhibitors mainly contribute in disruption of biofilms. They work primarily by quenching the quorum sensing. Different molecules are used as quorum sensing inhibitors. They may be from different sources [68].

2.3.3.1 Synthetic Quorum Sensing Inhibitors

Various synthesized chemicals were tested as quorum sensing inhibitors and shown to be helpful in combating infections e.g. penicillic acid have been shown to be advantageous against *Pseudomonas aeruginosa*. Baicalin hydrate was found to be beneficial in treating *Burkholderia* infections. Vancomycin is also helpful against *Candida elegans*. The XYD-11G2 antibody was found to be efficient against *Pseudomonas aeruginosa* [68].

2.3.3.2 Natural Quorum Sensing Inhibitors

A number of quorum sensing inhibitors has been obtained through natural sources such as methanolic extracts of Psoraleacorylifolia and Quercus plant extracts, which are effective against *Staphylococcus aureus*. *Escherichia coli* quorum sensing ability was inhibited by coumarin and *Castanea sativa* leaf extracts. Staphylococcal infections can be prevented by using hamamelitannin [69].

2.4 *Reynoutria japonica*

The medicinal plant *Reynoutria japonica* is native to Japan although it can also be found in South Korea, China, Europe and North America. It is also known as *Polygonum cuspidatum*. It is the member of the plant family Polygonaceae. It is herbaceous and its length is estimated around 1-2 meters. Its stem and root are used as medicinal purpose against various diseases. This species produces large, upright and hollow stems with distinct nodes that are very similar in appearance to those of bamboo but much softer and with large-leaved foliage as shown in figure 2.5 [70].

Stems are also marked with distinct purple speckles. Leaves are arranged oppositely, measure approximately 10-15cm long and form on a characteristically zig-zagging stem. Leaves are heart to shield-shaped, with notable flat bases and

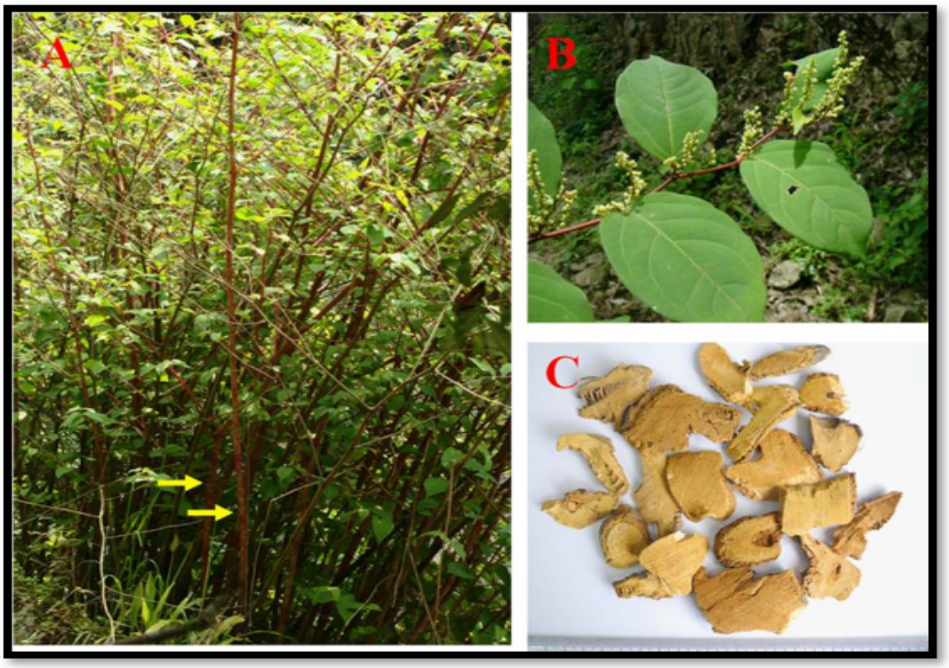


FIGURE 2.5: *Reynoutria japonica* (A) Whole plant, and the arrow represented the stems of Polygonum cuspidatum (B) flowers and leaves (C) dried roots of *Reynoutria japonica* [70] .

pointed tips. A crown of rhizomes may be visible at the base of the plant, these are bright orange inside and produce white shoots. It is capable of growing over 4m in height in a single season. . Flowers are small and usually cream-white in color, they measure between 6-15cm in length and are produced late in the summer to early autumn. It has invasive rhizomes that are very hard to remove [71]. It is used for medicinal, fodder and ornamental purposes. Young shoots are edible and a good source of Vitamin A [72]. Its hierarchical classification is shown in table 2.5.

TABLE 2.5: Hierarchical classification of *Reynoutria japonica* [73].

Serial No	**Domain**	**Eukarya**
1	Kingdom	Plantae
2	Phylum	Tracheophytes
3	Class	Angiosperms
4	Order	Caryophyllales
5	Family	Polygonaceae
6	Genus	Reynoutria
7	Species	*R. japonica*

2.4.1 Medicinal Uses of *Reynoutria japonica*

Reynoutria japonica is a rich source of functional metabolites. Its delicate stem has been used in everyday meals and its roots have been employed as a rice coloring agent. The root of this plant has also been used as a herbal medicine in the treatment of inflammation, infection, jaundice, skin burns, chronic bronchitis and hyperlipidemia problems in the form of granules, powders and extracts [74]. Quinones, stilbenes, flavonoids, coumarins and other polyphenolic compounds have all been identified from this plants and anti-inflammatory, anticancer, antiviral and antibacterial effects are known for these functionally essential phytochemicals as shown in figure 2.6 [75].

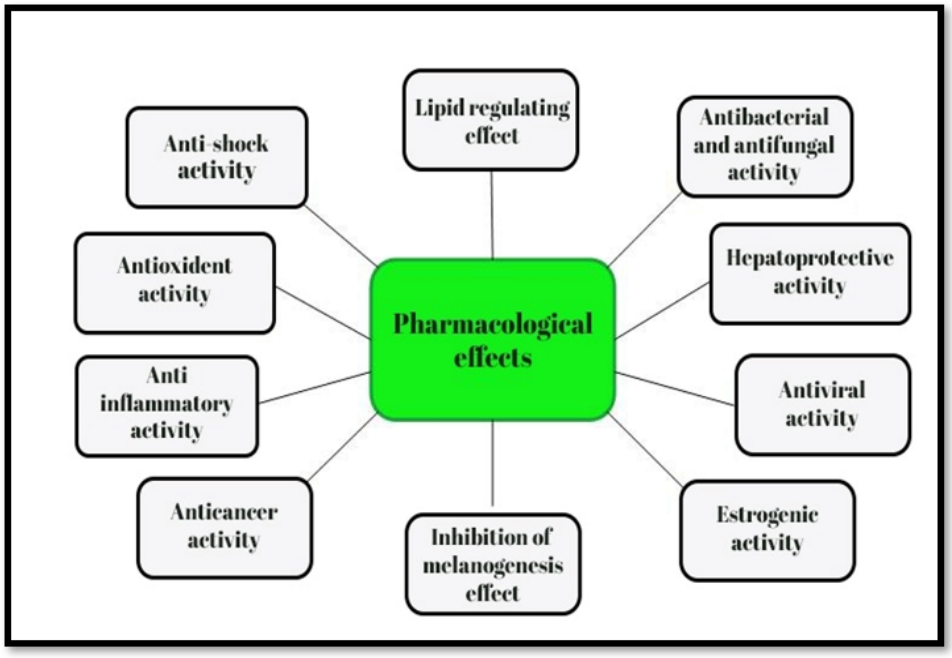


FIGURE 2.6: Given figure indicates multiple pharmacological effects of *Reynoutria japonica* [80].

Naphthoquinones are one of these secondary metabolites with a variety of biologically significant features. Many 1,4-naphthoquinones, particularly 5-hydroxy-1,4-naphthoquinones (juglone) and its derivatives have antibacterial [76], antifungal [77], antiviral, antiplatelet, antidiabetic [78], anticancer, and cytotoxic properties [79]. Quinones and closely related chemical compounds have been employed as antibiotics and chemotherapeutic agents in the past [80].

Five anthraquinones, two stilbenes and a 1,4-naphthoquinone derivative from the root portion of *Reynoutria japonica* have previously been reported for anti-*Helicobacter pylori* activity. As a natural chemical, the 2-methoxy-6-acetyl-7-methyl-juglone showed the most antibacterial activity among the isolates [81]. Due to the better efficacy of these bioactive compounds having anti-bacterial properties, the present study is focused on their positive contribution in controlling resistant strains of *Staphylococcus aureus*.

2.4.2 Special Bioactive Compounds Which Act as Inhibitors

Bioactive compounds have been described as compounds that cause a specific biological reaction in animals and humans. Bioactive compounds of *Reynoutria japonica* which can act as inhibitors are discussed below.

2.4.2.1 2-Methoxy-6-acetyl-7-methyljuglone

2-Methoxy-6-acetyl-7-methyljuglone is also called juglone-1 is a quinoid compound that has been isolated from several jugladaceae members. Furthermore it is a bioactive element found in specific plant parts and has been used in ethnomedicine to treat a variety of diseases including allergies and bacterial infections. Proteomics approach shows that it is found to reduce protein synthesis in *Staphylococcus aureus* [81]. Naphthoquinones are one of several secondary metabolites found in *Reynoutria japonica* and they have biologically important applications [82]. It showed remarkable therapeutic activity against bacteria, fungi and algae. It is seen effective in treatment of tumors and diabetes. It is also useful in human intestinal infections [83]. Many juglone derivatives have been found to be effective in the treatment of *Helicobacter pylori* infections in recent studies. These derivates can be synthesized from *Reynoutria japonica* by using the acetate-polymalonate pathway. Their positive results against *Helicobacter pylori* led to the hypothesis that naphthoquinones could be employed against other bacterial strains such as *Staphylococcus aureus*, to restrict growth and treat infections [84].

2.4.2.2 Emodin

Emodin is a naturally occurring anthraquinone derivative that is found in Chinese plants such as *Rheum palmatum*. Many cultures particularly in eastern Asia, have long employed these herbs as traditional treatments [85]. A lot of scientists are now studying the pharmacological effects of this substance. Previous studies confirm its anticancer and anti-inflammatory activities. Emodin has also been shown to have a wide range of pharmacological actions including antiviral, antibacterial, antiallergic, antidiabetic, immunosuppressive, neuroprotective and hepatoprotective properties [86]. It is good for weight loss and has antimicrobial qualities as well. Emodin was investigated as a cancer chemopreventive agent. It also possesses antibacterial properties. Emodin was examined as cancer chemopreventive agent [87] [89]. Emodin could respond tumour cells to chemotherapeutic drugs by blocking pathways. It has also been suggested that emodin could be used as a lead therapeutic drug in the treatment of SARS [89]. The gram-positive bacteria particularly *Bacillus* subtilis and *Staphylococcus aureus* as well as *Mycobacterium tuberculosis* showed a strong bacteriostatic activity with Emodin. Furthermore emodin was discovered as the main anti-MRSA component, which was linked to the degradation of the cell wall and cell membrane integrity [90]. Overall emodin like many other plant substances, possesses antibacterial activity. Emodin could be employed as a lead chemical in the development of new antibacterial drugs.

2.4.2.3 Emodin 8-o-b glucoside

Emodin 8-glucoside is a dihydroxyanthraquinone. It is glycosylated derivative of emodin. It is involved in phosphorylation of mitogen-activated protein kinase pathway and phagocytosis. It also stimulates the secretion of proinflammatory cytokines. It is an anthraquinone that can be found in the roots and barks of variety of plants. It has antiproliferative effect in cancer cells that are mediated by many signalling pathways. It has cancer-fighting, anti-depressant and antimicrobial properties [91]. It is also taken as an effective anti-aging, anti-hyperlipidaemia

and anti-inflammatory agent in pre-clinical and clinical therapy to improve immunomodulation, neuroprotection and the recovery of other diseases [92].

2.4.2.4 Polydatin

Polydatin is a stilbenoid monocrystalline compound isolated from *Polygonum cuspidatum* but is also detected in grape, peanut, hop cones, red wines, hop pellets, cocoa-containing products, chocolate products and many daily diets. Anti-platelet aggregation, anti-oxidative action of low-density lipoprotein, cardioprotective activity, anti-inflammatory, hepatoprotective effects and immune-regulating functions are among few biomedical properties of polydatin [93]. It also exhibited neuroprotective and lung protective effect [94]. Anti-tumor and anti-oxidative effects are also shown by polydatin. Effective anti-bacterial activity is also exhibited by polydatin. It inhibits *Streptococcus* mutans and greatly lowers glycolytic acid generation at a low level. Despite the fact that polydatin's bioactivities have been proven in laboratory animals, organs and cells but few molecular mechanisms of action are known and the definitive target proteins bound by polydatin are still unknown which require further clinical applications of polydatin [95] [96] [97].

2.4.2.5 Resveratrol

Resveratrol is a polyphenolic antioxidant found in nature that belongs to the stilbene family [98]. Resveratrol can be found in a variety of plants, including peanuts, blueberries, and cranberries. *Polygonum cuspidatum*, also known as Japanese knotweed is a traditional Asian herbal remedy as well as a natural source for human consumption in grapevines [99]. It is naturally found in different plants. It has anti-inflammatory properties. It is used as an antioxidant and has potential chemo-preventive activities. It is effective in lowering of blood pressure and cholesterol. It protects brain functions and improve cardiovascular health. Resveratrol is a plant polyphenol and present in large amounts in many plants and has been recommended in the treatment for hyperlipidemia as well as

useful in diabetes, atherosclerosis and aging [100]. It showed antifungal, antiviral and antibacterial properties [101]. Past researches showed that it reduced the growth of *Bacillus cereus, Helicobacter pylori, Vibrio cholerae, E.coli* and *Neisseria gonorrhoeae* [102]. The capacity of resveratrol to inhibit biofilm formation in diverse bacterial pathogens has been investigated. Resveratrol has also been shown to reduce the ability of *Vibrio vulnificus* to swarm [103] [104]. Few reports suggest that resveratrol inhibits toxin expression in some circumstances in *E.coli* and *Chromobacterium violaceum* [105] [106]. Resveratrol interferes with quorum sensing systems through an unidentified mechanism [107]. To determine the clinical potential of resveratrol as a monotherapy or in combination with standard antibiotics, more research in suitable animal models is required.

2.4.2.6 Physcion

Physcion is a dihydroxyanthraquinone and has been widely isolated from both terrestrial and marine sources. It has a role as an apoptosis inducer, an antineoplastic agent, a hepatoprotective agent, an anti-inflammatory agent, an antibacterial agent, an antifungal agent and a metabolite [108]. The anti-proliferative impact of physcion was investigated in one study. It activated the mitochondrial apoptotic pathway as seen by the loss of mitochondrial membrane potential. Furthermore, physcion caused a long-term stimulation of cytochrome c phosphorylation. Physcion induced the production of reactive oxygen species in some cells. All of these studies point to physcion inducing apoptosis through the mitochondrial route [109].

2.4.2.7 Citreorosein

Citreorosein is a trihydroxyanthraquinone. It is effective on cyclooxygenase dependent prostaglandin generation in mast cells, allergic reactions and other inflammatory diseases [110]. Citreorosein decreased nuclear translocation of nuclear factor subunits as well as its cognate DNA-binding activity which was associated with its

inhibitory effects on phosphorylation of specific genes. Furthermore, by reducing phosphorylation it greatly reduced the DNA binding of activator protein which regulates cyclooxygenase. Taken together the results indicate that citreorosein could be used as a therapeutic model to treat inflammatory diseases.

2.4.2.8 Quercetin

Quercetin is a polyphenolic flavonoid with potential chemopreventive activity. Quercetin is present in many plant food sources and a major bioflavonoid in the human diet, may produce antiproliferative effects. It also aids in the onset of G1 phase cell cycle arrest and the suppression of heat shock protein production. When coupled with chemotherapeutic medicines, this chemical is also effective in reversal of the multidrug resistant phenotype in vitro [111]. Quercetin also has anti-inflammatory and anti-allergy properties, which are mediated by inhibiting the lipoxygenase and cyclooxygenase pathways, which prevents the synthesis of pro-inflammatory mediators [112]. It contains antibacterial, antioxidant, protein kinase inhibitor and plant metabolite properties.

2.4.2.9 Hyperoside

Hyperoside is an active metabolite of *Reynoutria japonica* belonging to flavonoid family. It is quite efficient in the pathology of diabetes mellitus when it comes to oxidative stress and inflammation. It is a flavanone glycoside that has been linked to a variety of health advantages including antioxidant and anti-inflammatory characteristics. In rats the researchers wanted to see if this could help with diabetes induced cognitive impairment and identify a potential molecular pathway [113].

2.4.2.10 Coumarin

Coumarin and some of its metabolites have been shown to inhibit glucose-6-phosphatase in liver and in liver microsomal preparation. It interferes with excision

repair processes on ultra-violet damaged DNA and with host cell reactivation of ultra-violet-irradiated phage in *E.coli*. Coumarin is a colorless crystal, flakes or powder with a nice vanilla odour and a bitter pungent burning taste. The chemical name for coumarin is hydroxycinnamic acid. Many plants produce this pleasant smelling chemical which is released as they wilt. By competing with Vitamin K, it has anticoagulant properties. It functions as a luminous dye, a plant metabolite and a human metabolite [114].

2.5 Molecular Docking

Molecular Docking is technique used to estimate the strength of a bond between a ligand and a target protein through a special scoring function and to determine the correct structure of the ligand within the target binding site. The 3D structure of the target proteins and the ligands is taken as the input for docking. It represents a frequently used approach in structure-based drug design since it requires three-dimensional structure of a target protein [115]. It can be used to determine the correct structure of the ligand within the target binding site and to estimate the strength of the binding between the ligand and the target proteins through a specific scoring function. It also helps in the recognition of new small molecular compounds revealing the essential properties such as high interaction between binding with target protein having reasonable absorption, distribution, metabolism and excretion which help in the selection of lead compound for the target [116]. So the docking process include steps which are discussed below:

1. The docking process requires a three-dimensional structure of protein which is downloaded from protein data bank.

2. Minimum size of molecules or compounds or virtual compounds that contain a database is required.

3. A computational framework is also needed to perform the docking and find the scoring process.

Protein and ligand docking is one of the key areas of molecular docking, which obtained high popularity and appreciation due to its role in structure-based drug designing. Molecular dynamics, distance geometry method and genetics algorithm etc. are most widely used algorithm in molecular docking and the most frequent software used for molecular docking are Auto Dock vina, Auto Dock, CB Dock and ICM etc [117]. The target proteins and ligands for docking purpose are shown in table 2.6 A significant number of information is present on the effectiveness

TABLE 2.6: Table showing bioactive compounds with target proteins for Docking purpose.

Ligands	**Target Proteins**
• Juglone	• Agr A
• Emodin	• Agr B
• Emodin 8-o-b glucoside	• AgrC
• Polydatin	• TRAP
• Resveratrol	
• Physcion	
• Citreorosein	
• Quercetin	
• Hyperoside	
• Coumarin	

of quorum quenching techniques in the battle against infectious diseases till date. No doubt researchers have focused their attention on disruption of quorum sensing in this regard but the majority of their work is related to signal antagonism and very little research was done on signal enzymatic destruction, signal inhibition, or signal sequestration [118]. *Staphylococcus aureus* has adverse history related to clinical aspects and undoubtedly many antibiotics had been tried for treatment but there is very little search to control its signaling mechanism which could be more effective than antibiotics usage. Therefore, it is need to search innovate strategies to suppress signaling and non-toxic compounds preferentially from herbal sources for drug designing purpose [119]. Despite the significant research in this area and application of novel medicines there is need of more strong proofs of ideas. Also medical studies are obviously required before more quorum quenching related items may be seen in the market.

Chapter 3

Research Methodology

3.1 Methodology Flowchart

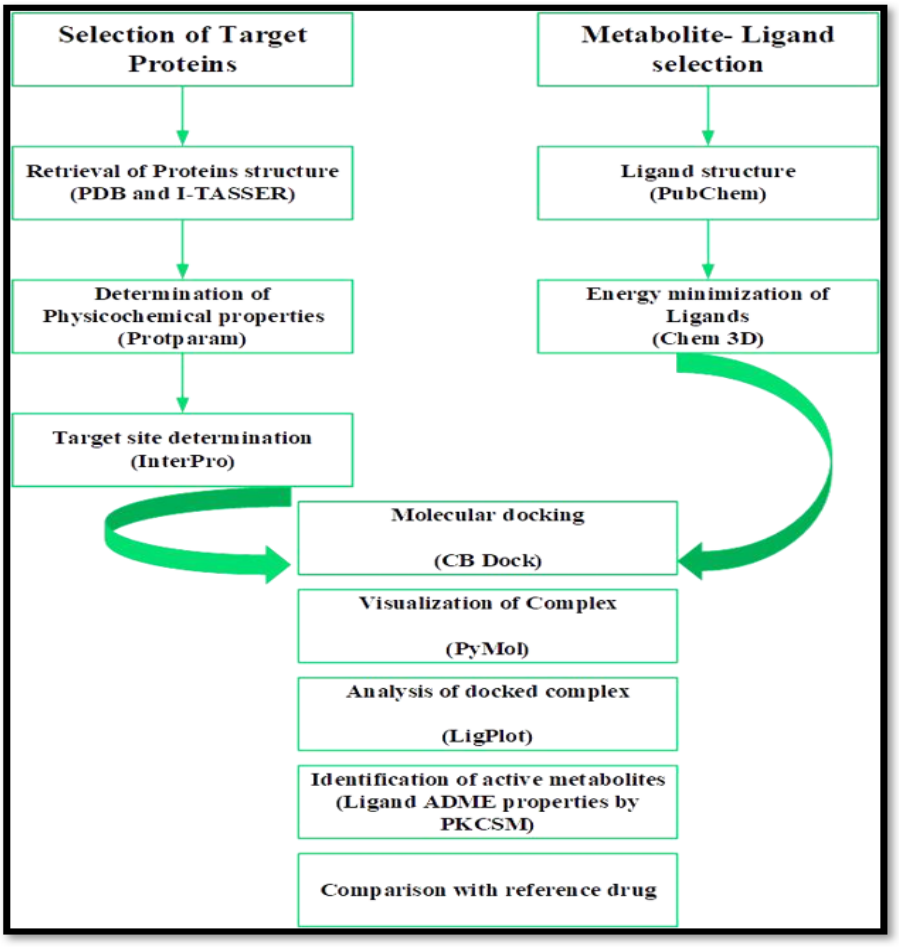


FIGURE 3.1: The flowchart of methodology.

3.2 Selection of Problem

In case of bacterial infections antibody treatment is recommended but it is seen that certain bacteria become resistant to those antibiotics. Due to which diseases are spreading on a larger scale. Alternative sources are required to lessen the extent of problem. In case of *Staphylococcus aureus*, the main contributor of pathogenicity are Agr and TRAP quorum sensing systems [119] [120].

3.3 Target Protein Selection

It is possible to manage bacterial infections, the key factor involved are several active metabolites in *Reynoutria japonica*. These metabolites are involved in the bacterial pathways which play a vital role to inhibit severe infections. Target proteins which were selected on basis of their virulence and pathogenicity factors are Accessory gene regulator protein A, B and C [121].

3.4 Primary Sequence Retrieval

Primary sequence of target proteins (AgrA, AgrB, AgrC and TRAP) was taken in FASTA format from protein sequence database UniProt http://www.uniprot.org/ under accession number POA017, POC1P7,007911 and Q84DC6 with residues length of 238,189,430 and 167 respectively [122].

3.5 Analysis of Physicochemical Properties of Protein

Physicochemical properties play an important role in determining the function of proteins. ProtParam https://web.expasy.org/protparam/ was used to predict these properties of AgrA, AgrB, AgrC and TRAP. The number of positively

charged residue (Arg+ Lys) and negative charged residue (Asp+ Glu), theoretical pI, molecular weight, Ext coefficient (Cys included), Ext coefficient (Cys not included), instability index, aliphatic index and grand average of hydrophobicity were computed through ProtParam [123].

3.6 3D Structure Prediction of Protein

The 3D structures can be predicted through PDB https://www.rcsb.org/. Alternatively, I-TASSER can be used if some structures are not available on PDB. I-TASSER https://zhanglab.ccmb.med.umich.edu/I-TASSER/ stands for Interactive Threading Assembly Refinement. It is an online server used for the prediction of the structure and function of the protein in three dimensions. This online server firstly identifies the structural model of the PDB through various strategies which include the atomic models of full length and they are built by using simulations of the different threading fragments. The I-TASSER server also predict the 3D structure of proteins and these server gives us five 3D structure of proteins so on the basis of C-score we can select the best 3D structure of the protein [124]. Alphafold https://alphafold.com/ is also an authentic protein structure database used for 3D structure prediction of proteins [125].

3.7 Structure Analysis by Use of PyMOL

PyMOL https://pymol.org/ is a cross platform molecular graphics tool that has been used world widely for the three-dimensional analysis and visualization of many proteins, small molecules including nucleic acids, densities of different electrons and varying surfaces and also the trajectories. It is also used for editing the molecules, tracing the ray and also to make animations and movies. This is software that is based on python and also contain many plugin tools in order to enhance its use and also facilitate the drug targeting and designing by the use of PyMOL software. After downloading the protein structure, the extra constituents

attached to the protein need to be removed which was done by the use of an open-source system PyMol [126].

3.8 Functional Domain Identification of Targeted Proteins

Interpro http://www.interpro.com/ is an online database which was used to identify the functional domains of targeted proteins AgrA, AgrB, AgrC and TRAP [127]. Conserved domains are involved in sequence/structure/relationship.

3.9 Retrieval of Chemical Structure of Ligands

PubChem https://pubchem.ncbi.nlm.nih.gov/ is the world's largest repository of easily accessible chemical information database. So the chemical compounds that were used as ligands were selected from PubChem database. The selected ligands were refined through Chem Draw Ultra version 12.0.2 software. Those ligands were selected that had previously shown some antibacterial properties. These include the juglone, emodin, emodin 8-o-b glucoside, polydatin, resveratrol, physcion, citreorosein, quercetin, hyperoside and coumarin. If in case the selected ligand structure was not available then our next attempt would be to download the canonical similes from PubChem and then insert them in the software ChemDraw and after repeat the energy minimization step using Chem pro software [128].

3.10 Bioactivity Analysis of Ligands and Toxicity Measurement

Chemical compounds that were used as ligand were selected from PubChem database. Selected compounds follow the lipinski rule of five and those are likely to be used

as active drug in humans. The potential success of a compound depends on its ADMET properties. PkCSM https://omictools.com/pkcsm-tool is an online tool that helps to find the ADMET properties of the compounds. The rules are described as under:

1. The logP value of most "drug-like" molecules should be limited to 5.
2. Maximum number of H-bond acceptor should be 10.
3. Maximum number of H-bond donor should be 5 [129].

3.11 Molecular Docking of Targeted Proteins

The purpose of molecular docking is to find the best conformational interaction between target proteins and compounds. The two essential requirements for docking are the target protein and the candidate ligand. Active metabolites of *Reynoutria japonica* found after the review of literature were used as the ligands and target proteins were accessory gene regulator protein A, B, C and signal transduction protein TRAP. CB dock http://clab.labshare.cn/cbdock/php/blinddock.php is an online docking server which automatically identifies binding sites and is used to perform docking. It can simplify docking procedures and improve accuracy by predicting target protein binding sites [130].

3.11.1 Process of Molecular Docking

The first step in performing the docking process is to create ligand and target protein files. Firstly the target protein file was compiled following a few steps. PDB file of target proteins was given to CB dock as input file one by one. After these amendments target protein file was saved in pdbqt format. After compilation of protein files, the ligands files were prepared by following the same procedure and saved in PDB format in same directory. Then setting up Grid box around protein ligand structure was performed. For this purpose, macromolecules option

was selected from Grid and pdbqt target protein file was opened then from set map type option ligand structure was opened [131]. After performing these steps from grid, grid box option was selected to design grid around protein- ligand complex. Grid box with all parameters appeared and file was saved as Grid Parameters File in same directory after selecting parameters. Utilizing docking related commands docking was performed. These commands help to get the directory path where the docking readable prepared protein ligand files have been saved along grid parameters file. Docking files were created for chosen data set after completion of this step and results were saved in pdbqt format [132].

3.11.2 Active Site Identification

The ligand shows maximum or highest interaction with the protein where the target protein has their active site. Amino acids are highly involved in the formation of complex of ligand to protein. Protein binding pockets were identified by CASTp software `http://sts.bioe.uic.edu/castp/` [133].

3.12 Protein Ligand Interaction

The interaction of the active pockets of the ligand and the protein are calculated for the interpretation of docking results. Two types of interactions are studied, hydrogen bonding and hydrophobic bonding. Using Ligplot plus (version v.1.4.5) the protein ligand interactions were studied. This software automatically generates schematic diagrams of the protein-ligand interaction of the given ligands in the PDB file [134].

3.13 Lead Compound Identification

After a detailed analysis of protein and ligand interactions, docking scores and toxicity studies, the most active inhibitor was identified. The selected compound

was our lead compound.

3.14 Reference Anti-Bacterial Drug Identification and Selection

This step was performed for identification of drugs that were used for antibacterial diseases treatment purpose. KEGG and Drug Bank https://go.drugbank.com/ databases were used for drug identification because it helps to analyze the disease in details with its pathway and drugs [135].

3.15 Prediction of Different Parameters of Selected Drug

The identified drugs must be filtered in order to select the most effective drug. This is done through a detailed study of identified drugs and most effective drug is identified setting parameters i.e. physiochemical properties, effective ADMET properties, effective mechanism of action and minimal side effects using PubChem, Drug Bank, pkCSM, and KEGG databases, respectively. The identified drug was then docked with target proteins to identify the inhibition efficiency. CB dock is an online docking server which was used to perform docking. It can simplify docking procedures and improve accuracy [136].

3.16 Reference Drug and Lead Compound Comparison

The comparison between reference anti-bacterial drug and the proposed lead compound was done through comparing docking values, physiochemical properties and ADMET properties.

Chapter 4

Results and Discussions

This chapter will explain the results that were obtained by following our methodological steps. The 3D structure of proteins and ligand was taken as input. After checking physiochemical properties and domain prediction the proteins were docked against selected ligands whose energy had already minimized. ADMET properties and lipinski rule helped in prediction of drug-like features of compounds. Further the validation of selected compound was checked by comparing its properties with available antibiotic drug. All these steps are described under headings sequentially.

4.1 Structure Modeling

Structure modeling includes primary sequence retrieval, physiochemical properties prediction, 3D structure prediction and functional domain identification of proteins.

4.1.1 Primary Sequence Retrieval

FASTA sequence of selected target proteins was retrieved through UniProt www.uniprot. These proteins were selected on basis of their pathogenicity and virulence causing

factors. The FASTA sequence of accessory gene regulator protein A, B, C and signal transduction protein TRAP were downloaded from uniport under accession number POA017, POC1P7,007911 and Q84DC6 with 238,189,430 and 167 residues length respectively.

>sp|P0A0I7|AGRA_STAAU Accessory gene regulator protein A OS=*Staphylococcus aureus* OX=1280 GN=AgrA PE=1 SV=1

MKIFICEDDPKQRENMVTIIKNYIMIEEKPMEIALATDNPYEVLEQAKNMNDIGCYFL
DIQLSTDINGIKLGSEIRKHDPVGNIIFVTSHSELTYLTFVYKVAAMDFIFKDDPAELRT
RIIDCLETAHTRLQLLSKDNSVETIELKRGSNSVYVQYDDIMFFESSTKSHRLIAHLDNR
QIEFYGNLKELSQLDDRFFRCHNSFVVNRHNIESIDSKERIVYFKNKEHCYASVRNVKK
I

>sp|P0C1P7|AGRB_STAAU Accessory gene regulator protein B OS=*Staphylococcus aureus* OX=1280 GN=AgrB PE=1 SV=1

MNYFDNKIDQFATYLQKRNNLDHIQFLQVRLGMQVLAKNIGKLIVMYTIAYILNIFLFT
LITNLTFYLIRRHAHGAHAPSSFWCYVESIILFILLPLVIVNFHINFLIMIILTVISLGVISV
YAPAATKKKPIPVRLIKRKKYYAIIVSLTLFIITLIIKEPFAQFIQLGIIIEAITLLPIFFIKED
LK

>tr|O07911|O07911_STAAU Accessory gene regulator C OS=*Staphylococcus aureus* OX=1280 GN=AgrC PE=4 SV=1

MELLNSYNFVLFVLTQMILMFTIPAIISGIKYSKLDYFFIIVISTLSLFLFKMFDSASLIILT
SFIIIMYFVKIKWYSILLIMTSQIILYCANYMYIVIYAYITKISDSIFVIFPSFFVVYVTISILF
SYIINRVLKKISTPYLILNKGFLIVISTILLLTFSLFFFYSQINSDEAKVIRQYSFIFIGITIFLS
ILTFVISQFLLKEMKYKRNQEEIETYYEYTLKIEAINNEMRKFRHDYVNILTTLSEYIRE
DDMPGLRDYFNKNIVPMKDNLQMNAIKLNGIENLKVREIKGLITAKILRAQEMNIPISI
EIPDEVSSINLNMIDLSRSIGIILDNAIEASTEIDDPIIRVAFIESENSVTFIVMNKCADDIP
RIHELFQESFSTKGEGRGLGLSTLKEIADNADNVLLDTIIENGFFIQKVEIINN

>sp|Q2G2F3|TRAP_STAA8 Signal transduction protein TRAP OS=*Staphylococcus aureus* OX=93061 GN=TRAP PE=1 SV=1

MKKLYTSYGTYGFLHQIKINNPTHQLFQFSASDTSVIFEETDGETVLKSPSIYEVIKEIGE
FSEHHFYCAIFIPSTEDHAYQLEKKLISVDDNFRNFGGFKSYRLLRPAKGTTYKIYFGFA
DRHAYEDFKQSDAFNDHFSKDALSHYFGSSGQHSSYFERYLYPIKE

FIGURE 4.1: Sequence Retrieval

4.1.2 Physiochemical Characterization of Proteins

ProtParam is a tool of Expasy which is used online for the prediction of different parameters including both physical and chemical properties of selected proteins. These several parameters calculate and estimate the following: molecular weight,

composition of amino acid, theoretical value of Protein index, atomic composition of protein, extinction coefficient, estimated half-life of protein instability, aliphatic index and grand average of hydropathicity which was abbreviated as GRAVY. The calculated PI greater than 7 represents the basic nature of the protein while less than 7 shows acidic nature of protein. Extinction coefficient represents light absorption. Instability index if less than 40 show stability of the protein while greater than 40 indicates the instability of protein [137]. The physiochemical properties of nucleoprotein are shown in Table 4.1, 4.2.

TABLE 4.1: Physicochemical properties of target proteins.

Target Proteins	**MW**	**PI**	**NR**	**PR**	**Ext. Co1**	**Ext. Co2**
AgrA	27906	5.8	37	31	15150	14900
AgrB	21930	9.9	8	19	18910	18910
AgrC	49897	5.2	45	38	38405	38280
TRAP	19547	6.1	22	18	20860	20860

The below table show target proteins with the properties.

TABLE 4.2: Target proteins properties.

Target Proteins	**Instability index**	**Aliphatic index**	**GRAVY**
AgrA	36.25	91.3	-0.379
AgrB	45.16	147.04	0.828
AgrC	39.15	127.16	0.494
TRAP	20.68	60.78	-0.58

The aliphatic index represents the aliphatic content of a protein. The high value of the aliphatic index indicates the thermo stability of the protein. Molecular weight contains both positive and negative charged residues of protein. Low GRAVY shows better interaction with water molecules. All these parameters which were selected for this research work were taken according to previous research work [138]. MW stands for molecular weight, pl for theoretical isoelectric point at which protein is neutral, without any charge), NR for total number of negatively charged residues (Asp +ve Glu),PR for total number of positively charged residues (Arg

+ve Lys), Ext.Co1 for extinction coefficients when assuming all pairs of Cys residues form cystines , Ext. Co2 for extinction coefficients when assuming all Cys residues are reduced and GRAVY for grand average of hydropathicity.

4.1.3 3D Structure Prediction of Proteins

3D Structures of targeted proteins can be downloaded from RCSB PDB in PDB format. Protein Data Bank may be a three-dimensional database of complex molecules of living organisms like proteins and nucleic acids. I-TASSER (Iterative threading Assembly Renement) may be a special procedure in order to predict the structure of the protein also structure and function of the target proteins.
This online server firstly identifies the templates of the given structure retrieved by the PDB obtained by applying different approaches including the multiple threading approach LOMETS, by involving atomic models of full length which are built by the simulations assembled by fragments that are template based.
This server has been widely used for protein structure and performance predictions in biological and biomedical investigations.
I-TASSER predicts regions of secondary protein structure which may include like alpha helix, beta sheet and coils obtained by the sequence of the organic compound [139]. I-TASSER server team mails complete results of job id with five models and on the base of C-score best 3D structural model is often easily selected. Alphafold https://alphafold.com/ is also a protein structure database used for 3D structure prediction of proteins. The 3D structures of accessory gene regulator protein A, B, C and signal transduction protein TRAP were taken from Alphafold in PDB file under IDs respectively.

1. **POA017**,
2. **P61637**,
3. **Q2FWM5** and
4. **Q2FFR1**.

The protein structures were prepared in PyMol by removing water molecules and ligands if existed. After the removal of ligands and other atoms the missing polar hydrogens were added. The energy of minimazation for structures was performed to get the stable conformation by preventing overlaps and saved the modified file in PDB format. The refined structures are shown in figures below.

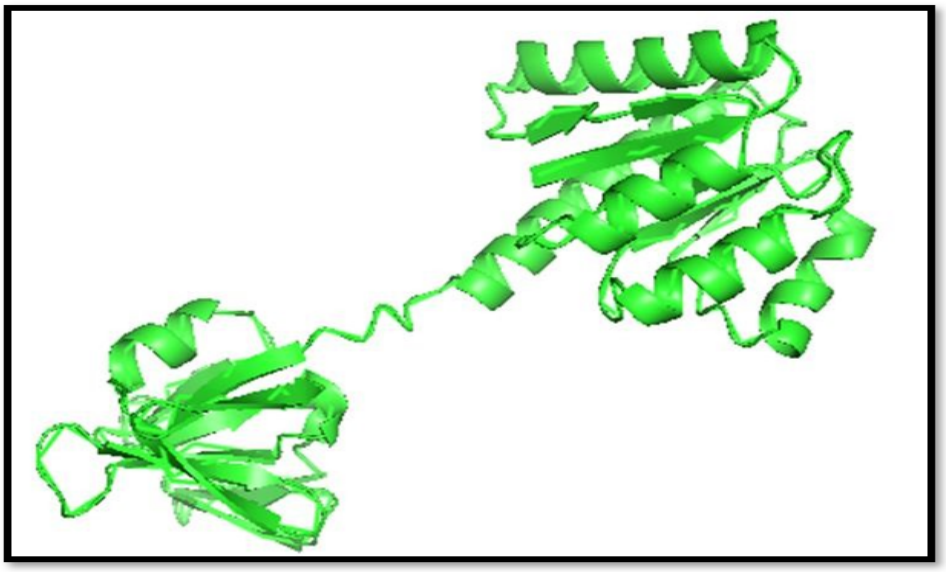


FIGURE 4.2: 3D Structure of accessory gene regulator protein A

Above figure representing three-dimensional structure of accessory gene regulator protein A which is part of Agr quorum sensing system and is required for high level post-exponential phase expression of a series of secreted proteins.

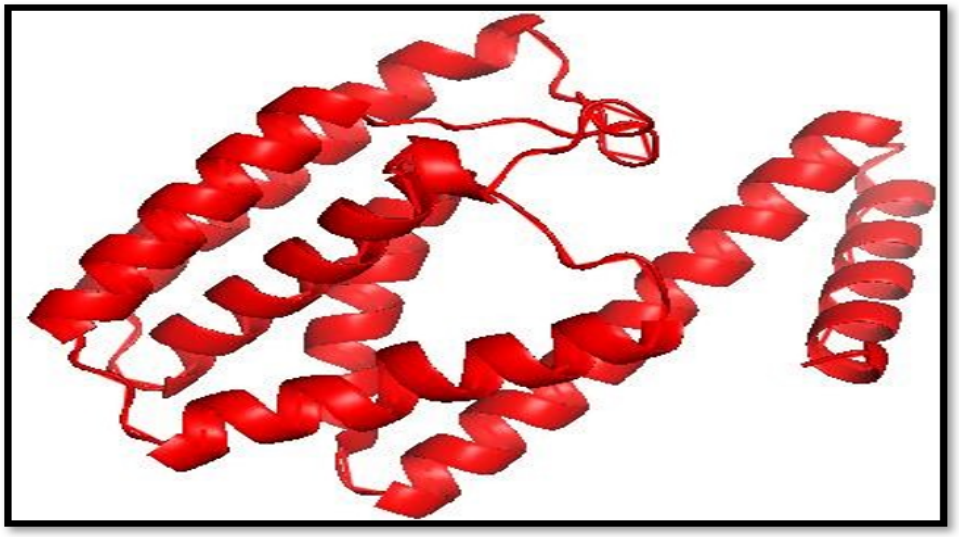


FIGURE 4.3: 3D Structure of accessory gene regulator protein B

Above figure representing three-dimensional structure of accessory gene regulator protein B which is part of Agr quorum sensing system of *Staphylococcus aureus* and is required for the generation of the autoinducing peptide, a quorum sensing system signal molecule. It also plays a role in the proteolytic processing of AgrD which is the precursor to AIP. The regulation of virulence factor gene expression is controlled by this quorum sensing system.

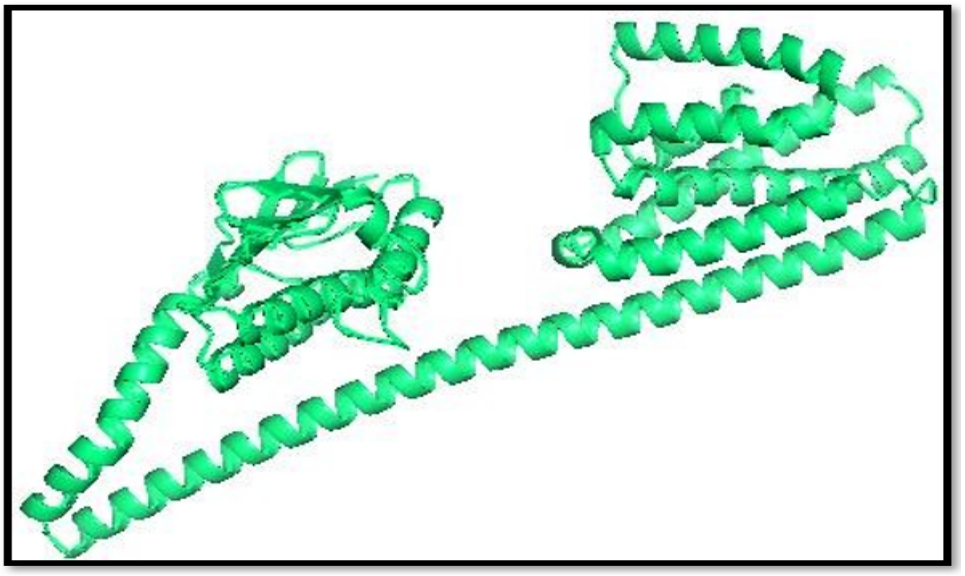


FIGURE 4.4: 3D Structure of accessory gene regulator protein C

Above figure representing the three-dimensional structure of accessory gene regulator protein C which is part of Agr quorum sensing system of *Staphylococcus aureus*. AgrC is the membrane-bound histidine kinase sensor. The binding of AIP to the AgrC receptor leads to histidine autophosphorylation.

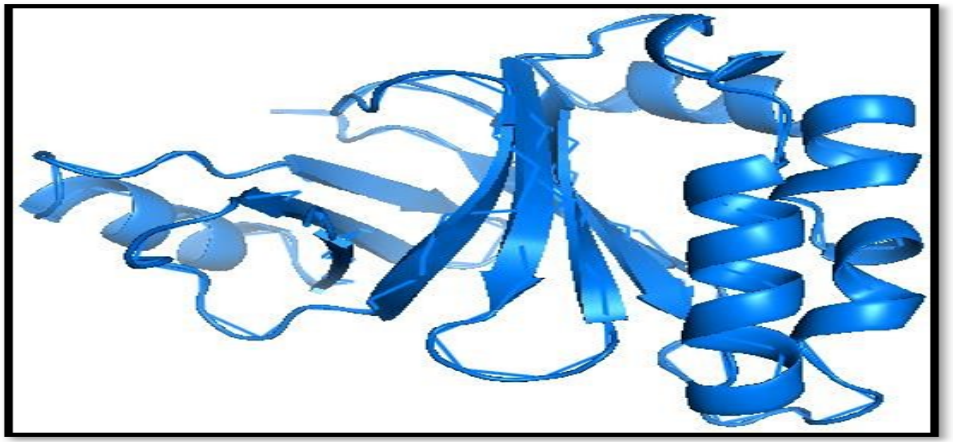


FIGURE 4.5: 3D Structure of signal transduction protein TRAP

Above figure representing three-dimensional structure of Signal transduction protein TRAP which is a main factor in staphylococcal pathogenesis. The Agr system is activated by phosphorylated TRAP, which results in the synthesis of RNAIII and the activation of several virulence factors. It upregulates the majority of toxins and genes known to be involved in biofilm formation.

4.1.4 Functional Domain Identification of Protein

Database Interpro was used to identify the domains and functional sites of selected proteins. Interpro is a resource for functional analysis of protein sequences. Conserved domains are involved in sequence/structure/relationship. Proteins can have more than one functional domain that perform different functions. Functional domain is the active part of a protein that is involved in interactions of proteins with other substances [140]. Figure 4.6,4.7, 4.8 and 4.9 show the functional domains of the protein. Accessory gene regulator protein A is 238aa long protein consisting of two domains. One is Lyt-TR DNA binding domain starting from residue 143 and ending at 238 while the other one is signal transduction response regulator, receiver domain starting at residue 1 and ending at 125. AgrB protein is 186aa long protein consist of single domain called accessory gene regulator B domain starting at residue 6 and ending at 186. AgrC protein with sequence of 430aa consist of sensor histidine kinase NatK, C terminal domain starting at residue 325 and ending at 427.

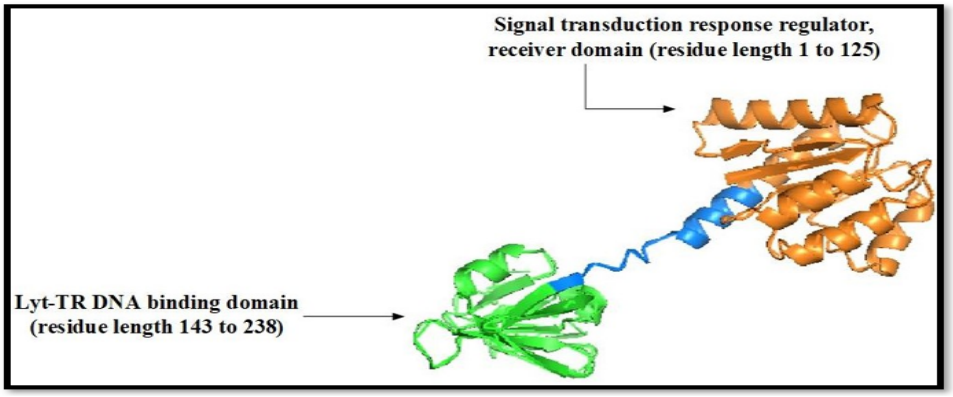


FIGURE 4.6: Figure showing functional domains of AgrA protein with residues length

Signal transduction protein TRAP with sequence of 167aa consist of single domain called antibiotic biosynthesis monooxygenase domain starting at residue 67 and ending at 158 [141]. The above figure 4.6 representing the domains of accessory gene regulator protein A. This is 238aa long protein consisting of two domains. Green color showing Lyt-TR DNA binding domain starting from residue 143 and ending at 238 while the orange color showing signal transduction response regulator, receiver domain starting at residue 1 and ending at 125.

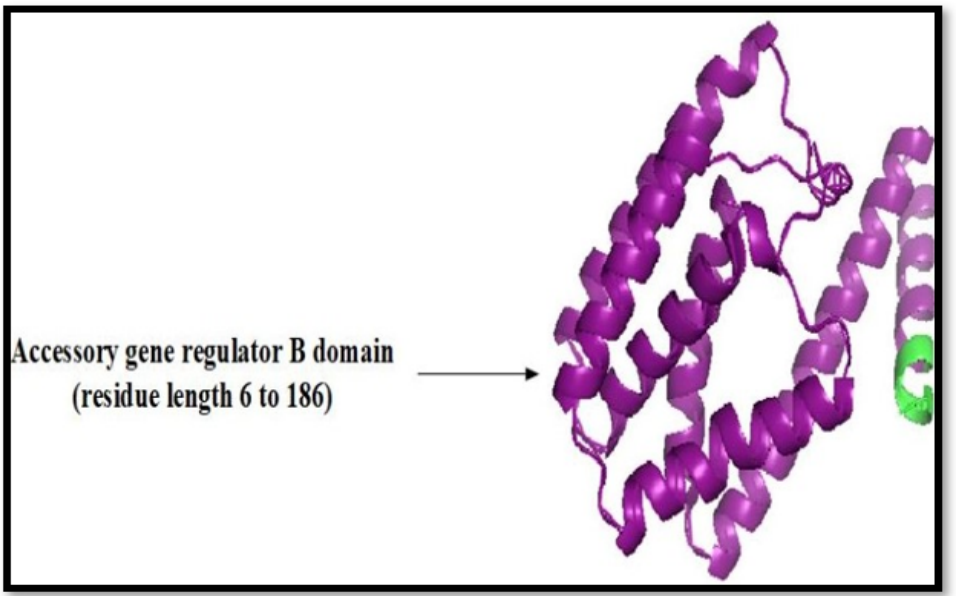


FIGURE 4.7: Figure showing domain of AgrB protein with residues length

AgrB is 186aa long protein consist of single domain shown in purple color called accessory gene regulator B domain starting at residue 6 and ending at 186.

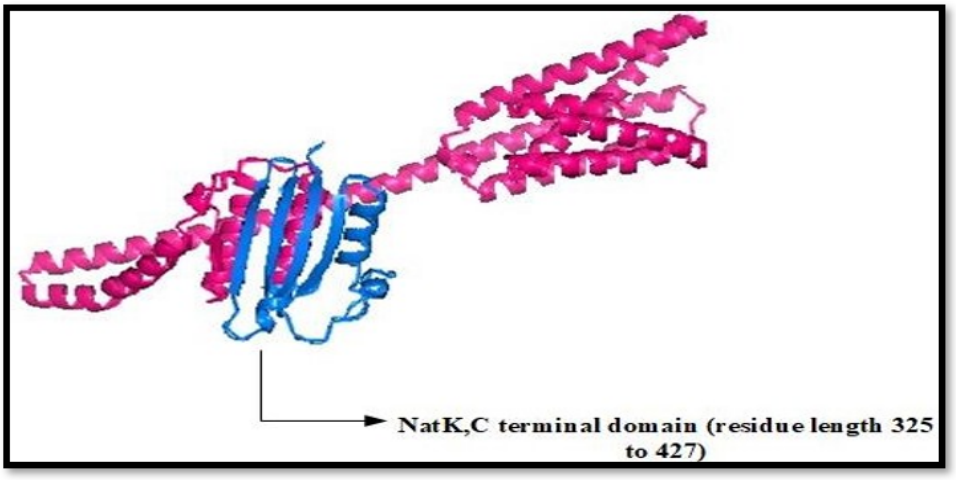


FIGURE 4.8: Figure showing domain of AgrC protein with residues length

Above figure representing accessory gene regulator protein C protein with sequence of 430aa consist of sensor histidine kinase NatK, C terminal domain shown in blue color starting at residue 325 and ending at 427.

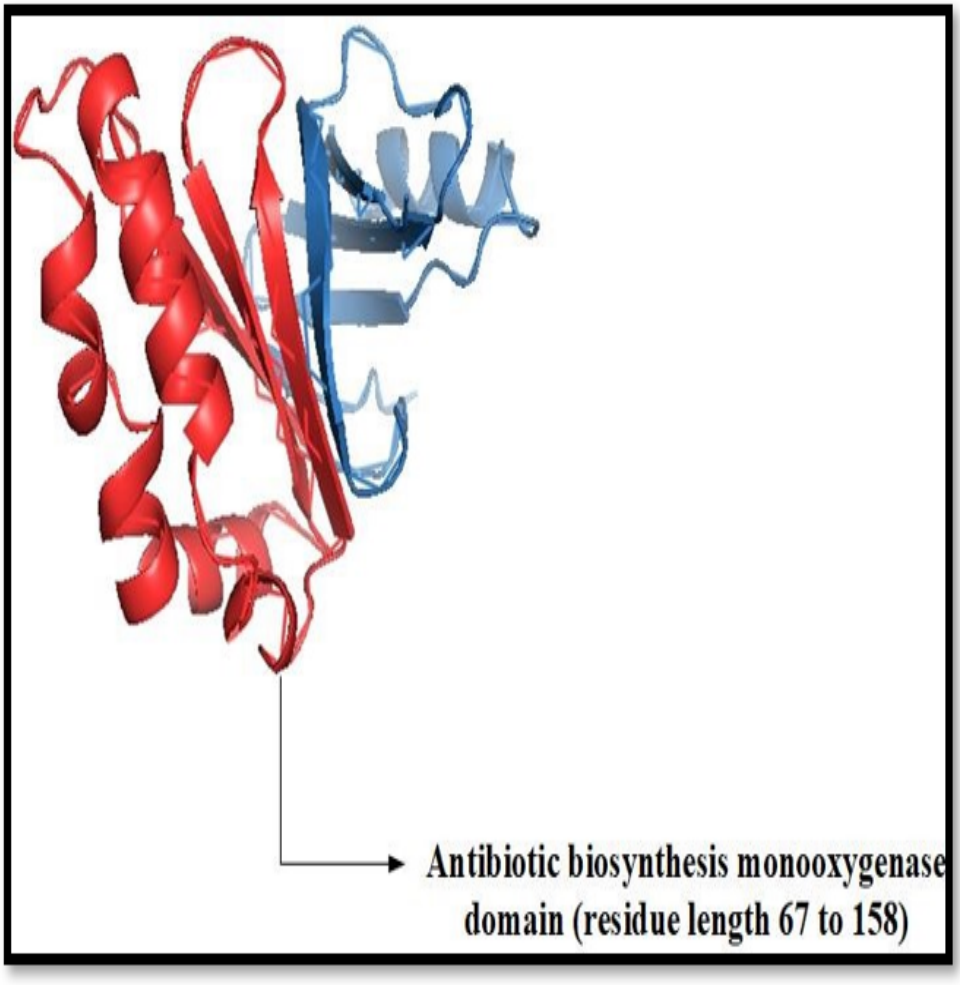


FIGURE 4.9: Figure showing domains of TRAP with residues length

Above figure representing signal transduction protein TRAP with sequence of 167aa consist of single domain called antibiotic biosynthesis monooxygenase domain shown in red color starting at residue 67 and ending at 158.

4.2 Ligand Selection

Protein data bank contains a large amount of protein ligand complex, especially for the protein target. Therefore, the selection of ligands is based on the best

resolution of the structure, the chemical class of the co-crystal ligand bound to the protein structure and the best binding affinity. Conformational selection is a process in which ligand selectively binds to one of these conformers, strengthening it and increasing its population with respect to the total population of the protein ultimately resulting in the observed complex [142].

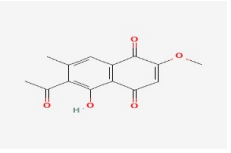
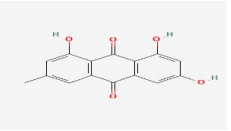
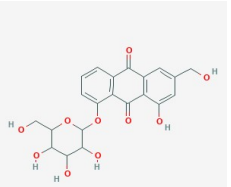
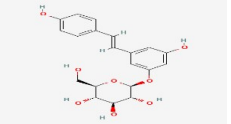
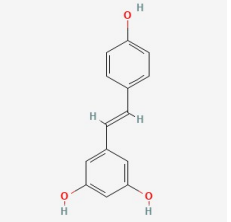
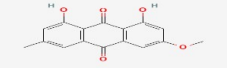
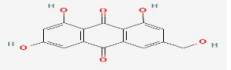
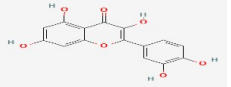
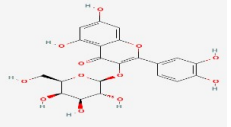
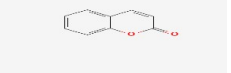
Ligands were searched out from the chemical information database PubChem https://pubchem.ncbi.nlm.nih.gov, from here the information can be accessed freely around the globe. Their 3D structures were downloaded from PubChem in SDF format. After selection of ligands, energy minimization was carried out by chem pro software (chem 3D v 12.0.2) [143]. The lipinski rule deals with certain parameters like Molecular weight which should be is less than or equal to 500, log P is less than or equal to5, Hydrogen bond donors is less than or equal to5, hydrogen bond acceptors is less than or equal to 10.

This was a mandatory step in the preparation of ligands for docking because unstable ligands will show unreliable vina scores in docking results. Bioactive antibacterial compounds of *Reynoutria japonica* were selected as ligands for the present study. These rules are to be followed by orally active compounds. The drug like is dependent on the mode of administration. As shown in the structures. The selected ligands were 2-methoxy-6-acetyl-7-methyljuglone, emodin, emodin 8-ob glucoside, polydatin, resveratrol, physcion, citreorosein, quercetin, hyperoside and coumarin. This ligand selection is based upon lipinski rule of five.

The lipinski rule deals with certain parameters like Molecular weight which should be is less than or equal to 500, log P is less than or equal to 5, Hydrogen bond donors is less than or equal to 5, hydrogen bond acceptors is less than or equal to 10 [144].

These rules are to be followed by orally active compounds. The drug like is dependent on the mode of administration. A compound is considered a drug when it follows 3 or more rules and if a compound violates two or more rules it is considered poorly absorbed. All ligands except hyperoside and coumarin obey lipinski rule of five. Selected ligands with molecular formula, molecular weight and chemical structure are represented in table 4.3.

TABLE 4.3: The following table represents Structure of ligands.

Sr.No	Ligands Name	Molecular Formula	Molecular Weight	Structure
1	2-methoxy-6-acetyl-7-methyljuglone	$C_{14}H_{12}O_5$	260.24g\mol	
2	Emodin	$C_{15}H_{10}O_5$	270.24g\mol	
3	Emodin 8-o-b glucoside	$C_{21}H_{20}O_{10}$	432.4g\mol	
4	Polydatin	$C_{20}H_{22}O_8$	390.4g\mol	
5	Reveratrol	$C_{14}H_{12}O_3$	228.24g\mol	
6	Physcion	$C_{16}H_{12}O_5$	284.26g\mol	
7	Citreorosein	$C_{15}H_{10}O_6$	286.24g\mol	
8	Quercetin	$C_{15}H_{10}O_7$	302.23g\mol	
9	Hyperoside	$C_{21}H_{20}O_{12}$	464.4g\mol	
10	Coumarin	$C_9H_6O_2$	146.14g\mol	

4.3 Molecular Docking

Molecular Docking is technique used to estimate the strength of a bond between a ligand and a target protein through a special scoring function and to determine the correct structure of the ligand within the target binding site. The 3D structure of the target proteins and the ligands is taken as the input for docking. It represents a frequently used approach in structure-based drug design since it requires a 3D structure of a target protein.

It can be used to determine the correct structure of the ligand within the target binding site and to estimate the strength of the binding between the ligand and the target proteins through a specific scoring function. It also helps in the recognition of new small molecular compounds, revealing the essential properties such as high interaction between binding with target protein having reasonable absorption, distribution, metabolism and excretion which help in the selection of lead compound for the target [145].

The docking was performed using accessory gene regulator proteins A, B, C and TRAP proteins and ligands were 2-methoxy-6-acetyl-7-methyljuglone, emodin, emodin 8-o-b glucoside, polydatin, resveratrol, physcion, citreorosein, quercetin, hyperoside and coumarin. Ligands with best binding score values with target proteins were represented in table 4.4, 4.5. To automatically predict binding modes without information about binding sites, a user-friendly blind docking web server called CB Dock was used.

CB dock predicts and estimates a binding site for a given protein and calculate centers and sizes with a novel rotation cavity detection method and perform docking with the popular docking program named Auto dock Vina [146]. CB dock gives five best interacting confirmations for each ligand molecule.

These confirmations were arranged based on binding affinity and then finest confirmation selection was done on the basis of highest affinity score of protein-ligand interaction. After docking process, the dock structures were selected for further analysis. On the basis of docking score, cavity size, Grid map, binding energy one can select best docked structure.

TABLE 4.4: Results of CB dock with ligands name , binding score and cavity size.

Sr.No	Ligands Name	Binding Score	Cavity Size
1	2-methoxy-6-acetyl-7-methyljuglone	-7.1	730
2	Emodin	-8.4	821
3	Emodin 8-o-b glucoside	-9.9	730
4	Polydatin	-8.8	730
5	Resveratrol	-8.9	1857
6	Physcion	-8.6	821
7	Citreorosein	-8.4	730
8	Quercetin	-8.8	730
9	Hyperoside	-9.1	4047
10	Coumarin	-6.6	730

TABLE 4.5: Results of CB dock with ligands name , grid Map, minimun Energy and maximum energy values.

Ligands Name	Grid Map	Mini Energy	Max Energy
2-methoxy-6-acetyl-7-methyljuglone	17	0.00	16E+00
Emodin	28	0.00	16E+00
Emodin 8-o-b glucoside	22	0.00	16E+00
Polydatin	23	0.00	16E+00
Resveratrol	34	0.00	16E+00
Physcion	20	0.00	16E+00
Citreorosein	20	0.00	16E+00
Quercetin	21	0.00	16E+00
Hyperoside	30	0.00	16E+00
Coumarin	17	0.00	16E+00

4.4 Active Site Identification

To identify active sites of protein, CASTp software was used which predicts available pockets for binding and also tells about surface area and volume of pockets [147]. Tables 4.6 4.7,4.8 and 4.9 illustrate the area and volume of accessory gene regulator A, B, C and TRAP binding pockets respectively.

TABLE 4.6: Area and volume of binding pockets of Agr A obtained by CASTp.

Pocket id	Area(SA)	Volume(SA)
1	60.457	81.841
2	78.492	33.244

Table 4.6 continued from previous page

Pocket id	Area(SA)	Volume(SA)
3	75.781	32.393
4	25.233	11.305
5	32.675	9.401
6	18.283	5.740
7	21.585	3.693
8	2.860	3.532
9	13.686	2.711
10	8.718	1.551
11	6.540	1.313
12	8.124	1.301
13	6.934	1.142
14	4.305	1.127
15	6.859	1.120
16	1.408	0.401
17	4.039	0.373
18	4.874	0.325
19	0.812	0.087
20	0.396	0.011
21	0.077	0.005
22	0.207	0.003
23	0.158	0.003
24	0.106	0.002
25	0.049	0.000
26	0.038	0.000
27	0.006	0.000
28	0.002	0.000

The above table representing binding pocket IDs with area and volume of accessory gene regulator protein A along with area and volume. It shows that there are

twenty-eight pockets available for protein AgrA. The largest binding pocket has surface area 60.457 whereas its volume is 81.841 while the smallest binding pocket has surface area 0.002 and 0.000 volume.

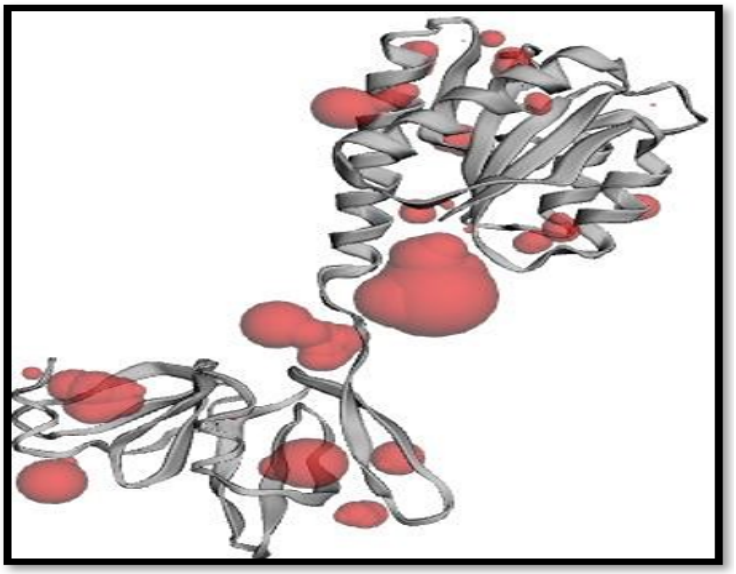


FIGURE 4.10: Structure of AgrA protein showing available pockets for ligands.

Above figure representing accessory gene regulator protein A structure. Red color showing the available binding pocket for protein. Binding pocket is the region where ligand can bind. The number of pockets with size and volume is already shown in above table.

TABLE 4.7: Area and volume of binding pockets of AgrB obtained by CASTp

Pocket id	**Area(SA)**	**Volume(SA)**
1	955.862	809.512
2	25.950	8.009
3	16.788	5.502
4	19.383	3.268
5	9.985	3.195
6	13.827	2.036
7	7.325	1.136
8	6.202	0.799
9	2.718	0.598

Table 4.7 continued from previous page

Pocket id	Area(SA)	Volume(SA)
10	3.322	0.528
11	4.131	0.422
12	0.561	0.052
13	0.997	0.032
14	0.760	0.018

The below figure representing accessory gene regulator protein B structure. Red color showing the available binding pocket for protein. Binding pocket is the region where ligand can bind. The number of pockets with size and volume is already shown in above table.

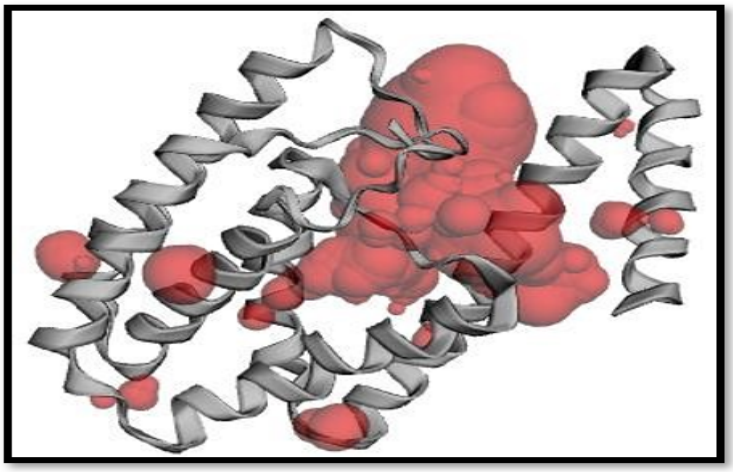


FIGURE 4.11: Structure of AgrB protein showing available pockets for ligands

TABLE 4.8: Area and volume of binding pockets of AgrC obtained by CASTp

Pocket ID	Area(SA)	Volume(SA)
1	1238.840	2518.565
2	394.397	540.006
3	591.799	444.747
4	301.740	219.820
5	64.084	30.397
6	48.272	13.229

Table 4.8 continued from previous page

Pocket ID	Area(SA)	Volume(SA)
7	28.103	24.835
8	25.606	13.229
9	34.867	12.409
10	12.892	12.113
11	23.604	10.489
12	13.035	3.619
13	15.503	2.688
14	14.438	2.055
15	12.452	1.670
16	8.885	1.666
17	8.981	1.247
18	6.079	1.147
19	9.416	1.105
20	3.440	0.864

CASTp data depicts seventy binding pockets for accessory gene regulator protein C. Above table representing twenty binding pockets with area and volume of accessory gene regulator protein C. The largest binding pocket has surface area 1238.840 whereas its volume is 2518.565 while the smaller binding pocket has surface area 3.440 and volume 0.864. The below figure representing accessory gene regulator protein C structure. Red color showing the available binding pocket for protein. Binding pocket is the region where ligand can bind. The number of pockets with size and volume is already shown in above table.

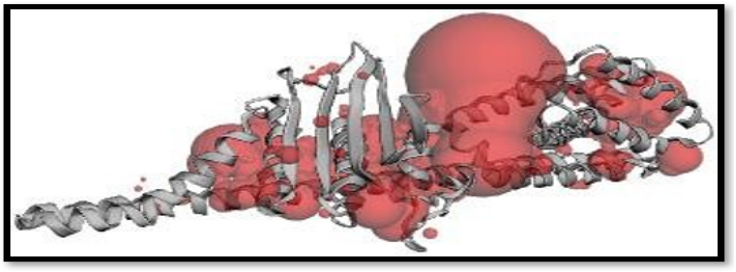


FIGURE 4.12: Structure of AgrC protein showing available pockets for ligands.

The below table representing binding pocket IDs with area and volume of signal transduction protein TRAP. It shows that there are twenty-five pockets available for this protein. The largest binding pocket has surface area 104.300 whereas its volume is 60.012 while the smaller one has surface area 0.184 and volume 0.003.

TABLE 4.9: Area and volume of binding pocket of TRAP obtained by CASTp

Pocket ID	**Area(SA)**	**Volume(SA)**
1	104.300	60.012
2	9.171	4.736
3	4.050	4.575
4	10.966	2.008
5	7.823	1.674
6	6.651	1.587
7	9.722	1.580
8	9.081	1.468
9	6.593	1.286
10	4.353	0.903
11	5.394	0.686
12	2.952	0.604
13	2.765	0.564
14	4.344	0.424
15	3.741	0.314
16	4.328	0.304
17	2.976	0.250
18	1.340	0.055
19	0.459	0.034
20	0.649	0.023
21	0.588	0.016
22	0.643	0.015
23	0.183	0.007
24	0.291	0.005
25	0.184	0.003

The below figure representing signal transduction protein TRAP structure. Red color showing the available binding pocket for protein. Binding pocket is the region where ligand can bind. The number of pockets with size and volume is already shown in above table.

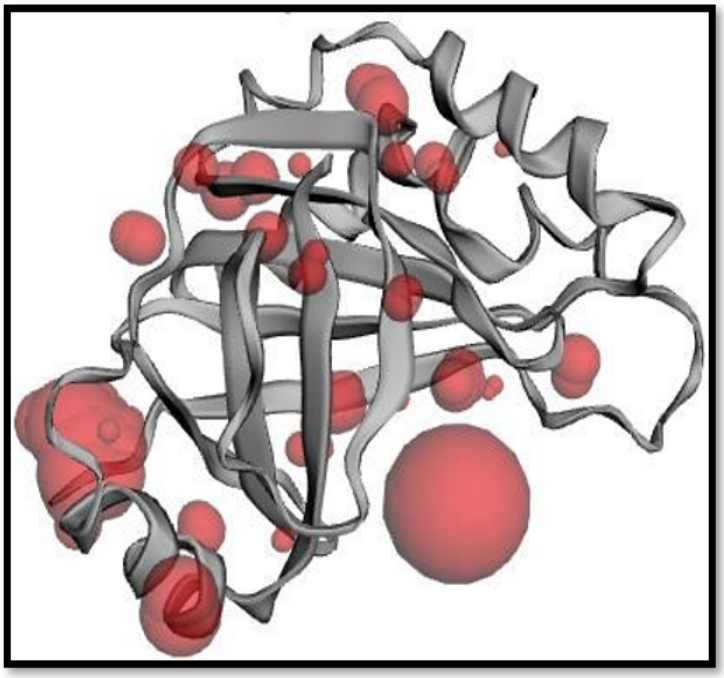


FIGURE 4.13: Structure of TRAP protein showing available pockets for ligands

4.5 Interaction of Ligands and Target Proteins

The interaction of the active pockets of the ligand and the protein were calculated for the interpretation of docking results. Two types of interactions were studied, hydrogen bonding and hydrophobic bonding interaction. Using Ligplot plus (version v.1.4.5) the protein ligand interactions were studied [148] [149] [150]. By using Ligplot plus the interaction of active confirmation of ligands and the target protein has been identified. The saved conformations for ligand receptor complex of each molecule were analyzed in detail [151]. This software automatically generates schematic diagrams of the protein-ligand interactions of the given ligands in the PDB file. The docked files were uploaded in PDB format to get hydrogen and hydrophobic bonding. A significant number of hydrophobic and hydrogen bond

interactions were observed between the ten ligands and the four target proteins. Ligand-receptor complex shows strong hydrogen bonding and hydrophobic interactions [152] [153]. Following diagrams showing the ligand-receptor interactions.

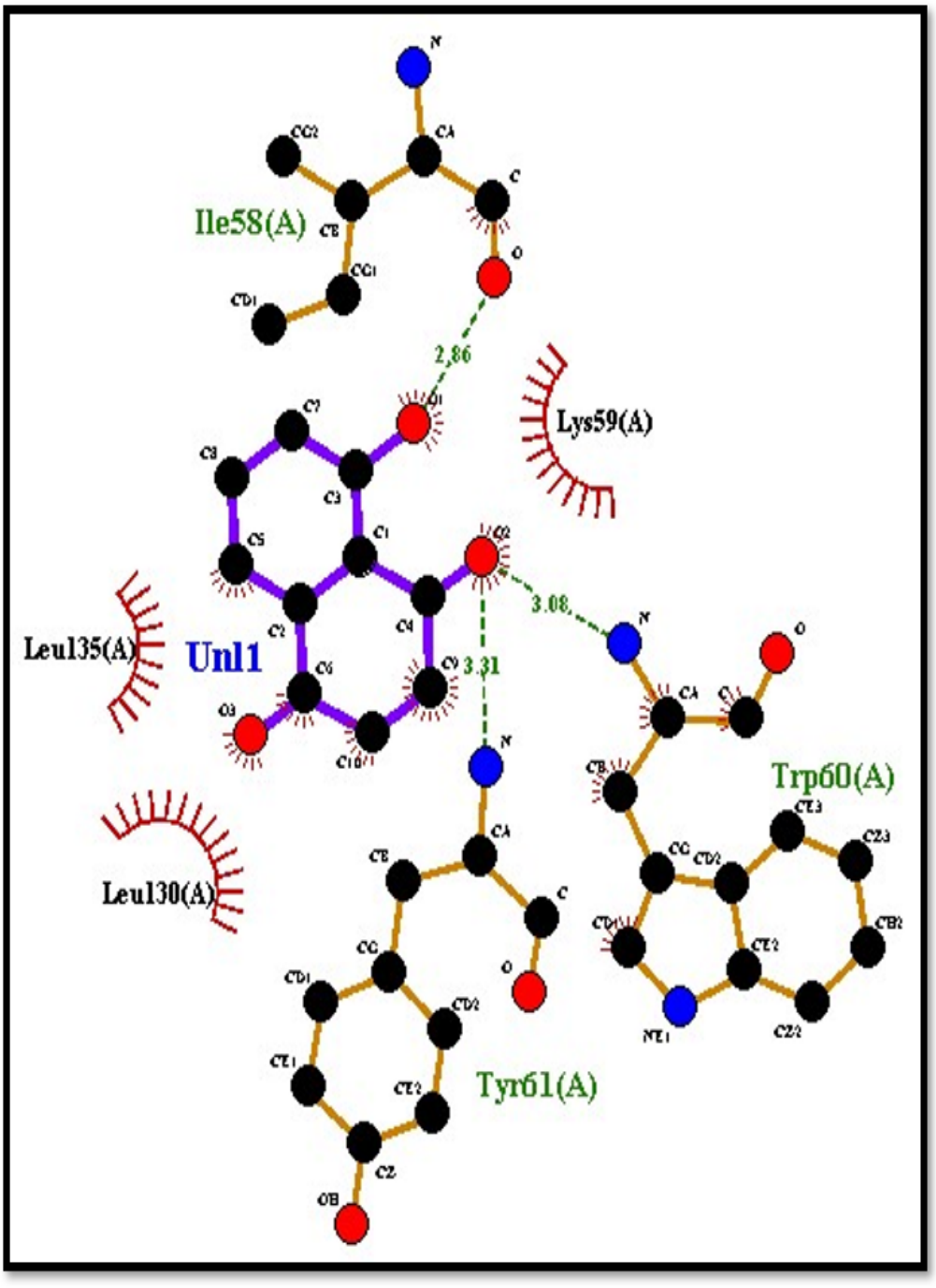


FIGURE 4.14: Interactions of 2-methoxy-6-acetyl-7-methyljuglone by ligplot.

4.14 shows the interaction of juglone with receptor protein. It shows juglone has formed three hydrophobic interactions and three hydrogen bonds.

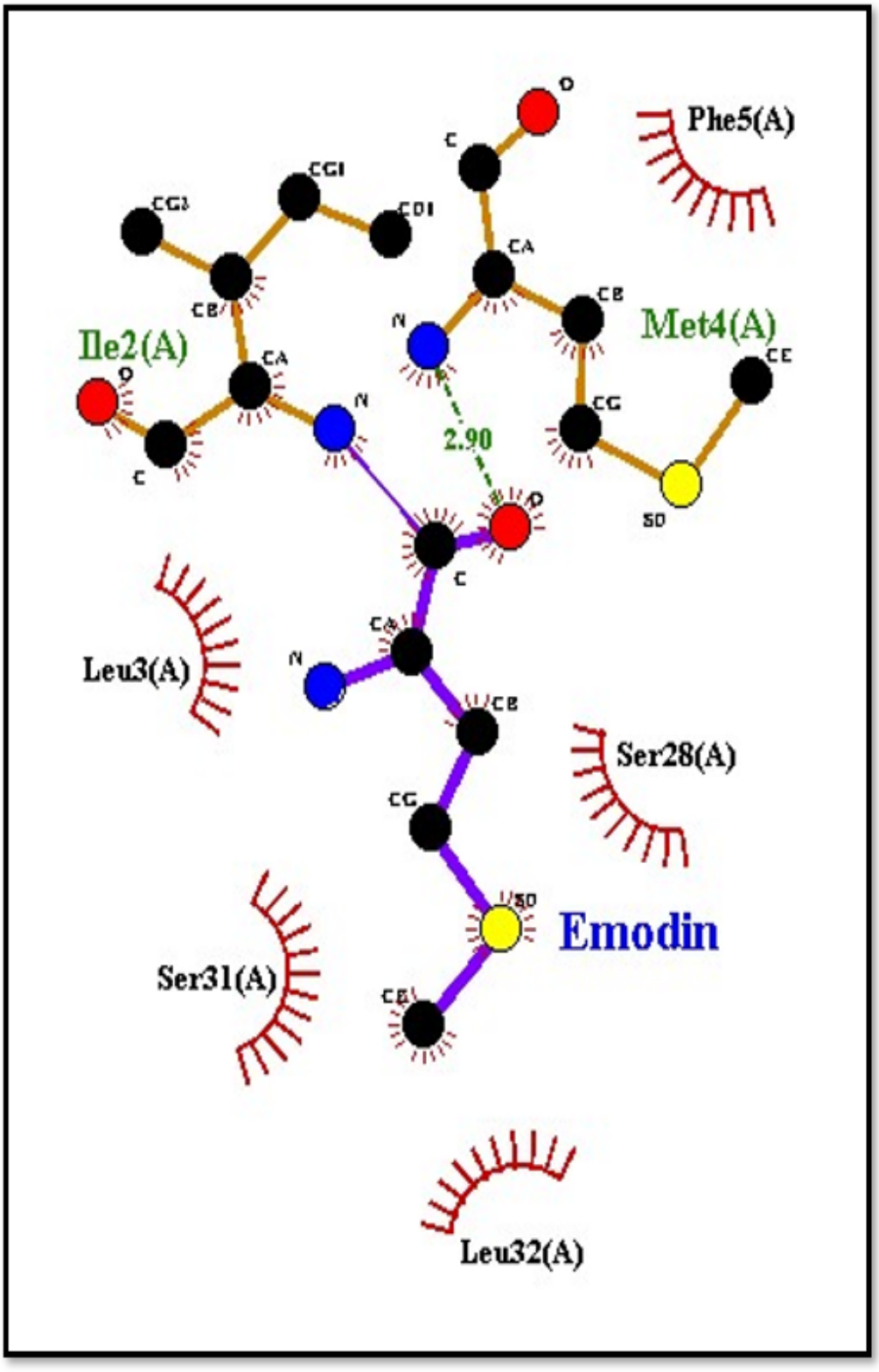


FIGURE 4.15: Interactions of Emodin by ligplot .

4.15 shows the interaction of emodin with receptor protein. It shows emodin has formed five hydrophobic interactions and two hydrogen bonds.

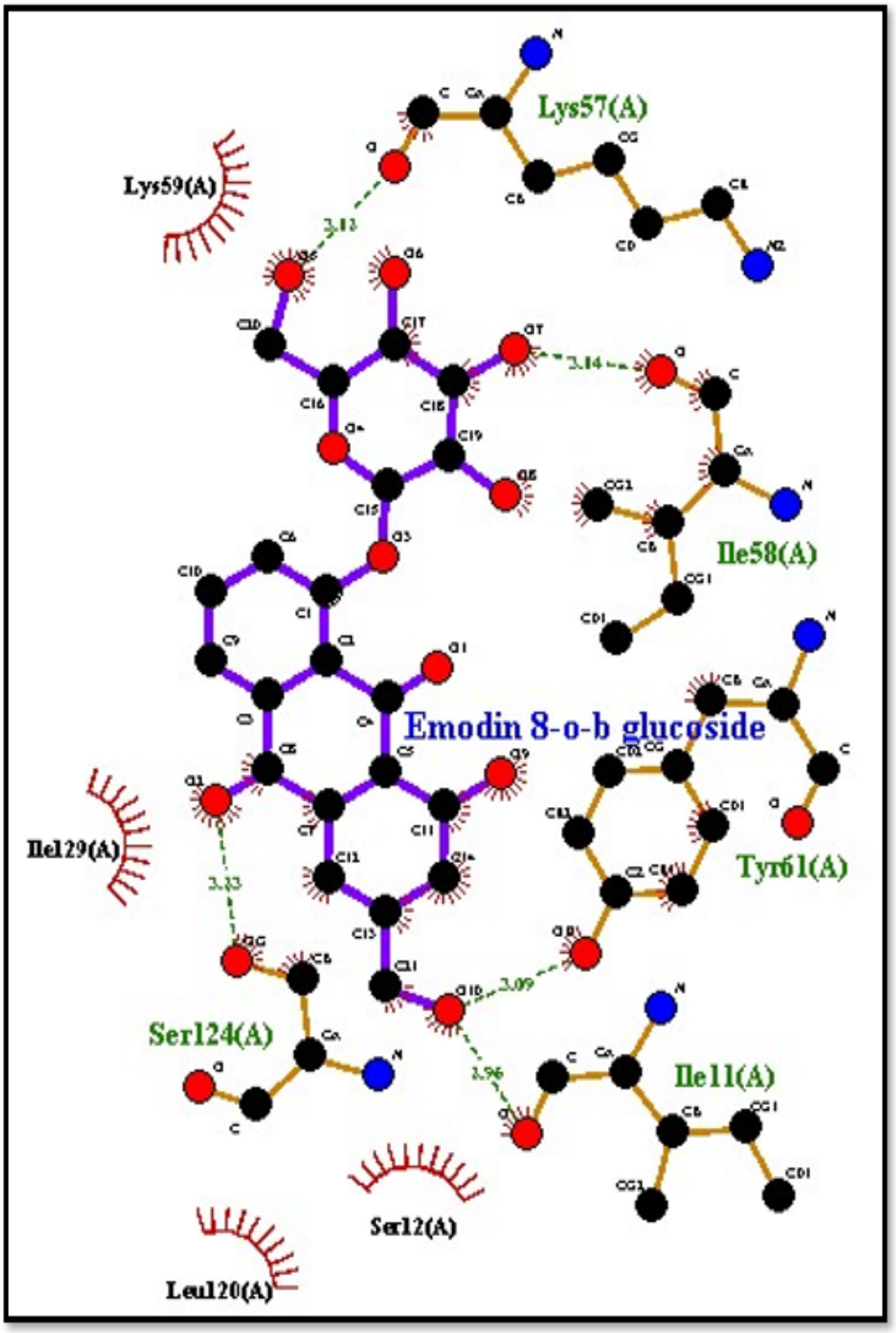


FIGURE 4.16: Interactions of Emodin 8-o-b glucoside by ligplot .

Figure 4.16 shows the interaction of emodin 8-o-b glucoside with receptor protein. It shows that emodin 8-o-b glucoside has formed four hydrophobic interactions and five hydrogen bonds.

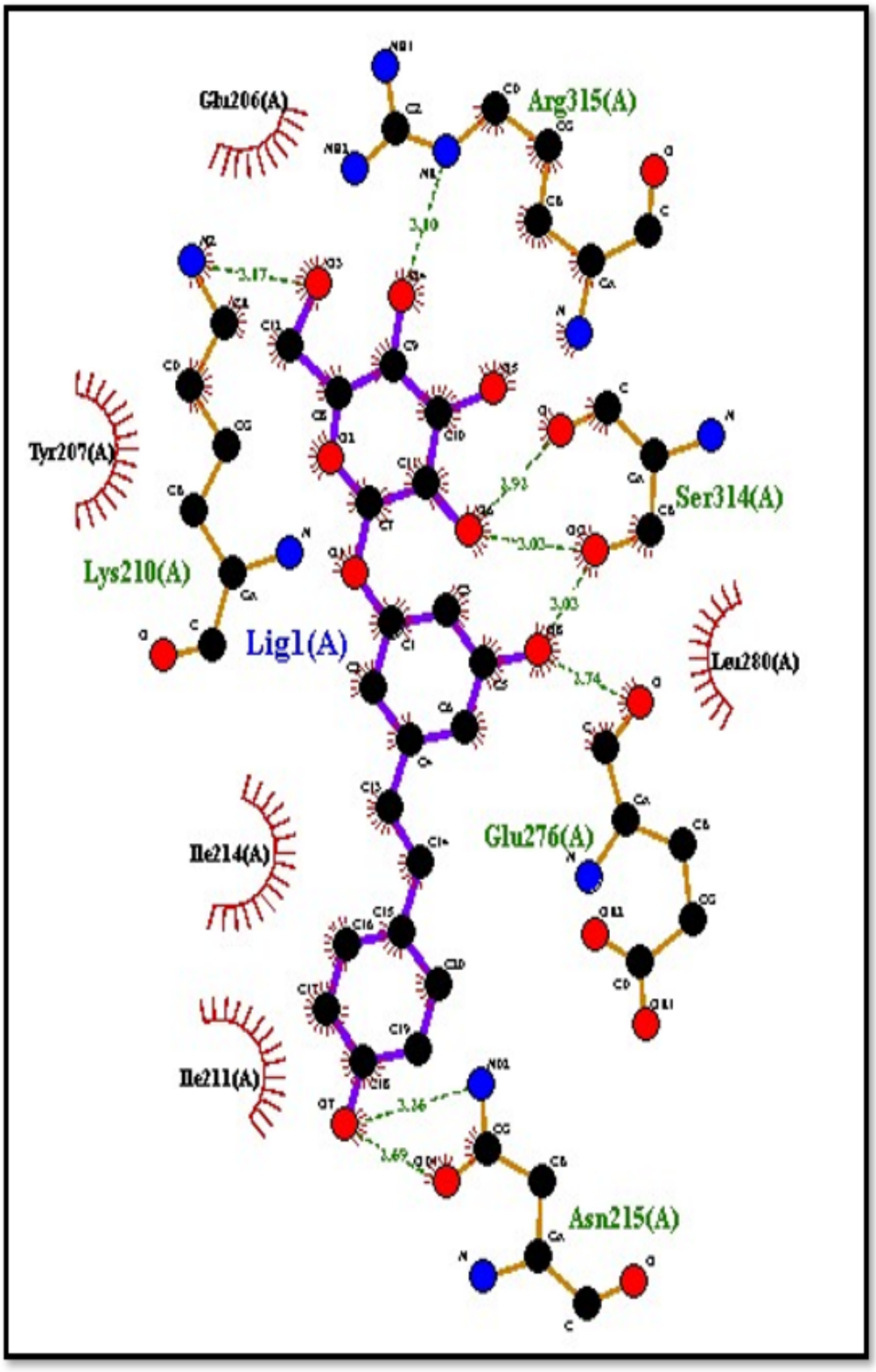


FIGURE 4.17: Interactions of Polydatin by ligplot

Figure 4.17 shows the interaction of polydatin with receptor protein. It shows that polydatin has formed five hydrophobic interactions and five hydrogen bonds.

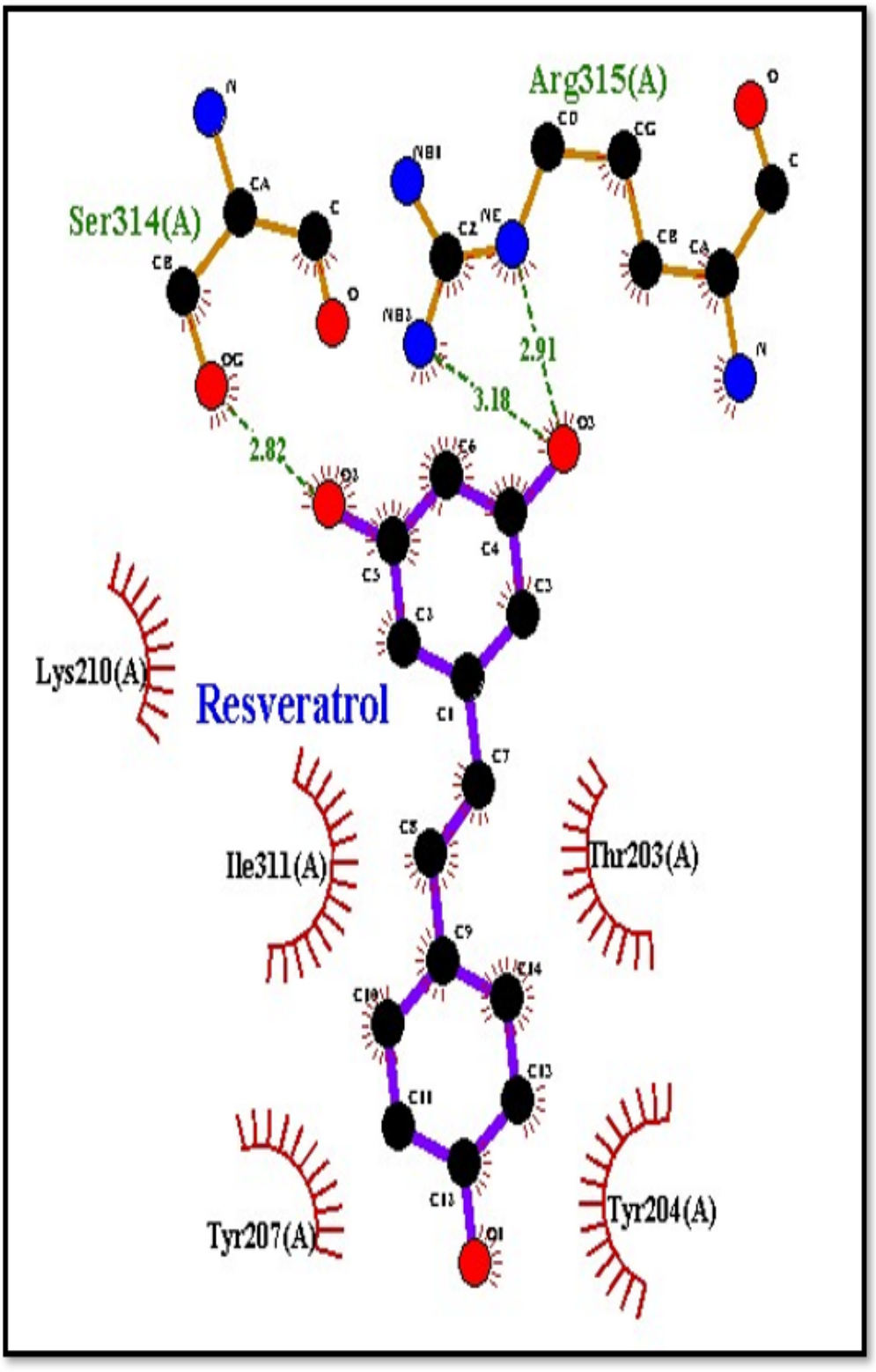


FIGURE 4.18: Interactions of Resveratrol by ligplot

Figure 4.18 shows the interaction of resveratrol with receptor protein. It shows that resveratrol has formed five hydrophobic interactions and two hydrogen bonds.

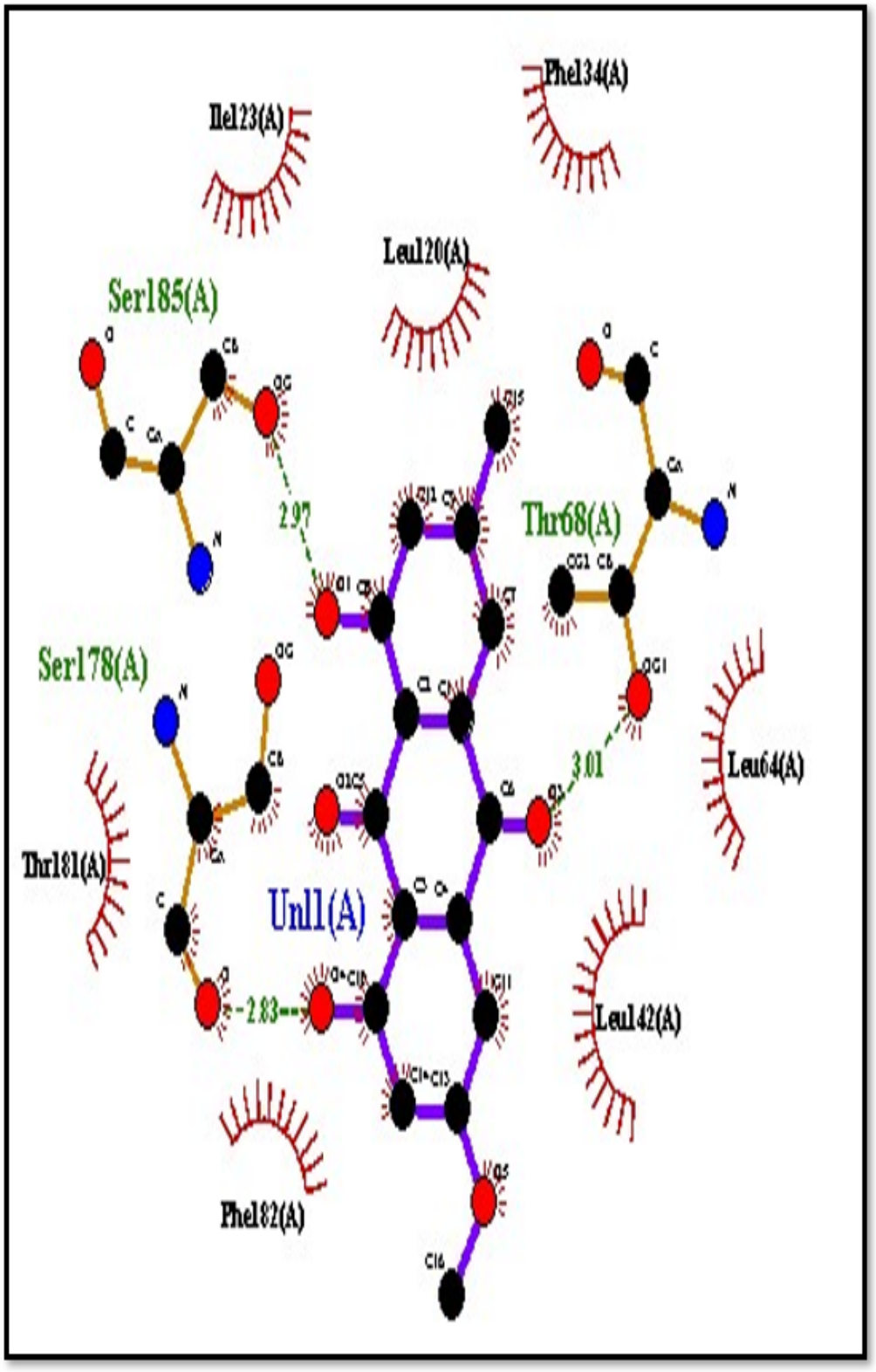


FIGURE 4.19: Interactions of Physcion by ligplot

Figure 4.19 shows the interaction of physcion with receptor protein. It shows that physcion has formed seven hydrophobic interactions and three hydrogen bonds.

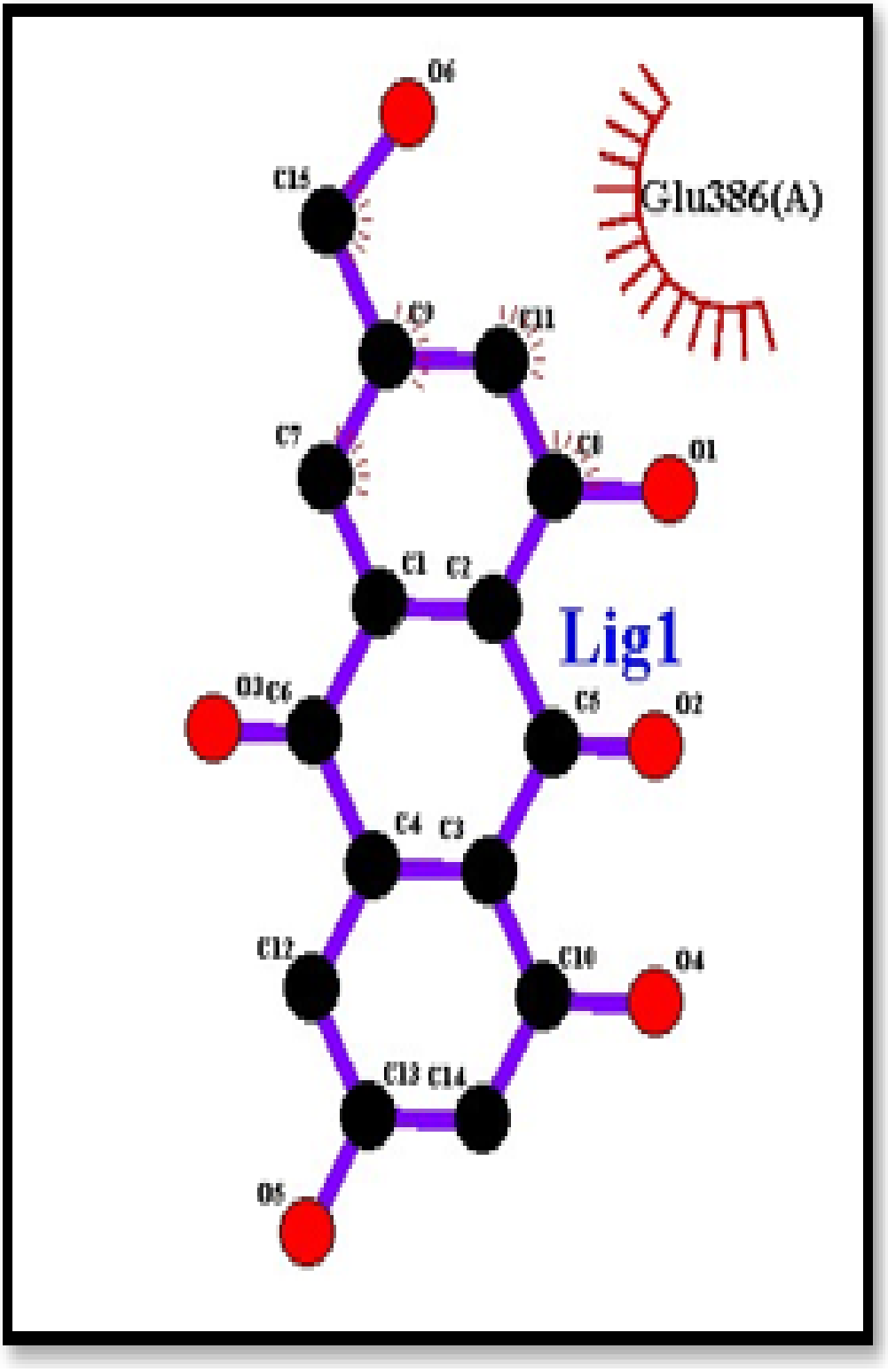


FIGURE 4.20: Interactions of Citreorosein by ligplot

Figure 4.20 shows the interaction of citreorosein with receptor protein. It shows that citreorosein has formed one hydrophobic interaction only.

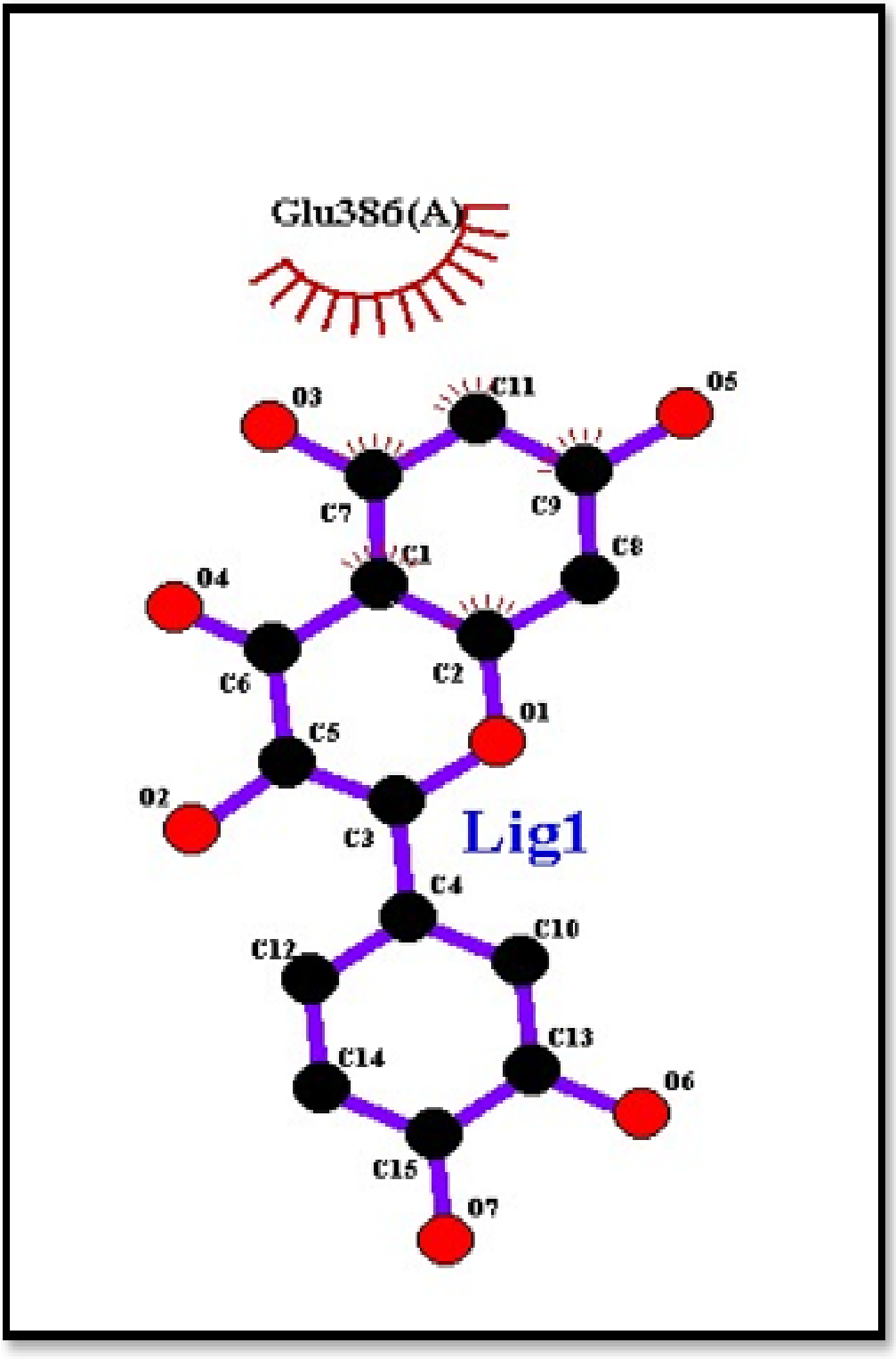


FIGURE 4.21: Interactions of Quercetin by ligplot

Figure 4.21 shows the interaction of quercetin with receptor protein. It shows that quercetin has formed one hydrophobic interaction only.

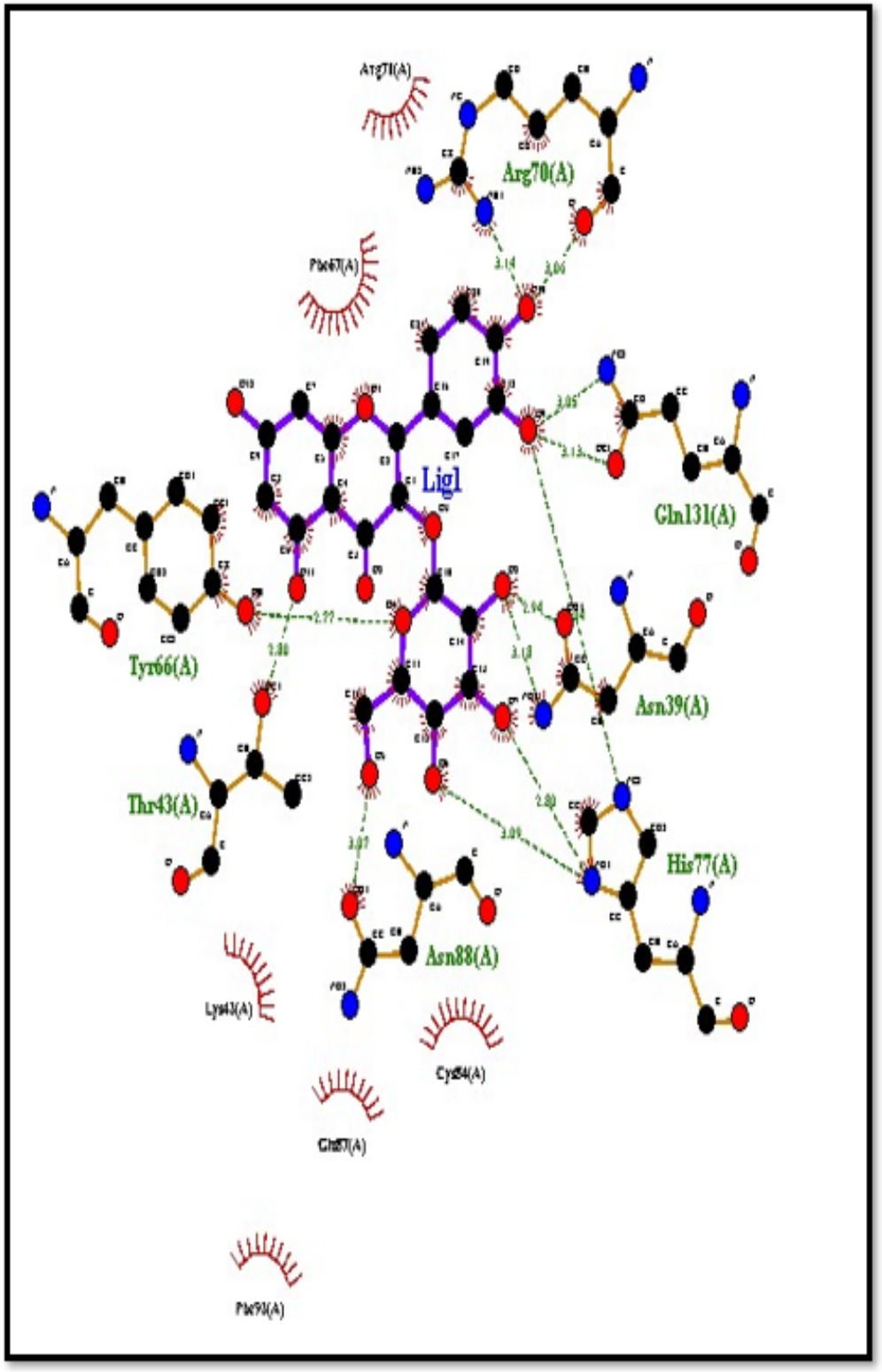


FIGURE 4.22: Interactions of Hyperoside by ligplot

Figure 4.22 shows the interaction of hyperoside with receptor protein. It shows that hyperoside has formed six hydrophobic interactions and seven hydrogen bonds.

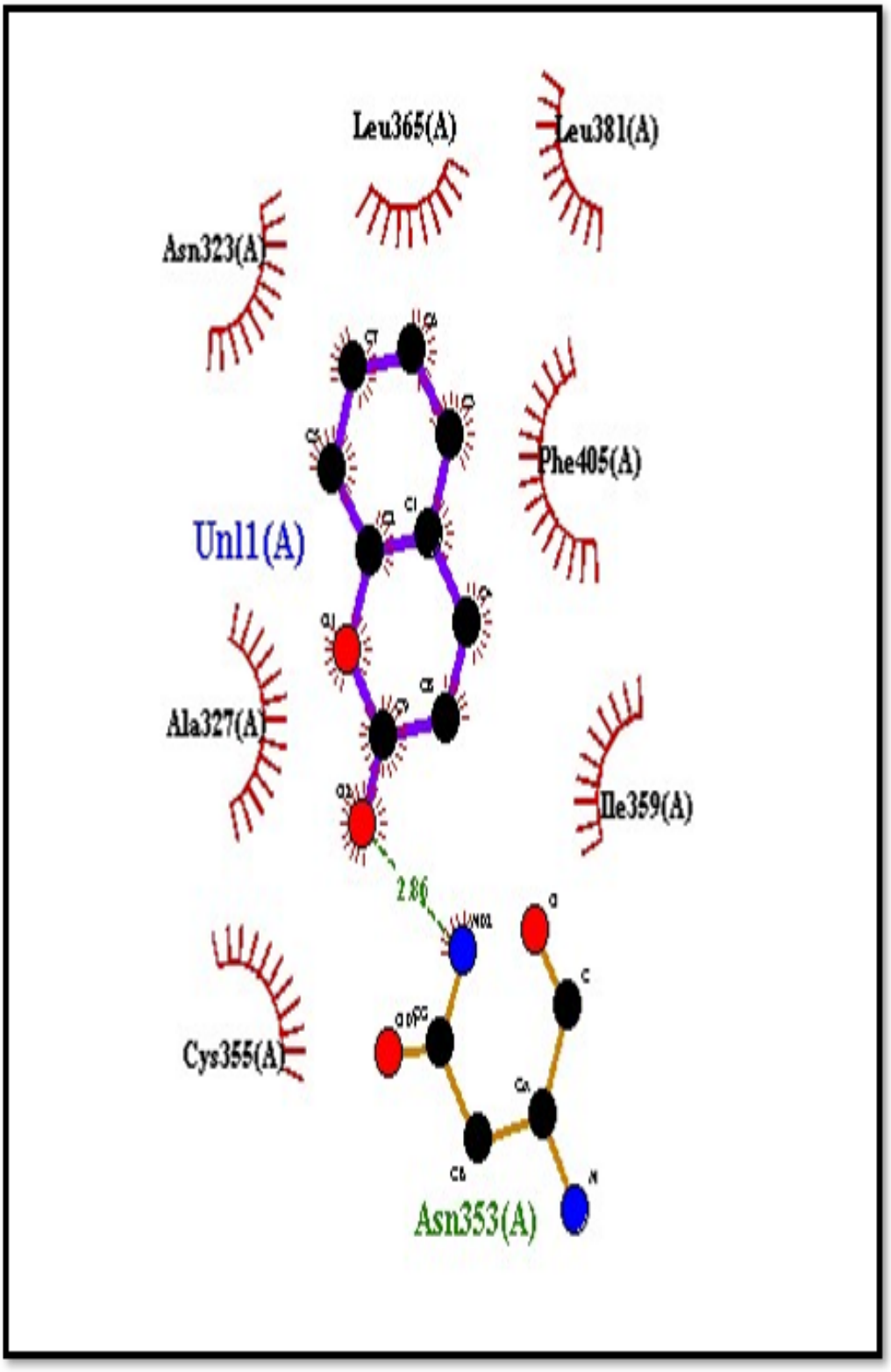


FIGURE 4.23: Interactions of Coumarin by ligplot

Figure 4.23 shows the interaction of coumarin with receptor protein. It shows that coumarin has formed seven hydrophobic interactions and one hydrogen bond.

4.6 ADMET Properties of Ligands

Lipinski's five-drug law used as a first step in assessing verbal bioavailability and artificial availability [154]. A second study was performed by calculating the ADMET properties of ligands as a measure of pharmacokinetics using the online tool pkCSM. In pharmacology there two broad terms the one is pharmacodynamics and pharmacokinetics [155].

4.6.1 Pharmacodynamics

Pharmacodynamics is a branch of pharmacology in which we study the effect of drugs on the body.

4.6.2 Pharmacokinetics

In pharmacokinetics we study the absorption of drugs, distribution of drugs, metabolism of the drug and excretion of the drugs.

4.6.3 Absorption Properties of Ligands

In pharmacology specifically pharmacokinetics, the transfer of a drug from the bloodstream into the tissues is called absorption. So the chemical composition of a drug, as well as the environment into which a drug is placed, work together to determine the rate and extent of drug absorption. A medicine must pass through cellular barriers such as epithelial or endothelial cells in order to be absorbed.
Only a few medications pass cellular barriers in an active manner that demands the use of energy and transports the drug from a low concentration to a higher concentration.
Most medications on the other hand pass past cellular barriers by passive diffusion in which they travel from a high-concentration area to a low-concentration area by diffusing through cell membranes. This sort of drug movement does not involve any

energy expenditure but it is controlled by the drug size and solubility [147]. Water solubility and skin absorption for all ligands is low while CaCO2 permeability is normal, intestinal absorption of juglone, physcion and coumarin is more than 90% while it is average for emodin and resveratrol and low for remaining ligands. Skin permeability for all ligands is low. Juglone shows negative value for p-glucoprotein substrate while other ligands show positive value for one factor. If a compound is positive for Pgp substrate then its means that it can be easily pumped out of the cells to reduce its absorption. Absorption properties of ligands were shown in following tables.

TABLE 4.10: Absorption properties of ligands table (1a)

Ligands Name	**Water Solubility**	**$CaCO_2$ Permeability**
Juglone	-0.835	1.232
Emodin	-3.271	0.259
Emodin 8-o-b glucoside	-2.972	0.367
Polydatin	-3.113	0.167
Resveratrol	-3.235	1.196
Physcion	-3.156	1.26
Citreorosein	-3.186	-0.368
Quercetin	-3.097	-0.277
Hyperoside	-2.894	-0.173
Coumarin	-1.486	1.642

4.6.4 Distribution Properties of Ligands

Distribution in pharmacology is a branch of pharmacokinetics which deals with the movement of drug within the body from one location to another location. Volume of distribution in human (VDss defined as log L/kg) is one of the ADMET properties that contains four models. Fraction unbound in humans (Fu), permeability of the blood-brain barrier (BBB) expressed as log BB, and permeability of the central nervous system expressed as log PS.

Table 4.11: Absorption properties of ligands table (1b)

Ligands Name	**Intestinal Absorption**	**Skin Permeability**
2-methoxy-6-acetyl-7-methyljuglone	94.085	-2.77
Emodin	71.316	-2.741
Emodin 8-o-b glucoside	43.072	-2.735
Polydatin	42.758	-2.735
Resveratrol	87.933	-2.748
Physcion	95.924	-2.8
Citreorosein	62.631	-2.74
Quercetin	76.081	-2.735
Hyperoside	44.847	-2.735
Coumarin	97.171	-1.911

Table 4.12: Absorption properties of ligands table (1c)

Ligands	**P-glycoprotein**	**P-glycoprotein I inhibitor**	**P-glycoprotei n II inhibitor**
2-methoxy-6acetyl-7-methyljuglone	No	No	No
Emodin	Yes	No	No
Emodin 8-o-b glucoside	Yes	No	No
Polydatin	Yes	No	No
Resveratrol	Yes	No	No
Physcion	Yes	No	No
Citreorosein	Yes	No	No
Quercetin	Yes	No	No
Hyperoside	Yes	No	No
Coumarin	Yes	No	No

Permeability of central nervous system expressed as log PS and shows that the total amount of drug that will be needed to be evenly distributed to provide the same concentration as in blood plasma. VDss is considered low if it is less than 0.71 L/kg and higher if it is above 2.81L/kg. If VDss is high it means that more of the drug is still distributed to the tissues than to plasma. If a compound shows more Fu value it means it is more effective. BBB protects the brain from exogenous compounds so BBB permeability is an important parameter. If predicted value of log BB greater than 0.3 then it means given substance can cross BBB and if value less than -1 then no harm to brain. Log PS is the product of blood brain permeability and surface area and its value greater than 2 considered to penetrate

the Central Nervous System and less than -3 considered as safe. VDSS of all ligands is low, Fu value of all ligands is in positive numbers. BBB permeability of all ligands is in range of -1. Log PS value of emodin 8-o-b glucoside, polydatin, citreorosein, quercetin and hyperoside is less than -3 while for other ligands it is in range of more than -3. The distribution properties of ligands are shown in table 4.13, 4.14.

TABLE 4.13: Distribution properties of ligands (1a)

Ligands Name	**VDss (Human) (L/kg)**	**Fraction unbound(Human) (Fu)**
2-methoxy-6-acetyl-7-methyljuglone	0.13	0.418
Emodin	0.313	0.159
Emodin 8o-b glucoside	0.488	0.195
Polydatin	0.103	0.177
Resveratrol	0.022	0.089
Physcion	0.206	0.133
Citreorosein	0.313	0.181

TABLE 4.14: Distribution properties of ligands (1b)

Ligands Name	**BBB Permeability(Human) (Log BB)**	**CNS Permeability (log PS)**
Juglone	-0.209	-2.203
Emodin	-0.861	-2.304
Emodin 8-o-b glucoside	-1.251	-4.376
Polydatin	-0.994	-3.862
Resveratrol	-0.152	-2.113
Physcion	-0.035	-2.278

Table 4.14 continued from previous page

Ligands Name	**BBB Permeability(Human) (Log BB)**	**CNS Permeability (log PS)**
Citreorosein	-0.996	-3.295
Quercetin	-1.363	-3.313
Hyperoside	-1.539	-4.721
Coumarin	-0.013	-1.992

4.6.5 Metabolic Properties of Ligands

CYP1A2, CYP2C19, CYP2C9, CYP2D6, and CYP3A4 models of the various isoforms of Cytochrome P450 which is an important cleansing enzyme found in the liver [149]. Metabolic properties of ligands are given in tables 4.15, 4.16.

TABLE 4.15: Metabolic properties of ligands (1a)

Ligands Name	**CYP-2D6 Substrate**	**CYP-3A4 Substrate**	**CYP-2D6 Inhibitor**	**CYP-2619 Inhibitor**
Juglone	No	No	No	No
Emodin	No	No	Yes	No
Emodin 8-o-b glucoside	No	No	No	No
Polydatin	No	No	No	No
Resveratrol	No	No	Yes	No
Physcion	No	No	No	Yes
Citreorosein	No	No	No	No
Quercetin	No	No	Yes	No
Hyperoside	No	No	No	No
Coumarin	No	No	Yes	No

TABLE 4.16: Metabolic properties of ligands (1b)

Ligands Name	**CYP-269 Inhibitor**	**CYP-2D6 Inhibitor**	**CYP-3A4 Inhibitor**
Juglone	No	No	No
Emodin	No	No	No
Emodin 8-o-b glucoside	No	No	No
Polydatin	No	No	No
Resveratrol	No	No	No
Physcion	No	No	No
Citreorosein	No	No	No
Quercetin	No	No	No
Hyperoside	No	No	No
Coumarin	No	No	No

4.6.6 Excretion Properties of Ligands

The organs involved in drug excretion are the kidneys, which play important role in excretion (renal excretion) and the liver (biliary excretion). Other organs may also be involved in excretion, such as the lungs for volatile or gaseous agents. Drugs can also be excreted in sweat, saliva and tears. Models of Excretion property are Total Clearance expressed as log (CL tot) in ml/min/kg and second one is Renal OCT2 substrate which predicts results as Yes /No.

TABLE 4.17: Excretion properties of ligands.

S.NO	**Ligands Name**	**Total Clearance (ml/day)**	**Renal OCT2 Substrate**
1	2-methoxy-6acetyl-7-methyljuglone	0.022	No
2	Emodin	0.352	Yes
3	Emodin 8-o-b glucoside	0.023	No
4	Polydatin	0.144	No

Table 4.17 continued from previous page

S.NO	Ligands Name	Total Clearance (ml/Kg)	Renal OCT2 Substrate
5	Resveratrol	0.094	No
6	Physcion	0.431	No
7	Citreorosein	0.386	No
8	Quercetin	0.457	No
9	Hyperoside	0.435	No
10	Coumarin	0.96	No

4.6.7 Toxicity Properties of Ligands

PkCSM is an online tool used to find the ADMET (Absorption, Distribution, Metabolism, Excretion, and Toxicity) properties of bioactive compounds and drugs. The maximum tolerated dose (MRTD) provides a measure of toxic chemical on individuals. This will help in directing the first recommended dose of the treatment regimen in phase 1 clinical trials. MRTD is expressed in the form of logarithms (log mg / kg / day). In a given compound MRTD less than or equal to 0.477 log (mg / kg /day) is considered to be lower and higher if it is higher than 0.477 log (mg / kg / day). Table 4.18 shows toxicity properties of ligands.

Table 4.18: Toxicity properties of ligands (1a).

S.No	Toxicity	Juglone	Emodin	Emodin 8-o-b glucoside
1	Max tolerated dose	0.658	0.166	0.331
2	hERG1 Inhibitor	No	No	No
3	hERG II inhibitor	No	No	No
4	Oral rat acute toxicity	1.845	2.281	2.54
5	Oral rat chronic	2.673	1.78	3.785
6	Hepatoxicity (log ug/L)	Yes	No	No
7	Skin Sensitization	No	No	No

Table 4.18 continued from previous page

S.No	Toxicity Properties	Juglone	Emodin	Emodin 8-o-b glucoside
8	T. pyriformis activity	0.509	0.62	0.285

TABLE 4.19: Toxicity properties of ligands (1b)

Toxicity	Polydatin	Resveratrol	Physcion
Max tolerated dose	0.37 4	0.561	0.293
hERG1 Inhibitor	No	No	No
hERG II inhibitor	No	No	No
Oral rat acute toxicity	2.39 7	2.216	2.152
Oral rat chronic	3.81 7	1.761	1.694
Hepatoxicity (log ug/L)	No	No	No
Skin Sensitization	No	No	No
T. pyriformis activity	0.28 5	0.982	0.629
Minnow toxicity	0.86 4	1.367	1.191

TABLE 4.20: Toxicity properties of ligands (1c)

Toxicity	Citreorosein	Quercetin	Hyperoside	Coumarin
Max tolerated dose	0.205	0.742	0.508	0.253
hERG1 Inhibitor	No	No	No	No
hERG II inhibitor	No	No	Yes	No
Oral rat acute toxicity	2.178	2.516	2.624	2.082
Hepatoxicity (log ug/L)	1.82	2.616	4.07	1.816
Skin Sensitization	No	No	No	No
T. pyriformis activity	No	No	No	No
Minnow toxicity	2.232	1.999	2.706	1.074

The hERG I and II inhibitors model is reported to generate chronic QT syndrome and fatal ventricular arrhythmia by inhibiting potassium channels induced by the

hERG (human ether-a-go-go gene). Many pharmaceuticals have been withdrawn from the market due to the inhibition of hERG channels. The amount of a substance that kills 50% of experimental animals such as mice is known as the LD50. The LD50 (mol/kg) predicts toxicity of a probable compound where as LOAEL aims to identify the lowest dosage of a compound with a significant adverse effect. Exposure to low to moderate chemical doses for a long time is very important in medicine and is expressed in a log (mg/kg - bw/day). Hepatotoxicity reveals drug-induced liver damage and is a major safety concern for drug development. Skin sensitivity is a potential adverse effect of skin care and applied products. T. pyriformis is a protozoan bacterium, whose toxin is often used as a toxic endpoint (IGC50) and inhibits 50% growth. p IGC50 (negative concentration logarithm required to prevent 50% growth) in log ug / L predicted value greater than - 0.5 log ug / L is considered toxic. The lethal concentrations (LC50) represent the concentration of molecules needed to cause the death of 50% 4 of Flathead Minnows (small bait fishes). In Minnow toxicity LC50 values below 0.5 mM (log LC 50 less than -0.3) are regarded as high acute toxicity [129]. Toxicity predicted values of selected ligands were listed in table 4.20. The maximum tolerated dose of juglone, resveratrol, quercetin and hyperoside is high. All ligands claimed no for hERGI and hERGII inhibitor. Hepatotoxicity is shown by only 2-methoxy-6-acetyl-7-methyljuglone while no one is skin sensitive. No ligand showed T. pyriformis activity less than- 0.5 log ug / L. Minnow toxicity value of all ligands is more than 0.5m M which is considered safe.

4.6.8 Lipinski Rule of Five

Lipinski's rule of five are as follow:

1. The logP value of most "drug-like" molecules should be limited to 5.
2. Molecular weight should be under 500.
3. Maximum number of H-bond acceptor should be 10.

4. Maximum number of H-bond donor should be 5 [129].

So, following rules are applied on our compound and hence analysis of different ligands of *Reynoutria japonica* is checked and results are shown in table 4.21.

TABLE 4.21: Applicability of Lipinski rule on ligands.

Ligands	**logP value**	**Molecular Weight**	**H-bond acceptor**	**H-bond donor**
Juglone	1.3274	174.155	3	1
Emodin	1.88722	270.24	5	3
Emodin 8-o-b	-1.1614	432.381	10	6
Polydatin	0.4469	390.388	8	6
Resveratrol	2.9738	228.247	3	3
Physcion	2.19022	284.267	5	2
Citreorosein	1.0711	286.239	6	4
Quercetin	1.988	302.238	7	5
Hyperoside	-0.5389	464.379	12	8
Coumarin	1.793	146.145	2	0

The table shows molecular weight, logP values, hydrogen bond acceptor and donor values of ligands of *Reynoutria japonica*. These rules are to be followed by orally active compounds. The drug-like is dependent on the mode of administration. A compound is considered a drug when it follows 3 or more rules and if a compound violates two or more rules it is considered poorly absorbed. Except hyperoside and coumarin nearly all the ligands followed lipinski rule of five.

4.7 Lead Compound Identification

Physicochemical and pharmacokinetics properties determine the final destiny of compounds as drug or non-drug compounds. Physicochemical properties or lipinski rule of five works as primary filter and pharmacokinetics studies as secondary

filter in screening of potential compounds. Emodin 8-o-b glucoside, polydatin, hyperoside and coumarin did not obey lipinski rule of five so they knock out in primary screening. On the basis of binding score, ADMET properties, physico-chemical properties and lipinski rule of five, resveratrol was selected as lead compound which could inhibit target proteins.

4.8 Reference Anti-Bacterial Drug Identification

The selection of most efficient anti-bacterial drug is based on the physiochemical, ADMET properties along mechanism of action with side effects. For physiochemical properties PubChem online database is used and for ADMET properties of drugs pkCSM online tool is used. Mechanism of action is identified through Drug Bank and KEGG databases.

4.8.1 Penicillin (Beta-Lectam Antibiotic)

Penicillin is selected as reference drug because of its repeated use and effectiveness against bacterial infections. It is used to treat infections caused by gram-positive bacteria especially staphylococcal and streptococcal infections. Due to low oral absorption it is given intravenously or intramuscularly. Natural penicillins can be used as a first or second line antibiotic against gram-positive bacteria. Natural penicillins only have a limited effect on gram-negative bacteria. Anaerobic infections are rarely treated with them. The patterns of resistance, susceptibility and treatment options differ by region [154].

4.8.2 Penicillin Mechanism of Action

By binding to specific penicillin-binding proteins located inside the bacterial cell wall, penicillin inhibits the third and last stage of bacterial cell wall synthesis. Cell lysis is then mediated by bacterial cell wall autolytic enzymes such as autolysins.

It is possible that penicillin interferes with an autolysin inhibitor. Penicillin is stable against hydrolysis by a variety of beta-lactamases, including penicillinases, cephalosporinases and extended spectrum beta-lactamases [155].

4.8.3 Drug ADMET Properties

ADMET properties (Absorption, Distribution, Metabolism, Excretion and Toxicity) of FDA approved antibacterial drug were explored by pkCSM online prediction tool.

4.8.3.1 Absorption Properties

The absorption properties of selected drug penicillin are shown in table 4.22.

TABLE 4.22: Absorption properties of drug

Properties	**Predicted Values**
Water Solubility(mol\L)	-2.199
CaCO2 permeability (cm\S)	0.293
Intestinal absorption (Human)%	58.344
Skin permeability (log\Kp)	-2.735
P-glycoprotein substrate	Yes
P-glycoprotein I inhibitor	No
P-glycoprotein II inhibitor	No

4.8.3.2 Distribution Properties

Distribution properties of selected drug penicillin are shown in table 4.23.

TABLE 4.23: Distribution properties of drug

Properties	**Predicted Values**
VDss (Human) (L\kg)	1.681
Fraction unbound (Human)(Fu)	0.32
BBB permeability (Human)(log BB)	-0.741
CNS permeability (logPS)	-2.936

4.8.3.3 Metabolic Properties

Metabolic properties of selected drug penicillin are shown in table 4.24

TABLE 4.24: Metabolic properties of drug

S.no	**Properties**	**Predicted Values**
1	CYP-2D6 Substrate	No
2	CYP-3A4 Substrate	Yes
3	CYP-2D6 Inhibitor	No
4	CYP-2619 Inhibitor	No
5	CYP-269 Inhibitor	No
6	CYP–2D6 Inhibitor	No
7	CYP-3A4 Inhibitor	No

4.8.3.4 Excretion Properties

Excretion properties of selected drug penicillin are shown in table 4.25.

TABLE 4.25: Excretion properties of drug

S.NO	**Drug Name**	**Total Clearance**	**Renal OCT2 Substrate**
1	Penicillin	0.02	No

4.8.3.5 Toxicity Properties

Toxicity properties of selected drug penicillin are shown in table 4.26

TABLE 4.26: Toxicity properties of drug

S.No	Toxicity Properties	Predicted Values
1	Max tolerated dose (Human) (mg/kg)	1.284
2	hERG1 Inhibitor	No
3	hERG II inhibitor	No
4	Oral rat acute toxicity (mol/kg)	2.04
5	Oral rat chronic toxicity (mg/kg)	2.63
6	Hepatoxicity (log ug/L)	Yes
7	Skin Sensitization	No
8	T. pyriformis activity (log ug/L)	0.285
9	Minnow toxicity (log mM)	2.255

4.8.3.6 Lipinski Rule of Five

Table 4.27 showing properties of selected drug penicllin according to lipinski rule of five.

TABLE 4.27: Application of lipinski rule on drug.

Ligands	logP value	Molecular Weight	H-bond acceptor	H-bond donor
Penicillin	0.8608	334.397 g/mol	2	1

4.8.3.7 Penicillin Docking

For the docking purpose CB Dock online docking tool was used. It gives 5 best confirmation results and finest is selected. Penicillin is used as ligand and accessory gene regulator proteins A, B, C and signal transduction protein TRAP are selected

as receptors. As the mechanism of action shows that penicillin inhibits those proteins which involve in infection. So docking help us to find out the inhibition value, the values were shown in Table 4.28.

TABLE 4.28: Results of CB dock of Penicillin.

Drug Name	**Binding Score**	**Cavity Size**	**Grid Map**	**Minimum energy (Kcal/mol)**	**Maximum energy (Kcal/mol)**
Penicillin	-6.7	86	23	0.00	1.6E+00

4.9 Penicillin and Lead Compound Comparison

The comparison between penicillin and resveratrol help us to identify the better treatment for infectious diseases. Comparison was being performed through parameters like ADMET properties and physiochemical properties of both compounds.

4.9.1 ADMET Properties Comparison

ADMET properties include the values regarding to drug absorption, distribution, metabolism, excretion and toxicity. These values help us to determine the drug activity and efficiency.

4.9.1.1 Absorption Properties Comparison

Penicillin and resveratrol absorption properties are given in table 4.29

TABLE 4.29: Absorption properties comparison

S.No	**Properties**	**Penicillin**	**Resveratrol**
1.	Water solubility	-2.199	-3.233

Table 4.29 continued from previous page

S.No	Properties	Penicillin	Resveratrol
2.	CaCO2 permeability	0.293	1.196
3.	Intestinal absorption on Human	58.344	87.933
4.	Skin permeability	-2.735	-2.748
5.	P-glycoprotein substrate	Yes	No
6.	P-glycoprotein in I inhibitor	No	No
7.	P-glycoprotein in II inhibitor	No	No

So it is clear that resveratrol is easily soluble in water as compared to penicillin and it is permeable to skin. The intestine absorption of resveratrol is greater as compared to penicillin.

4.9.1.2 Distribution Properties Comparison

The distribution properties of penicillin drug and resveratrol are given in Table 4.30.

TABLE 4.30: Distribution properties comparison.

S.No	Properties	Penicillin	Resveratrol
1.	VDss Human (L\kg)	1.681	0.022
2.	Fraction unbound Human (Fu)	0.32	0.089
3.	BBB Permeability (logBB)	-0.741	-0.152

Table 4.30 continued from previous page

S.No	Properties	Penicillin	Resveratrol
4.	CNS Permeability (log PS)	-2.936	-2.113

So it is clear that distribution properties of bioactive compound resveratrol lies better than drug penicillin.

4.9.1.3 Metabolic Properties Comparison

The metabolic properties of penicillin drug and resveratrol are given in Table 4.31.

TABLE 4.31: Metabolic properties comparison.

Compounds Name	CYP-2D6 Substrate	CYP-3A4 Substrate	CYP-2D6 Inhibitor	CYP-2619 Inhibitor	CYP-269 Inhibitor
Penicillin	No	Yes	No	No	No
Resveratrol	No	Yes	Yes	No	No

The CYP-3A4 substrate presentin both resveratrol and penicillin but CYP1A2 inhibitor present only in resveratrol that help in the metabolism of drug.

4.9.1.4 Excretion Properties Comparison

The excretion properties of penicillin drug and resveratrol are given in Table 4.32.

TABLE 4.32: Excretion properties comparison

S.NO	Compounds Name	Total Clearance (ml/day)	Renal OCT2 Substrate
1	Penicillin	0.02	No

Table 4.32 continued from previous page

S.NO	Compounds Name	Total Clearance (ml/Kg)	Renal OCT2 Substrate
2	Resveratrol	0.094	No

The total clearance value of resveratrol in the body is greater that helps in the excretion of drug from the body.

4.9.1.5 Toxicity Properties Comparison

The toxicity ranges of penicillin and reseratrol were given in table 4.33. The max tolerated dose for penicillin is 1.284 and for resveratrol is 0.561 and oral acute toxicity rat of resveratrol is greater.

TABLE 4.33: Toxicity properties comparison,

S.No	Toxicity Properties	Penicillin	Resveratrol
1	Max tolerated dose (Human) (mg/kg)	1.284	0.561
2	hERG1 Inhibitor	No	No
3	hERG II inhibitor	No	No
4	Oral rat acute toxicity (mol/kg)	2.04	2.216
5	Oral rat chronic toxicity (mg/kg)	2.63	1.761
6	Hepatoxicity (log ug/L)	Yes	No
7	Skin Sensitization	No	No
8	T. pyriformis activity (log ug/L)	0.285	0.982
9	Minnow toxicity (log mM)	2.255	1.367

4.9.1.6 Lipinski Rule of Five

Penicillin and resveratrol lipinski rule of five are given in Table 4.34.

TABLE 4.34: Penicillin and Resveratrol lipinski rule of five .

S.No	Compounds Name	logP value	Molecular Weight	H-bond acceptor	H-bond donor
1	Penicillin	0.8608	334.397 g/mol	4	2
2	Resveratrol	2.9738	228.247 g/mol	3	3

So it is seen that resveratrol bioactive compound showed better result over penicillin with respect to logP value and hydrogen bond donors and acceptors.

4.9.2 Docking Score Comparison

Both the standard and the lead compound were docked against the target proteins and the docking result gives us the best binding score. Table 4.35 shows that the lead compound resveratrol has higher vina score than that of the standard drug which is penicillin.

TABLE 4.35: Docking results comparison.

S. No	Compounds Name	Binding Score	Cavity Size	Grid Map	Minimum energy (Kcal/mol)	Maximum energy (Kcal/mol)
1	Penicillin	-6.7	86	23	0.00	1.6E+00
2	Resveratrol	-8.9	1857	34	0.00	1.6E+00

Chapter 5

Conclusions and Recommendations

The aim of this research was to identify compound using computational method for the treatment of infectious diseases that could be used in near future as an efficient drug. After performing data mining studies on literature databases ten ligands were selected for the current research work. The proteins used for virtual screening were accessory gene regulator protein A, accessory gene regulator protein B, accessory gene regulator protein C and signal transduction protein TRAP. CB Dock automated version of Auto Dock vina was used for the docking studies. Protein ligand interactions of these ligands were analyzed using Ligplot plus version v.1.4.5.

After the detailed analysis of their binding score, physiochemical properties and ADMET properties, four best scoring phytocompounds namely as, 2-methoxy-6-acetyl-7-methyljuglone, emodin, resveratrol and physcion were identified as hit compounds. Physicochemical and pharmacokinetics properties determined the final destiny of compounds as drug or non-drug. Resveratrol was identified as lead compound by virtual screening results. From the above mentioned physiochemical and ADMET values it is concluded that the resveratrol showed best binding ability with accessory gene regulator protein C and its activity is also better in comparison to synthetic drug penicillin. All the software's and tools used in the

current research study are reliable and authentic.

These finding suggest that resveratrol, a bioactive molecule found in the root of *Reynoutria japonica* could be a promising choice for treating *Staphylococcus aureus* infections. More research is needed to explore the exact mechanisms of action, as well as the impact on the human body and safety concerns. Furthermore, *Reynoutria japonica* is commonly used in combination with other herbs in traditional Chinese medicine, but modern experiments show that this plant alone has significant pharmacological effects, making it interesting and important to investigate the medical effects and molecular mechanisms of this plant combined with other herbs using modern disease pathophysiology concepts.

The root of this plant has also been utilized as an effective agent in the past and the aerial part of this plant is frequently discarded in landfills without being used. There are currently few studies on this component therefore it is needed to evaluate the chemical constituents and pharmacological effects of the aerial part as well as to find new chemical ingredients in order to reuse the aerial part as a future value-added product of *Reynoutria japonica*.

Most previous studies on *Staphylococcus aureus* infections have concentrated on in vitro tests, but the effects of natural products on quorum sensing must be evaluated in complex organisms. Both quorum sensing and quorum quenching are research hotspots right now. We should pay more attention to the use of quorum sensing inhibitors and agonists in the future. Future study will aim on the extensive application of quorum sensing to clinical therapies, production and living. The new findings have the potential to revolutionize our understanding of *Staphylococcus aureus* pathogenicity and provide novel anti-*staphylococcal* therapeutic targets. Among these most applications are related to antagonism of the quorum sensing signals and only a few deals with signal enzymatic destruction, the inhibition of signal synthesis or signal sequestration.

Although anti-quorum sensing medications are thought to generate a lower evolutionary pressure for bacterial antimicrobial resistance but resistance may evolve over time. A pathogen's ability to overcome the mechanisms of action of a medicine is supported by the quick rate of bacterial evolution. Constant observations and

basic research on bacterial virulence and intercellular signalling will continue to promote the development of new and effective medications. There will likely remain an ongoing battle between microbes and their hosts in pathogenic associations. The anti-quorum sensing strategies developed so far have not yet been applied on a broad scale clinical trial and hence it is difficult to determine their full potential and limitations at this stage. However, it is clear that we need to broaden our antimicrobial targets and approaches and interference with intercellular signalling appears as a viable and promising option for drug development.

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An Appendix

Table 5.1: Properties of Compounds obtained by ligplot such as amino acid, hydrogen bonding and hydrophobic bonding (1a)

Ligands Name	**Amino Acids**	**H Bonding Distance**	**Hydrophobic Interaction**
2-methoxy-6acetyl-7-methyljuglone	Ile58	2.86	Lys59
	Trp60	3.08	Leu135
	Tyr61	3.31	Leu130
Emodin	Met4	2.90	Ser28
	Ile2	2.91	Ser31
	Lys57 Ile58	3.12	Lys59
Emodin 8-o-b glucoside	Tyr61	3.14	Ile129
	Ile11	3.09 2.96	Leu120
	Ser124	3.23	Ser12

Table 5.1 continued from previous page

Ligands Name	**Amino Acids**	**H Bonding Distance**	**Hydrophobic Interaction**
	Arg315	3.10	Glu206
	Lys210	3.17	Tyr207
Polydatin	Ser314	3.03	Leu280
	Glu276	2.76	Ile214
	Asn215	3.26	Ile211
			Lys210
			Ile311
			Thr203
		2.91	
Resveratrol	Arg315 Ser314		Tyr207
		2.82	
			Tyr204
			Phe 134
			Leu120

Table 5.1 continued from previous page

Ligands Name	**Amino Acids**	**H Bonding Distance**	**Hydrophobic Interaction**
Physcion	Ser185	2.97	Ile123
	Thr68	3.01	Leu64
	Ser178	2.83	Leu142
			Thr181
			Phe182
Citreorosein	-	-	Glu386
Quercetin	-	-	Glu386
Hyperoside	Arg70	3.06	Arg78
	Gln131	3.13	Phe67
	Asn39	2.94	Cys54
	His77	2.80	Lys43
	Asn88	3.02	Glu37
	Thr43	2.80	Phe92
	Tyr66 Leu381	2.22	

Table 5.1 continued from previous page

Ligands Name	**Amino Acids**	**H Bonding Distance**	**Hydrophobic Interaction**
Coumarin	Asn353	2.86	Asn323 Phe405 Ile359 Ala327

TABLE 5.2: Properties of Compounds obtained by ligplot (1b)

S.NO	**Ligands Name**	**Binding Energy**	**No of HBs**
1	2-methoxy-6acetyl-7-methyljuglone	-7.1	3
2	Emodin	-8.4	2
3	Emodin 8-o-b glucoside	-9.9	5
4	Polydatin	-8.8	5
5	Resveratrol	-8.9	2
6	Physcion	-8.6	3
7	Citreorosein	-8.4	0
8	Quercetin	-8.8	0
9	Hyperoside	-9.1	7
10	Coumarin	-6.6	1