# Cheminformatics approach to identify bioactive inhibitors against type I dehydroquinase (DHQ1) enzyme of typhoidal *Salmonella typhi.*

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By

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A thesis submitted in the partial fulfilment of the requirement for the degree of

**Master of Science in Industrial Biotechnology**

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# National University of Sciences & Technology

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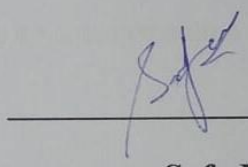

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**Signature of Supervisor**

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| | |
|---|---|
| AMR | Antimicrobial Resistance |
| Å | Angstom |
| ADMET | bsorption, distribution, metabolism, excretion, and toxicity |
| $Azith^R$ | Azithromycin-resistant |
| BBB | Blood-brain barrier |
| CYP | Cytochrome P |
| DHQ1 | Dehydroquinase dehydratase type 1 |
| DHQase | Dehydroquinase enzyme |
| Glu | Glutamic acid |
| GPCR | G protein-coupled receptors |
| HBA | Hydrogen bond acceptor |
| HBD | Hydrogen bond donor |
| HIA | gastrointestinal absorption |
| His | Histidine |
| kDa | Kilo Dalton |
| Ki | Inhibition constant |
| Lys | Lysine |
| MD | Molecular Dynamic Simulation |
| MDR | Multi-drug Resistance |
| nOHNH | hydrogen bond donors |
| nON | hydrogen bond acceptors |
| Nrotb | Number of rotatable bonds |
| ns | Nanoseconds |
| PASS | Prediction of activity spectra for substances |
| PDB | Protein Data Bank |
| PDBQT | Protein Data Bank, Partial Charge (Q) |
| PGP− | non-substrate of P-gp |
| P-gp | P-glycoprotein |

| | |
|---|---|
| PGP+ | substrate of P-gp |
| RMSF | Root means square fluctuation |
| SBVS | Structure-based virtual screening |
| SF | scoring function |
| TPSA | topological polar surface area |
| XDR | Extensively-drug Resistance |

# ABSTRACT

The 21st century's foremost public health concern is the widespread occurrence of antibiotic resistance. The chronic carriage of *Salmonella typhi*. is the primary source of enteric fever transmission and perforation of resistant typhoid especially among children in developing countries. However, the paradigm to 'disarm' the bacteria instead of killing it directly can augment our dwindling antimicrobial arsenal with limited resistance. In this context, the Shikimate pathway exclusive to bacteria is a desirable target for the generation of antibacterial therapies. The enzyme 3-dehydroquinate dehydratase type 1 (DHQ1) in *Salmonella typhi.* is a validated and crucial therapeutic target because of its key role in the bacterial aromatic amino acid production pathway. The goal of the current investigation is to ascertain which bioactive substances have inhibitory action against the DHQ1 enzyme. The protein data bank (PDB) provided the protein's three-dimensional structure. To identify potential anti-typhoidal compounds, structure-based virtual screening (SBVS) was performed using 551 natural and anti- infection compounds from ApexBio database. About 206 compounds out of 551 were filtered based on physicochemical properties and Lipinki's rule and went through the molecular docking process. The top ten ligands (Cabotegravir, Imatinib, Prulifloxacin, Limonin, Silibinin, Atovaquone, Betamethasone Valerate GSK1324726A, Isavuconazole, and Raltegravir) were chosen based on their greatest binding affinities, which ranged from -9.8 to -9.1 (kcal/mol). Moreover, PASS analysis was used to prioritise the best inhibitory chemical by performing

ADMET analysis, bioactivity prediction, and pharmacokinetic property prediction. Additionally, RMSF values (root mean square fluctuation) from molecular dynamic modelling demonstrated that the docked protein-ligand combination was stable. Experiments conducted in vitro and in vivo will also help to clarify the safe and effective therapeutic role of the substances that are prioritized.



Keywords: Typhoid, anti-typhoidal compounds, Structure-based virtual screening (SBVS), PASS analysis, ADMET analysis, molecular dynamic modelling.


## 1. INTRODUCTION

### 1.1. Global Antimicrobial resistance

Antimicrobial resistance is the capability of micro-organisms such a bacteria, virus, fungi, and protozoans to continue their perpetual growth despite being treated with anti-microbial substances

(Wagenlehner & Dittmar, 2022). From an anthropogenic standpoint, AMR is a classical evolutionary process centered on bacteria's specific response (natural selection) to antibiotic exposure. It happens in a variable eco-biological system, nested in complex biological networks, from genes to larger communities (Baquero et al., 2021). Bacterial evolution, at the ontological level, advances by random (mutation and drift), directional processes (natural selection) as well as sequential pathways of adaptive evolution (Frlan, 2022).

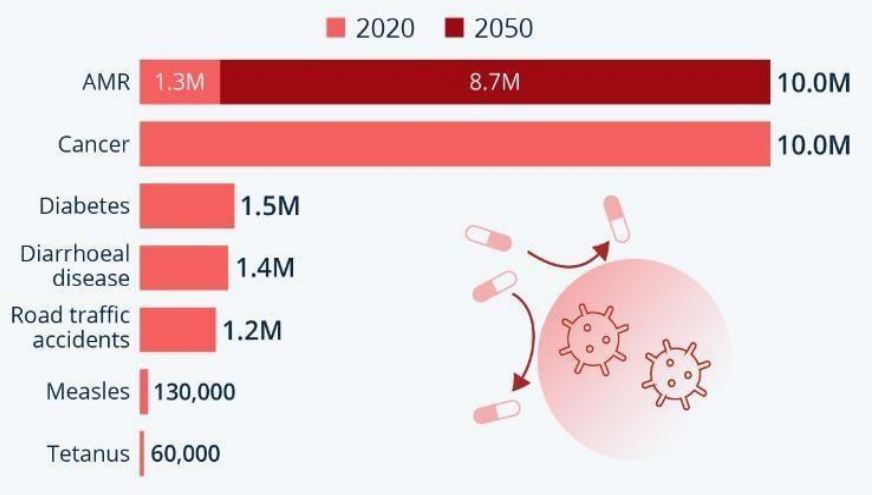



**Figure 1**. Predicted mortality from antibiotic resistant infections (AMR) versus today's common causes of death. Source: Bracing for superbugs 2023 (UN environmental program).

### 1.2. Driving factors for Antibiotic resistance

The antimicrobial resistance phenomenon is exacerbated by several reasons. The major driving factors include the abuse of antibiotics in human prescription medicine as well as the horrendous misuse of antibiotics in animal husbandry and farming (Wagenlehner & Dittmar, 2022). According to studies, antibiotics are not only selectors but also drivers of bacterial genetic variation. Antimicrobials generate stress response (transcription/translation) response in susceptible organisms continuously in subinhibitory concentration during bacterial growth phase. The stress-induced mutagenesis tops the list as the main driver of bacterial evolution. Including, the development of microbial resistance mechanisms and numerous faltering environmental factors (Zhu et al., 2022).

Importantly, the inappropriate use of antibiotics has radically changed bacterial evolution and led to unprecedented selection pressure on bacterial genomes. This has culminated in the mobilization and horizontal transfer of a huge number of resistance genes (ARGs) in bacterial pathogens (Ma et al., 2021). Bacterial pathogens display a remarkable ability to endure antibiotic exposure through an assortment of phenotypic and genetic-resistant mechanisms. Genetic factors involve the acquisition of mobile genetic elements and mutations/modifications in the chromosomes, whereas a phenotypic mechanism that confers resistance is microbial biofilm formation (Kok et al., 2022).

### 1.3. Combating Antibiotic Resistance

Mechanisms of rapidly increasing antibiotic resistance and the multi-resistance of these pathogens are multifaceted (Kok et al., 2022). The latest technologies allow scientists to explore if a particular bacterial gene or protein is critical for the proliferation of bacteria, as well as if bacterial transcription is triggered during infection (Srivastava, 2022). As the genomic and bio- informatics revolution has become an impetus for the quest for novel antimicrobial targets, a plethora of antimicrobial targets are now evidenced and accessible (Yang et al., 2022). This provides an interesting insight into the *in-silico* investigation of the putative drug targets and their binding affinity towards novel bioactive ligands for the development of rational design of effective drugs.

Antibiotics that are currently available in the global market are already overstretched because of multi-resistance by pathogens (de Kraker & Harbarth, 2022). However, there are multiple strategies for mitigating antibiotic resistance. The main approach to circumvent microbial resistance by the pharmaceutical industry is modifying and repurposing the existing classes of antibiotics. Another approach is the production of novel classes of drugs for which exists no prior resistance amongst the microbial population (Hofer, 2022; Zhu et al., 2022).

In the control and management of antibiotic resistance to multiple niches and the proliferation of multi-resistant pathogens, multidisciplinary, collaborative, and constant monitoring is required (Swami, 2014). Such as ensuring the rational use of antimicrobials, monitoring upon over-the-counter availability of antibiotics, introducing precision medicine and making sure of its availability to LMIC's (WHO,2021). Establishing infection prevention measures and standard hygiene practices effectively will mitigate the burden of hospitalization (Hofer, 2022). A cut-above and an improved collaboration between government, non-governmental organizations, and international agencies are required to understand the AMR phenomena and innovative drug

development. Global surveillance programs on antibiotic usage and AMR development serves as a benchmark for reinforcing the existing programmes to control and counterfeit the abuse of antimicrobials (Saleem et al., 2022). Also, antimicrobial stewardship programmes (ASPs), hospital-based initiatives, must be organized worldwide to improve antibiotic use (Murugaiyan et al., 2022).

### 1.4. Typhoid fever and its implications:

According to Aiemjoy et al. (2022) and Khanam et al. (2022), typhoid fever is a febrile infection carried on by the gram-negative bacteria *Salmonella enterica* subspecies enterica serovar *Typhi*. (*S.typhi*), which is a member of the Enterobacteriaceae family. In many low- and middle-income countries (LMICs), typhoid infection is widespread (Masuet-Aumatell & Atouguia, 2021). Typhoid fever is spread by the fecal-oral pathway, which is triggered by eating or drinking water infected with *S. typhi*. (Hoffman & Luby, 2023). Only humans may absorb contaminated food and water, acting as a host and reservoir for typhoid bacteria (Frempong et al., 2018). Step ladder-high-grade fever, myalgias, nausea, weakness, irregular bowel movements, and bradycardia are among the classic signs and symptoms of typhoid fever. Early digestive distress is the first symptom of the condition, and it can cause other consequences (Carey et al., 2022).

#### 1.4.1. Etiology:

It can be disseminated via contaminated water, uncooked food, infected patient surfaces, and places with inadequate sanitary facilities. Cross-contaminated poultry, eggs, and meat products are the main sources of *Salmonella* (Baquero et al., 2021). But the only way it may spread is from one ill individual to another (Basnyat and others, 2021). The four Fs—flies, fingers, feces, and fomites—are how *Salmonella* is spread (Hoffman and Luby,2023). Although the native gut flora of humans provides protection against *S.typhi* infection, medications like streptomycin destroy this natural gut flora and increase the risk of *S. typhi* infection (Duy et al., 2020).

#### 1.4.2. Epidemiology:

According to WHO estimations, there are 11–20 million cases of typhoid fever worldwide each year, which results in 128,000–16,1000 fatalities (WHO, 2021). Typhoid fever is more frequent in LMICs, primarily in Asia and Africa. It is especially prevalent in South Asian nations including Bangladesh, India, Nepal, and Pakistan (Tharwani et al., 2022). Most typhoid fever outbreaks—

93% of them—occur in Asia, sparking widespread alarm and fear, particularly among medical professionals and government officials (Farah et al., 2020). According to recent surveillance studies, Pakistan has the highest frequency of typhoid fever among South Asian nations, with 493.5 cases per 100,000 cases in 2018. The majority of cases were recorded in children between the ages of 2-4 (Andrews et al., 2020).

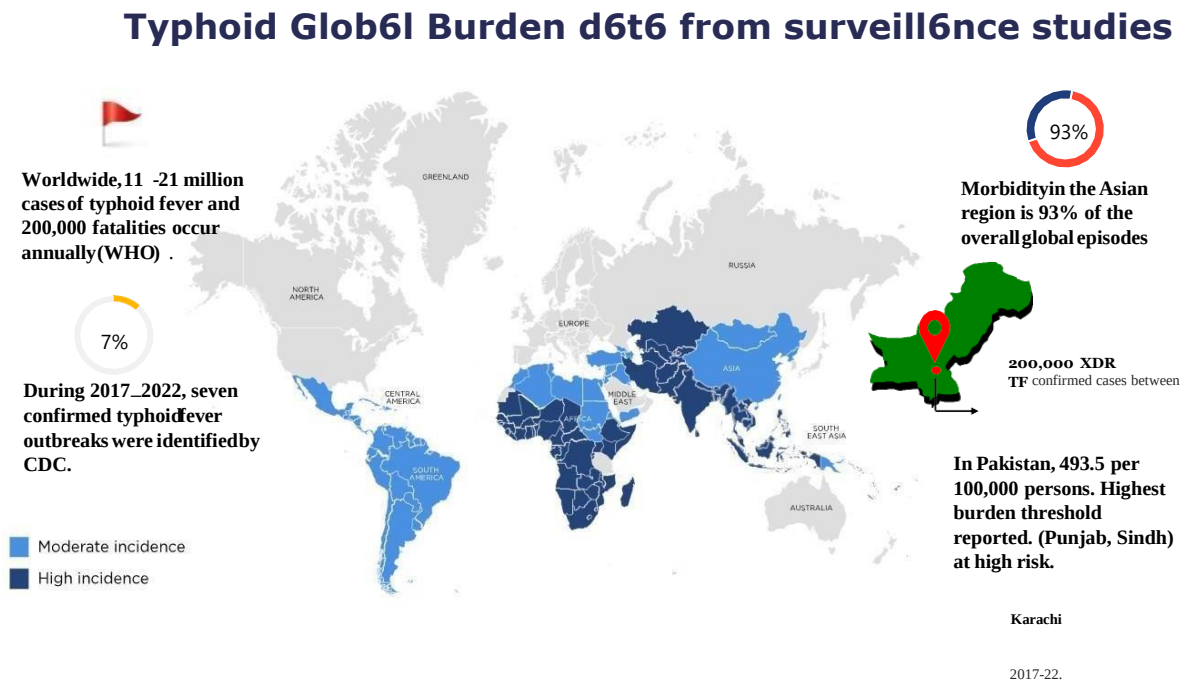



**Figure 2.** Worldwide, 10-21 million cases of typhoid fever and 200,000 casualties occur annually. The map legend indicates high incidence rate in dark blue and low incidence in light blue color. However, morbidity in the Asian region is approximately 93% of the o

### 1.5. Statistical significance:

The disease's occurrence has increased over the cutoff in many populations in South Asian nations (100 per 100000 cases-years) (Mejia et al., 2020; Yousafzai et al., 2020). Typhoid is predominantly a public health problem in Africa, with an overall prevalence estimated at 7.6%. This rate is higher

than that of reported in America (1.8%), and Oceania (0.7%) combined, estimated at 112.1/100,000 person-year (Kim et al., 2022). The 2016 epidemic of extensively resistant (XDR) typhoid fever in Hyderabad, Pakistan's Sindh region, presented the globe with concerning circumstances. According to the latest reports, about 10,365 XDR typhoid cases have occurred in Hyderabad and Karachi alone. Typhoid fever has also been reported in USA, United Kingdom, and Canada in 2017-2018, mostly in children aged 4-12 years having a travel history to Pakistan (Amber et al., 2021).

### 1.6. Pathophysiology of *Salmonella typhi.*:

A variety of factors influence the pathogenesis of typhoid such as the infectious species, its virulence, host immunity, and the infectious load itself (Zhu et al., 2022). *Salmonella* has devised a strategy to disrupt the host's typical cellular processes, enabling itself to proliferate and reproduce within the host cell (Bhat, 2022). *Salmonella* must have a set of characteristics called as virulence factors to cause successful infection. These comprise of the potential to enter cells, the presence of a full lipopolysaccharide coat, the capacity to replicate inside cells, and the ability to secrete toxin(s) effectively. (Browne et al., 2020).

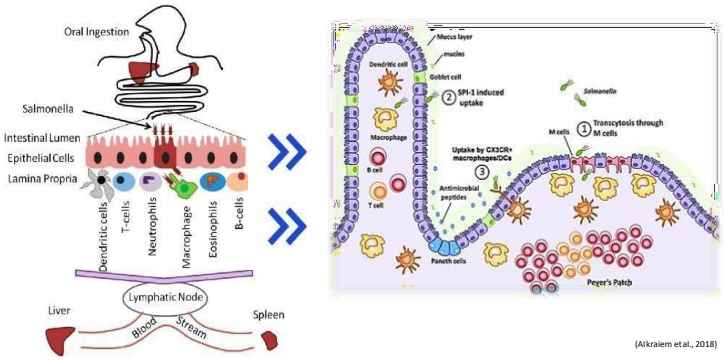



**Figure 3.** The pathophysiology of Salmonella typhi. (Al kraiem et al., 2018)

Upon consumption of food or water contaminated by *Salmonella typhi.*, the bacteria enter the epithelial layer of the intestinal wall. Subsequently, as an immune response, they are digested by the host's macrophages and delivered to lymphoid tissues in the small intestine (Peyer's patch). *Salmonella typhi*. induces proliferation in Peyer's patch via the mobilization of mononuclear cells and lymphocytes. Through the lymphatic system and bloodstream, the pathogen reaches and aggregates to the organs of reticuloendothelial. The Peyer's patch responds with an intense

inflammatory reaction via releasing lytic lysosomal enzymes, obstruction of microcirculation, and secretion of other inflammatory mediators. Ultimately, this causes necrosis and eventually ulceration of Peyer's patches, which clinically manifests itself as bleeding. Perforation is reported to occur in duodenum, colon, gall bladder, and appendix, However, terminal ileum is the most common site. Hence, the *S.typhi.* alters and modifies the host cell signaling pathway functions to promote its intracellular survival and replication (Schultz et al., 2021). The clinical onset of disease symptoms occurs in the secondary bacteremia phase (Theuretzbacher & Piddock, 2019).

### 1.7. Antibiotic resistance in Typhoidal *Salmonella*:

The evolution of multidrug resistance in *Salmonella typhi.* has created a major barricade for the successful therapy of typhoid fever (Amber et al., 2021). The Multidrug-resistant (MDR) *Salmonella* is resistant against three 1$^{st}$ line drugs i-e ampicillin, chloramphenicol, trimethoprim-sulphamethoxazole. By acquisition of resistance against fluoroquinolones and ceftriaxone, MDR strain is gradually becoming Extremely drug-resistant (XDR) (Katiyar et al., 2020). The MDR strain of *Salmonella typhi*. has caused several disease outbreaks in Southeast Asia, as well as occasional instances of third-generation cephalosporin resistance, which have also been documented globally (Ishaque et al., 2022).

The WHO report recorded 5274 cases of XDR typhoid fever among the 8188 cases of typhoid fever that have been recorded in Pakistan (Akram et al.,2020). Researchers have pointed out COVID-19 infection along with other secondary infections such as XDR-typhoid can simultaneously result in co-infections or co-endemics, putting more burden on already burdened healthcare infrastructure (Butt et al., 2022). When *S.typhi* develops resistance to five antibiotics—fluoroquinolones, ampicillin, trimethoprim-sulphamethoxazole, and third generation cephalosporins like ceftriaxone—it is known to as XDR *S. typhi*. (Jamilah et al., 2020; Saeed et al., 2019).

### 1.8. Limitations with existing treatments:

Increased prescriptions of azithromycin due to its availability in oral form has increased the chances of resistance in future. In the last decade, a high reliance on azithromycin for enteric fever has led to the development of azithromycin-resistant (Azith$^{R)}$ *S.typhi* in South Asia (Khanam et

al., 2022). In October 2017, Typbar-TCV vaccine was prequalified by WHO, however, its post-licensure population impact and data have not yet been extensively reported (Yousafzai et al., 2021). The vaccine has limitations such as it only targets children aged between 6 months to 10 years old, following several adverse responses (Qamar et al., 2020). Lack of hygiene, absence of mass vaccination, and soaring antimicrobial resistance due to indiscriminate prescription of antibiotics have made XDR-typhoid a challenge for low-and middle-income countries like Pakistan.

### 1.9. Targeting bacterial virulence- An alternative antimicrobial therapy.

We are clearly losing the long-running fight to develop new antimicrobials to restock our depleting supply of antibiotics (Bakkeren et al., 2022). The antibiotics that target cellular viability have shown to be very beneficial in the past. But these underlying processes of drug- pathogen interactions also impose selection pressure, which promotes the growth of bacteria resistant to antibiotics (Chaudhari et al., 2022). Many efforts are being made to target bacterial pathogenicity by interfering with its metabolic system in order to get around this problem (Lee et al., 2020). This strategy has a number of upsides, including a wider range of potential bacterial targets, preservation of the host endogenous microbiome, and a reduction in the selective pressure exerted on bacteria (Clatworthy et al., 2007;Maneiro et al., 2019).

### 1.10. Shikimate Pathway Enzymes as antibacterials:

Contemporary antibiotics commonly work by suppressing exclusively the cellular activities of bacteria, such as DNA replication, protein synthesis, and cell wall biosynthesis. This results in bacteria evolving enriched with resistant mechanisms upon the continuous selective pressure by such antibiotics (Frlan, 2022). Worldwide, the effectiveness of using the bacterial core metabolic pathway as a pharmacological target area for the forthcoming therapeutics is being studied with increased attention. (Gao et al., 2021). Among all the promising strategies to combat bacterial pathogen, the one which appears repeatedly in academic literature, however, not exploited by the existing drugs is targeting the essential metabolic pathway (Frlan, 2022).

Since the enzymes involved in the shikimate pathway are unique to bacteria, plants, and fungi, they are significant in this context and make sense as targets for the development of antimicrobials. The shikimate pathway is essential to the metabolism of certain species because it consumes

around 20% of the carbon released after the breakdown of carbohydrates (Sahu et al., 2020). According to Maneiro et al. (2019), the route synthesizes every necessary aromatic molecule used in plants' and microbes' main metabolism. The genomic investigations also validate the potential for creating broad-spectrum antibiotics by taking advantage of pathogenic bacteria's shikimate pathway (Escalante et al., 2021).

The shikimate pathway offers a promising avenue for the development of antimicrobial medicines and herbicides, given that the enzymes involved are conserved across algae, plants, bacteria, cyanobacteria, parasites, except mammals (Ogrodniczuk & Fuanta, 2021;Wu et al., 2022). To produce vitamin K, ubiquinone, p-aminobenzoate, and the aromatic acids L- tryptophan, L-phenylalanine, and L-tyrosine and their respective derivatives, chorismate is a crucial branch point (Stogios et al., 2022). Knock-out experiments have shown that the shikimate pathway's enzymes are essential for maintaining the proliferation and pathogenicity of bacteria.
(Wu et al., 2022).

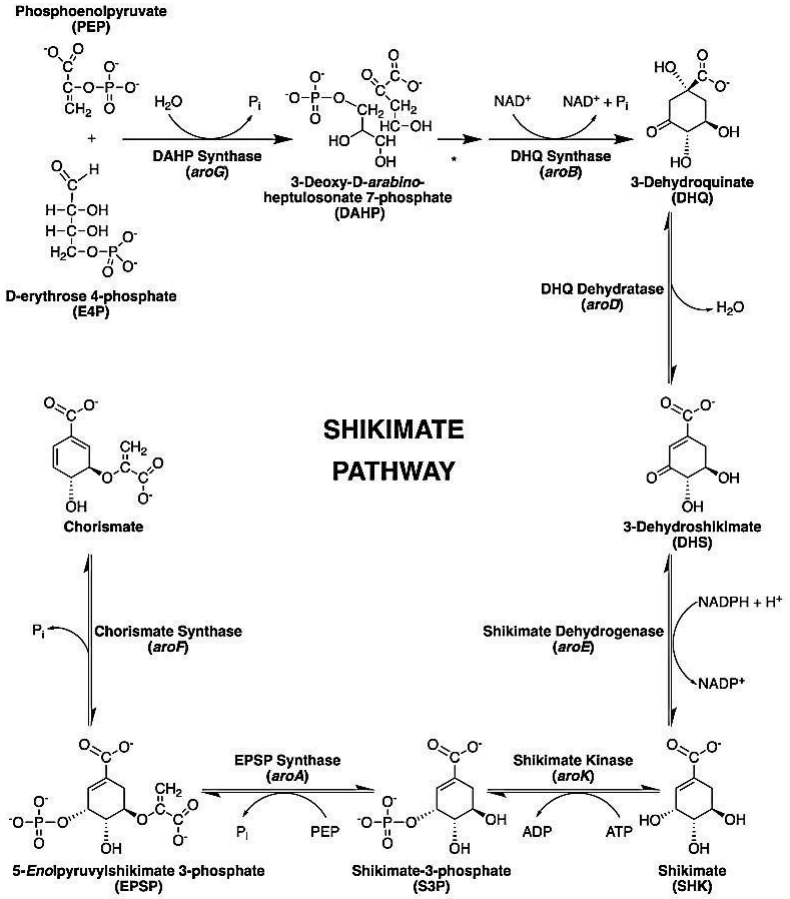


**Figure 4.** Shikimate pathway in bacteria (Nunes et al., 2020)

Seven enzymes are used in the shikimate pathway to catalyse the successive conversion of phosphoenol pyruvate and eythrose 4-phosphate to chorismite, a crucial metabolic intermediate. Chorismate is a precursor of iron-scavenging siderophores, p-aminobenzoic acid, vitamin K, napthoquinones, ubiquinone, and aromatic amino acids (tryptophan, phenylalanine, and tyrosine). The seven enzymes are catalysing the following reactions: dehydroquinate, dehydroshikimate, shikimate, shikimate 3-phosphate, 5-enolpyruvylshikimate 3-phosphate (EPSP) synthase, chorismite synthase, 3-deoxy-D-arabino-heptulosonate 7-phosphate (DAHP) synthase, 3-dehydroquinate synthase, 3-dehydroquinate dehydratase (dehydroquinase, DHQase), shikimate dehydrogenase, shikimate kinase, and 3-dehydroquinate synthase (Rapp et al., 2021).

### 1.11. Dehydroquinase (DHQase):

The third enzyme in the pathway leading to the production of aromatic amino acids is called dehydroquinase, or 3-dehydroquinate dehydratase (Tizón et al., 2015). The reversible dehydration of 3-dehydroquinic acid to 3-dehydroshikimic acid is catalysed by the enzyme DHQase. DHQase is classified into two basic groups, type I and type II, that allow the elimination of H2O through completely different methods (Maneiro et al., 2019). In evolutionary terms, the two types of DHQase are unrelated to each other, possessing an entirely different structure with no sequence homology. Genomic studies suggest that by comparing the two types of DHQases, it is concluded that the two enzymes arose by convergent evolution (Kleanthous et al., 1992).

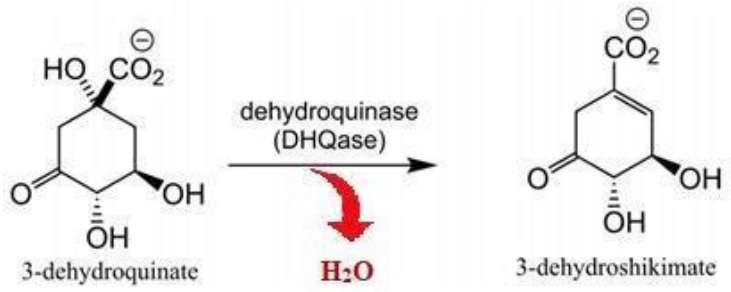



**Figure 5**. The DHQase reaction. 3-dehydroquinate into 3-dehydroshikimate conversion.

Type II DHQases function in both catabolic and biosynthetic biopathways, but type I DHQase is exclusive to the biosynthetic shikimate pathway. The possibility of developing specific enzyme

inhibitors is suggested by the existence of two DHQase forms with different processes and structures. This is important for the targeted antimicrobial therapy of *Helicobacter pylori* and *Mycobacterium tuberculosis*, which contain type II enzyme, while *Salmonella typhi* and *E. Coli* only have type I enzyme (Nunes et al., 2020). As there is no human analogue or equivalent for DHQ1. Research indicates that DHQ1 could function as a virulence factor in vivo, given that it has been demonstrated that deleting the aroD gene in *Shigella flexneri* and *Salmonella typhi*. results in effective live oral vaccines (González-Bello, 2014).

### 1.12. Structural topology of Protein

The aldolase (dehydratase) enzyme DHQ1 is present in many infectious bacteria including *Salmonella typhi, Staphylococcus aureus, and Escherichia coli*. The unique, monofunctional enzyme known as type 1 DHQase possesses a dimeric 28 kDa subunit. It catalyses the reversible syn dehydration of water in 3-dehydroquinic acid through a multi-step mechanism involving the production of Schiff base species. Each chain of it contains eight lysine residues (Gonzalez- Bello, 2015). The table displays the DHQ1 X-ray crystal structures that are available with bound ligand.

**Table 1**. Available X-ray Crystal structure of 3-dehydroquinase dehydratase type 1 enzyme in pdb databank.

| **PDB ID** | **Structure Title** | **Year** | **Resolution (Å)** | **Active Structure with Bound Ligands** | **Reference** |
|---|---|---|---|---|---|
| 4CNN | Structure of the *S. typhi* dehydroquinase type I | 2015-02-18 | 1.00 Å | N/A | (Maneiro et al., 2014) |
| 4UIO | Structure of the Type I Dehydroquinase of *S.typhi* Covalently inhibited by a derivative of 3-dehydroquinic acid | 2015-07-15 | 1.35 Å | CL, NA, VAU | (González-Bello et al., 2015) |

| | | | | | |
|---|---|---|---|---|---|
| 6SFG | *S.typhi*. DHQ1 covalently modified by by compound 9 | 2020-04-15 | 1.23 Å | FQZ, FSQ | (Lence et al., 2020) |
| 6H5C | Crystal structure of DHQ1 from *S.typhi* covalently modified by ligand 1 | 2019-07-24 | 1.14 Å | FSQ | (Maneiro et al., 2019) |
| 6H5D | Crystal structure of DHQ1 from *Salmonella typhi* covalently modified by ligand 2 | 2019-07-24 | 1.25 Å | WPL | (Maneiro et al., 2019) |
| 6H5G | Crystal structure of DHQ1 from *Salmonella* typhi covalently modified by ligand 3 | 2019-07-24 | 1.04 Å | FQZ | (Maneiro et al., 2019) |
| 6H5J | Crystal structure of type I DHQ from *S.typhi* covalently modified by ligand 4 | 2019-07-24 | 1.4 Å | FT5 | (Maneiro et al., 2019) |
| 4CLM | Structure of *S.typhi* DHQ1 irreversibly inhibited with a 1,3,4-trihydroxyciclohexane-1-carboxylic acid derivative | 2014-11-05 | 1.4 Å | CL, LI, WPL | (Tizón et al., 2015) |

| | | | | | |
|---|---|---|---|---|---|
| 4CNO | Structure of the *S.typhi* DHQ1 dehydroquinase inhibited by a 3-dehydroquinic acid derivative | 2014-10-08 | 1.5 Å | 9PY | (Maneiro et al., 2014) |

### 1.13. The proposed strategy:

An unconventional strategy in the age of widespread antibiotic resistance would be an approach that impairs the ability of bacteria to spread illness (virulence). Considering this, the important idea to lessen the issue of enteric fever is the creation of an entirely novel arsenal of powerful selective small molecule inhibitors against such vital enzymes. The present study employs a range of in silico methodologies to investigate the DHQ1 protein antagonists, hypothesis, and molecular underpinnings of interactions with the protein via Structure-based virtual screening (SBVS).

### 1.14. Objectives

1. To predict the optimal conformation between ligands and protein through molecular docking.
2. To predict binding energies between ligand and target protein.
3. To probe important 3D interaction features of top protein-inhibitors complexes via MD simulation.

# Chapter 2

## 2. LITERATURE REVIEW

### 2.1. Structure-based studies

### 2.1.1. Structure of DHQase

Type 1 DHQase contains approx.50% α-helix in its secondary structure and exists as a dimer with $M_r$= 46000 +2000. The single domain of type 1 DHQase from *S.typhi* folds into an eight- stranded α/β (or TIM) barrel, which is blocked by two short anti-parallel strands that are positioned at the N-terminal end of the barrel. Via the barrel's other end, the substrate can get to the enzyme's active site. The average length of the small loops that join the α-helices and β- strands is six residues. The hH loop, on the other hand, has 13 residues, Gln236 of which comes into direct contact with the substrate. For the enzyme's active site to be completed, the lid-like loop must close. Water is eliminated via the production of imines containing a highly conserved lysine residue in the active site (Lys-170) (Lence et al., 2020).

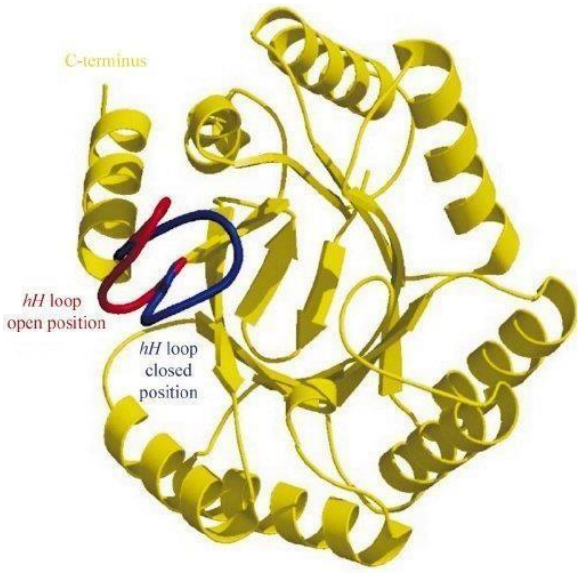



**Figure 6**. A general fold of DHQase polypeptide chain (yellow color). An open hH lid loop shown in (red) and hH closed position (blue) (Lee et al., 2002).

Light et al., 2011, reported a dynamic loop behavior (closing/opening above the enzyme's active site) in three crystal structures of type I *Salmonella enterica typhi*. The protein's crystal structure and kinetic analyses demonstrated that loop is essential to catalysis and that Gln236 is necessary for loop closure. This loop closure facilitates the substrate binding by induction of structural rearrangement in Arg213. Numerous studies suggest the development of such molecules which can prevent the loop closure or stabilize the open position of the loop, may be an effective allosteric DHQase inhibitor. X-ray diffraction analysis suggests that hH loop upon closure acts as an extra hydrophobic layer above the hydrophilic one, composed of residues such as Phe145, Ala172, Met 205 (Lee et al., 2002).

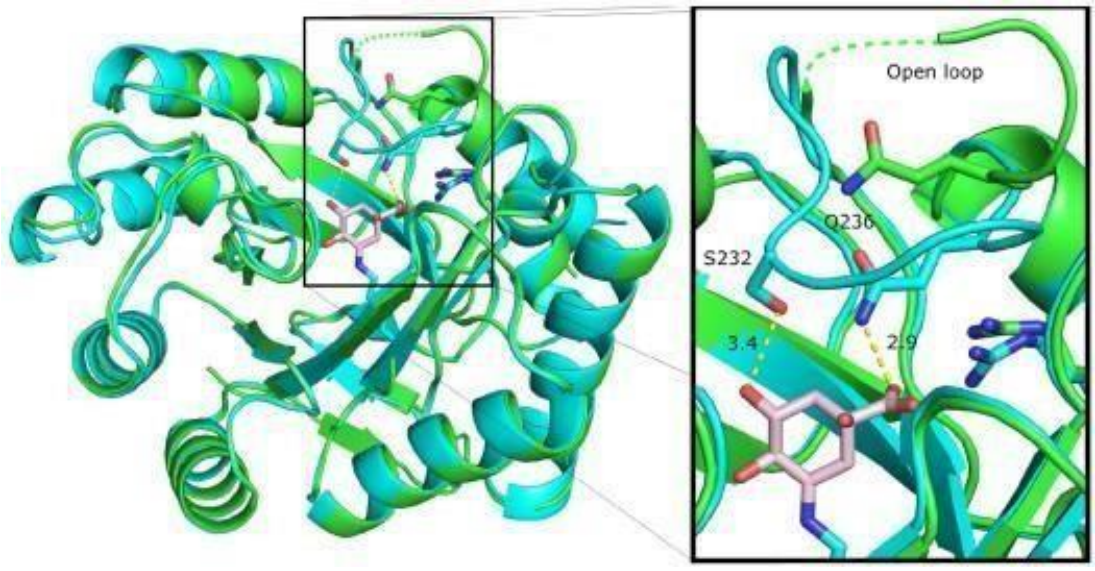



**Figure 7**. Dynamic loop dynamics in DHQD. The intermediate bound of pre-hydration (cyan) and the open loop (green). The inset emphasizes the interaction of closed loop residues with the reaction intermediate (shown in pink) and focuses on the differences in loop.

According to MD simulation studies, the conserved amino acids lysine and histidine play a crucial part in the process when they are present in the active sites. Histidine acts as a base to remove the

substrate's C-2 pro-R proton and produce an intermediate carbanion, while the lysine residue (Lys 171) ε-amino group forms a Schiff base with the substrate (Lence et al., 2020).

Upon substrate contact, the conformation changes to a co-planar alignment of the pro-R hydrogen with the imine's -acceptor orbital. This improves the syn-stereo chemistry of the elimination. Experimental evidence indicates that the carboxylate initiates the conformational modifications that result in syn-elimination and is essential for substrate reactivity (González-Bello, 2014).

DHQase dehydrates water by generating imines with the highly conserved active site residue lysine (Lys-170). The process involves the use of histidine (His143 in *S. typhi*), which acts as a proton donor, suggesting that it is involved in the synthesis and hydrolysis of the Schiff base intermediates. The crystallographic data places His-143 in the middle of a hydrogen-bonded trio with Lys-170 and Glu-86, which is consistent with His-143's involvement in the Schiff base production process (Tizón et al., 2015; Servos et al., 1991).

### 2.2. Inhibition studies of DHQase

Inhibition studies of DHQase

In several scientific pieces of research, the inactivation of genes of the shikimate pathway has led to the attenuation of bacterial virulence and loss of viability including species such as *Mycobacterium.tuberculosis* and *Salmonella typhimurium* (Almihyawi et al., 2022). Especially, DHQase which is an evolutionary conserved enzyme that catalyzes biosynthetic pathway to generate dTTP (used for synthesis of bacterial cells (Chaudhary et al., 2021). Cheung et al., 2014, in their experimental design using high throughput screening identified several polyketide- based antimicrobial inhibitors against DHQase of Enterococcus faecalis of the shikimate pathway. The compound marein, a flavonoid polyketide inhibited DHQase with 256 MIC values (mg/mL). It both retarded the growth as well as inhibited the *E.* faecalis. The team also found satisfactory inhibitory action of glycosides, coumarin, and chalcones against metabolic enzymes i-e DHQase.

In their work, González-Bello et al. designed a type-specific mechanism-based inhibitor against DHQase 1 using mechanistic dichotomy. They discovered that by producing an imine intermediate, (2 R)-2-bromo-3-dehydroquinic acid, (2 R)-2-fluoro-3-dehydroquinic acid, and 2-bromo-3-dehydroshikimic acid function as suicide inhibitors DHQ1. The competitive inhibitor (2R)-2-bromo-3-dehydroquinic acid was found (K i = 3.7 mM). It could be brought on by bromine

sterically thickening up in 3-dehyquinic acid's deleted pro-R hydrogen. It was demonstrated that each of these type II dehydroquinase substrates inhibited type 1 DHQase irreversibly (González-Bello, 2014; González-Bello, 2015).

Three non-covalent DHQ1 inhibitors from *Clostridium difficile* with IC50 values ranging from 31 to 35 nm were found by Ratia et al. in their experimental study. NMR research showed that these inhibitors compete with the product's active location. These inhibitor compounds, however, were discovered to be ineffective against other DHQ1 enzymes, including the *S. enterica* enzyme. The study could not give details about the binding action of these compounds (Ratia et al., 2014). Many dehdroquinate analogues have been synthesized against enzyme DHQ1.

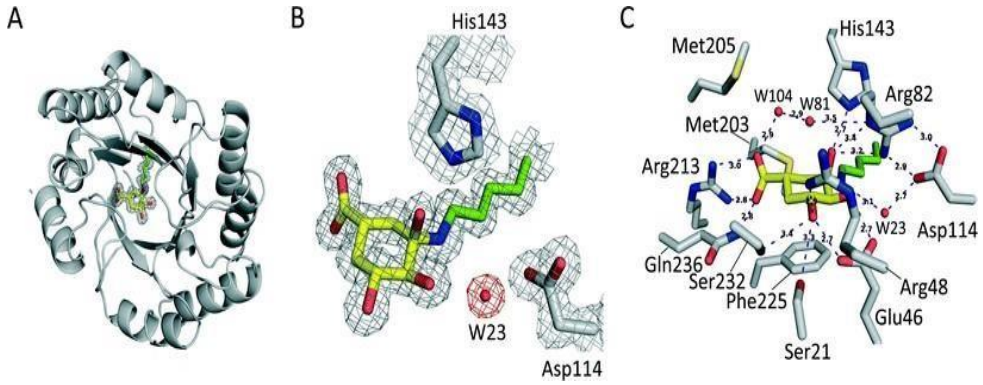



**Figure 8.** (A) 3 dimensional structure of S.typhi DHQ1 covalently modified by ligand (B) Electron density for altered ligand (yellow) and its covalent binding to Lys170 (green) of DHQ1 (C) The dotted lines represent the hydrostatic and electrostatic interactions

For the first time, Maneiro et al. showed that hydroxylammonium derivatives based on quinic acid may specifically covalently modify an enzyme's catalytic and sterically inaccessible lysine residue. According to computational research, the change takes place when a histidine residue deprotonates lysine and releases NH2OH owing to a direct nucleophilic assault on the ε-amino acid group of lysine (Maneiro et al., 2019). Lence, 2015, and his co-workers succeeded on hindering the bacterial facility to cause infection by designing ammonium derivative of epoxide which acted as a novel lysine targeted irreversible inhibitors. In 1974, Butler et al. originally reported the inactivation of 3-dehydroquinase of *E.coli*. with the action of 3-dehydroquinate and NaBH4.

### 2.3. Structure-based Virtual Screening (SBVS- An early-stage drug-discovery campaign):

Virtual screening (VS) is an approach that is complementary to high-throughput screening. It has the potential to boost the number of newly discovered potential active druggable compounds with high potency and chemical diversity (Tabrez et al., 2022). VS has revolutionized pharmaceutical research by being a fast, resource-efficient, and cost-effective technique (Morro et al., 2018, Singh et al., 2021 (Ghislat et al., 2021). Structure-based virtual screening (SBVS) is a computational technique employed in initial phase of drug discovery process in order to search for the bioactive compound library for novel molecules against a specific drug target is preferred in computational biology and pharmaceutical research because its success rate is 400 times higher, less costly, less laborious than the traditional in vitro screening techniques (Sefika Feyza et al., 2022). SBVS provides prediction of the likely binding conformations as well as an easy method of ranking the docked compounds based on the scoring functions. SBVS strategies follow a particular sequence of processes i-e, (1) Selection and preparation of target molecule, (2) Ligand database/ library assortment (3) ligand preparation (4) molecular docking (5) Result interpretation (Srivastava, 2022).

VS approach employs a target receptor (protein/enzyme) and a large collective library of small compounds (Gupta, 2022). A library is a rich source of annotated compounds, chemically diverse sets of ligands such as metabolites, natural compounds, trace amines, neurotransmitters, peptides, antibodies, small molecule inhibitors, and protease cocktails etc. This virtual technique is deemed as the *in-silico* equivalent of *in vitro* methods such as high-throughput screening (HTS), which filters the potential ligand depending on its pharmacokinetics, molecular weight, number of hydrogen donor and acceptors etc (Peón et al., 2019). Jalal et al., 2021, screened ZINC library 1000 of natural compounds focusing on a plant source to develop inhibitor compounds against shikimate dehydrogenase and colonic acid biosyn thesis acetyltransferase by virtual screening methods.

Tizon and his co-workers (2011), utilized a pro-drug approach to develop potent inhibitors against DHQase. By employing SBVS in their chemoinformatic research, they reported propyl esters to be the most effective target against the bacterial enzyme. Peek et al., 2014, employed a similar screening approach in the experiment to hamper shikimate pathway in bacteria. Their studies revealed that phenolic compounds such as epigallocatechin gallate as an important inhibitor (IC=

3μM) of Shikimate dehydrogenase *in Pseudomonas putida.* Hsu et al., exploited virtual screening and site-moity maps to identify putative inhibitors of shikimate dehydrogenase, a shikimate pathway enzyme, with IC50 values around 2μM (Hsu et al., 2013).

Souza et al., in their research proposed 3-dehydroquinate dehydratase, a putative target to vitiate *M. tuberculosis* survival by impairing its essential shikimate pathway. By using SBVS, about 216 million compounds were reduced to 460,000 chemical compounds by filtering their toxicity and molecular weights. The best inhibitor displayed stable binding affinity and strong molecular contacts with the binding pocket residues of the enzyme DHQase (Souza et al., 2022). Almihyawi and his co-workers (2022), published a study on in silico identification of inhibitors from natural marine organisms against *Acinetobacter baumannii* 5-enolpyruvylshikimate-3- phosphate (EPSP) synthase of shikimate pathway. By employing various servers and virtual screening, three compounds CMNPD31561, CMNPD28986, and CMNPD28985 were classified as primarily the EPSP synthase blockers., hence, might stop the growth and survival of *A.baumannii* (Almihyawi et al., 2022)

**2.4. Molecular docking studies:**

The orientation of ligands or small molecules within the target protein's active site (receptor) are ascertained via an in-silico method called molecular docking (Rebehmed et al., 2022). It offers an estimate ligand bio-affinities with the protein and aims to anticipate the ligand-protein complex and improves docking efficiency by computing the ligands' conformational space inside the protein's binding site residues. Numerous docking applications, including AutoDock, Dock, FlexX, Glide, Gold, Surflex, ICM, Ligand, Fit, HexDock, Drugster, and eHiTs, have been created (Ghislat et al., 2021). Many web tools, such as GRID, POCKET, SurfNet, PASS, DoGSiteScorer, and others, are useful for pocket identification. (Stanzione et al., 2021). A unique scoring function (SF) guided by a regression model, evaluates the free binding energy between ligands and the protein in every docking pose. The top hits are then subjected to post- processing by calculating the binding scores, validating the generated pose, desired and undesired chemical moieties and metabolites, and physiochemical properties.

The selected compounds are also evaluated based on their toxicity levels, lead-likeliness, and chemical diversity. Such selected compounds can be processed for in vitro assays for further experiments (Wong et al., 2022). A common scoring function used by docking software is Force

field-based functions. It approximates the binding free energy of the complex by summing up the intermolecular van der Waals forces, electrostatic interactions, and hydrogen bonding. Solvation and entropy energy estimates among each atom of the binding partner in a complex are also taken into consideration. Top ranked molecules by docking indicate that the binding partners in the complex are strongly attached and hence have higher binding affinity (Rebehmed et al., 2022).

Using AutoDock, Isa et al. (2019), reported three compounds which make the best ligands: ZINC633887 (binding energy= -10.29 kcal/mol), ZINC08983432 (-9.34 kcal/mol), and PubChem73393 (-8.61 kcal/mol). (DHQS). These compounds were deemed possible treatment of *M.tuberculosis.* In another effort by Souza et al., novel putative inhibitors of 3- dehydroshikimate dehydratase were distinguished by large scale docking strategy along with ligand based chemoinformatic methods. About 300 hits were subjected to docking in GOLD after screening 216 million compounds. GOLD also selected the best molecule which made strong interactions with the target protein active site loop by consensus ranking (Souza et al., 2022).

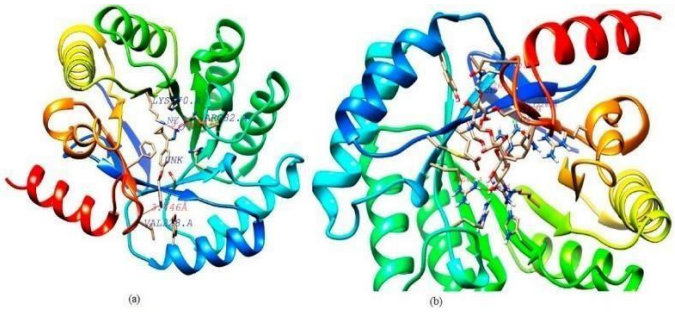


**Figure 9**. 3D conformation of bound ligand within active site residues by hydrogen bonding of target protein 3-dehydroshikimate dehydratase crystal structure (Souza et al., 2022).

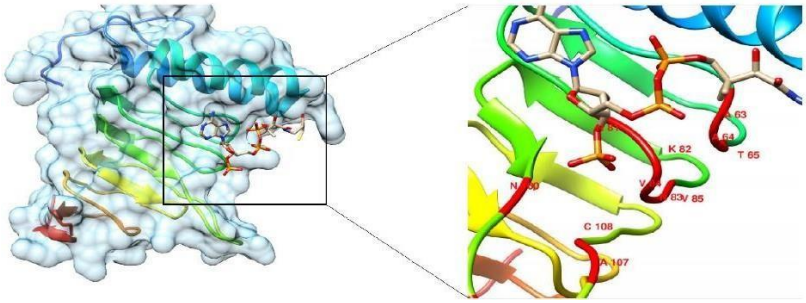


**Figure 10**. Ligand and protein 3D complex (Jalal et al., 2021)

Using AutoDock Vina software, Sarkar et al. looked for possible inhibitors against the shikimate pathway enzymes 3-dehydroquinate synthase (3N76) and 3-dehydroquinate dehydratase (3QBE) as drug targets. They evaluated the two enzymes against 11 phytochemicals of Achyranthes aspera. Docking showed that Ecdysterone 2,3-acetonide 22-O-benzoate had the highest binding affinity with enzyme 3-dehydroquinate synthase (3N76), and 2,3,14,20,25-Pentahydroxy-6- oxocholest-7-en-22-yl benzoate) had the highest binding affinity with 3QBE. Both compounds also show druglikeliness by adhering to Lipinski's rule of 5. (Sarkar et al., 2022).

In 2021, Chaudhary and colleagues synthesised 4-alkoxy/aryloxyphenyl cyclopropyl methane oxime derivatives 2(a–k) and investigated their antimicrobial properties. The chemical was docked to *S.typhi* 3-Dehydroquinate (1GQN) in order to explain its efficacy. One conformation with -7.16 kcal/mol was chosen from among the 10 docked conformations and suggested as a potential therapeutic option (Chaudhary et al., 2021). In order to create an antibacterial agent, Neetu et al. discovered that chlorogenic acid inhibited the 3-dehydroquinate synthase. (Neetu et al., 2020).

# 3. Chapter 3: METHODOLOGY

## 3.1. Virtual Screening (VS)

Virtual screening (VS) was employed in the hunt for ligands that might interact to *Salmonella typhi's* DHQ1. In the present stusy, a total of five hundred compounds from ApexBio database. However, 551 FDA approved small-molecule compounds were considered in the research to identify if any of them had good inhibitory interaction with the stated bacterial enzyme. These drugs were additionally passed through a filter for physiochemical features manually. The compounds with desirable properties were shortlisted and docked using AutoDock Vina.

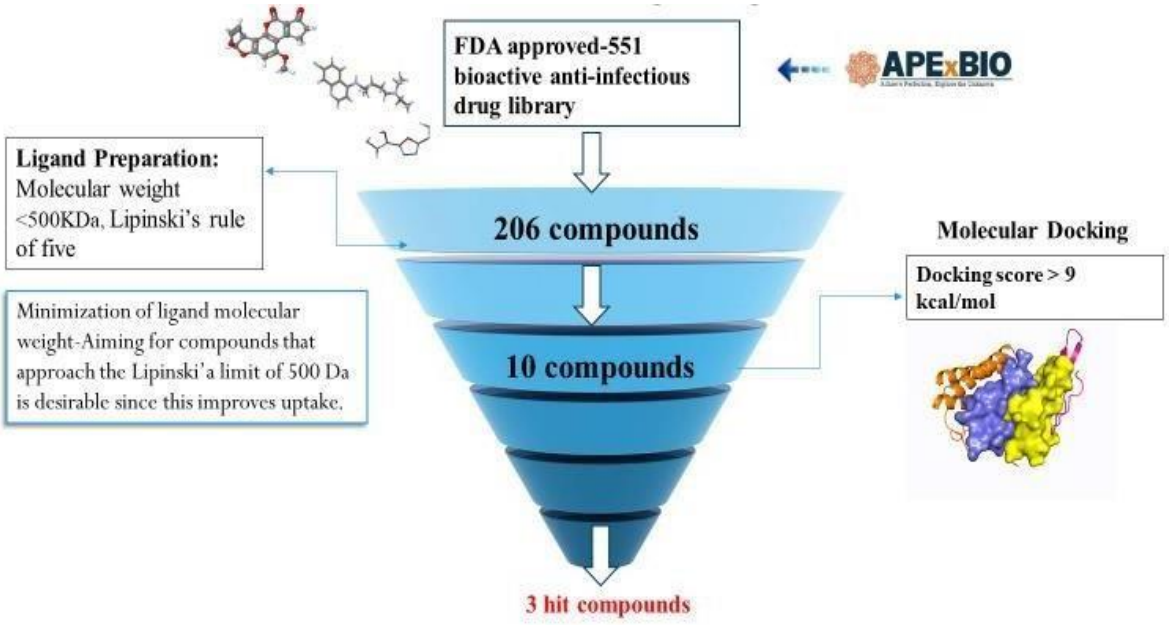



**Figure 11**. Schematic overview of methodology Structure based virtual screening (SBVS).

The RSCB Protein Data Bank (http://www.rscb.org) provided the 3-dehydroquinase dehydratase from *Salmonella typhi*. (PDB ID:4CNN). The X-ray diffraction approach yielded a high resolution of 1.00 Å for the crystal structure 4CNN (Maneiro et al., 2014). PDB format download of the protein structure was made. Software called AutoDock Vina was used to prepare the protein structure for docking process. The original enzyme structure was stripped of the associated ligands, C6 H8 O7, Cl, and Na ions. Next, to standardize DHQ1, polar hydrogens, Kollman charges, heteroatoms had to be removed, and crystallized water molecules had to be cleared out. Protein Data Bank (PDB) to PDBQT file format conversion was done using AutoDock Tools.

**Table 2.** The high-resolution X-ray crystal structure of protein 4CNN is accessible in the PDB databank.

| **PDB ID** | **Structure** | **Release Date** | **Resolution (Å)** | **Enzyme with bound ligands** | **Reference/citation** |
|---|---|---|---|---|---|

| | | | | | |
|---|---|---|---|---|---|
| 4CNN | Crystal structure of *S. typhi* type I dehydroquinase DHQ1 | 2015-02-18 | 1.00 Å | 1) CIT<br>2) CL | (Coderch et al., 2014) |

### 3.2. Ligand library preparation:

In order to construct the ligand library, ApexBio's natural and chemical bioactive chemicals were screened. The library of ligands consists of 551 chemicals in total. It was, however, trimmed to 206 compounds by using the most effective physicochemical filters utilising Lipinski's rule of 5. The SDF (Spatial data file) format of each ligand was obtained from the PubChem database (https://pubchem.ncbi.nlm.nih.gov/). With BIOVIA Discovery Studio Visualizer, the sdf format was subsequently converted to pdb format. In AutoDock Vina, the ligand pdb files were opened and made ready for the docking process. After AutoDock translated the files into pdbqt format, they were considered ready for molecular docking.

### 3.3. Docking studies:

Using the PyRx GUI and AutoDock Vina software, the ligands were docked (Halder et al., 2023). In order to give ligands at the binding site and binding scores every conceivable conformation and orientation, docking was finished. The grid box's centre was fixed at (11.463, 0.454, 29.442) Å for (centre x, centre y, and centre z) in this investigation. In the meanwhile, the grid box's dimensions were given as (40,40,40) Å (centre x, centre y, and centre z). The specified energy range was 4, and the exhaustiveness was 8. The docking procedure was carried out via an encoded bash script file. The Vina programme and a few necessary parameters, together with input and output parameters, were stored in the script file (Mukhtar et al., 2023). Once the programme was run, the binding energy score was presented for each ligand molecule and the protein. Every ligand molecule's grid box parameters were determined and then stored in a configuration file with .txt extension. The docking procedure used was in line with earlier research (Mukhtar et al., 2023, Jalal et al., 2021; Meena, 2021).

### 3.4. 3D Visualizations:

The PDB structures of the protein and ligand macromolecules were stored in PyMol in pdb format to enable 3D visualisation of the protein-ligand molecular interaction. The Protein-Lipid Profiler (PLIP) (projects.biotec.tu-Dresden.de/plip-web), a new online server for identifying and dynamically visualising non-covalent protein-ligand connections, received the PDB file. The complex images were saved in pse format (to be visualized further in PyMol software). The molecular interaction profiles of the best-fit model from the docking experiments were visualized in 2D and 3D in PyMol and Discovery Studio v2021 for evaluation. To further visualize the structural complexes, LigPlot$^{+}$ was used for automatic generation of 2D ligand-protein interaction diagrams.

### 3.5. Prediction of activity spectra for substances (PASS) analysis:

Prediction of activity spectra for substances (PASS) analysis is a substitute to clinical studies *in-silico*. It uses the structure-activity conjunction of the ligand molecule to predict the molecular characteristics, pharmacological features, bioactivity, drug-likeness, and side effects. PASS analysis was carried out for the ligands in the current study to assess their possible inhibitory actions using a variety of online servers and tools, which are listed below (Siddiqui et al., 2022).

#### 3.5.1. Lipinski's rule of five:

Lipinski's rule of five uses pharmacokinetic measures including absorption, metabolism, distribution, and excretion to assess the molecular characteristics and drug-likeness of ligand molecules. In the current study, ten ligand compounds were evaluated for drug-likeness utilising the Lipinski rule of five. This was achieved through an online software Molinspiration v2021.03 (https://www.molinspiration.com/). Molinspiration computed the following parameters: molecular weight (MW 500), number of hydrogen donors (NOHNH ≤5), number of hydrogen bond acceptor sites (NON ≤10), topological polar surface area (TPSA ≤140 Å2), number of rotatable bonds (≤10), and logarithm of the compound partition coefficient between n-octanol and water (log P 5). For an oral medicine to remain bioavailable, it must not have more than one Lipinski violation (Zaki et al., 2022).

#### 3.5.2. Bioactivity score (BAS) prediction:

According to Siddiqui et al. (2022), BAS levels indicate the likelihood that a chemical would be a promising therapeutic candidate. To get detailed information on the bioactivity score of the

selected ligands about human receptors, such as G protein-coupled receptors (GPCRs), kinases, proteases, ion channels, enzymes, and nuclear receptors, a web tool called Molinspiration version 2021.03 was utilized. A molecule's likelihood of being active generally increases with its bioactivity score. In 2023, Mukhtar et al. A molecule is regarded as active if its bioactivity score is greater than 0.0; ligands are regarded as moderately active if their bioactivity score is between -5.0 and 0.0; and inactive or idle if their bioactivity score is less than -5.0. (Lata et al., 2023).

### 3.5.3. Toxicity potential study

An essential phase in the development of new drugs is the assessment of toxicity. However, computational toxicity estimation does not only save time but also reduces the number of animal testing. Here, in the study we employed Protox-II web server, a freely available tool which takes 2D chemical compound input to generate the possible toxicity profile of the compound. It incorporates machine learning and molecular similarity to predict numerous toxicity endpoints such as carcinogenicity and immunotoxicity etc (Malhotra, 2022). The prioritized compounds were also estimated for their $LD_{50}$ value (expressed mg $^{kg-1}$ body weight) and Drug toxicity class. The median lethal dose, or $LD_{50}$ refers to the amount of drug used to kill half of the subjects of tested population after a specific time frame. The input files in the Protox-II web server were submitted via PubChem name.

### 3.5.4. Pharmacokinetic property prediction

The SWISS-ADME programme was utilised to systematically predicted the ADMET parameters, which include absorption, metabolism, excretion, and toxicity, as well as physiocochemical descriptors. For the top ten compounds, it predicted the pharmacokinetic characteristics such as lipophilicity for plasma membrane absorption, distribution, GI absorption, metabolism as a P-glycoprotein (P-gp) substrate, and cytochrome P450s such as CYP1A2, CYPC19, CYP2D6, and CYP3A4 inhibitor. According to Lipinski et al., a material has to possess at least three of the five physiochemical characteristics in order to be categorized as drug-like (Iqbal et al., 2023, Ameji et al., 2023).

### 3.6. Molecular dynamic simulation:

The CABS-flex 2.0 server was utilised to evaluate the flexibility of the best-docked ligand- protein complexes using molecular dynamic simulations. The root mean square fluctuation (RMSF) of the optimal docked complex among the docked structures that were prioritised was verified by the online server. The default settings e, protein rigidity:1.0, number of cycles:50, number of cycles between trajectories:50, temperature range:1.40, and random number of generator seed of 6912 were used to run the simulations in CABS-flex. CABS-flex is a robust online server for predicting protein structure fluctuations, offers high resolution (10ns) protein flexibility simulation as a substitute for traditional, all atom molecular dynamics (Badaczewska- Dawid et al., 2020; Kuriata et al., 2018).

# 4. Chapter 4: RESULTS

## 4.1. Protein preparation:

The PDB format of the protein structure was obtained, and AutoDock Vina was used to prepare the protein structure. From the original structure, the associated ligands C6 H8 O7, Cl, and Na ions were eliminated. Next, to standardise DHQ1, polar hydrogens had to be added, heteroatoms had to be removed, Kollman charges had to be added, and crystallized water molecules had to be removed. The file format was changed from Protein Data Bank (PDB) to PDBQT using AutoDock Tools.

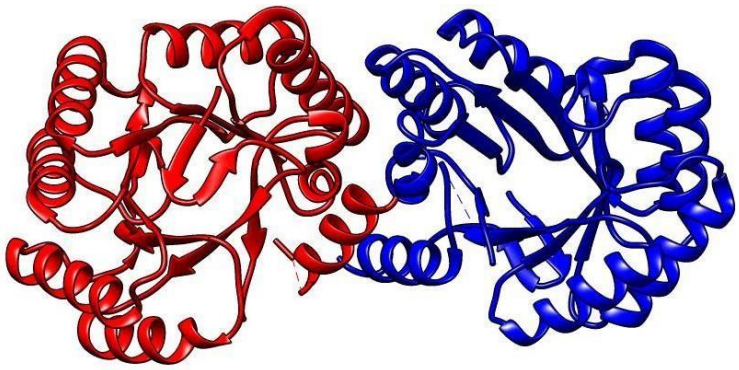


**Figure 12**. The three-dimensional crystal structure of bacterial protein DHQ1 after removing irrelevant ions, ligands, and water molecules.

## 4.2. Virtual screening of ligands:

Virtual screening was applied to 551 substances retrieved from ApexBio, both natural and chemical in origin. Following the inclusion of the filter, 206 compounds were excluded from the remaining 551, indicating that the compound's molecular weight has to be less than or equal to 500 kDa to facilitate improved absorption (Bos & Meinardi, 2000). Therefore, 206 little molecules that had been screened out were sent to molecular docking studies.

## 4.3. Molecular docking experiments:

To calculate the binding affinity score of the 206 ligands against the DHQ1 bacterial shikimate pathway protein, AutoDock tools 1.5.6 was utilized. The protein was considered a "rigid" molecule, but the ligands were made "flexible." (Shidi et al.2022). The grid box's centre was changed for this inquiry to (11.463, 0.454, 29.442) Å for (centre x, centre y, centre z). At the same time, the measurements of the grid box were given as (40,40,40) Å (centre x, centre y, and centre z). Eight was selected as the exhaustiveness, and the energy range was changed to 4. A bash script file that was encoded was used to carry out the docking process.

The docking procedure was initiated, and the output scores were provided by entering a sequence of codes, or instructions, into the command prompt file (Dien-Yu Adrianne et al., 2022). The values of the Gibbs free energy (G) or binding affinity score are stated in kcal mol-1. By monitoring the maximum negative value of the ligand molecule, the target protein's greatest binding affinity was ascertained, and vice versa (Chaudhary et al., 2021). Ten best-fit models from the nine distinct conformations were selected out of 206 ligands. The 3D docked structure of the ligands and enzyme were visualized using the Discovery Studio v2021, LigPlot$^{+}$ (Mukhtar et al., 2023).

**Table 3.** Docking results of DHQ1 with ligands of good binding energy, molecular weights, interacting amino acids, binding energies and structure of ligands.

| **S/No.** | **Chemical identifier (ID)** | **Ligands** | **Interacting amino acid residues** | **Binding energy (kcal/mol)** |
|---|---|---|---|---|
| 1. | PubChem ID 54713659 | Cabotegravir | LYS 178, GLU 217, ALA222 | -9.8 |
| 2. | PubChem ID 5291 | Imatinib | ALA 227, GLN 236, PHE 225 | -9.8 |
| 3. | PubChem ID 65947 | Prulifloxacin | SER21, MET23, HIS143, LYS 170 | -9.6 |

| | | | | |
|---|---|---|---|---|
| 4. | ZINC ID 4096134 | Limonin | SER21,MET 205, ALA127, LYS170. PHE 225. | -9.3 |
| 5. | DB09298 | Silibinin | PRO234, HIS143, ALA 127, GLN 236, ARG82. | -9.3 |
| 6. | PubChem ID 74989 | Atovaquone | LYS170, SER21, ARG28 | -9.1 |
| 7. | DB00443 | Betamethasone Valerate | SER21, MET23, HIS143,LYS 170 | -9.1 |
| 8. | PubChemID 52912222 | GSK1324726A | GLN 192, GLU217, VAL218 | -9.1 |
| 9. | ChemSpider ID32701987 | Isavuconazole | GLU208, LYS 207, LYS178 | -9.1 |
| 10. | DB06817 | Raltegravir | ARG82, GLU46, SER21 | -9.1 |

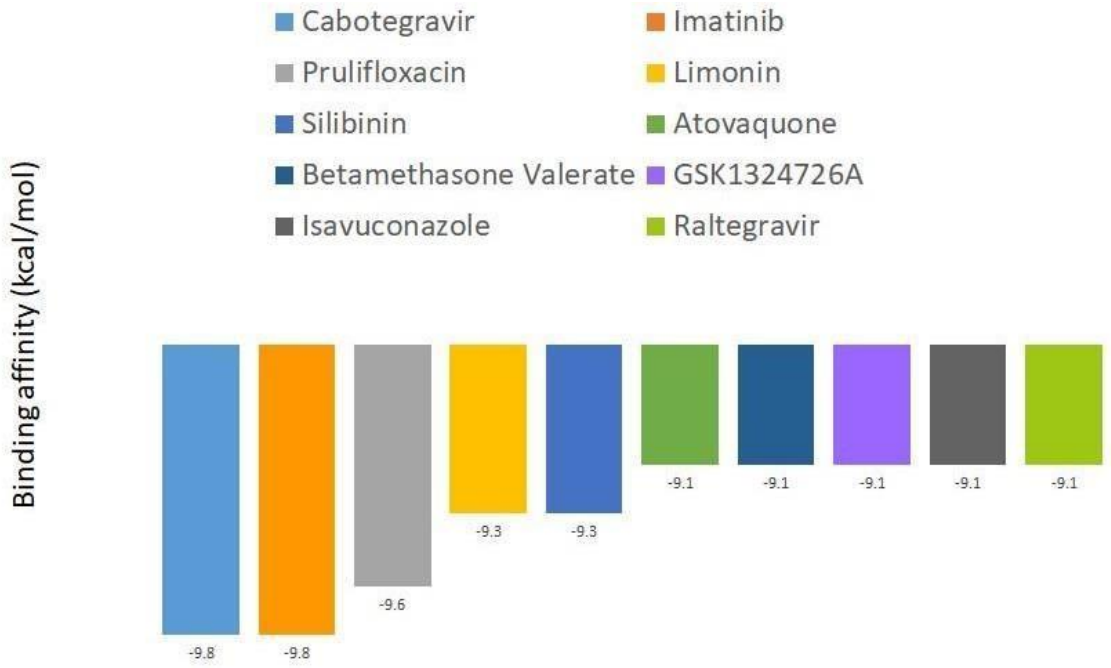


**Figure 13**. Based on their molecular docking scores—obtained from the AutoDock Vina software—the chart shows the protein DHQ1-ligands' best-docked complexes. From -9.8 to -9.1 kcal/mol are the docking energy ratings.

### 4.4. Lipinski's rule of five:

Ten ligands with the highest binding scores had their physicochemical properties assessed using PASS analysis and Lipinski's rule of five. Raltegravir and GSK1324726A both showed one Lipinski violation. The miLogP value of GSK1324726A was 5.6, which is higher than the normal value of 5. Additionally, the pharmacokinetic study of Raltegravir revealed 11 nON (hydrogen bond acceptors), compared to the standard value of 10. None of the other ligand compounds showed any violations. Eight out of ten ligand molecules successfully met the requirements, as the perfect lead compound should not show any violations.

**Table 4.** The ligand molecules that showed the highest scores for binding energy with the protein DHQ1 followed PASS analysis (Lipinski's rule of five).

| S/No. | Ligands | miLogP (≤5) | TPSA (≤140 Å2) | MW (≤500) | nON (≤10) | nOHNH (≤5) | Nrotb (≤10) | Lipinski's rule nviolations |
|---|---|---|---|---|---|---|---|---|
| 1. | Cabotegravir | 0.65 | 100.87 | 405.36 | 8 | 2 | 3 | 0 |
| 2. | Imatinib | 3.89 | 86.28 | 493.62 | 8 | 2 | 7 | 0 |
| 3. | Prulifloxacin | 0.16 | 109.13 | 461.47 | 9 | 1 | 4 | 0 |
| 4. | Limonin | 2.53 | 104.58 | 470.52 | 8 | 00 | 1 | 0 |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| 5. | Silibinin | 1.47 | 155.15 | 482.44 | 10 | 5 | 4 | 0 | |
| 6. | Atovaquone | 4.96 | 54.37 | 366.84 | 3 | 1 | 2 | 0 | |
| 7. | Betamethasone Valerate | 4.18 | 100.90 | 476.58 | 6 | 2 | 7 | 0 | |
| 8. | GSK1324726A | 5.61 | 69.64 | 434.92 | 5 | 2 | 4 | 1 | |
| 9. | Isavuconazole | 2.96 | 87.63 | 437.48 | 6 | 1 | 6 | 0 | |
| 10. | Raltegravir | -0.81 | 152.25 | 444.42 | 11 | 3 | 6 | 1 | |

**4.5. Prediction of Bioactivity:**

Except for Raltegravir, which was only moderately active, all of the ligands were biologically active as GPCR ligands, according to the bioactivity prediction. With the exception of atovaquone, which was extremely active, all of the ligands showed only modest biological activity as ion channel modulators. Nuclear receptor ligands and kinase inhibitors with modest activity included cabotegravir, isavuconazole, raltegravir, and limonin. It was shown that imatinib, ciprofloxacin, and silibinin were effective kinase inhibitors. Because the reading was below the threshold, betamethasone valerate was deemed inactive (-0.74). As protease and enzyme inhibitors, Cabotecvir, Limonin, Silibinin, Atovaquone, Betamethasone Valerate, and Raltegravir were shown to be physiologically active, but the remaining ligands had only modest action. The highest value was demonstrated by betamethasone valerate as an enzyme and protease inhibitor (i.e., 0.77 and 0.78, respectively). As a GPCR ligand, ion channel modulator, kinase inhibitor, nuclear receptor ligand, protease, and inhibitor, atovaquone was shown to be active. Table 4.3 provides the details of the anticipated bioactivity score for each understudy ligand. **Table 5**. Prediction of Bioactivity score of ligands:

| S/No. | Ligands | GPCR ligand | Ion channel | Kinase inhibitor | Nuclear receptor | Protease inhibitor | Enzyme inhibitor |
|---|---|---|---|---|---|---|---|

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | **modulator** | | | **ligand** | |
| 1. | Cabotegravir | 0.07 | -0.31 | -0.03 | -0.14 | 0.06 | 0.08 | |
| 2. | Imatinib | 0.10 | -0.09 | 0.59 | -0.40 | -0.08 | 0.07 | |
| 3. | Prulifloxacin | 0.12 | -0.22 | 0.00 | -0.08 | -0.21 | 0.13 | |
| 4. | Limonin | 0.18 | -0.11 | -0.45 | -0.45 | 0.06 | 0.34 | |
| 5. | Silibinin | 0.07 | -0.05 | 0.01 | 0.16 | 0.02 | 0.23 | |
| 6. | Atovaquone | 0.04 | 0.17 | -0.01 | 0.04 | 0.05 | 0.23 | |
| 7. | Betamethasone Valerate | | 0.08 | -0.15 | -0.72 | 1.38 | 0.78 | 0.77 |
| 8. | GSK1324726A | | 0.05 | -0.13 | -0.42 | 0.05 | -0.20 | -0.29 |
| 9. | Isavuconazole | 0.00 | -0.07 | 0.00 | -0.18 | -0.07 | -0.00 | |
| 10. | Raltegravir | -0.03 | -0.43 | 0.00 | -0.39 | 0.11 | 0.13 | |

**4.6. Pharmacokinetic property prediction of ligands:**

The only substance that could penetrate the blood-brain barrier was atovaquone, according to the ADMET investigations of the designated ligands. As permeability glycoprotein substrates (P-gp substrates), it was discovered that imatinib, ciprofloxacin, betamethasone valerate, GSK1324726A, isavuconazole, and raltegravir were positive, whereas the remaining ligands were negative. The results suggest that because the ligands are not Pgp substrates, their pharmacokinetic efficacy will increase due to their prolonged half-lives in the cells. All ligands, with the exception of silibinin, isavuconazole, and raltegravir, were shown to have substantial gastrointestinal absorption.

For incessant plasma concentration and enhanced bioavailability, it was anticipated that the ligands would block the following five classes of cytochromes: P450, i.e., CYP1A2, CYP2C19, CYP2C9, CYP2D6, and CYP3A4. There was no inhibition of any cytochrome P450 by cabotegravir. On the other hand, imatinib inhibited every P450 class save CYP1A2. Silibinin, limonin, and pulifloxacin did not inhibit CYP1A2, CYP2C19, or CYP2D6. Except for CYP2D6, all P450 classes were inhibited by atovaquone. All ligands, apart from limonin and cabotegravir, were shown to inhibit CYP3A4. The table lists the ligands' particular pharmacokinetic characteristics.

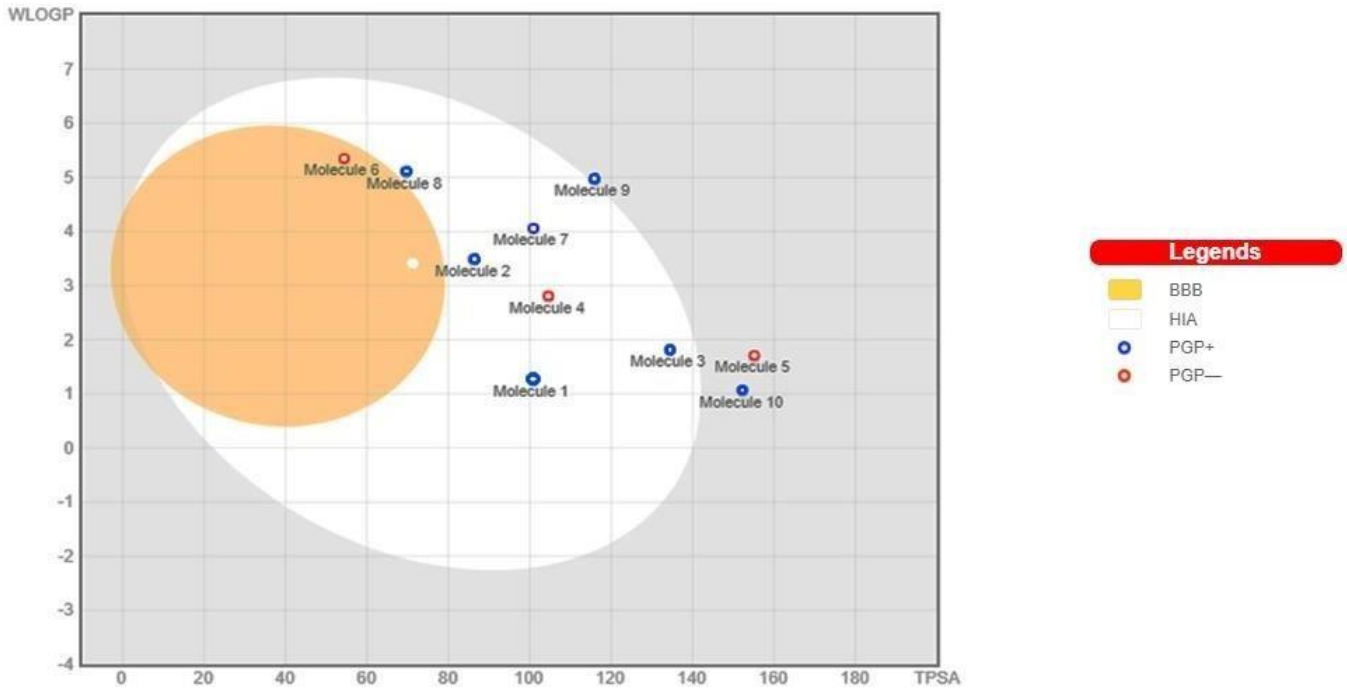
**Table 6**. The table depicts the pharmacokinetic properties prediction including cytochrome P450 classes, blood-brain barrier (BBB), P-gp and Gastrointesinal absorption of the understudy ligands.

| **S/No.** | **Ligands** | **CYP1A2 inhibition** | **CYP2C19 inhibition** | **CYP2C9 inhibition** | **CYP2D6 inhibition** | **CYP3A4 inhibition** | **P-gp substrate** | **BBB permeant** | **GI absorption** |
|---|---|---|---|---|---|---|---|---|---|
| 1. | Cabotegravir | No | No | No | No | No | No | No | High |
| 2. | Imatinib | No | Yes | Yes | Yes | Yes | Yes | No | High |
| 3. | Prulifloxacin | No | No | Yes | No | Yes | Yes | No | High |
| 4. | Limonin | No | No | No | No | No | No | High | |
| 5. | Silibinin | No | No | No | Yes | No | No | Low | |
| 6. | Atovaquone | Yes | Yes | Yes | No | Yes | No | Yes | High |
| 7. | Betamethasone Valerate | No | No | No | No | Yes | Yes | No | High |
| 8. | GSK13247 No 26A | Yes | Yes | No | Yes | Yes | No | High | |
| 9. | Isavuconaz | No | No | Yes | No | Yes | Yes | No | Low |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | ole | | | | | | | | |
| 10. | Raltegravir | No | No | No | No | Yes | Yes | No | Low |

As a graphical model for drug discovery and exploration, the BOILED-Egg analysis offers an easy-to-use, repeatable, and statistically sound technique to forecast blood-brain barrier (BBB) penetration and passive gastrointestinal absorption (HIA) (Alyar et al., 2023; Daina et al., 2017). Using the BOILED-Egg approach as an accurate prediction model, the link between the lipophilicity and polarity of the examined compounds 1–10 was analysed (Morak-Młodawska et al., 2023; Maliar et al., 2023). The yellow area (yolk) only contains molecule 6 (atovaquone), which indicates a high probability of brain penetration and absorption.

The remaining molecules, 1, 4, 2, 7, and 9, which are located in the egg white area, indicate a high probability of passive absorption by the digestive system. Moreover, P-gp (PGP+) is actively effluxing the ligands, as indicated by the blue dots in the yolk and white zone, but P-gp (PGP-) is not a substrate for them, as indicated by the red points. Moreover, the dots are colored red when P-gp is considered to be non-substrate (PGP−) and blue when P-gp is expected to be actively effluxed (PGP+).



**4.7. Interactions between target protein & ligands- 3D visualization.**

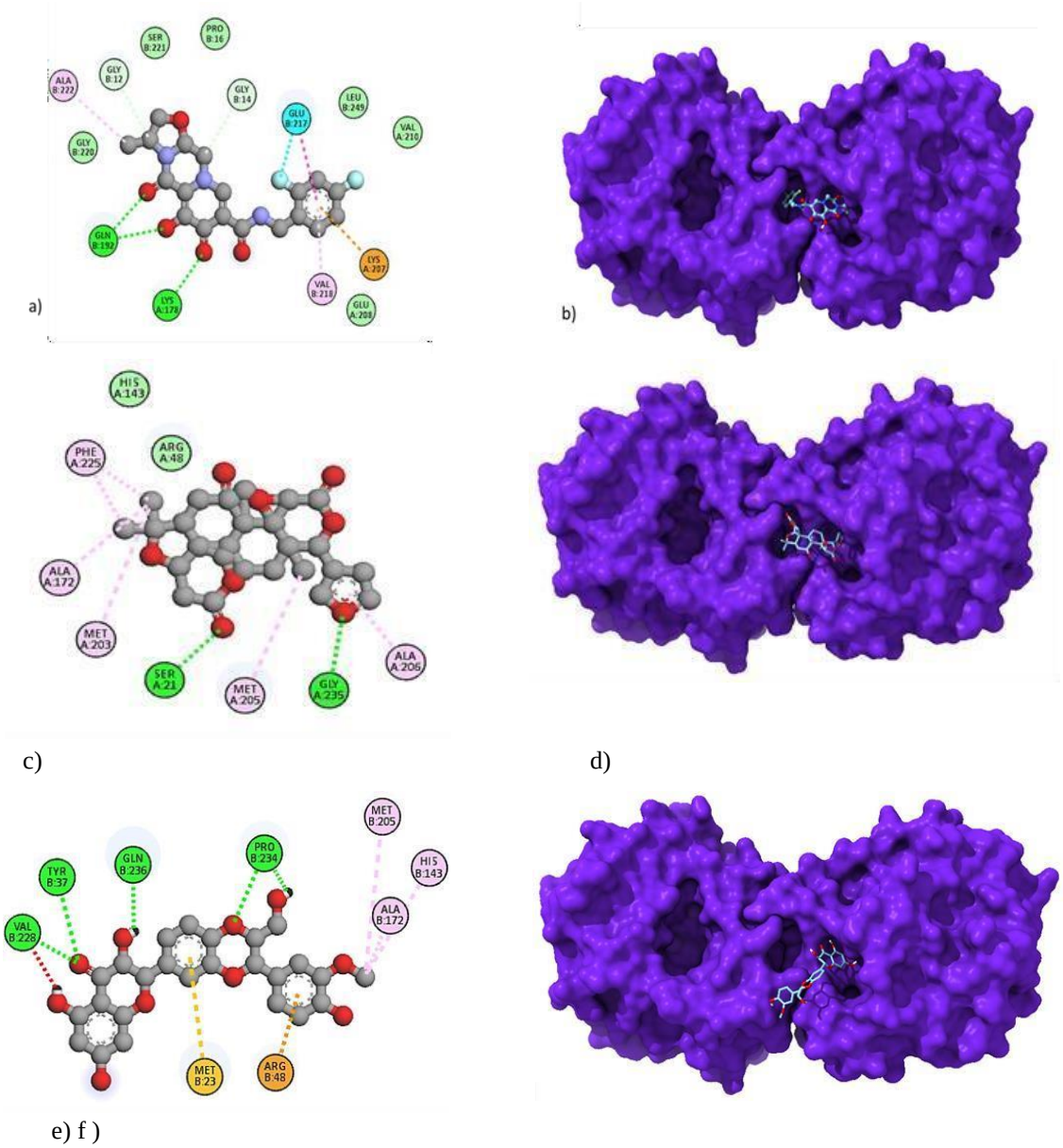



**Figure 14.** Docking positions of DHQ1 with best three ligands (a): Cabogravir (b) Limonin (c) Silibinin represented as 3-D models next to their 2D structures.

**4.8. MD simulation results:**

The DHQ1 protein tethered complex's structural stability and flexibility were assessed using Cabs-flex 2.0. The plot shows that the free state DHQ1 protein (positive control) has RMSF values of 6.04 Å. After binding with cabotegravir at residue ASP 54 chain A, the value decreased to 3.12 Å. In this case, the protein complex's stability was demonstrated by the decreased RMSF value.

Likewise, the Limonin-DHQ1 complex's RMSF value decreased to 3.06 Å at residue GLY 88 chain B, indicating stability in each atomic residue. The Silibinin-DHQ1 complex's RMSD plot revealed a reduced fluctuation pattern with strong stability, with a value of 4.04 Å at residue 87 in chain B. A lower value of RMSF indicates greater stability and less flexibility.

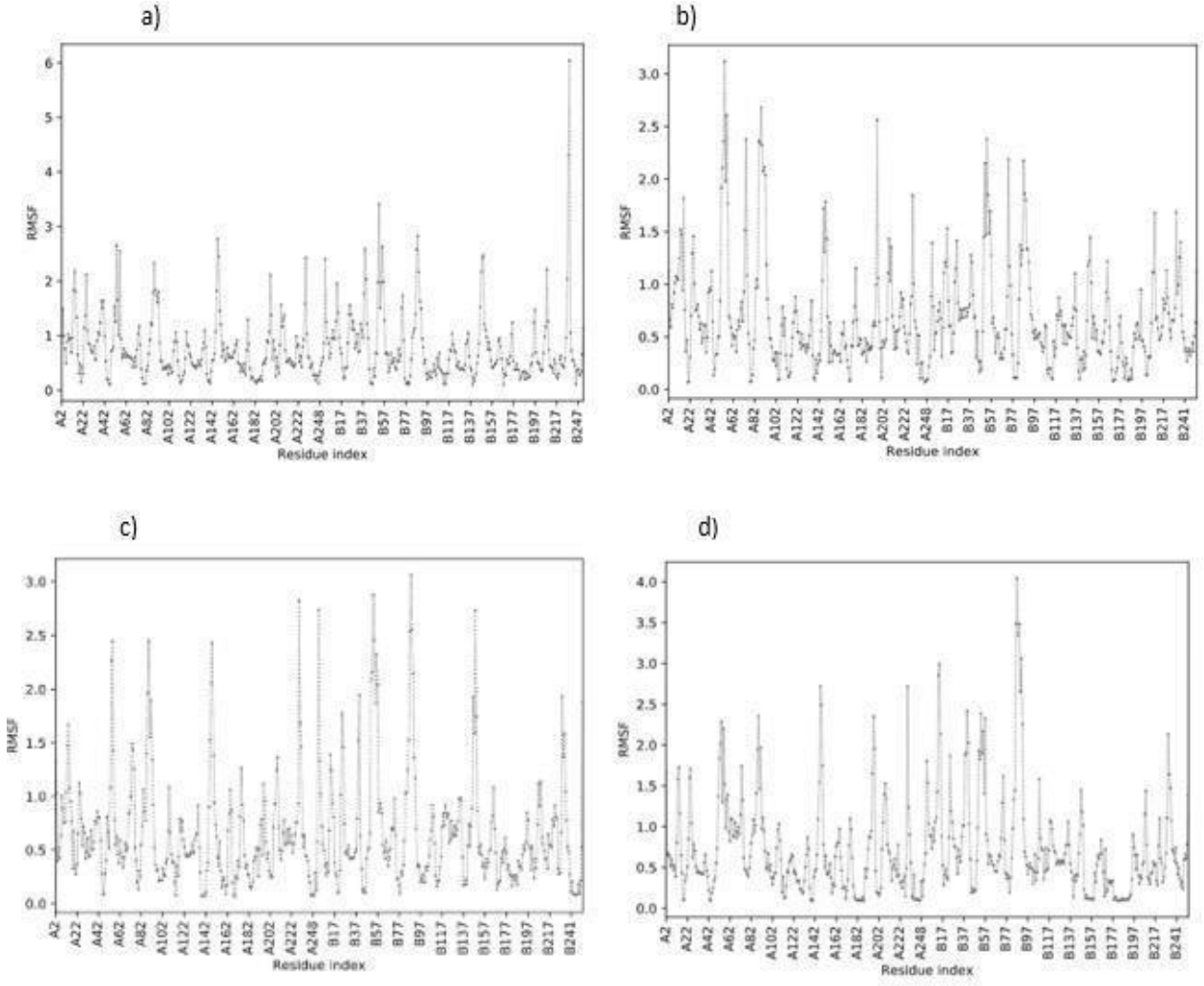



**Figure 15**. The value of the root mean square fluctuation (RMSF) of protein and ligand complex. (A) RMSF plot of bacterial protein DHQ1 6.04 Å; (B) RMSF plot of DHQ1-Cabotegravir docking complex (3.12 Å) at residue ASP 54 chain A. (c) RMSF plot of DHQ1-Limonin docking

# 5. Chapter 5: DISCUSSION

## 5.1. Discussion:

The Shikimate pathway enzyme is the focus of this study because it is essential for the synthesis of aromatic amino acids in bacteria, which promote the formation of biofilms and link SPI2 expression to surface-associated proliferation in *S.typhi* (Hamilton et al., 2020). The current study used pharmaceutical repurposing to investigate possible antibacterial compounds against

*Salmonella typhi's* highly conserved 3-dehydroquinate dehydratase type 1 (DHQ1) gene. This method is used to identify new chemical compounds that prevent a variety of serious viral, bacterial, and fungal infections (Joshi et al., 2022; Liu et al., 2021; Sencanski et al., 2020). DHQ types I and II have been the focus of several bacterial species' possible anti-virulence treatment research efforts (Rodríguez et al., 2023; Isa & M, 2020; Maneiro et al., 2019; González-Bello, 2014; Light et al., 2011).

Several investigations have demonstrated that anti-virulence therapeutics may target Type I DHQ (EC 4.2.10). This is due to the following: i) DHQ1 has the capacity to produce potent live oral vaccines and acts as a virulence factor in vivo after the deletion of the aroD gene, which codes for this enzyme (Rodríguez et al., 2023; Cunningham et al., 2020; Racz et al., 2013; Revolledo & Ferreira, 2012; Servos et al., 1991) ii) The aroD mutation makes bacteria auxotrophic, compromises the integrity of the cell wall and outer membrane, and prevents the production of biofilms (Sebkova et al., 2008; Chatfield et al., 1993), iii) In small colony variations, the DHQ1 enzyme is increased, which promotes recurrent infections. (Zhang et al., 2017; Malcova et al., 2009), iv) DHQ1 is present in various infective bacteria i-e, *Clostridium difficile, Escherichia coli, Staphylococcus aureus. v*) Since DHQ1 has no known mammalian homologues, it is a desirable target for the development of bactericidal drugs. (Lence et al., 2020).

To identify drug candidates for *S. typhi* that act as inhibitors of DHQ1, we also used a Virtual Screening (VS) technique based on successive screening. Lipinski's rule of five was applied to 551 FDA-approved compounds that may bind to DHQase and were obtained ApexBio database. Rule MW< 500 was used to select 206 ligand compounds before they were put via molecular docking. Based on the binding affinities, the top 10 compounds were determined to be Cabotegravir, Imatinib, Prulifloxacin, Limonin, Silibinin, Atovaquone, Betamethasone Valerate,

GSK1324726A, Isavuconazole, and Raltegravir. The ligands exhibited binding energies for DHQ1 ranging from -4.6 kcal mol−1 to -9.8 kcal mol−1. Based on the binding affinities, the top 10 compounds against DHQ1were determined to be: Cabotegravir=Imatinib>Prulifloxacin>Limonin=Silibinin>Atovaquone=BetamethasoneValerate =GSK1324726A=Isavuconazole=Raltegravir.

Additionally, using Molinspiration, the bioactivity score of ligands was calculated (https://www.molinspiration.com/), an online server. Atovaquone was found to be highly active as

cation channel modulator, GPCR ligand, nuclear receptor ligand, and enzyme and protease inhibitor whereas the rest of ligands depicted good to moderately biological activity. The bioactivity levels of a molecule reveal its overall potential as a lead compound. It effectively distinguishes between inactive structures and those with strong drug likeness. (Jeelani et al., 2021). Based on Lipinski's rule of five, which was computed using Molinspiration, the physiocochemical characteristics of the ligands revealed that only GSK1324726A and Raltegravir had one Lipinski violation. There were no violations observed in other ligand compounds. As of this point in the inquiry, every ligand fulfilled the criteria needed to be regarded as an exceptional lead chemical overall.

The pharmacokinetic properties were calculated to determine if the selected compounds were drug-like (Edache et al., 2021). Out of all the ligand compounds, only atovaquone was shown to be able to successfully cross the blood-brain barrier, according to the research conducted using Swiss-ADME. It was investigated if the proposed ligands could block the five kinds of cytochromes. P450, namely CYP1A2, CYP2C19, CYP2C9, CYP2D6, and CYP3A4, to understand the toxicity of drugs and their constant plasma concentration. With its highest binding affinity for DHQ1, Cabotegravir did not inhibit any cytochrome P450 enzymes. This indicates a minimal chance of toxicity and negative consequences. (Samuels & Sevrioukova, 2018). Except CYP2D6, atovaquone inhibited all P450 classes, which can result in drug aggregation and intoxication. All ligands—aside from limonin and cabotegravir—were intended to inhibit CYP3A4. Except silibinin, isavuconazole, and raltegravir, all the ligands had significant gastrointestinal absorption. Whereas Imatinib, Prulifloxacin, Betamethasone Valerate, GSK1324726A, Isavuconazole, Raltegravir were shown to be positive as permeability-enhancing glycoprotein substrates (P-gp substrates), the remaining ligands, however, were negative.

Analysis of the relationship between the lipophilicity and polarity of the compounds 1–10 under investigation was also done using the BOILED-Egg method, which serves as a credible prediction model for determining penetration into the intestines or brain. The compounds under examination exhibit both great permeability through the blood-brain barrier (BBB) and adequate binding to human blood albumin (HIA) (yellow area). Many of the chemicals under study are not able to pass across the blood-brain barrier (BBB) i-e, Cabotegravir, Imatinib, Prulifloxacin, Limonin, Silibinin, Betamethasone Valerate, GSK1324726A, Isavuconazole, Raltegravir. One potential explanation

might be that generic medications, especially tiny compounds, are not able to pass the blood-brain barrier (BBB) (Pardridge, 2005). Atovaquone is the only chemical detected in the yolk that suggests a high probability of brain penetration. There is a substantial probability that the gastrointestinal tract will passively absorb molecules 1, 4, 2, 7, and 9.

Using Discovery Studio, the post-docking analysis was carried out on the three molecular docking hit compounds that were selected. Hydrophobic and hydrogen bond formation were expected to be present in the active site residues of *S. typhi* DHQ1, Lys 178, Glu 217, Phe225, Ser21, Arg48, Arg82, His143, Lys170, Arg213, Gln236, Met205, and Ala227. (Maneiro et al., 2019). Previous studies have shown that *S.typhi* DHQ1 undergoes chemical modification by an epoxide ligand, which leads to the formation of a Schiff base containing Lys 170, highlighting the requirement for His143 in catalysis. (Tizón et al., 2015). In a research study, quinic acid- based hydroxylammonium derivatives were created to covalently alter the catalytic lysine residue of DHQ1 by directly attacking the ε-amino group of the lysine with NH2OH, followed by the necessary histidine residue deprotonating the lysine adduct. (Maneiro et al., 2019). In another investigation, the hydrolytic release of product from the active site was stopped and catalytic activity was reduced by 106 times when alanine was substituted for the His143 residue. In particular, the attachment of the substrate to the active site is facilitated by salt-bridges between its carboxylate group and Arg213 (González-Bello, 2014).

Imatinib (-9.8 kcal mol−1) interacted with Phe225, Ala227, Gln236, Ser 21, while Atovaquone with binding affinity of -9.1 kcal $mol^{-1}$ interacted with residues i-e. Ser21, Arg48, Lys170, Ala172, Phe225, Met205. GSK1324726A (-9.1 kcal mol−1) formed binding links with Lys207 as Pi-Cation, and Val218 as a Pi-Sigma bond, while forming conventional hydrogen bonds with Gln192, Gly12, Glu 217. An in-depth analysis of the interaction between *S.typhi.* DHQ1 and each ligand indicate a substantial contribution from hydrogen bonding, alkyl interactions, Pi- Cation interactions, Pi-Sigma bond, and carbon-hydrogen bonding (Kleanthous et al., 1990). The stability of the ligand-protein structure is mostly determined by hydrophobic interactions, with hydrogen bonding interactions playing a less significant role. The number of hydrogen bonds affects the docking scores since, among the other criteria, it maintains the most negative weighted factor value (Mukhtar et al., 2023).

The DHQ1's protein-ligand docked complexes structural flexibility and stability was estimated by CABS-flex 2.0 (Joshi et al., 2022). Throughout a 10-ns simulated period, changes in the protein-ligand complex system and macromolecule conformational stability were evaluated using RMSF (Kakhar Umar et al., 2023). The pharmacokinetic characteristics and bioactivity score of the ligands (Cabotegravir, Limonin, Silibinin) were taken into consideration while choosing them for MD simulation. The DHQ1 in a free state (positive control) RMSD plot indicates a higher RMSF value of 6.04 Å. The terminal amino acid residues (residues 227 to 239), or the hH loop, displayed a high RMSF value. Loops often allow for more conformational flexibility in proteins because they are less restricted by hydrogen bonds and other interactions that stabilise the structure. The ability of loop sections to move freely to raise RMSF values. Rosmalen et al., 2017).

After binding of Cabotegravir, the RMSF value lowered to 3.12 Å. Based on this, MD simulations confirmed the structural stability of each amino acid in the DHQ1/Cabotegravir docking complex. A loop region in the protein (ASP 54 to THR58) appears to be the source of the variation in the RMSF plot at residue ASP 54 chain A. According to Michelitsch and

Weissman (2000), ASP-rich areas often form rather unstructured loops that link α-helices and β-sheets. The residue GLY 88 chain B of the DHQ1-Limonin docking complex showed a peak at 3.06 Å on the RMSF plot. Analysis verified that the glycine residues may provide the flexibility needed for the enzyme to change into its active state. Because GLY loop sequences are so flexible, nearby residues can also be made to be flexible. Furthermore, compared to other amino acids, glycine residues show substantially faster loop formation (Krieger et al., 2005). Plot values mainly indicated that the complex's interacting amino acid residue was stable. The stability of each amino acid was disclosed by the DHQ1-Silibinin docking complex based on the RMSF plot (4.04 Å) during the 10-ns MD simulation. The study indicates that the GLY residue, which formed the loop region in the absence of hydrogen bonds, provides the flexibility of the protein structure. Variations may be seen at GLY 87 chain B in the plot. GLY loop sections provide protein structural flexibility, which causes atomic-level variations (Çınaroğlu & Biggin, 2023).

An FDA-approved antiretroviral medication called cabotegravir suppresses HIV infection by causing virologic suppression (Hodge et al., 2021). However, strains of S. aureus and E. coli have been shown to respond well to cabotegravir's antibacterial properties (Rawat et al., 2023). The in-vitro toxicity and antibacterial activity of the encapsulated cabotegravir with gold nanoparticles in

the research indicated that it was safe. Nevertheless, no investigation found that capegravir has anti-*Salmonella* properties. Tetracyclic triterpenoids like limonin, the second component, have significant biological action in plants. It is extracted from Rutaceae and Meliaceae plants and separated from fruits and traditional Chinese medicines. Limonin is a versatile substance with pharmacological applications including anti-oxidation, anti-cancer, anti- inflammatory, analgesic, and even anti-bacterial and anti-viral (Dong et al., 2016). Research also showed that after receiving limonin therapy, indicators of the toxins and Staphylococcus aureus infection were inhibited (Jia et al., 2023). Numerous investigations have documented the bacteriostatic impact of limonin on a range of pathogens, including *Shigella species, Escherichia coli, Salmonella, Micrococcus luteus, Bacillus thuringiensis, and Bacillus cereus*. Furthermore, limonin's A ring alteration at C-7 location strengthened its antibacterial action. (Tavares et al., 2015).

A significant bioactive component of milk thistle extract Silymarin is silibinin. For thousands of years, people have utilized milk thistle (Silybum marianum L. Gaernt.), a medicinal herb in the Asteracea family, to cure liver ailments (Li et al., 2023). Among silibinin's many pharmacological properties are its anti-apoptotic, anti-fibrotic, anti-oxidative, anti-diabetic, anti- inflammatory, and neuroprotective properties. Milk thistle is widely recognised as safe (GRAS) and does not show toxicity in animals (Khalid & Naseem, 2023). Studies on plant extracts that can block the efflux pump in Gram-negative bacteria that are resistant to several medications, including curcumin against *Pseudomonas aeruginosa*, plumbagin against *E. coli*, and thymol against *Salmonella enteritidis*, are also scarce. It has been demonstrated that milk thistle seed extract inhibits the MDR efflux pump STY4874 of *Salmonella typhi.* (Tariq et al., 2019).

# 6. Chapter 6: Conclusion:

Herein, drugs approved by the FDA were used in molecular docking and MD simulation studies to treat growing XDR typhoid by targeting the *S.typhi* DHQ1 shikimate pathway enzyme. The three high affinity ligands with the best pharmacokinetic characteristics were selected from 551 by use of simulation and molecular docking experiments. Utilizing repurposing techniques, we employed a successful approach to repurpose already existing, low-risk medications for the typhoid crisis. Molecular docking studies, PASS analysis, Lipinski's rule of five, bioactivity score (BAS) prediction, pharmacokinetic property prediction, and molecular dynamic simulation were used to confirm the drug potential of cabotegravir, limonin, and silibinin (binding affinities: -9.8,-9.3, and 9.3 kcal/mol, respectively). Consequently, extensive study suggests that drug molecules' capacity for binding may have bactericidal effects and might be used as a suitable therapeutic agent to treat *S.typhi* infection.

## 6.1. Future Prospects:

It is possible to confirm a compound's inhibitory activity by in vitro and in vivo testing to further assess the therapeutic potential of drugs against *S.typhi*. Very strong inhibitors for DHQ1 may be produced by developing catalytic residues, such as lysine and histidine targeted irreversible inhibitors, to stop the enzyme's catalytic pocket activity. Additionally, artificial intelligence may significantly improve medication development by leveraging big data to get access to international genetic data banks in the pharmaceutical sector, which can accelerate drug discoveries and lower R&D expenses. This may lead to the development of more precise and targeted sets of ligands with superior therapeutic qualities against a range of important pathogens that fuel the AMR phenomenon.

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