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Original Article

IN SILICO IDENTIFICATION OF NATURAL PRODUCTS WITH ANTITUBERCULOSIS ACTIVITY FOR THE INHIBITION OF INHA AND ETHR PROTEINS FROM *MYCOBACTERIUM TUBERCULOSIS*

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ABSTRACT

Objective: The rise of *Mycobacterium tuberculosis* (MT) strains that are resistant to antibiotics poses a serious threat to public health, particularly in middle and low-income countries. The important role of natural products (NPs) in the discovery of new drugs to treat infectious diseases is driving the success of synthetic chemistry in the production of new drugs. In our study, 15 NPs were selected to be investigated for their anti-TB properties by *in silico* method.

Methods: Molecular reverse docking approach to predict the interaction of NPs as a drug lead against the regulatory proteins (InhA, EthR) of MT. For each mycobacterial target, the docking scores/binding free energies were predicted and calculated using AutoDock Vina along with the physicochemical and structural characteristics of the NPs, and they were compared to the established inhibitor (control) drugs.

Results: The specific interactions of luteolin, piperine, butein, tiliacorinine against the targets InhA and EthA (-9.1 and-6.7 kcal. mol^{-1};-9.1 and-8.4 kcal. mol^{-1};-8.4 and-6.6 kcal. mol^{-1};-8.3 and-7.6 kcal. mol^{-1}) had significantly superior docking scores compared to controls.

Conclusion: Our research proposed these compounds as potent therapeutic agents for the development of anti-tuberculosis medications; however, additional *in vitro* and *in vivo* testing is required to confirm their potential as novel therapeutics and mechanisms of action.

Keywords: Tuberculosis, *Mycobacterium tuberculosis,* Multidrug resistance, Antibiotic, Natural products, Reverse docking

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INTRODUCTION

The most common infectious cause of death is *MT*, which causes the chronic infectious disease known as tuberculosis (TB). Approximately 10 million people are infected each year, making it a serious danger to public health around the world. It became increasingly challenging to stop the prevalence of TB infections when multidrug-resistant TB (MDR-TB), extensively drug-resistant TB (XDR-TB), and HIV co-infection emerged [1-4].

For many years, it has been believed that the first line in the fight against TB is made up of traditional anti-TB medications like isoniazid, rifampicin, ethambutol, and pyrazinamide. Recent years have seen an increase in the number of new anti-TB medications after a lengthy absence [5-7]. The two most recent anti-TB medications to be licensed in nearly 50 y are bedaquiline and delamanid. Resistance to bedaquiline and delamanid did, however, emerge with increased use in clinics. Despite the significant advancements made, the problem of rising drug resistance means that there is still an urgent need for new anti-TB drugs that are highly effective and toxic-free. In order to create new anti-TB medications, innovative pharmacological targets are still required [8-10].

In the rare case of failure, the infection begins to spread through lymphatic channels and blood circulation, which can result in serious harm to the lungs (pulmonary TB) and other body parts, such as the brain, spinal cord, lymph nodes, abdomen, bones and joints, intestinal system, and genitourinary system [11, 12]. In general, some of the clinical signs of active TB include continuous coughing for longer than three weeks, bloody sputum (hemoptysis), chest pain, fever, exhaustion/weakness, weight loss, anorexia, dyspnea, etc. Although the individual with latent tuberculosis infection (LTBI) may not exhibit any symptoms and cannot infect others with Mtb, they could yet acquire active TB [13, 14].

Its distinctive cell envelope shape, which has an abundance of lipids and special long-chain, strongly hydrophobic-alkyl-hydroxy fatty acids, mycolic acids (MAs), is one of the main obstacles to therapeutic interference against Mtb. By serving as a very effective permeability barrier, MA maintains the mycobacterium's structural integrity and vitality and increases its inherent resistance to host bactericidal agents or several kinds of antibiotics [15, 16]. Additionally, it defends against oxidative stress and causes macrophages to differentiate into foaming macrophages. As a result, we decided to focus on enoyl-acyl carrier protein reductase (InhA), a crucial enzyme involved in the production of MA, in our *in silico* method; furthermore, the negative transcriptional regulator EthR, which lowers the production of the EthA enzyme [17, 18]. The inhA gene encoding enoyl-acyl carrier protein (ACP)-reductase, an enzyme involved in the formation of long chain fatty acids (mycolic acids) and the reduction step of fatty acid synthesis. The inhA gene is an appropriate target for drug development since mutation of the inhA gene promotes resistance to a number of first-line medications, such as isoniazid [19-21]. The transcriptional repressive EthR, a member of the tetR/CamR family, controls the synthesis of the EthA enzyme, which is necessary for the stimulation of the thiocarbamide-containing medication ethionamide. The probability of Mycobacterium tuberculosis developing drug resistance is increased by the EthA enzyme's poor activation of ethionamide. This process suggests that it is important as a therapeutic target for drug resistance to these drugs [22-24].

The development of multidrug resistance in TB due to its low efficacy, toxicity, and higher expense associated with the extended administration of second-line medications for around 20 mo, TB made the treatment of TB more difficult. Furthermore, the treatment has become more difficult due to the limited efficacy of available medications due to an estimated 6.2% incidence of extensively drug-resistant TB (XDR-TB) cases among patients with MDR-TB cases worldwide in 2016 [25-27].

TB is a manageable infection, but the widespread rise of drug resistance makes it a worldwide problem. It is evident that shorter, easier, more cost-effective, tolerant, and safe medication regimens are required in order to advance the existing TB treatment and circumvent the rise in drug resistance. Our *in silico* research explores the performance of novel drugs in an effort to lessen the impact of potential drug resistance scenarios. A total of fifteen drug-like compounds were selected to evaluate their efficiency in the clinical management and treatment of TB. The InhA (PDB ID: 3FNG) and EthR (PDB ID: 3G1M) receptor proteins were used in our

experiment to assess the binding affinity of fifteen ligands using two separate docking technologies, Autodock Vina software.

MATERIALS AND METHODS

Materials

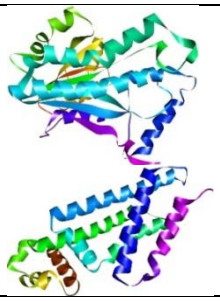
Softwares and tools

AutoDockVina 1.1.2, ChemBio3D, admetSAR, PROTOX-II, Avogadro, Open Babel, Protein Data Bank (PDB), PubChem.

Protein preparation

Selected target proteins with the InhA (PDB ID: 3FNG) and EthR (PDB ID: 3G1M) receptor protein properties (table 1) had their 3D crystal structures downloaded from the RCSB PDB (http://www.rscb.org/pdb). All proteins contained co-crystallized ligands (X-ray ligands) in their binding sites. The target receptor molecule was freed of these complexes, which contained heteroatoms and extra water molecules. Finally, hydrogen atoms were acquired by the target receptor molecule [21].

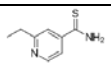
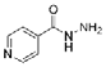
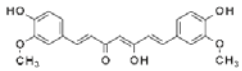
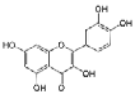
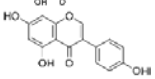
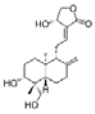
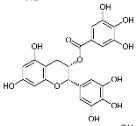
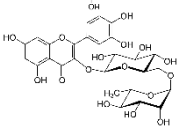
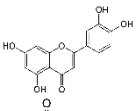
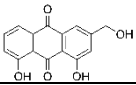
Table 1: Target proteins in regulators (InhA, EthR) of *M. tuberculosis*

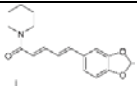
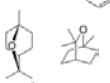
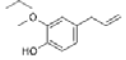
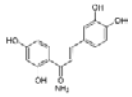
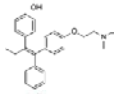
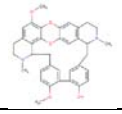
PDB ID	Name of protein	3D structure
3FNG	MT of InhA (enoyl-acyl carrier protein reductase) is a crucial enzyme of the fatty-acid synthase II system involved in the production of mycolic acid. It is the main target of the first-line anti-tuberculosis medication isoniazid.	
3G1M	EthR is a transcriptional repressor from the tetR/CamR family that inhibits the production of the enzyme needed to activate the thiocarbamide-containing medication ethionamide.	

Ligand preparation

In order to evaluate the efficiency of these compounds in the clinical care of TB, a total of 15 NPs of drug-like compounds were identified; curcumin, quercetin, genistein, andrographolide, epigallocatechin gallate, rutin, luteolin, aloe emodin, piperine, eucalyptol, eugenol, butein, cytarabine, tamoxifen, tiliacorinine (table 2). Antibiotics such as isoniazid (INH) and ethionamide (ETH) were used as the standard of control. The 2-dimensional structures of all the NPs and antibiotics were retrieved in the. sdf format from Pubchem (www. pubchem. com). The compounds. sdf file was additionally translated to PDB format using Open Babel [21].

Table 2: Molecular structure of selected natural products and standard drugs

Drug identifiers	Natural products/Standard drugs	Molecular structures
Control 1	Isoniazid (INH)	
Control 2	Ethionamide (ETH)	
1	Curcumin	
2	Quercetin	
3	Genistein	
4	Andrographolide	
5	Epigallocatechin Gallate	
6	Rutin	
7	Luteolin	
8	Aloe Emodin	

Drug identifiers	Natural products/Standard drugs	Molecular structures
9	Piperine	
10	Eucalyptol	
11	Eugenol	
12	Butein	
13	Cytarabine	
14	Tamoxifen	
15	Tiliacorinine	

***In silico* drug-likeness and toxicity predictions**

Lipinski *et al.* predicted the existence of drug-like effects in NPs based on a well-established theory. The NPs structures were converted into their typical simplified molecular-input line-entry system (SMILE). The number of hydrogen donors and acceptors, the number of rotatable bonds, and the total polar surface area of a chemical were among the information they submitted to the SwissADME and PreADMET tools to compute *in silico* pharmacokinetics. PreADMET was utilized for predicting the organ toxicity and toxicological endpoints of the isolated compounds. Compounds were chosen as drug candidates using a criterion known as the drug score. With increasing drug score values, a chemical has a higher possibility of being considered a drug candidate. The website's server has the molecular PDB structures available at http://www.scfbio-iitd.res.in/utility/lipinskiFilters.jsp.

Docking studies using autodock vina

The target proteins' energy-minimized structures for NPs and ligands (positive control) were docked using AutoDock Vina 1.1.2. The receptor and ligand files, definitions of ligand atom types, and topological features (rotatable bonds) were all represented in the PDBQT file format, a modified version of pdb, which also included the atomic charges. The entire receptor was enclosed in a grid box for docking, with a grid spacing of 1, keeping the ligand flexible and the receptor rigid. The ligand's side chain and backbone could be altered, enabling interaction with the receptor in any possible arrangement. Docking runs were initiated using the command prompt after the receptor-ligand and binding site preparations were completed. The interaction energy between the ligand and receptor at the whole binding region was calculated and reported as affinity (kcal/mol). For the ligand and receptor, the total binding site interaction energy was calculated and reported as affinity (kcal/mol).

RESULTS

***In silico* pharmacokinetics (Drug-likeness) and toxicity analysis**

Based on Lipinski's rule of five, the SwissADME tool was used to estimate *in silico* pharmacokinetic features (drug-likeness attributes). The structures of bioactive compounds were converted to their standard simplified molecular-input line-entry system (SMILE). Lipinski's five-parameter rule, which states that hydrogen-bond acceptors (HBAs) should be less than 10, hydrogen-bond donors (HBDs) should be less than 5, molar refractivity (MR) should be between 40 and 130, log P shouldn't be less than 5, and molecular mass should be less than 500 Da, should be adhered to by the drugs and/or candidates [21].

The docking-ready ligands were used to validate Lipinski's rule of five. Using the described or readily available peptide inhibitors or inducers as positive controls, the target proteins were also assessed (table 2). The "drug-likeness" hypothesis predicts if a certain organic compound demonstrates properties typical of an orally active medication. SwissADME predicted that the examined compounds in this study would be orally active since they followed Lipinski's rule of five.

Table 3: Lipinski's rule-screened ligand molecule physiochemical characteristics

Drug identifiers	MW (g/mol)	NHD	NHA	Log P	MR	Lipinski's rule
Control 1	137.14	2	11	0.78	35.13	Yes
Control 2	166.24	1	13	1.98	49.47	Yes
1	368.38	2	26	3.37	102.8	Yes
2	302.24	5	16	1.99	78.04	Yes
3	270.24	3	14	2.58	73.99	Yes
4	350.45	3	35	1.96	95.21	Yes
5	458.37	8	29	2.23	112.06	Yes
6	610.52	10	45	1.69	141.38	Yes
7	286.24	4	15	2.28	76.01	Yes
8	270.24	3	15	1.37	69.92	Yes
9	285.34	0	23	2.94	85.48	Yes
10	154.25	0	19	2.74	47.12	Yes
11	164.2	1	14	2.13	49.06	Yes
12	272.25	4	17	2.41	74.34	Yes
13	243.22	4	20	-1.98	55.85	Yes
14	371.52	0	31	6.0	119.72	Yes
15	576.68	1	43	6.71	174,11	Yes

Abbreviations: NHD, number of hydrogen donor; NHA, number of hydrogen acceptor; MR, molar refractivity

ADMET properties

Swiss ADMET was employed to forecast the outcomes of studies on the assimilation, distribution, metabolism, excretion, and toxicity (ADMET) of bioactive substances. The skin's permeability value (Kp), which is quantified in cm/s, reveals how well the skin can absorb compounds. Skin permeability, or Kp, values for all compounds ranged from-3.50 to-9.30 cm/s *in silico*, showing weak skin permeability. Additionally, the penetration of the GI system and the blood-brain barrier (BBB) show that medication molecules are absorbed and distributed. The outcomes of the *in silico* analyses for the tested drugs' absorption, distribution, metabolism, and excretion (ADME) are displayed in (table 3). Only compounds (5, 6, 13, 14) showed low gastrointestinal (GI) absorption, according to ADME Swiss prediction parameters, whereas the other compounds showed high absorption. Similarly, Swiss ADME predictions revealed that only bioactive compounds (9, 10, 11) showed blood-brain barrier (BBB) penetration. Additionally, a variety of cytochromes (CYPs) control how drugs are metabolized, with CYP1A2, CYP2C19, CYP2C9, CYP2D6, and CYP3A4 being particularly important for the biotransformation of drug compounds. Thus, *in silico* SwissADME predictions, only compounds (4, 6, 14) have glycoprotein (P-gp) permeability.

Table 4: ADME predictions of bioactive compounds

No	Skin permeation value (log Kp) cm/s	GI absorption	BBB permeability	Inhibitor interaction (SwissADME/PreADMET)					
				P-gp substrate	CYPIA2	CYP2CI9	CYP2C9	CYP2D6	CYP3A4
C 1	-7.63	High	No	No	No	No	Yes	No	No
C 2	-7.63	High	No	No	No	No	No	No	No
1	-6.05	High	No	No	No	No	Yes	No	Yes
2	-7.05	High	No	No	Yes	No	No	Yes	Yes
3	-6.05	High	No	No	Yes	No	No	Yes	Yes
4	-6.09	High	No	Yes	No	No	No	No	No
5	-8.27	Low	No	No	No	No	No	No	No
6	-10.26	Low	No	Yes	No	No	No	No	No
7	-6.25	High	No	No	Yes	No	No	Yes	Yes
8	-6.66	High	No	No	Yes	No	No	No	Yes
9	-5.58	High	Yes	No	Yes	Yes	Yes	No	No
10	-5.30	High	Yes	No	No	No	No	No	No
11	-5.69	High	Yes	No	Yes	No	No	No	No
12	-5.96	High	No	No	Yes	No	Yes	No	Yes
13	-9.30	Low	No	No	No	No	No	No	No
14	-3.50	Low	No	Yes	No	Yes	No	Yes	No
15	-5.51	High	No	No	No	No	No	No	No

Abbreviations: GI, gastro-intestinal; BBB, blood brain barrier; P-gp, P-glycoprotein; CYP, cytochrome-P

According to the outcomes of acute toxicity prediction, such as toxicity class categorization and LD_{50} values, none of the discovered compounds have acute toxicity. The toxicological prediction gives information on endpoints like hepatotoxicity, carcinogenicity, mutagenicity, and cytotoxicity. The outcomes of the PreADMET and ProTox property explorer prediction evaluations are shown in table 4. Consequently, based on ADMET prediction analysis, bioactive substances might be suitable candidates for this investigation.

Table 5: Toxicity prediction of compounds computed by PreADMET and ProTox

No	LD_{50} (mg/kg)	Toxicity class	Organ toxicity				
			Hepatotoxicity	Carcinogenicity	Immunotoxicity	Mutagenicity	Cytotoxicity
C 1	133	3	Active	active	Inactive	Inactive	Inactive
C 2	1000	4	Inactive	Inactive	active	Inactive	Inactive
1	2000	4	Inactive	Inactive	active	Inactive	Inactive
2	159	3	Inactive	active	Inactive	active	Inactive
3	2500	5	Inactive	Inactive	Inactive	Inactive	Inactive
4	1890	4	Active	Inactive	active	Inactive	Inactive
5	1000	4	Inactive	Inactive	Inactive	Inactive	Inactive
6	5000	5	Inactive	Inactive	active	Inactive	Inactive
7	3919	5	Inactive	active	Inactive	active	Inactive
8	5000	5	Inactive	Inactive	Active	active	Inactive
9	330	4	Inactive	active	Inactive	Inactive	Inactive
10	2480	5	Inactive	Inactive	Inactive	Inactive	Inactive
11	1930	4	Inactive	Inactive	Inactive	Inactive	Inactive
12	1000	4	Inactive	active	active	Inactive	Inactive
13	826	4	Active	active	Inactive	Inactive	Inactive
14	1190	4	Active	Inactive	Active	Inactive	Inactive
15	1700	4	Inactive	Inactive	Active	Active	Inactive

Docking study with AutoDock vina

The docking algorithm provided by Auto Dock Vina was used to analyze the best-docked configuration between the ligand and protein, table 5 shows the docking scores (binding affinity) of the ligands in kcal/mol. The review of the molecular docking revealed that the binding affinities of all the chemicals range between (-1.1 and-9.1 kcal/mol).

For this kind of docking, a higher docking score is preferable. For the purpose of researching how ligands interact with the target receptor, the Discovery Studio Visualizer found the conformations with the most beneficial (least) free binding energy.

For this kind of docking, a higher docking score is preferable. For the purpose of researching how ligands interact with the target receptor, the Discovery Studio Visualizer found the conformations with the most beneficial (least) free binding energy.

Table 6: The score of the binding affinities of fifteen ligands and the control drugs for the receptors (InhA, EthR)

Drug identifier	Drug name	H-bond		Binding affinity (kcal/mol)	
		InhA	EthR	InhA	EthR
Control 1	Isoniazid (INH)	4	-	-4.2	-
Control 2	Ethionamide (ETH)	-	2	-	-4.6
1	Curcumin	3	-	-5.5	-4.5
2	Quercetin	3	2	-6.7	-5.8
3	Genistein	1	2	-4.9	-6.2
4	Andrographolide	1	-	-5.6	-4.9
5	Epigallocatechin gallate	1	2	-2.4	9.1
6	Rutin	4	5	-7.7	-7.1
7	Luteolin	2	2	-9.1	-6.7
8	Aloe emodin	1	3	-6.2	-4.8
9	Piperine	1	3	-9.1	-8.4
10	Eucalyptol	-	1	-1.1	-1.2
11	Eugenol	1	2	-4.9	-5.3
12	Butein	2	2	-8.4	-6.6
13	Cytarabin	2	1	-5.2	-4.7
14	Tamoxifen	2	2	-6.4	-5.5
15	Tiliacorinine	3	2	-8.3	-7.6

DISCUSSION

Computational techniques, especially when using the molecular docking methodology, have become incredibly helpful in pharmaceutical research because of their ability to find and make novel, promising molecules. These techniques have been utilized by scientists from many research groups to identify potential new chemicals that have the potential to treat a variety of diseases [21].

The proteins InhA and EthR are linked to critical functions. InhA is involved in the production of mycolic acids, which are necessary components of the mycobacterial cell wall, and EthR is a monooxygenase EthA repressor that causes resistance to ethionamide [28, 29]. In our investigation, molecular docking analysis was used to carry out virtual screening of high-affinity ligands. For the initial docking assessment, 15 NPs were separately docked via AutoDock Vina against the InhA and EthR receptor proteins. In order to choose two ligands for the InhA protein and two ligands for the EthR protein, lower binding scores were utilized to calculate binding energy and bound conformation using the Autodock Vina software. Both the InhA and EthR proteins perform crucial roles; InhA is involved in the production of mycolic acids, a component of the mycobacterial cell wall, and EthR is a monooxygenase EthA repressor that results in resistance to ethionamide [30].

Our studies showed the efficacy of all NPs (except compounds number 5, and 10) in terms of their pharmacokinetic or binding affinity. These compounds show a more dominant binding affinity to *M. tuberculosis*'s InhA (enoyl-acyl carrier protein reductase) receptor targets compared to isoniazid as the first-line anti-tuberculosis medication. These results indicate that the active compound has an inhibitory mechanism for an important enzyme of the fatty acid synthase II system which is involved in the production of mycolic acid, which is the main target of first-line anti-tuberculosis treatment. The InhA gene is a suitable target for drug discovery since mutations in it promote resistance to a number of first-line medications, including isoniazid [19]. Aiming to confirm the mode of action of the compounds with their discovered anti-mycobacterial activity, they were docked against the InhA enzyme because the MTB inhibitory action suggested that InhA could be a possible molecular target for the compounds mentioned in the title. In addition, all NPs (except compounds 1 and 10) also showed inhibitory activity against transcriptional repressors of the tetR/CamR family that inhibit the production of enzymes necessary to activate thiocarbamide-containing ethionamide drugs (ETH) [31, 32].

The technique referred to as "drug-likeness" assesses a drug-like molecule's solubility, chemical stability, bioavailability, and distribution profile qualitatively. A method of analysis known as Lipinski's Rule of Five was developed to assess the "drugability" of novel chemical compounds possessing specific pharmacological or biological properties. The 15 NPs fulfills all requirements of the ADMET and Lipinski Rule of 5 [33-35].

By using a variety of bioinformatics approaches, our work intends to establish a connection between tuberculosis and antibacterial medications that are presently on the market. This method can get over the experimental challenges of screening tens of thousands of ligands for TB. Our findings need to be validated by *in vivo* study using an animal model. The curated dataset can be of considerable interest to researchers in this sector who are looking for new anti-TB medications.

CONCLUSION

Docking studies on 15 NPs provide information that luteolin, piperine, butein, tiliacorinine have specific interactions against the targets InhA and EthA with significantly superior docking scores compared to controls. Our research proposed these compounds as potent therapeutic agents for the development of anti-tuberculosis medications; however, additional *in vitro* and *in vivo* testing is required to confirm their potential as novel therapeutics and mechanisms of action.

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AUTHORS CONTRIBUTIONS

All the authors contributed equally.

CONFLICT OF INTERESTS

Declared none

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