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Identification of novel scaffold using ligand and structure based approach targeting shikimate kinase

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ABSTRACT

Tuberculosis (TB) remains a major global health problem. It causes ill-health among millions of people each year and rank as the second leading cause of death from an infectious disease worldwide, after the human immunodeficiency virus (HIV). Shikimate kinase is one of the major enzymes targeted for TB. Most approaches to overcome TB were based on synthesis and screening of a known compounds to obtain a few representatives with desired potency. In this study, we have applied a virtual screening approach which combines ligand- and structure-based approaches to screen a large library of compounds as a starting point for the identification of new scaffolds for the development of shikimate kinase inhibitors. The combined approach has identified **2 new scaffolds** as potential inhibitors of shikimate kinase. To prove the approach, few of the molecules and their derivatives, a total of **17 compounds**, were synthesized. The compounds were tested for biological activity and shows moderate activity against shikimate kinase. The shikimate kinase enzyme inhibition study reveals that the compounds showed inhibition (IC_{50}) at concentrations of 50 μg/mL (Compounds **21, 22, 24, 25, 26, 27, 30, 32, 34**) and 25 μg/mL (**14, 19, 23, 31, 33**).

1. Introduction

Tuberculosis (TB) remains a global health concern with about 10 million cases in 2018 [1]. TB remains the top infectious killer worldwide. Although some countries are significantly accelerating their TB response, most World Health Organization (WHO) regions and many high-burden countries are still not on track to reach the 2020 goal of the End TB strategy. TB is a communicable disease that is a major cause of ill health, also one of the top 10 causes for death worldwide and the leading cause of death for a single infectious agent ranking above Human Immuno Virus (HIV). It is caused by the bacillus *Mycobacterium tuberculosis* [2], which is spread when people who are sick with TB expel bacteria into the air.

There are an estimated 1.2 million TB deaths among HIV-negative people in 2018 and an additional 251,000 deaths among HIV-positive people. Globally 7.0 million new cases of TB were notified in 2018 [1], an increase from 6.4 million notified 2017. Most of the increase in global notifications of TB cases since 2013 is explained by trends in India and Indonesia, the two countries that rank first and third world wide in terms of estimated cases per year. The recommended standard chemotherapeutic regimen for TB treatment is prescribed under Directly Observed Treatment Short-course (DOTS), lasting for minimum 6 months [3].

Currently there are 23 drugs for the treatment of drug-susceptible TB, multidrug-resistant TB are in phase I, II and III trials. These drugs consist of 13 new compounds, three other drugs (Bedaquiline, Delamanid and Pretomanid) that have already got the regulatory approval and 7 repurposed drugs [4]. FDA approved TB drug Bedaquiline (Sirturo) for the treatment of Multi Drug Resistant – TB (MDR-TB) [5,6] Sirturo precisely inhibits mycobacterial adenosin 5-triphosphate synthase, an enzyme that is essential for the final step in ATP production by oxidative phosphorylation [7]. Its related risk of potentially lethal heart problems has emphasized the unmet and urgent need for the development of safer antitubercular drugs with new targets and new mechanisms of action to treat resistant forms of the TB disease.

One promising mechanism for antitubercular agent is the inhibition of shikimate kinase (SK) in the shikimate pathway [8]. The shikimate pathway is used in a variety of bacteria, including *Mycobacterium*

Abbreviations: TB, Tuberculosis; DOTS, Directly Observed Treatment Short-course; MDR-TB, Multi Drug Resistant Tuberculosis; MtSK, Mycobacterium tuberculosis shikimate kinase; MACCS, Molecular access system; MOE, Molecular operating environment

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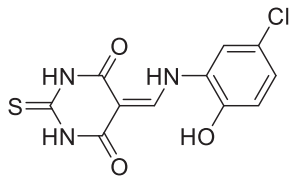
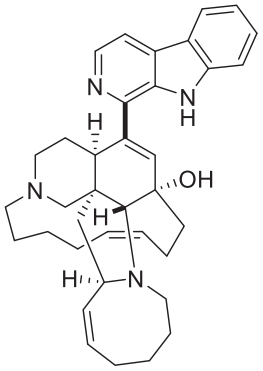


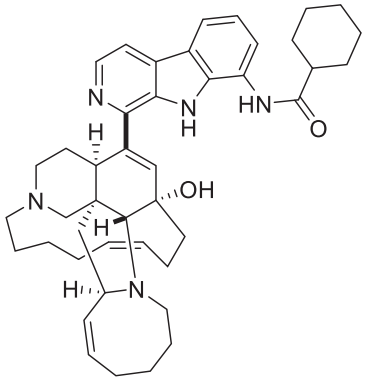
1

IC50 = 5.10 μM
(Rajput VS et al., 2016) [15]



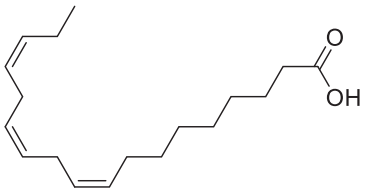
2

Manzamine A
IC50 = 10 μM
(Simithy J et al., 2018) [16]



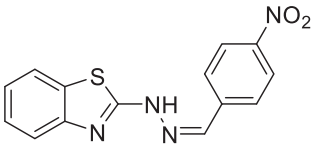
3

6-cyclohexamidomanzamine A
IC50 = 0.06 μM
(Simithy J et al., 2018) [16]



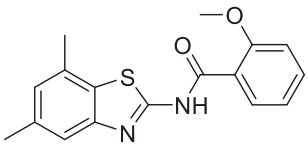
4

Alpha linolenic acid
IC50 = 3.7 μg/ml
(Masoko P, et al., 2016) [17]



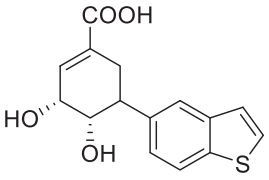
5

IC50 = 10.69 μM
(Mehra R et al., 2016) [18]



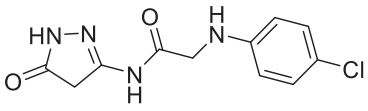
6

IC50 = 46.22 μM
(Mehra R et al., 2016) [18]



7

MIC value of 4 μg/mL
(Prado V, et al., 2016) [19]



8

IC50 = 5.84 μM
(Krishnasamy SK et al., 2016) [20]

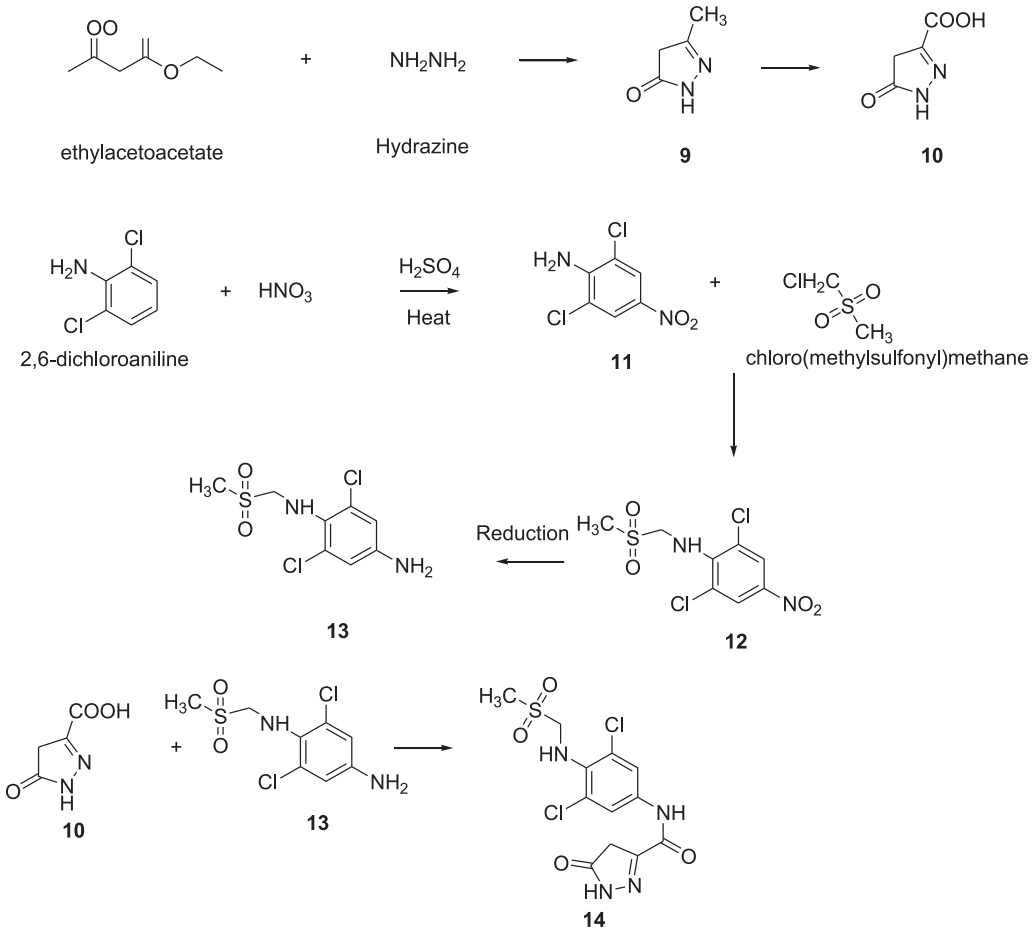
Fig. 1. List of shikimate kinase inhibitors reported in the literature.

tuberculosis, for the production of chorismite, a precursor for aromatic amino acids [9] and other aromatic compounds [10]. Mammals do not have the shikimate pathway enzymes necessary for de novo synthesis of these amino acids but rather obtain them from the diet [10]. Therefore, inhibitors of SK are anticipated to be discriminating antitubercular drugs. Shikimate kinase is the one of the seven enzymes involved in the shikimate pathway.

Shikimate Kinase in *Mycobacterium tuberculosis* (MtSK), is the fifth enzyme in the pathway, catalyzes the phosphorylation of shikimate (SA) using ATP as a phosphoryl donor to form shikimate 3-phosphate (S3P) and ADP [11]. With no mammalian counterpart, MtSK represents a favorable target for the design of drugs specific to the M. tuberculosis pathogen with reduced risk of toxicity in the human host [12,13]. Shikimate kinase is encoded by aroK and is essential for the survival of *Mycobacterium tuberculosis* [14].

1.1. List of shikimate kinase inhibitors

To identify new scaffolds as inhibitors of shikimate kinase, we selected two computational approaches the ligand-based screening method, similarity search and the structure-based approach, molecular docking. The ligand-based screening approach is based on the hypothesis that molecules with similar structure possibly have similar activity. Thus, for the initial screening and understanding of current available scaffolds, we collected the shikimate kinase inhibitors which were reported in the literature. Then the similar structures are screened using the crystal structure of shikimate kinase for identifying the potential interaction required for the scaffold to act as inhibitors of shikimate kinase. As a first step, the list of potent shikimate kinase inhibitors reported in the literature is shown in Fig. 1 [15–20].



Scheme 1. Procedure for synthesis of Compound **14**.

2. Materials and methods

2.1. Compound dataset

Enamine database is a commercial supplier of chemicals with more than 15 million small molecules which are categorized into building blocks, fragments and screening compounds (www.enamine.net). They have introduced one of the largest databases which contains more than 720 million synthetic accessible drug-like molecules called as Readily Accessible (REAL) database. The compounds in the database are synthesizable using one-pot synthesis protocols. The REAL database is further classified into drug-like, lead-like, fragments, covalent modifiers, chemical classes and natural product-like compounds. For our hit-identification, we have downloaded REAL Diverse drug-like database with over 15 million diverse compounds. The compounds in the database comply with "rule of 5" and Veber criteria with molecular weight maximum of 500, SlogP contains less than 5, Hydrogen bond acceptors and donors with less than or equal to 10 and 5, respectively and TPSA $\leq$ 140. In addition, the database is with the absence of PAINS and toxic compounds.

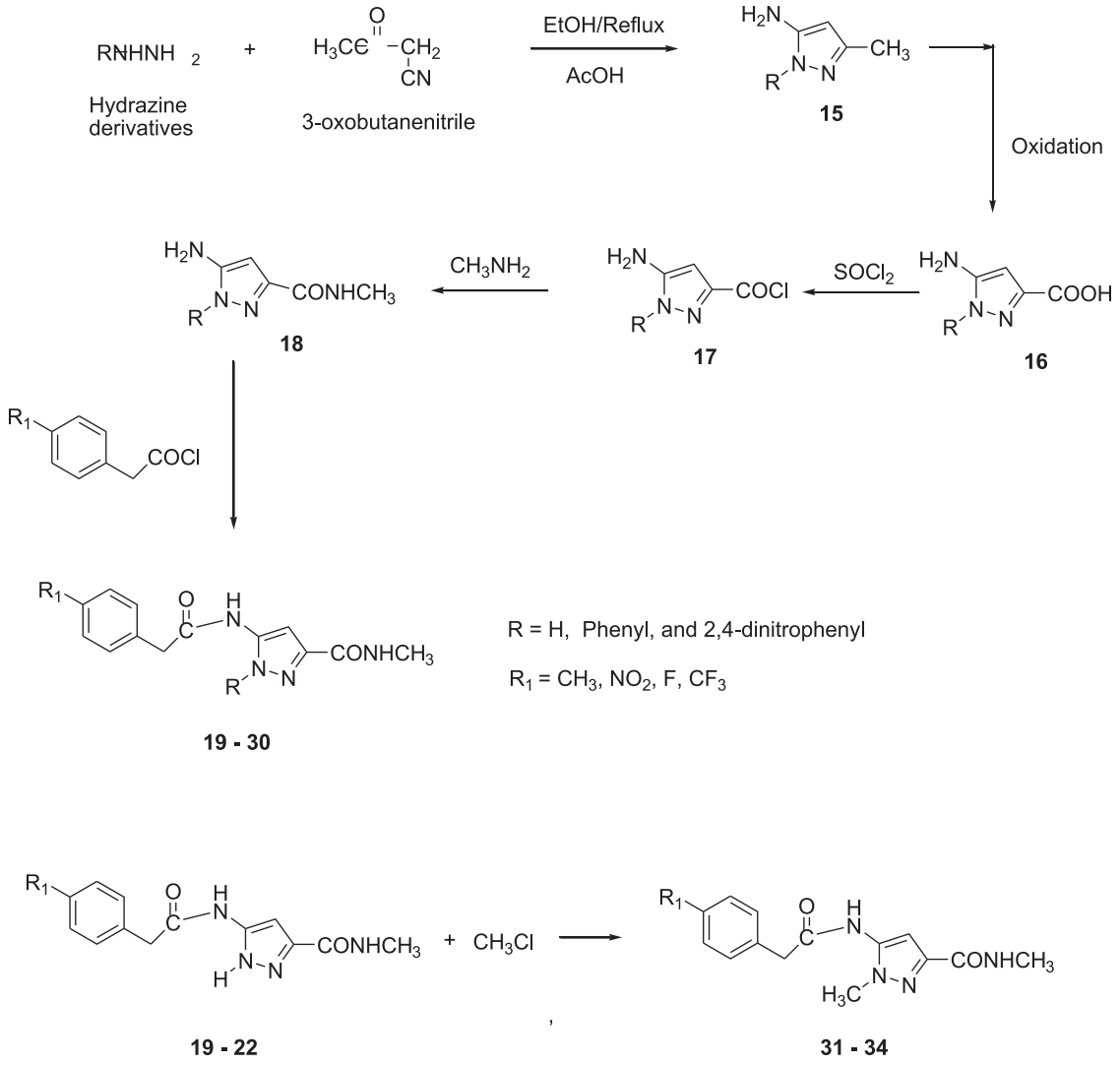
2.2. Similarity search

The molecules similar to the selected query molecule are screened against the downloaded Enamine dataset using FTrees similarity search from BiosolveIT GmbH FTrees (http://www.biosolveit.de/FTrees) [21,22]. FTrees-based approach is a complex feature tree instead of linear fingerprint representations such as MACCS [23] or PubChem fingerprints (http://pubchem.ncbi.nlm.nih.gov). Each of the feature trees represents hydrophobic fragments and functional groups of the query molecule and the way these groups are linked together. Each node in the tree is labelled with a set of features representing chemical properties of the part of the molecule corresponding to the node. The comparison of feature trees is based on matching subtrees of two feature trees onto each other. Unlike fingerprint-based similarity search, the minimum FTrees similarity score between the query and the target molecules called as similarity threshold was set to a fixed value as 0.8. The output of FTrees visual similarities is a particular similarity score for each query-target pair.

2.3. Molecular docking

The docking studies were performed using MOE-Dock module available in Molecular Operating Environment (MOE) 2018.01 [24]. The molecular docking approach is a two-stage process with pose generation and scoring the complexes. For the pose generation, we have selected the triangle matcher algorithm which was aligned using the ligand triplets on the alpha sphere triplets of the protein. Then the generated poses were scored on the interaction with amino acids in the binding site of the protein using two rescoring functions, London dG and GBVI/WSA dG.

For our docking studies, we have selected the crystal structure of shikimate kinase in complex with ADP and shikimate (2IYQ.pdb) was downloaded from Protein Data Bank (PDB) [14]. Firstly, the enzyme was prepared by the following steps, removing water molecules, adding hydrogen atoms, assigning the partial charges to all atoms and energy minimization by adopting the default parameters in each module implemented in MOE 2018.01. The co-crystallized ligand shikimate was used to define the binding site for docking the ligands. Secondly, the accuracy and suitability of MOE for the present system was validated by



Scheme 2. Procedure for synthesis of Compounds **19–34**.

reproducing the conformation of shikimate which resulted in a RMSD value of 0.43 Å. Finally, the selected hit molecules identified from virtual screening were docked into the binding site of shikimate kinase. The top 10 docked poses for each ligand was selected and analyzed the interactions with shikimate kinase using 2D ligand interaction diagram and visual inspection.

Chemical synthesis

The selected identified compounds were synthesized using the above Scheme 1. The pyrazole (**9**) ring is synthesized by reacting ethylacetoacetate and hydrazine. The Pyrazole (**9**) was oxidized to form the corresponding acid derivative (**10**). In another process, 2,6-dichloroaniline is nitrated and then further coupled with chloro(methylsulfonyl)methane, which on reduction yielded compound **13**. Pyrazole acid (**10**) and Compound **13** were combined using amide bond in presence of DCI to form the final product (Compound **14**).

Some more derivatives of the selected compounds were synthesized using Scheme 2. Substituted hydrazines and 3-oxobutanenitrile were cyclised to form the pyrazole (**15**). Pyrazole **15** was oxidised in presence of sodium hydroxide to form the carboxylic acid derivative of the pyrazole (**16**), which on treatment with thionyl chloride yielded the acid chloride derivative (**17**). This compound **17** reacted with methyl amine forming compound **18**. Compound **18** on reaction with substituted benzoyl chlorides yielded the derivatives **19 – 30**. The non-substituted pyrazole derivatives (**19 – 22**) were methylated using methyl chloride to yield compounds **31 – 34**.

A total of seventeen derivatives of the selected compounds from the library were synthesized using Schemes 1 and 2.

The structured of the synthesised compounds were characterized by spectral analysis (IR, NMR and Mass). IR spectra of compound **14** demonstrated disappearance of primary amine (N-H_2) peak present in the intermediate compound **13**, and appearance of amide (CON-H) characteristic absorption band at 3430 and 1698 cm^{-1} respectively. Compound **14** showed characteristic absorption band of pyrazolone cyclic amide (CON-H) at 1705 cm^{-1}; characteristic of arylchloride moiety (Ar-Cl) absorption band at 1089 cm^{-1}. Compounds **20, 24, 28, & 32** showed appearance of NO_2 (Ar—C—N—O) characteristic stretching bands at 1550–1475 cm^{-1}. Compounds **21, 25, 29, & 33** exhibited aryl halide (Ar-C-F) characteristic stretching bands at 1069 – 1073 cm^{-1}. The second series of synthesized pyrazole compounds **19 – 34** were confirmed by the presence of –NH/-N, and –N=C pyrazole ring system stretching bands at 3505 – 3675, 1652 – 1694 cm^{-1} respectively. Synthesized final compounds **19 – 34** demonstrated disappearance of primary amine (N-H_2) from the intermediate compound **18**, and appearance of amide (CON-H) characteristic absorption band at 3430, and 1705–1698 cm^{-1} respectively. Proton assignments in ^{1}H NMR spectra for the titled pyrazole compounds **19 – 34** showed signal at δ 12.10 to

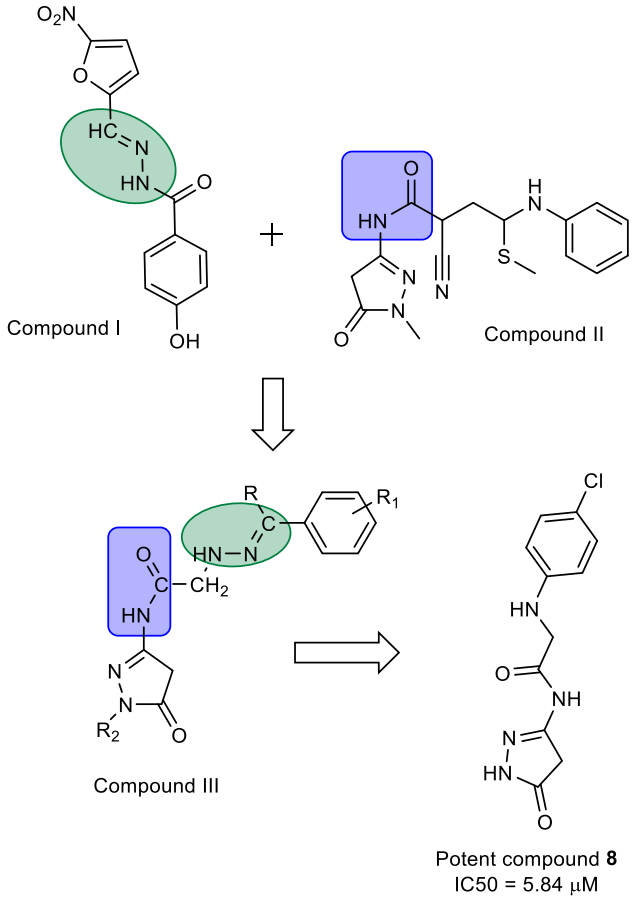


Fig. 2. Development of novel scaffold pyrazolone derivatives using the combined pharmacophore features from known hydrazone and amide derivatives which acts as shikimate kinase inhibitors.

12.16 (s, 1H, NH, D_2O exchangeable) and carboxamide (d, 1H, CONH-CH_3) demonstrated characteristic signals at δ 7.56 – 7.60. All the synthesized compounds **19 – 22 & 31 – 34** showed signal at δ 7.0 – 7.45 (*m*, 5H, Ar-H), while compounds **23 – 30** showed peak at 6.9 – 7.45 (*m*, 4H, Ar-H). All the synthesized compounds **19 – 34** showed characteristic peak of methylene (–CH_2-) and methyl (N-CH_3) signal at δ 3.96 – 4.10 & 2.96 – 3.05 respectively. On the other hand, Compounds **19, 23, 27** and **31** showed additional typical aromatic methyl signal at δ 2.56 – 2.60. Compounds **31 – 34** showed the characteristic peaks of methyl group in pyrazole ring at δ 3.86. Furthermore, the titled compounds were confirmed by mass spectra (*m/z* values).

2.4. Biological testing

The shikimate kinase enzyme inhibition studies were performed using the method described by Simithy et al. [25], with a slight modification. Test compounds at different concentrations *viz.*, 100, 50, 25, 12.5, 6.25 μg/mL, and 15 nM of shikimate kinase were preincubated for 15 min in a micro-centrifuge tube containing 455 mL of assay buffer (100 mM Tris-HCl pH 7.5, 50 mM KCl, and 5 mM $MgCl_2$) at 25 °C. The reaction was initiated by the addition of an aqueous solution of shikimic acid and ATP (2 and 0.2 mM, final concentrations, respectively) and quenched after 5 min by the addition of 2 mL of 100% formic acid. The total volume of the reaction mixture was 500 mL. The presence of shikimate-3-phosphate after incubation indicates enzyme activity. The shikimate-3-phosphate in the final solution was determined by using HPLC. Inhibitory concentration is the conc. at which the compounds inhibit the enzyme and the absence or decrease in the amount of shikimate-3-phosphate in the final solution.

3. Results and discussion

3.1. Similarity search

In similarity searching, compounds with the known biological activity are utilized as reference to search against the molecules in a database. The fingerprint representations calculated for the query and database molecules are compared in a pair-wise manner to identify novel scaffolds as inhibitors of shikimate kinase. Recently Timo and his coworkers reported the inhibitors targeting *M. Tuberculosis* identified using *in silico* approaches from ligand- and structure-based drug design methodologies [26]. Among the reported molecules, one of the pyrazolone derivatives is the potent inhibitors of shikimate kinase (Fig. 2) is selected for our virtual screening approach. This lead pharmacophore combines the features of the available shikimate kinase inhibitors mentioned in Fig. 1 i.e., the hydrazide, amide, and cyclic structure with two nitrogen (molecule 1, 5, 6). Briefly, the pyrazolone derivative was identified by screening the molecules that are designed on the basis of combination of pharmacophore features from hydrazone and amide pharmacophore features which is known to inhibit shikimate kinase efficiently [20] (Fig. 2).

Thus, in our study, one of the most potent pyrazolone derivatives **8** (Fig. 2) is used as the query molecule. The selected query molecule has to be searched against the molecules from Enamine database. This database is one of the largest suppliers of molecules which provide high quality of purchasable compounds with a size of 43.6 million. The MACCS fingerprint was calculated for the molecules in the database and the search using the query molecule 8. The top 1000 molecules identified from the similarity search using MOE-similarity was utilized for the molecular docking studies.

3.2. Structure analysis and docking studies

Currently in RCSB Protein Data Bank (PDB) [27] a total of 49 crystal structures have been reported for shikimate kinase. The structures of shikimate kinase have been complexed with nucleotides include ATP and ADP, the endogenous ligand shikimate and its product shikimate 3-phosphate, inhibitors and also available in different states explaining their mechanism of action in detail. The shikimate kinase enzyme transfers the phosphate group from ATP to shikimate and forms as shikimate 3-phosphate. The phosphate group transfer mechanism and the conformation movement of the enzyme can be understood clearly from the crystal structures in apo and complex states. The shikimate kinase consists of three domains including SB (shikimate binding domain), a core domain containing highly conserved phosphate binding loop (P-loop), and the “lid”, a highly flexible domain in open and closed conformation [28,29,30]. The reported structures were analyzed in detail and the complex with ADP and shikimate (PDB ID: 2IYQ) was selected for the docking study because the conformations of SB domain in open and closed states of the enzyme were similar [6]. Among these three domains in shikimate kinase, the SB is responsible for the transfer mechanism of phosphate group and the substrate analogs have shown inhibition of shikimate pathway. Shikimate is found to be stabilized within the binding site through hydrogen bond interactions with the amino acid residues D34, R58, G80, and R136. Furthermore, residues P11, I45, F49, F57, E61, G79, G81, P118, and L119 contributed within to form the binding site for shikimate [14] (Fig. 3). The docking studies of the reference compound 8 shows that it occupy the same binding site as the shikimate and form interaction with D34, R58, G81, and R117,

The selected crystal structure and the docking approach were validated by redocking studies. The shikimate structure obtained from the crystal structure was redocked and found to have a top rank pose with an RMSD value of 0.43 Å. During the docking simulations, the entire amino acid residues in the enzyme were considered rigid. This confirms the selected docking approach and the scoring function is appropriate for the structure and the binding site.

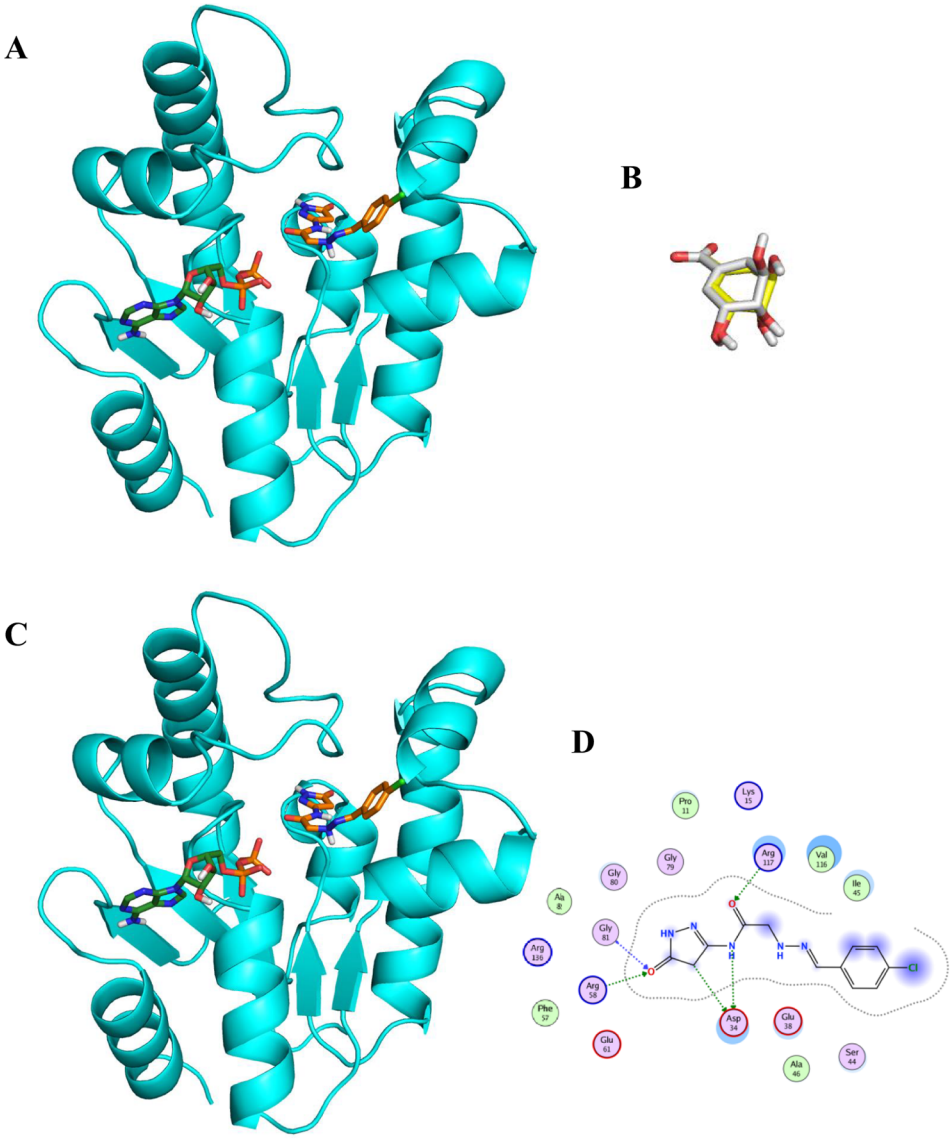


Fig. 3. (A) The crystal structure of shikimate kinase along with ADP (colored green) and shikimate (colored blue) are shown in stick representation. The crystal structure of shikimate kinase is represented in ribbon and colored in cyan. **(B)** The comparison of docked pose (colored blue) and crystal structure (colored yellow) of shikimate. **(C)** The putative docked pose of the reference compound, **8** and **(D)** 2D interaction diagram of **8** with the residues in the binding pocket of shikimate kinase. Oxygen atoms are colored in red, nitrogen atoms in blue, hydrogen in silver white, and phosphor atoms in orange. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

The hit molecules identified from similarity search was docked into the binding pocket (SB) of the shikimate kinase. The process was divided further into two step process. In the first step, the entire 5000 molecules identified from similarity search were docked using MOE-Dock by considering the binding site of the enzyme as rigid. In the second step process, the top 1000 compounds identified from rigid docking were subjected to an additional docking step with flexible binding of the enzyme.

The top 5000 molecules obtained from similarity search were processed and energy minimized in MOE and docked by considering the binding site as rigid. Then the resulted binding poses obtained for molecules from rigid docking were analyzed. The energy values of the molecules were found in the range from −8.22 to −5.31 kcal/mol. On the basis of the docking score the top 1000 molecules were selected and subjected to flexible docking. In this step, the binding site of the enzyme was considered flexible. During the docking simulation the side chains of the amino acids were flexible and allow the ligand molecules to dock deeper into the binding pocket. The energy values of the resulted ligand poses were found to be in the range from −9.50 to −6.63 kcal/mol. In the docking process, the energy values of the ligand molecules were improved due to the flexibility of the binding pocket. From the docking results of flexible binding site and ligand, the top 100 diverse molecules were analyzed. The interaction pattern of these 100 diverse molecules was analyzed in detail by visual inspection. On the basis of interaction and we selected 10 molecules found interesting and possibly show inhibition against the shikimate kinase. The 2D interaction of the 10 selected molecules with the shikimate kinase is provided in Fig. 4 (A-J).

On the basis of the docking results from query molecules **8** and the amino acids in the binding pocket we search for compounds forming key electrostatic interacting residues include Lys15, Asp32, As34, Arg58 and Arg136. Thus, in our docking results we explored for the ligands interacting with the specified important amino acid residues need to interact with the hit molecules. The assumed hypothesis is that the molecules forming interaction similar to the selected query molecules and possibly form similar potency obtained as Compound **8**. The selected ten compounds are pyrazole amides comprising of hydrogen bond acceptors, one aromatic ring, electron rich halogens and the aromatic ring substituted with carbonyl (C═O) or sulphonyl (O═S = O) groups. Possibly the selected ten molecules could be synthesized in our lab. The selected ten molecules (**I-X**) were shown in Fig. 5.

3.3. Chemistry

From the selected 10 compounds the compounds which are possible to synthesize in our lab were explored. Among the 10 compounds, **III** and **X** were selected for synthesis along with some of their derivatives

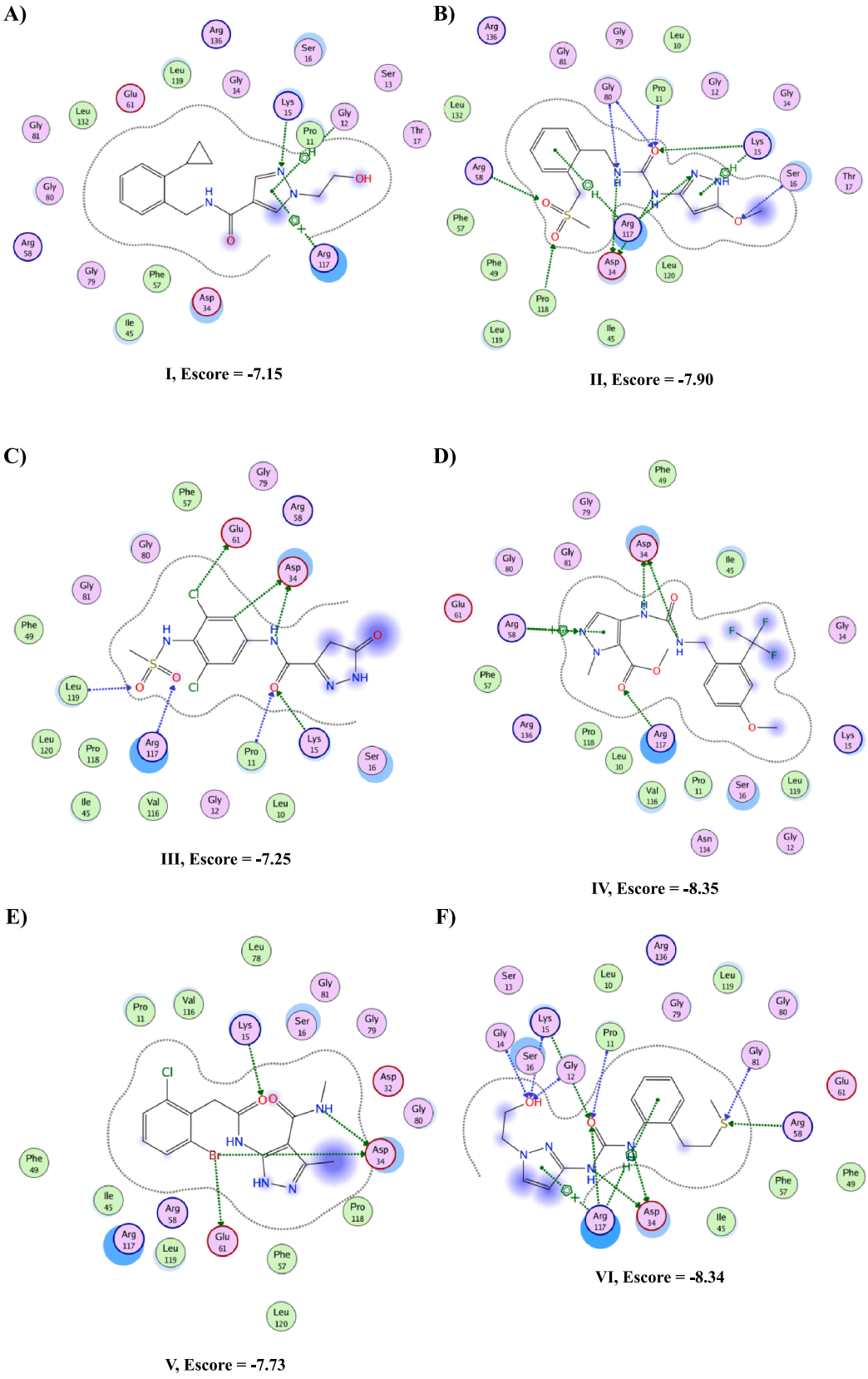


Fig. 4. The selected ten molecules from the virtual screening and their 2D interaction diagram specifying the interaction of the molecules with the binding pocket of shikimate kinase. Their docking score obtained from the flexible docking are also provided.

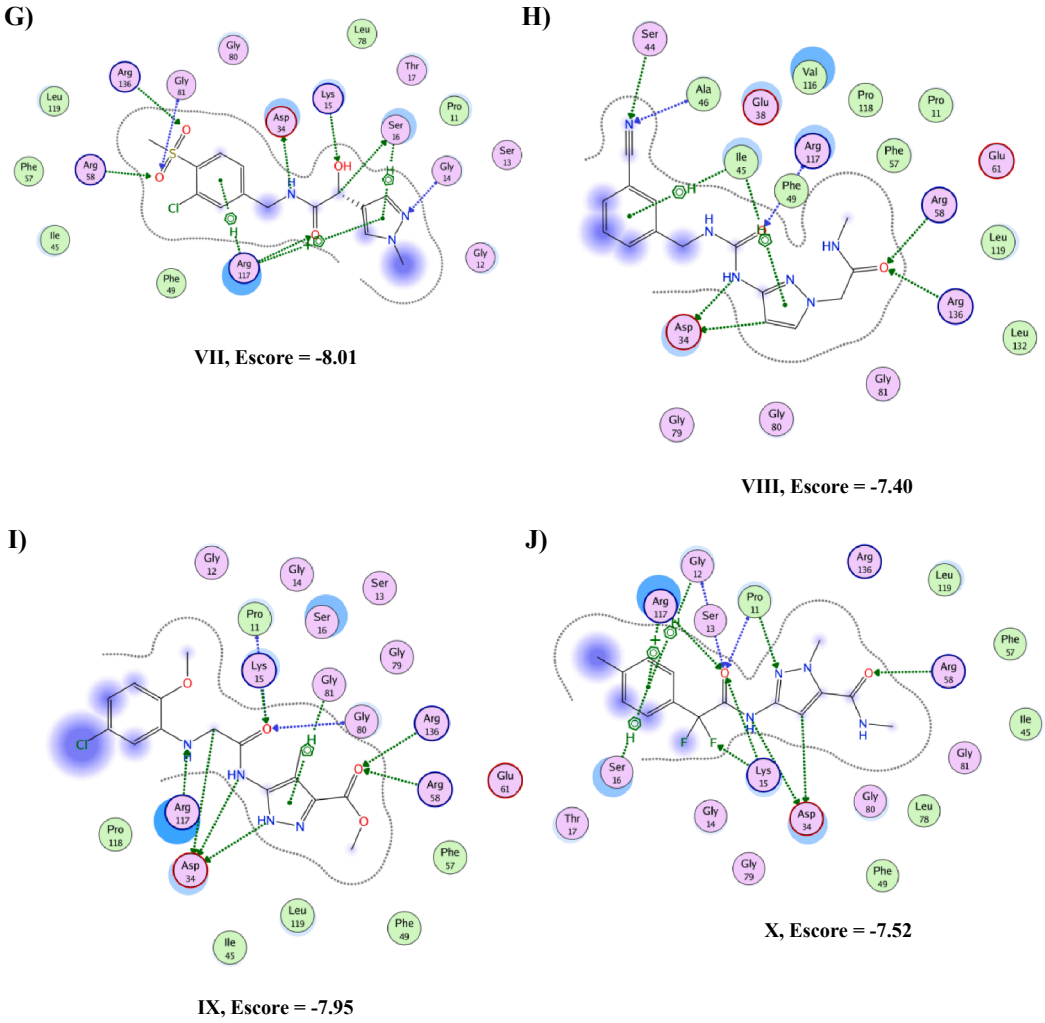
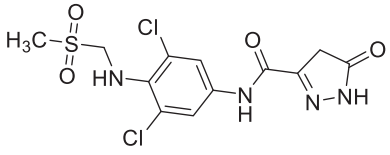
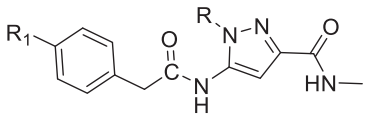


Fig. 4. (*continued*)

(total 17 compounds, **14, 19 to 34**). The selected identified compounds and their analogs were synthesized using Schemes 1 and 2 as described in the materials and methods section and tested for their enzyme inhibition study. The reaction progress and purity of the synthesized compounds were monitored by thin layer chromatography (TLC). The compounds were synthesized in good yields with high purity (Table 1). Structures of the synthesized compounds were established on the basis of spectral data (IR, ^{1}H NMR, and mass).



Compound 14



Compound 19–34

3.4. Biological testing

The synthesized compounds were tested for their potential for inhibiting the shikimate kinase enzyme using the method described earlier by Simithy *et al.* [16], with slight modification. Test compounds were tested at different concentrations *viz.*, 100, 50, 25, 12.5, 6.25 μg/mL, and 15 nM of shikimate kinase. The shikimate kinase enzyme inhibition study reveals that the compounds showed inhibition (IC_{50}) at concentrations of 50 μg/mL (Compounds **21, 22, 24, 25, 26, 27, 30, 32, 34**) and 25 μg/mL (**14, 19, 23, 31, 33**). The molecules identified from ligand- and structure-based virtual screening shows promising shikimate kinase inhibition activity. The activity data of the synthesized compounds suggests the importance of pyrazole moiety and amide bond linking the pyrazole with the aryl group. The methyl substitutions at R1-position is favorable for the activity and with small or large alkyl substitutions at R-position also, it is clear that substitution at the pyrazole nitrogen with bulky groups decreases the activity. This confirms the proposed methods have identified new scaffolds as inhibitors of shikimate kinase. However, the IC_{50} values obtained for the new scaffolds were not as comparable with that of the lead molecule used for the similarity search. This clearly suggests that further modification in the molecule is required to improve the biological activity.

4. Conclusions

The aim of this study is to identify novel scaffold as inhibitors of shikimate kinase on the basis of computational approach that can predict putative inhibitors from a database of chemical compounds. Utilizing ligand- and structure- based approaches similarity search and molecular docking we selected ten compounds as inhibitors of

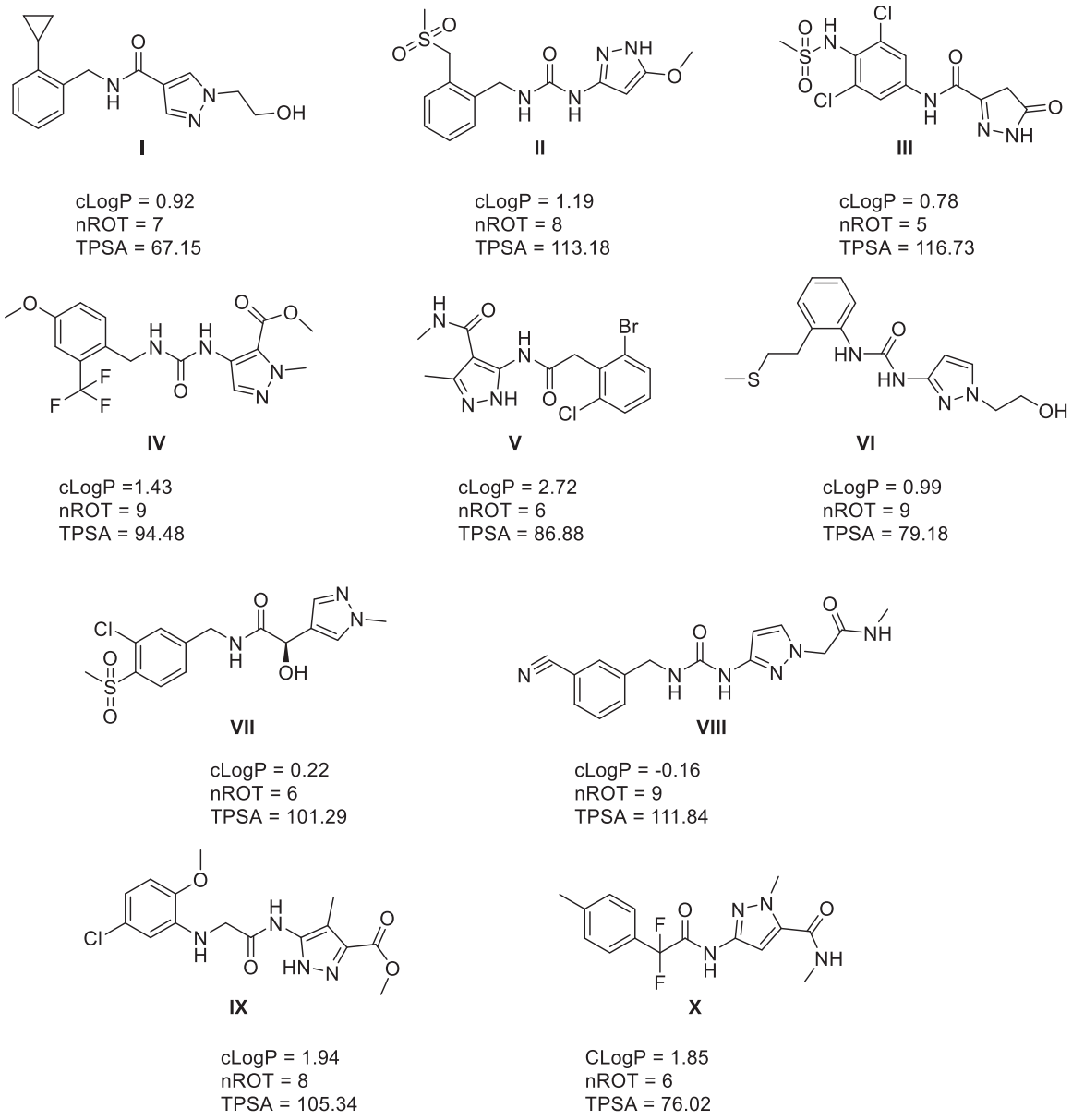


Fig. 5. List of selected compounds that can be a promising candidate for the inhibition of shikimate kinase.

Table 1
Derivative compounds which are synthesized and shikimate kinase enzyme inhibition activity of the synthesized compounds.

Compound No.	R	R_1	Activity (IC_{50} in μg/mL)
14	–H	$–CH_3$	25
19	–H	$–CH_3$	25
20	–H	$–NO_2$	100
21	–H	–F	50
22	–H	$–CF_3$	50
23	$–C_6H_5$	$–CH_3$	25
24	$–C_6H_5$	$–NO_2$	50
25	$–C_6H_5$	–F	50
26	$–C_6H_5$	$–CF_3$	50
27	$-2,4\text{-di}NO_2C_6H_3$	$–CH_3$	50
28	$-2,4\text{-di}NO_2C_6H_3$	$–NO_2$	greater than100
29	$-2,4\text{-di}NO_2C_6H_3$	–F	100
30	$-2,4\text{–di}NO_2C_6H_3$	$–CF_3$	50
31	$–CH_3$	$–CH_3$	25
32	$–CH_3$	$–NO_2$	50
33	$–CH_3$	–F	25
34	$–CH_3$	$–CF_3$	50

shikimate kinase. The compounds are selected on the basis of synthetic feasibility and can be further explored along with their derivatives. These new scaffolds identified confirm that the ligand- and structure-based virtual screening based combined approach can possibly identify promising candidates as inhibitors of shikimate kinase for the treatment of tuberculosis.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bioorg.2020.104083.

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