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Computational screening, docking and simulation analysis of phytochemicals from *Senna auriculata* against multiple targets of *Mycobacterium tuberculosis*

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Tuberculosis is an infectious disease caused by potential pathogenic bacteria *Mycobacterium tuberculosis* (Mtb) that causes more than ~1.5 million deaths every year. The reason for its successful infection rate is owed to its resilient, tough mycolic acid-rich cell wall that makes the antibiotics hard to penetrate into the cell and its ability to manipulate the immune system. In addition, drug resistance has become a major concern. For the above-mentioned reasons, incessant attempts are being made to identify novel drug targets and newer natural anti-tubercular drugs to control the spread of TB. In the previous study, ethnobotanically important medicinal plants *Trachyspermum copticum* and *Senna auriculata* were evaluated for anti-mycobacterial potential against *M. smegmatis*. The crude extracts were analyzed in Gas Chromatography-Mass Spectrometry (GC-MS) to identify potential anti-TB compounds. In the current study, a total of 53 phytochemicals identified and mentioned in literature from medicinal plants *Trachyspermum copticum* and *Senna auriculata* in addition to the phytochemicals obtained from the GC-MS analysis were subjected to *in silico* docking evaluation against important drug targets of *Mycobacterium tuberculosis.* Most targets chosen in the study contribute to cell wall metabolism of *Mtb*. From the exhaustive docking analysis, lupeol and stigmasterol are identified as potential multitargeted anti-TB compounds and proposed as drug candidates. Molecular dynamic simulation studies revealed stable interaction of stigmasterol with *FbiB*, *MmpL3* and *EmbC* making it a potential multi-target compound.

Keywords: Exhaustive docking, Lupeol, Multiple target approach, Simiarenol, Stigmasterol

Tuberculosis (TB) is a bacterial infection that mainly affects the lungs but can also affect other body organs. The World Health Organization lists TB as one of the top 10 killers worldwide, making it a serious global health issue. Tuberculosis infection has been hard to eradicate since the evolution of drug-resistant strains of *Mtb* which arises mainly because of improper drug administration. Treatment of drug-resistant tuberculosis is associated with a complex and long drug regimen with one or more health complications. The co-infection of HIV/AIDS has also increased the risk of TB treatment. For the aforementioned reasons, there is an urgent need to come up with alternative drugs that are less toxic, drugs that can shorten the treatment regimen, lower drug dosage, and target resistant strains of *Mtb*[1].

In the study done earlier, the plants *Senna auriculata* and *Trachyspermum copticum* were chosen as they are Already studied extensively for their medicinal properties[2,3]. Various extracts of the plants were screened for anti-mycobacterial activity. Ethyl acetate and methanol extracts of the plants were studied for using agar well diffusion method. The ethyl acetate extracts of both the plants and the methanol extract of *Senna auriculata* were further taken for Gas Chromatography-Mass Spectroscopy (GC-MS) analysis where many phytochemicals were identified which might be responsible for the anti-mycobacterial activity[4]. Some important compounds isolated include 2-Butenediamide-(E), Piperazine, Oxirane, Benserazide, Aminopyrrolidine, N-8-Guanidino spermidine, 2H-Pyran-2one, (Z)-2-Hydroxyimino-3-oxobutyric acid along with other compounds. All these compounds have been proven to rich source of bio-activity due to their anti-bacterial, anti-diabetic, anti-cancer, anti-helminthic properties, *etc*., in previous studies[5].

The inclusion of natural compounds is preferred in drug development in order to reduce the side effects of existing synthetic drugs for tuberculosis. Severe side effects like hepatotoxicity are seen associated with drug intake in patients. In this situation, natural compounds are preferred because they can be less

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toxic hence, the development of new drugs with natural compounds and/or its components as adjunct therapy is relevant. The selected plant compounds belong to various classes of phytochemicals including terpenoids, triterpenoids, sterols and many more. Based on various literature studies, some of these compounds have already been identified with various medicinal properties. However, detailed molecular docking analysis were not conducted to confirm the activity against tuberculosis[6]. The use of *in silico* when coupled with *in vitro* methods is efficient, less time-consuming and affordable as compared to the traditional methods of drug discovery[7]. Earlier, the selected plants were studied against *M. smegmatis* using *in vitro* techniques. It is a non-virulent species that is ~90% similar in genetic makeup to drug resistant *Mtb* and widely used in initial stages of drug discovery[8]. In order to get more details about the mechanism and exact target of the identified ligands with that of *M. tuberculosis* proteins, *in silico* methods are undertaken. In view of finding the targets of the compounds identified from *S. auriculata* and *T. copticum,* screening and molecular docking techniques are followed. Important proteins that serve as target for front-line drugs owing to their importance in arresting the growth of the organism are considered in the study as targets for natural compounds from *S. auriculata* and *T. copticum.* Various proteins play significant roles in different metabolic pathways which makes them possible drug targets. A multi-targeted approach of natural plant compounds against the front-line drugs is attempted in this study. The target proteins taken include – *RpoB*, *Alr*, *EmbC*, *MmpL3*, *InhA*, *PncA*, *FbiB*, and *AtpC*. Among them protein *InhA* (drug target for isoniazid), *RpoB* (drug target for rifampicin), *PncA* (drug target for pyrazinamide) and *EmbC* (target for ethambutol) are known targets for frontline drugs. Some proteins like *Alr* (proposed drug target for cycloserine), *AtpC* (drug target for bedaquiline), *FbiB* (proposed target for delamanid) and *MmpL3* (proposed target for SQ109) are also included as they form potential targets for emerging drugs that are in various phases of clinical trials[8]. Most of the proteins taken for the study are involved in the cell wall or mycolic acid metabolism.

In the present study, we aim to identify new compounds and mechanisms in a multi-targeted approach that can act as starting point for novel drug candidates. For this, well known approaches such as virtual screening and molecular docking are done. With the above-mentioned methods, 3 natural products are identified which act effectively on multiple proteins of *Mtb* that can alter the growth of the organism.

Materials and Methods

Protein preparation

The three-dimensional structures of the proteins considered for this study are taken from PDB based on their resolution and residues (https://www.rcsb.org/). The details of the proteins are given in the (Table 1 below). For the genes without any co-crystallized ligand structures, the validation was done with the help of the commercial drugs themselves. All the drug compound structures were obtained from PubChem. Rifampicin was taken as the ligand for validating *RpoB*. Similarly, ethambutol was used as the ligand for *EmbC*, SQ109 for *MmpL3*, and pyrazinamide was used for *PncA*[9,10].

Ligand preparation

For this study, 53 phytochemicals were taken among which 14 compounds were selected from the previously done GC-MS analysis and the rest were from various literature and databases (Supplement Table 1). The phytochemicals with molecular weight < 500 and hydrogen bond donors < 5 were selected by following Lipinski's filters. After removing duplicate compounds and applying Lipinski's filters, the number of phytochemicals was reduced to 50. It is said that the compounds that obey the rules of Lipinski are most likely to be drug molecules. The 3D structures of all the ligands were downloaded in SDF format from PubChem and were converted to Pdbqt format. All the above steps were done in open babel 3.3.1.

Validation study-self docking

The protein structures having the best resolution and with no mutation were taken from PDB for docking. *M. tuberculosis* was considered as the source organism.

Table 1 — Selected target protein and PDB ID

Sl. No.	Protein code	Protein name	PDB ID
1	*RpoB*	RNA polymerase β subunit	4KBJ
2	*Alr*	Alanine racemase	1XFC
3	*EmbC*	Arabinosyltransferase C- terminal domain	3PTY`
4	*AtpC*	ATP synthase	4V1F
5	*FbiB*	Cofactor F420 synthesis enzyme	6BWH
6	*MmpL3*	Mycobacterial membrane protein Large 3	6AJG
7	*InhA*	Enoyl-acyl carrier protein	4DRE
8	*PncA*	Pyrazinamidase	3PL1

Proteins with co-crystallized ligands were taken in most of cases. For some protein structures such as *RpoB*, *EmbC*, *MmpL3*, and *PncA*, where the co-crystallized ligands were missing, validation study was performed with the corresponding drug structure themselves. The active sites were referred from various literature sources and the validation study was performed.

In this study, we performed self-docking of the selected eight protein targets using Autodock 4.2. The co-crystallized ligands and water molecules were initially removed and after that Kollman charges and polar hydrogens were added using Autodock tools. The active site for docking was determined from various literature sources. The protein and ligand were loaded and converted to Pdbqt format. Grid boxes were created concentrating at the active site residues to set a boundary for docking and for improving the accuracy. A maximum of 100 poses were generated for each protein in rigid form using the Lamarckian genetic algorithm. The RMSD value was calculated for checking the reliability of docking. The interaction patterns developed between the ligand and protein were analyzed using Discovery studio version 2021[11].

Fast rank screening

The 50 compounds that passed the Lipinski's filter and were taken for virtual screening using AutoDock Vina version 1.1.2. Grid parameters were maintained the same as in validation study in order to improve the accuracy of docking. The results were obtained and the hit compounds were selected based on their binding energy and interaction with the key residues of the active site. All the interactions were observed using Discovery studio version 2021[11].

Exhaustive docking

The top three compounds identified from the fast-rank screening were further taken for exhaustive docking to identify a potential drug molecule that can interact with multiple target proteins. The docking procedure was done using Autodock 4.2 and 100 runs were carried out for each compound. The grid parameters from the validation studies were applied for the exhaustive docking. The results were analyzed for the number of hydrogen bonds along with other bonds and the binding energy. The interactions were analyzed using Discovery Studio version 2021.

Molecular dynamic simulation

Molecular dynamic simulation was performed using CABS Flex 2.0 (http://biocomp.chem.uw.edu.pl/CABSflex2/) which is an open access web server with 100 cycles and 100 trajectory frames[12,13]. The boundaries for atom pairing within the defined space during simulation was set using default parameters. This webserver is a coarse-grained modelling approach. No additional distance constrains with respect to the process were modified. The solvent probe radius was set at 1.4 Å, minimum atomic radius 1 Å and temperature was 1.4 K in order to analyze the interaction between the complex of stigmasterol and proteins (*EmbC*, *FbiB* and *MmpL3*) of *Mtb.* This process was validated and compared with simulation of apo-protein alone under the same conditions. The fluctuations of each residue of the hit complex could be explained using the root mean square fluctuation (RMSF) values obtained.

Results

Validation study

The binding sites from the literature were compared with the residues that were obtained from the docking procedure. These amino acid residues are considered to be important for determining stable and proper interaction with ligands.

RpoB protein (PDB ID- 4KBJ) having a resolution of 2.45 Å was considered for the study. After molecular docking analysis, -9.25 kcal/mol binding energy was obtained in self-docking which was similar as referred in literature. For *RpoB*, the residues Gly-203, Ala-204, Leu-293, Asn-381, and Val-385 showed bonding during the validation.

For *EmbC* protein (PDB ID: 3PTY) having a resolution of 2.00 Å was taken for the study. Binding energy obtained was -4.96 kcal/mol with interaction in important aminoacids such as sites Ala-922, Asn-928, and Gly-1058 and Asp-1056.

The protein *Alr* (PDB ID- 1XFC) having a resolution of 1.9 Å was considered for the study. Binding energy of -8.06 kcal/mol was obtained post docking. Interaction was seen in sites of catalytic pocket such as Lys-42, His-172, and Arg-228, Tyr-364 were involved in the binding.

For *InhA* protein (PDB ID: 4DRE), with resolution 2.4 Å was taken for the study. Validation study showed binding energy of -11.45 kcal/mol with binding at sites Gly-14, Ile-21, Asp-64, Gly-96, Tyr-158, Lys-165, Ile-194 and Phe-41.

The protein *PncA* protein (PDB ID: 3PL1) with resolution 2.2 Å was taken for the study. In the validation study, Asp-8, Ile-133, Ala-134, Cys-138 which are some of the amino acids in the active site,

displayed interaction. The binding energy obtained by docking was -4.33 kcal/mol which was compared to literature.

FbiB gene (PDB ID: 6BWH) having a resolution of 2.18 Å was considered for this study. Validation study showed interactions with Ala-22, Thr-24, and Asp-190 with a binding energy of -7.14 kcal/mol.

MmpL3 protein structure (PDB ID:6AJG) with a resolution of 2.60 Å was considered in this study. After validation study, interactions were found at sites Pro-433, Leu-607, Tyr-622, Leu-627, Met-631 and Phe-611 with binding energy of -5.75 kcal/mol.

AtpC gene in *M. tuberculosis* (PDB ID: 4V1F) having a resolution of 1.70 Å was considered for the study. Interaction was observed in key residues Ala-66, Ala-67, Met-75 and Val-72 with a binding energy of -4.12 kcal/mol. The obtained data was compared with literature for docking procedure and confirmed. The consolidated representation of validation study of various protein targets and their associated ligands obtained during re-docking are given in supplement (Table 2).

Fast rank screening

Virtual screening was performed for the selected 50 compounds using AutoDock Vina version 1.1.2[14]. Compounds with binding energy ranging from -9.7 to -2 kcal/mol were observed. It is also known that when binding energy is low, the binding affinity is high. Hence the compounds with the lowest binding energy ranging from -9.7 kcal/mol to -7.0 kcal/mol for all the proteins were selected for further analysis.

RpoB

The docking interaction analysis of *RpoB* with ligands showed that lupeol had the best binding energy of -9.3 kcal/mol. The second-best binding ligand simiarenol showed interactions at key residues of the active site with binding entropy of -8.8 kcal/mol. Yohimbine had a binding energy of -8.4 kcal/mol. It can collectively be seen that all the top 3 hit compounds has Gly-203 and Val-385 as their common residues which are closely situated in the active site of the protein.

EmbC

EmbC revealed best interaction with the ligand simiarenol with the least binding energy of -9.2 kcal/mol and interacted with almost all the residues of the catalytic pocket. The second-best interaction with *EmbC* protein was lupeol with a binding energy of -8.7 kcal/mol. Stigmasterol had the binding energy of -8.3 kcal/mol. Overall analysis of the interaction pattern of *EmbC* with ligands showed involvement of residues Asn-740, Leu-744, Leu-751, Leu-986, Leu-987, Ala-990, Pro-1013, Leu-1049, Asp-1051 in common proving that these residues are closely related and has steady binding with ligands.

Alr

In case of *Alr* protein, the ligands with best interactions were stigmasterol, simiarenol, and ethyl 5-[(methylamino)acetyl]-10,11-dihydro-5H-dibenzo [b,f]azepin- 3-yl carbamate with binding energy -7.9 kcal/mol, -7.3 kcal/mol, and -7.3 kcal/mol, respectively. It can be noted that stigmasterol had a better binding with *RpoB* and *EmbC* behaved similar to *Alr* protein. The important amino acids that interacted with the protein were all lying close to each other in the catalytic pocket.

InhA

In this study, *InhA* showed best interaction with ligands simiarenol, lupeol, and stigmasterol among all the selected ligand molecules. *InhA* interacted with simiarenol and showed the highest binding energy of -9.7 kcal/mol. In this study, *InhA* showed best interaction with ligands simiarenol, lupeol, and stigmasterol among all the selected ligand molecules. *InhA* interacted with simiarenol and showed the highest binding energy of -9.7 kcal/mol. All the residues that interacted with the ligand were lying in the catalytic pocket of the protein as identified from the literature[15]. Ligand lupeol which had a good interaction with *RpoB* and *EmbC* was seen to interact with *InhA* in a stable manner with a binding energy of -9.6 kcal/mol. Stigmasterol had a binding energy of -9.3 kcal/mol when it interacted with *InhA* protein.

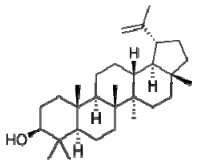
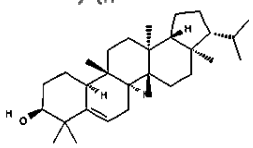
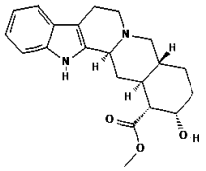
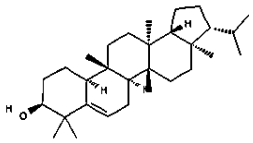
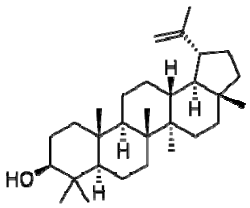
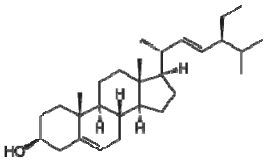
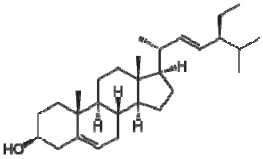
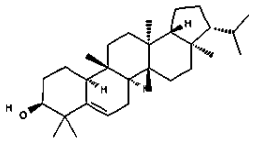
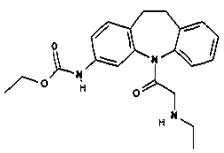
PncA

The protein *PncA* showed better binding affinity for chrysin, thujopsene-(12) and Stigmasterol. The binding energy of chrysin with *PncA* was -9.5 kcal/mol and showed conventional hydrogen bonding with Asp-8, Asp-49 and Ala-134. The protein *PncA* interacted with the ligand thujopsene-(12) and stigmasterol with a binding energy of -8.4 kcal/mol and -8.3 kcal/mol, respectively. Both the ligands showed large number of Van der Waal's interaction. Most of these residues are in the same catalytic pocket of protein as identified from literature.

FbiB

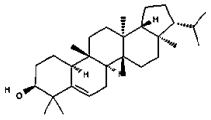
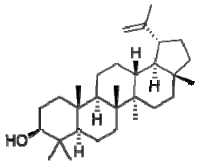
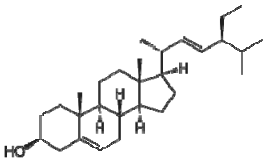
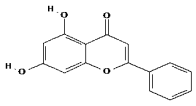
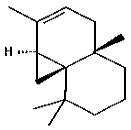
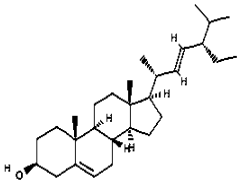
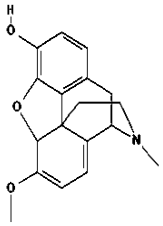
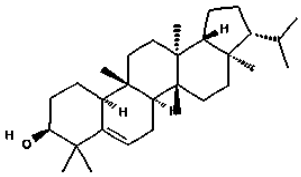
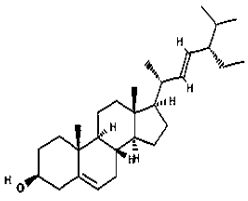
The protein *FbiB* interacted with the ligand morphinan-3-ol,6,7,8,14-tetrahydro-4,5-epoxy-6-methoxy-17-methyl-(5alpha)- with a high binding energy of -8.2 kcal/mol. Simiarenol bound with *FbiB* showed a binding energy of -8.0 kcal/mol. *FbiB*

Table 2 — Representation of binding energy of hit compounds with selected *Mtb* targets

S. No	Protein	Ligand	Structure	Binding Energy
		Lupeol		-9.3 kcal/mol
1	*RpoB*	Simiarenol		-8.8 kcal/mol
		Yohimbine		-8.4 kcal/mol
		Simiarenol		-9.3 kcal/mol
2	*EmbC*	Lupeol		-8.7 kcal/mol
		Stigmasterol		-8.3 kcal/mol
		Stigmasterol		-7.9 kcal/mol
3	*Alr*	Simiarenol		-7.3 kcal/mol
		Ethyl 5-[(methylamino)acetyl]-10,11-dihydro-5H-dibenzo[b,f]azepin-3-yl carbamate		-7.3 kcal/mol

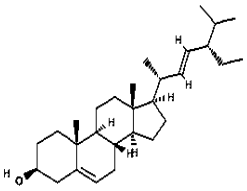
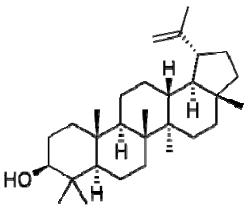
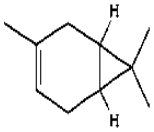
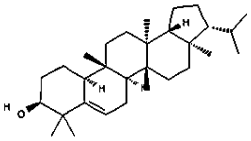
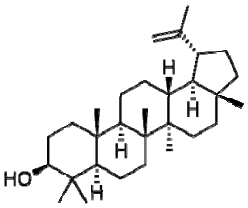
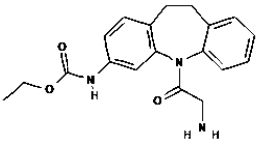
(*Contd.*)

Table 2 — Representation of binding energy of hit compounds with selected *Mtb* targets

S. No	Protein	Ligand	Structure	Binding Energy
		Simiarenol		-9.7 kcal/mol
4	*InhA*	Lupeol		-9.6 kcal/mol
		Stigmasterol		-9.3 kcal/mol
		Chrysin		-9.5 kcal/mol
5	*PncA*	Thujopsene-(12)		-8.4 kcal/mol
		Stigmasterol		-8.3 kcal/mol
		Morphinan-3-ol,6,7,8,14-tetrahydro-4,5-epoxy-6-methoxy-17-methyl-(5alpha)-		-8.2 kcal/mol
6	*FbiB*	Simiarenol		-8.0 kcal/mol
		Stigmasterol		-7.7 kcal/mol

(*Contd.*)

Table 2 — Representation of binding energy of hit compounds with selected *Mtb* targets

S. No	Protein	Ligand	Structure	Binding Energy
		Stigmasterol		-9.0 kcal/mol
7	*MmpL3*	Lupeol		-8.9 kcal/mol
		3-carene		-8.8 kcal/mol
		Simiarenol		-7.6 kcal/mol
8	*AtpC*	Lupeol		-7.2 kcal/mol
		Ethyl 5-[(methylamino)acetyl]-10,11-dihydro-5H-dibenzo[b,f]azepin-3-yl carbamate		-7.2 kcal/mol

interacted with stigmasterol with a binding energy of -7.7 kcal/mol. Overall, the amino acids Lys-17, Arg-18, Pro-64, Asp-82, Thr-84, Pro-85, Pro-91 and Leu-92 were seen commonly interacting with the ligands which shows that these compounds have firm interaction with the protein. All the compounds had firm binding with the residues of the active site of the protein.

MmpL3

The protein *MmpL3* interacted with stigmasterol with the highest binding energy of -9.0 kcal/mol. *MmpL3* interacted with lupeol and 3-carene with a binding energy of mild difference of -8.9 kcal/mol and -8.8 kcal/mol, respectively. The amino acids Ile-249, Ala-690, Phe-693, Met-700 and Leu-627 were seen commonly present in the catalytic pocket of the protein while interacting with all the mentioned ligands confirming the importance of these residues in maintaining the protein.

AtpC

The protein *AtpC* interacted with simiarenol with the highest binding energy of -7.6 kcal/mol. It showed a binding energy of –7.2 kcal/mol with lupeol and interacted with ethyl 5-[(methylamino)acetyl]-10,11-dihydro-5H-dibenzo[b,f]azepin-3-yl carbamate with a binding energy of -7.2 kcal/mol. The residues Ala-38, Leu-39, Gly-42, Leu-53, Pro-56, Phe-57, Thr-60 were

seen common in all three ligands with the protein *AtpC* showing their importance in maintaining the dynamicity of catalytic pocket. The ligand structure and the binding energy of the top 3 hits binding with the target proteins are represented in (Table 2).

Exhaustive docking and analysis

Molecular interaction of selected natural compounds against mycobacterial proteins that form targets for front line drugs and targets that are newly exploited for upcoming drugs are studied using various bio-informatic tools. Among the 50 natural compounds selected from GC-MS and literature survey of the plants *S. auriculata* and *T. copticum*, lupeol, stigmasterol and simiarenol were found to have vivid anti-TB activity based on their firmness and ability to interact with multiple target proteins with good binding energy. Other factors such as Van der Waals interactions, hydrogen bonds, Pi-Pi bonds and Pi-sigma bonds were considered important. These compounds also passed the Lipinski's rule of 5 implying that they can be drug candidates.

Exhaustive docking procedure was done using Autodock 4.2 and 100 runs were carried out for each compound. The results were visualized using Discovery studio 2021 and PyMol. All the Hit compounds showed binding energy of more than -7.0 and at least one conventional hydrogen bond was observed in each case.

In our study, Lupeol (Hit 1) has interaction with protein *RpoB*, *InhA* and *PncA* which are the important targets for drugs rifampicin, isoniazid and pyrazinamide, respectively, lupeol binds with *RpoB* protein in its catalytic pocket with binding energy of -9.0 kcal/mol displaying two conventional hydrogen bonds and one hydrophobic interaction and some Van der Waals forces. The binding sites are Gly-203, Ala-204, Asp-221, Arg-276, Leu-289, Leu-293 and Val-385, respectively. Hit 1 interacts with *InhA* at catalytic domain with binding energy of -11.48 kcal/mol showing hydrogen bond at one site, two alkyl bonds and other Van der Waals forces which might alter the ability of protein from binding to NADH and involve in reduction of fatty acid. It shows one hydrogen bond and three hydrophobic interactions with protein *PncA* and had a binding energy of -7.49 kcal/mol. Some common interaction sites found are Leu-293, Val-385 and Arg-395. The interaction of Lupeol with *RpoB*, *InhA* and *PncA* are represented in (Figs 1A-1C).

Stigmasterol (Hit 2) has interactions with three upcoming drug targets in this study namely, *EmbC*, *FbiB*, and *MmpL3*. *EmbABC* genes are arranged on a single operon code for arabinosyl transferase protein. It interacted with *EmbC* showing one hydrogen bond, seven hydrophobic bonds and some Van der Waals interaction and had a binding energy of -10.30 kcal/mol. Interaction were observed in Trp-1057, Ala-990, Phe-991, Pro-1013 and Leu-1049, respectively. Interaction of Hit 2 with *FbiB* showing binding energy of -9.58 kcal/mol with two hydrogen bonds and four hydrophobic bonds and Van der Waals interaction. The interaction sites were similar to that of *EmbC*. Stigmasterol interacts with *MmpL3* with binding energy of -14.04 kcal/mol showing six hydrophobic interactions and some Van der Waals forces. The interaction of stigmasterol with *EmbC*, *FbiB* and *MmpL3* are represented in (Figs 2A-2C).

Simiarenol (Hit 3) is seen interacting with *Alr* and *AtpC* which are recently identified drug targets in *Mtb*. *Alr* codes for D-alanine racemase is responsible for synthesis of peptidogly can in the cell wall of *Mtb*. Simiarenol bound with *Alr* protein with one hydrogen bond and two hydrophobic interactions and had a binding energy of -7.91 kcal/mol. The interaction sites were noted as Ser-237, Gly-243, Gly-345, His-363, and Asp-354. Simiarenol interacts with *AtpC* with a binding energy of -7.51 kcal/mol showing one hydrogen bond and Van der Waals interactions. The binding pattern for the proteins were similar. The representation of simiarenol interaction with *Alr* and *AtpC* proteins are given in (Fig. 3A & B).

The proteins taken for the study belongs to previously known and emerging drug targets. Among the eight proteins, six are involved in the cell wall synthesis mechanism of *Mtb* directly or indirectly. The hit compounds from this study lupeol and stigmasterol are found to be interacting well with the multiple drug targets associated with cell wall metabolism, which augments their role as potential anti-TB compounds. Targeting these proteins with the use of these phytochemicals might bring down the survival chances of both replicating and non-replicating *Mtb* as they show effects on the cell wall synthesis, which is the major reason for their resistance towards the existing drugs. To ensure the behavior of the hit compounds, a comprehensive toxicity filter approach was incorporated in the study using DataWarrior[16]. The results confirmed that the hit compounds are all non-toxic. The results are given in (Table 3).

Molecular dynamic simulation

Based on the results obtained in exhaustive docking, stigmasterol (Hit2) is taken for further molecular

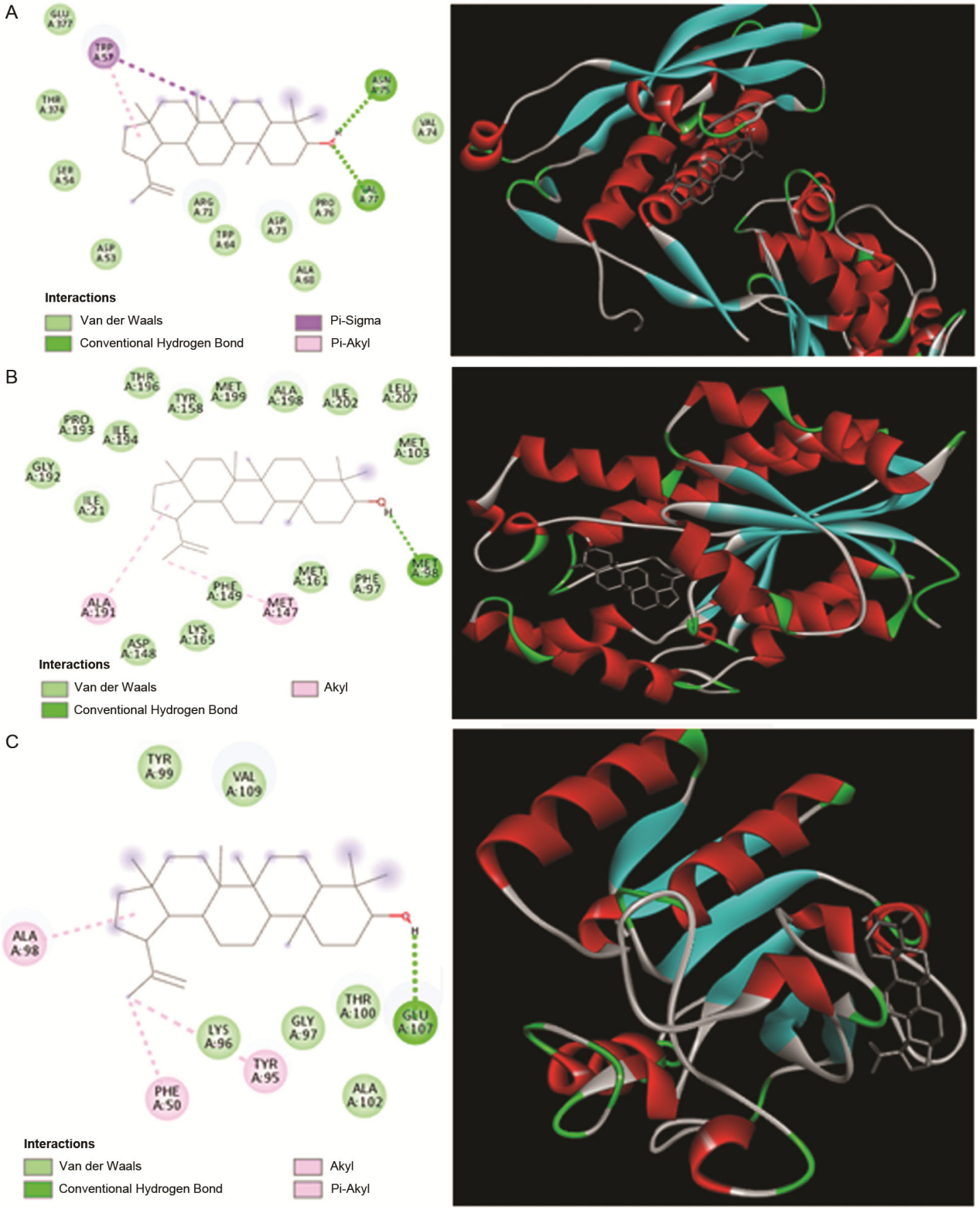


Fig. 1 — 2D and 3D representation of the interaction of Lupeol with (A) *RpoB*; (B) *InhA*; and (C) *PncA*

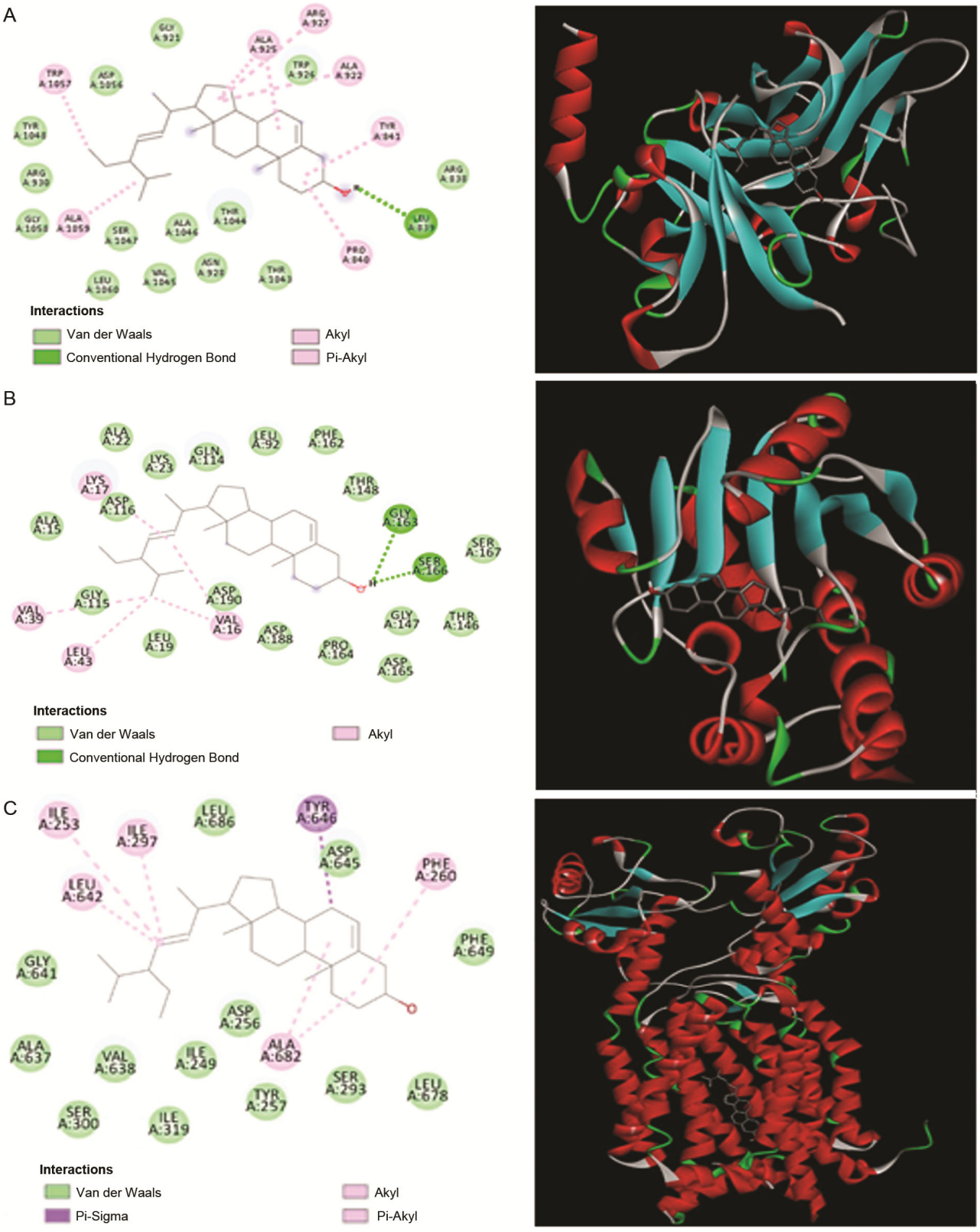


Fig. 2 — 2D and 3D representation of the interaction of Stigmasterol with (A) *EmbC*; (B) *FbiB*; and (C) *MmpL3*

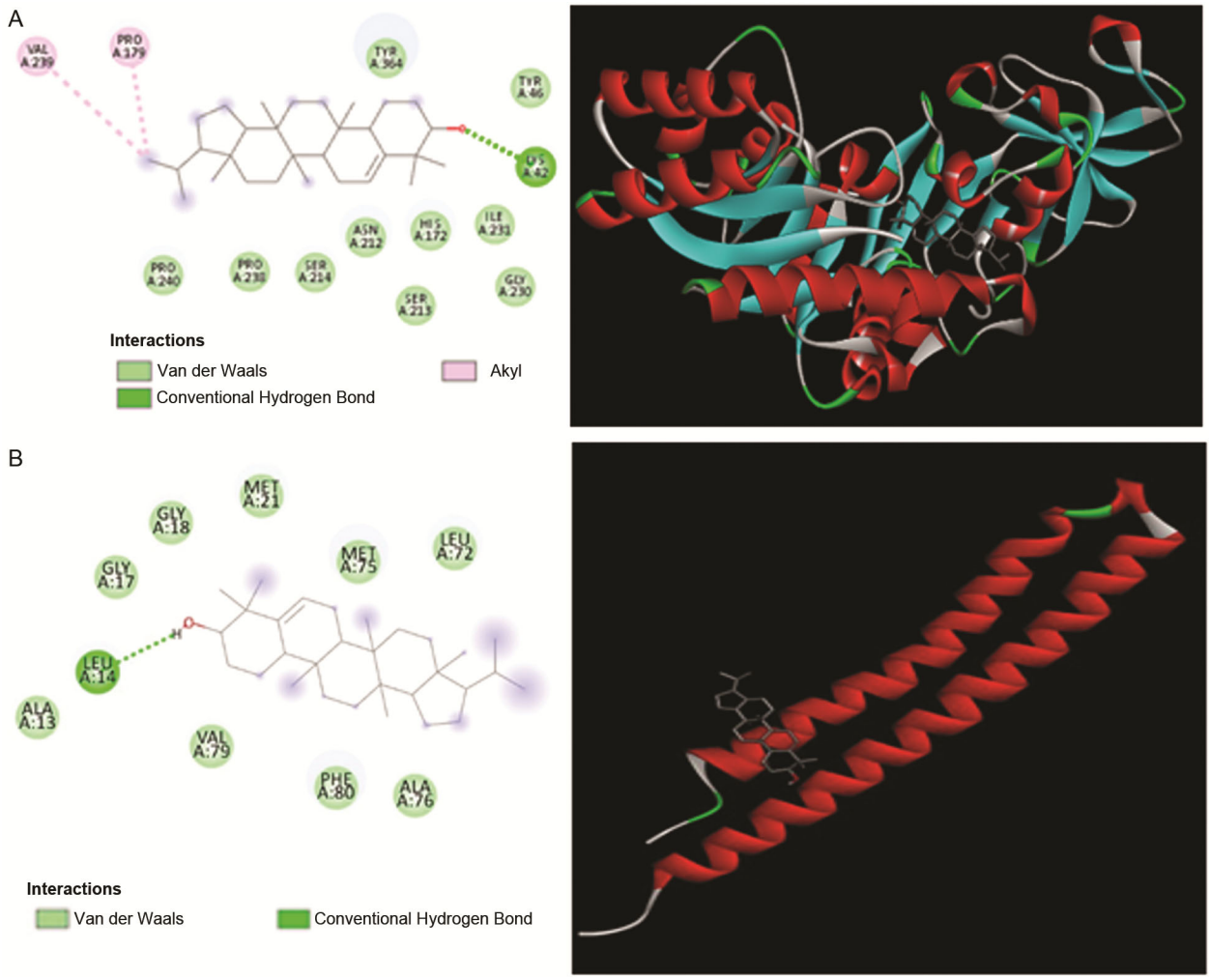


Fig. 3 — 2D and 3D representation of the interaction of Simiarenol with (A) *Alr*; and (B) *AtpC*

Table 3 — Representation of toxicity filters for potential hit compounds

Compound name	Tumorigenic	Mutagenic	Reproductive Effective	Irritant
Lupeol	None	None	None	None
Stigmasterol	None	None	None	None
Simiarenol	None	None	None	None

dynamic simulation analysis using web server named CABS Flex 2.0. The fluctuation in each amino acid of the protein with respect to the time in ns is plot and given in the (Fig. 4). It is to be noticed that as the RMSF value gets higher, the system shows maximum flexibility. Also, the RMSF values tend to be higher in amino acid regions of the loops rather than in helices and strands.

On analyzing the results, stigmasterol showed better interaction with *EmbC* protein by interacting stable with its amino acids in the regions 920 to 1060 with negligible fluctuations in loop areas. The interaction with *MmpL3* showed mild fluctuations mainly due to the presence of large number of loop structures, but later stages attained stability and maintained especially in the active site areas *viz.* 300 to 700 amino acid regions which lie in the catalytic pocket. RMSF plot of stigmasterol with *FbiB* displayed the most stable interaction with almost no fluctuations throughout the simulation.

Discussion

Validation study is done in order to confirm the correctness of the docking protocol. This is done by

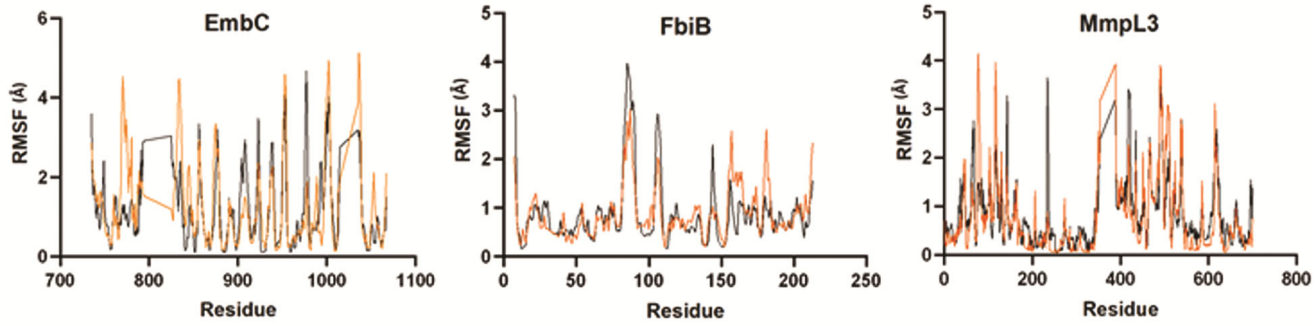


Fig. 4 — RMSF plot of *Mtb* proteins *EmbC*, *FbiB* and *MmpL3* with stigmasterol

matching the docking patterns and active site residues from various literature sources. In this study, for the chosen proteins of interest, co-crystallized ligands were docked. In cases where ethe protein missed a co-crystallized ligand, commercially available drug that targets the protein is docked. The active sites of the protein identified from various literature are as given.

RpoB gene codes for the β subunit of the RNA polymerase enzyme in *Mtb*. Mutation at specific sites of *RpoB* give rise to rifampicin resistance[17]. The active sites for the protein *RpoB* identified from the literature are Trp-57, Trp-64, Asp-73, Val-74, Asn-75, Pro-76, Val-77, Glu-82, Ser-201, Arg-202, Asp-301, Arg-371, Thr-374 and Glu-377, respectively[18].

The gene *EmbC* codes for a protein of arabinosyltransferase family that is involved in the cell wall synthesis that is targeted by ethambutol[19]. The active sites for the protein are Gly-921, Gly-1058, Tyr-841, Ala-922, Pro-1013, Ala-1046, Trp-1057, Ala-1059, Asn-928, Ser-1047, Arg-930, Asp-1014, and Asp-1056 as referred from the literature[20].

The protein *Alr* is the enzyme alanine racemase involved in cell wall synthesis of *Mtb*[21]. The active sites as obtained from literature are Lys-42, Trp-88, His-172, Arg-228, and Tyr-364[22].

The *InhA* protein is a well-known target involved in the synthesis of fatty acids required for the biosynthesis of mycolic acids which is a part of *Mtb* cell wall. The front-line drug isoniazid shows activity against this gene[15]. The active sites of protein Gly-14, Ile-21, Phe-41, Leu-63, Asp-64, Val-65, Ser-94, Gly-96, Phe-97, Ile-122, Met-147, Asp-148, Lys-165 and Ala-191 were referred from literature[23].

PncA codes for the enzyme pyrazinamidase which converts the prodrug pyrazinamide to its active form pyrazinoic acid. Mutations in this gene lead to pyrazinamide drug resistance[24]. The active sites for *PncA* are Asp-8, Asp-49, His-51, His-57, His-71, Ile-133, Ala-134, and Cys-138[25,26].

Protein *FbiB* is involved in the biosynthesis of co-factor 420 which is involved in oxidative stress management in *Mtb*. The drug delamanid is an emerging drug in clinical trials that is activated by this co-factor. The active sites Thr-148, Gly-163, and Ser-166 were referred from PyMol and literature sources.

The gene *MmpL3* is responsible for the transport of trehalose mono mycolate (TMM) which is the precursor of the trehalose di mycolate (TDM), an outer membrane component of *Mtb*[27]. Drug SQ109 targets this gene. This is an upcoming drug that is in clinical trials phase -3. The active sites Ile-249, Asp-256, Phe-260, Asp-645, Tyr-646, and Phe-649 were identified from literature[27].

The protein *AtpC* codes for the F0 rotor ring in ATP synthase and thus can inhibit ATP synthesis. An upcoming drug Bedaquiline that currently passed clinical trials successfully targets the gene. The active sites Gly-62, Leu-63, Glu-65, Ala-66, Ala-67, Tyr-68, Phe-69, Ile-70, and Leu-72 were referred from literature[28]. The consolidated representation of validation study of various protein targets and their associated ligands obtained during re-docking are given in supplement (Table 2).

From virtual screening approach, it can be seen that collectively, most of the proteins chosen for the study interact with the ligands lupeol, stigmasterol and simiarenol with binding affinity and stable interaction on comparison with other ligands. Based on this, exhaustive docking analysis was performed for further confirmation of the stability in interaction.

The compound Lupeol (Hit 1) is known for its anti-microbial enhancing efficiency and was found to have anti-mycobacterial effect against *H37Rv in vitro* alone and in combination with β-amyrin[29,30]. It has antibiotic enhancing effect treating MRSA infections. Additionally, lupeol has been found to

possess hepatoprotective properties[31,32]. In our study, Lupeol has interaction with protein *RpoB*, *InhA* and *PncA* which are the important targets for well-established first line drugs rifampicin, isoniazid and pyrazinamide, respectively. Rifampicin binds to the β-subunit of RNA polymerase (coded by *RpoB*) and inhibits the elongation of m-RNA and hence the translation process. Mutation and resistance of the genes *InhA* and *RpoB* are often seen together making them a prominent marker for MDR-TB related studies. *InhA* is targeted by Isoniazid (INH) and plays an important role in reduction step of biosynthesis of fatty acid. However, it is still unclear on how the *InhA* and *RpoB* gene products interact[33,34]. In the case of pyrazinamide (PZA) which targets pyrazinamidase encoded by *PncA* gene, and gets converted into its active form pyrazinoic acid, widely accepted mechanism is that accumulation of pyrazinoic acid causes fluctuation in cell wall integrity. Though PZA fails to directly involve in cell wall synthesis, its analog compound 5-Cl-PZA binds directly with FAS-I (fatty acid synthase enzyme) and causes inhibition[35,36]. Interaction of Hit 1 with the proteins *InhA*, *RpoB* and *PncA* were strong with many common interaction points.

Stigmasterol, an unsaturated phytosterol, possesses cholesterol-lowering effects, anti-osteoarthritic benefits, inhibitory effects on thyroid function, antiviral properties, and anti-inflammatory activity. Furthermore, stigmasterol has demonstrated antibacterial activity against *Mycobacteria*[37]. Stigmasterol (Hit 2) has interactions with three upcoming drug targets in this study namely, *EmbC*, *FbiB*, and *MmpL3*. *EmbABC* genes are arranged on a single operon code for arabinosyl transferase protein. *EmbC*, which is the C terminal of the protein[38], is considered in this study. Arabinogalactan expression in *Mtb* changes the cell wall synthesis rate and alters active state of bacteria to switch them into non-replicating (latent) state[39]. *FbiB* protein has a suggested role on the cofactor F-420 synthesis and co-ordinates with glucose-6-phospate to protect the cells from macrophage induced oxidative stress under aerobic conditions[40,41]. The complete role of delamanid (upcoming drug that targets *FbiB*) in inhibiting the protein is yet to be uncovered, however in this study *FbiB* interacts with stigmasterol with a good binding affinity.

MmpL3 is a recently identified cell wall protein target that is inhibited by the compound SQ-109. *MmpL3* transports trehalose monomycolate units across the *Mtb* cell as a unit of mycolic acid. Studies have shown that genetic inactivation of the protein causes aggregation of trehalose monomycolate that is lethal to the cell[42]. Stigmasterol interacts with three of the target proteins with stable interaction and good binding energy which makes it a potential therapeutic compound.

Simiarenol, a pentacyclic triterpenoid, has exhibited notable leishmanicidal activity against *Leishmania donovani* promastigotes *in vitro* [43]. Additionally, 3B-simiarenol has shown anti-oxidant, hepatoprotective and antiviral properties, specifically against Human coronavirus (HCoV)[44,45]. Simiarenol (Hit 3) is seen interacting with *Alr* and *AtpC* which are recently identified drug targets in *Mtb*. *Alr* codes for D-alanine racemase is responsible for synthesis of peptidoglycan in the cell wall of *Mtb*. *Alr* is an emerging drug target for compound D-cycloserine (cyclic analog of D-alanine)[21]. The ε subunit of ATP synthase that is coded by *AtpC* gene is targeted by the drug bedaquiline. ATP synthesized is transported across the cell for mycolic acid synthesis and other cellular metabolic pathways. Alterations in ATP synthase protein is associated with defective ATP synthesis and hence disrupted or stunt cell wall in the organism[46,47]. Simiarenol interacts with *AtpC* with a binding energy of -7.51 kcal/mol showing one hydrogen bond and Van der Waals interactions.

A coarse grained molecular dynamic simulation was done to the Hit 2 (stigmasterol) with proteins *FbiB*, *EmbC* and *MmpL3* using CABS Flex 2.0. *FbiB* protein is involved in the activation the prodrugs ethambutol, delamanid and pretomanid. It is shown that mutation in *FbiA*, *FbiB* and *FbiC* renders impaired F420 subunit production involved in ATP synthesis and methoxy and keto mycolic acid synthesis. It is interesting to note that the protein cluster *EmbA*, *EmbB* and *EmbC* are also targeted by the same drugs delamanid and pretomanid. Here the *EmbC* intergenic region is required for the proper activation of the other 2 proteins which pose resistance to ethambutol drug[48]. So it can be postulated that the regulation of protein clusters *EmbC* and *FbiB* are interdependent in a manner that affects the synthesis and metabolism of mycolic acid in *Mtb*[39]. *MmpL3* is a huge transmembrane protein that aids in the transport and arrangement of mycolic acid to the cell wall. An analogue of ethambutol

(SQ109) is in clinical trials against *MmpL3*[49]. It is also to be noted that proteins interacting with stigmasterol are targeted by the antibiotics of the same class.

Conclusion

In the current study, natural compounds identified from the plants *S. auriculata* and *T. copticum* were chosen effective against *Mycobacterium tuberculosis* proteins. Among the compounds, potential hits lupeol, simiarenol, and stigmasterol were found to be majorly binding with most of the target proteins chosen. Stigmasterol displayed excellent binding affinity towards the proteins *EmbC*, *FbiB*, and *MmpL3*. The number of bonds and the type of bonds show that it associates with the protein in firm manner. Further RMSF analysis confirms a stable receptor ligand interaction. However, the above mentioned phyto-chemicals are contributing to the disruption of the cell wall metabolism and hence they might be taken into consideration as a multitargeted approach for treating TB. Moreover, we can also potentiate that the selected phytochemicals have scope of controlling the disease by crippling the growth of both replicating and non-replicating bacteria since the phytochemicals bind with some of the emerging drug targets that aim to control latent tuberculosis. However further validation of the hit stigmasterol must be done by molecular dynamic simulation, *in vitro* and *in vivo* to identify its potential to control the disease.

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Conflict of interest

All authors declare no conflict of interest.

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