4. Experimental Work

4.1. Homology modeling

Three-dimensional (3D) structure of *Mtb* NDH-2 was constructed using X-ray crystal structure of S. aureus NDH-2 (PDB code: 4XDB)through homology modeling using ModFOLD web server version 6.0 [147, 148]. The amino acid sequence of *Mtb* NDH-2 available in the uniprot database (http://www.uniprot.org/) was used for the purpose. The modeled 3D structure was further validated using Verify 3D [149], RAMPAGE [150], PROCHECK [151], ERRAT [152], and Global model quality score. Then, it was refined using PDB2PQR and Dockprep utility [153], and visualized with help of Chimera.

4.2. Off-target virtual screening and filtering of designed molecules

The designed molecules were screened against dopamine D2 (PDB: 6CM4) and D3 (PDB: 3PBL) receptors using Maestro 10.5.014 application (Schrödinger, LLC, USA, 2016-1). The whole process involved protein preparation, ligand preparation, receptor grid generation and finally ligand docking. The process of protein preparation and energy minimization of 6CM4 and 3PBL was performed using protein preparation wizard of Maestro. The active site amino acid residues were selected and receptor grid was generated encompassing these residues, using the grid generation module of Maestro. Ligands were then subjected to ligand preparation using the ligand preparation wizard in Maestro. During this, ionization and tautomeric states of ligands were generated using the Epic module. The prepared ligands were then screened against the energy minimized protein through high-throughput virtual screening in Schrodinger suite [154]. Docking score of $\leq$ 2.0 Kcal/mol was fixed as the cut-off, to filter the molecules with least interaction with dopamine receptors (D2 & D3).

4.3. Virtual screening of ZINC database molecules

The purchasable subset of ZINC Database was downloaded and structure-based virtual screening was performed against Mycobacterial ATP synthase (PDB code: 4V1F). Virtual screening was performed using Maestro 10.5.014 application (Schrödinger, LLC, USA, 2016-1). It involved three consecutive steps *viz*. receptor grid generation, ligand preparation and ligand docking. The protein 4V1F was subjected to protein preparation and receptor gird generation with grid size of 10Å. Later, the ZINC database molecules were subjected to ligand preparation, followed by High-throughput virtual screening [155].

4.4. Superimposition of protein structures of NDH-2 from different species

Protein structures of NDH-2 from different species were superimposed using Blosum-62 scoring matrix and Needleman-Wunsch alignment algorithm in UCSF chimera matchmaker. Both the active motif and complete protein of *C. thermarum* NDH-2 (PDB code: 4NWZ) and *S. aureus* NDH-2 (PDB code: 4XDB) were superimposed against the *Mtb* NDH-2 protein obtained through homology modeling [156].

4.5. Alignment of amino acid sequence of selected ATP synthase c-subunits

Amino acid sequence of ATP synthase c-subunits of *M. smegmatis, M. pheli, Mtb* and human were retrieved from UniProt database. The obtained sequences were then subjected to alignment using Basic Local Alignment Search Tool (BLAST) with Blosum-62 scoring matrix in NCBI protein database. The drug-binding/active site residues of ATP synthase of *M. pheli viz*. Gly62, Leu63, Glu65, Ala66, Ala67, Tyr68, Phe69, Ile70, and Leu72 were analyzed against their corresponding residues of ATP synthase from human and *Mtb*, to understand the residues that were conserved in all the cases. Thereby, the specificity of lead compounds can be predicted [157, 158].

4.6. Extra precision molecular docking

The hit molecules obtained from off-target virtual screening and filtering steps (section 4.2) were further docked against *Mtb* NDH-2 or Mycobacterial ATP synthase (4V1F) through Glide XP (extra precision) mode in Maestro, to study the different ligand poses for a molecule and weed out the false positives to afford a better correlation between good score and good poses [159].

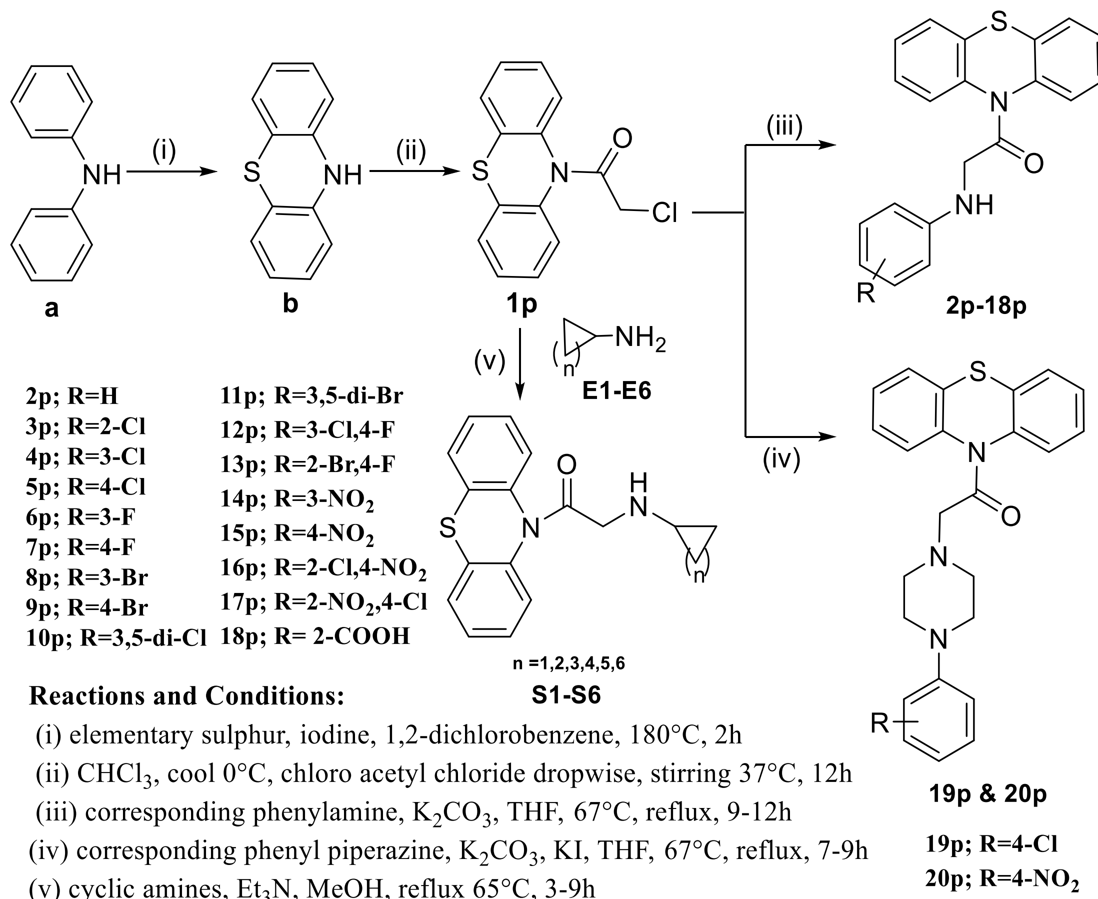
4.7. Molecular property and toxicity prediction

Molecular properties *viz*. cLogP, cLogS, hydrogen bond donor, hydrogen bond acceptor and drug-likeness were analyzed according to Lipinski's rule of five, using OSIRIS DataWarrior. Toxicity of the molecules in the study *viz*. mutagenicity, tumorigenicity and irritancy were also predicted through OSIRIS DataWarrior [160].

4.8. Synthesis and Characterization

Reagents, obtained from commercial suppliers, were used without further purification. Completion of reaction was monitored using pre-coated aluminium GF254 silica gel chromatographic plates. The compounds were purified by column chromatography and flash chromatography. Elution of the compounds in thin-layer chromatography was visualized in ultraviolet (UV) and iodine chamber. Melting point of the compounds was determined in Stuart-SMP10 melting point apparatus using closed-end glass capillary tubes. ^{1}H and ^{13}C NMR spectra were recorded in the Bruker DRX-500 FT-NMR at 500 and 125 MHz. Peaks in ^{1}H NMR spectra are reported as s(singlet), d(doublet), t(triplet), m(multiplet). Chemical shifts and coupling constants were reported in parts per million (ppm) and Hertz (Hz), respectively. ESI-MS spectrum was recorded in Micromass Quattro II spectrometer. Molecular ion and base peaks were reported as mass to charge ratio (*m/z*). Elemental composition was performed in Exeter CE-440 elemental analyser.

4.8.1. Synthesis of phenothiazine derivatives



Scheme 1: Synthesis of phenothiazine derivatives

*4.8.1.1. Synthesis of 10H-phenothiazine (**b**)*

Diphenylamine (**a**) (5.0 g, 29.54 mmol), elementary sulfur (1.9 g, 59.09 mmol), and iodine (0.37 g, 2.95 mmol) were taken in 1-2-dichlorobenzene (ODCB) used as a solvent and heated at 180°C for 2 hours. The evolved hydrogen sulfide was trapped in an aqueous solution of NaOH. The solvent was removed under vacuum and the crude obtained was subjected to flash column chromatography (SiO_2, EtOAc: hexane at 1:9 ratio) to afford pure phenothiazine. Further, it was recrystallized twice from aqueous ethanol to get light yellow crystals.

*4.8.1.2. Synthesis of 2-chloro-1-(10H-phenothiazin-10-yl) ethan-1-one (**1p**)*

10H-phenothiazine **(b)** (10.0 g, 59.09 mmol) was taken in chloroform ($CHCl_3$) (150 mL) and cooled to 0°C, dropwise chloroacetyl chloride (6.0 mL, 75.2 mmol) was added

to it and the reaction mixture was stirred for 12 hours at 37°C. It was allowed to cool to room temperature and concentrated under reduced pressure to obtain crude product. Water (100 mL) was added to it and extracted with dichloromethane (DCM) (2 × 100 mL). The obtained organic layer was dried over anhydrous Na_2SO_4 and solvent was evaporated to get white solid compound.

*4.8.1.3. Synthesis of substituted-phenyl analogs of phenothiazine **(2p-18p)***

2-Chloro-1-phenothiazine-10-yl-ethanone **(1p)** (0.2g, 0.73 mmol), potassium carbonate (K_2CO_3) (0.2 mg, 1.45 mmol) and corresponding phenyl amines were taken in 10 mL tetrahydrofuran (THF) and refluxed at 67°C for 9-12 hours. The crude material obtained was subjected to flash column chromatography (SiO2, EtOAc:hexane at 1:9 to 4:6 ratios) to afford different substituted-phenyl analogs of phenothiazine.

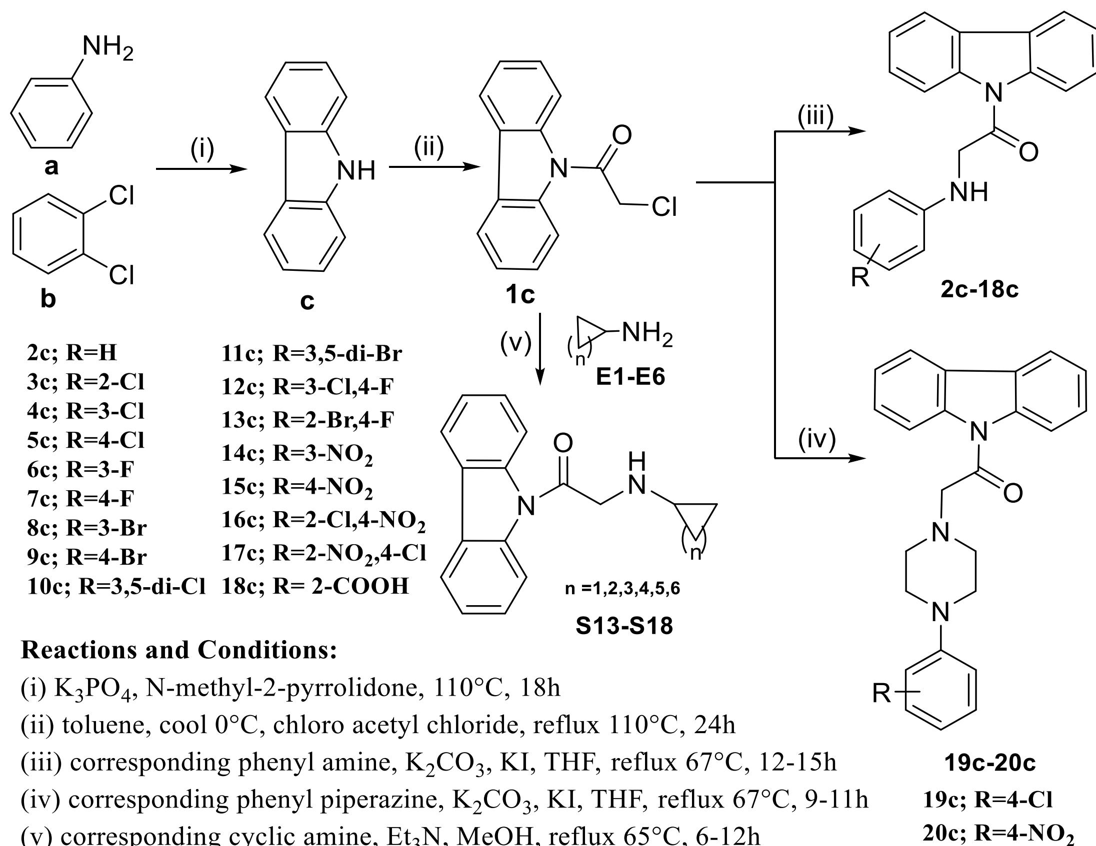
*4.8.1.4. Synthesis of substituted-phenylpiperazine analogs of phenothiazine **(19p, 20p)***

2-Chloro-1-phenothiazine-10-yl-ethanone **(1p)** (0.2g, 0.73 mmol), potassium carbonate (K_2CO_3) (0.2 mg, 1.45 mmol), potassium iodide (KI) (0.06 mg, 0.365 mmol) and corresponding phenyl piperazines were taken in 10 mL tetrahydrofuran (THF) and refluxed at 67°C for 7-9 hours. The crude material obtained was subjected to flash column chromatography (SiO2, EtOAc:hexane at 1:9 to 4:6 ratios) to afford different substituted-phenyl piperazine analogs of phenothiazine.

*4.8.1.5. Synthesis of cycloalkane analogs of phenothiazine **(S1-S6)***

2-Chloro-1-phenothiazine-10-yl-ethanone **(1p)** (0.2 g, 0.73 mmol), triethylamine (Et_3N) (0.1 mL, 0.72 mmol) and appropriate cyclic amine **(E1-E6)** (0.87 mmol) were taken in 10 mL methanol (MeOH) and refluxed at 65°C for 3-9 hours. The solvent was then evaporated to get the crude product. The crude material was subjected to flash column chromatography (SiO_2, EtOAc:hexane at 1:9 to 3:7 ratios) to afford different cycloalkane analogs of phenothiazine.

4.8.2. Synthesis of carbazole derivatives



Scheme 2: Synthesis of carbazole derivatives

*4.8.2.1. Synthesis of carbazole (**c**)*

Aniline (**a**) (5.35 g, 57.48 mmol), 1, 2-dichlorobenzene (**b**) (5 mL, 44.21 mmol), and tripotassium phosphate (22.52 g, 106 mmol) were taken in N-Methyl-2-pyrrolidone (100.0 mL) used as a solvent. The reaction mixture was heated at 110°C for 18 hours. The crude material obtained was purified by column chromatography over silica gel using n-pentane: Et_2O in 9:1 ratio, as eluent to afford a colourless solid of carbazole.

*4.8.2.2. Synthesis of 1-(9H-Carbazol-9-yl)-2-chloroethan-1-one (**1c**)*

Carbazole (**c**) (5.0 g, 29.90 mmol) and chloroacetyl chloride (3.56 mL, 44.85 mmol) were taken in toluene (75 mL) at 0ºC and refluxed at 110ºC for 24 hours. The reaction mixture was allowed to cool to room temperature and concentrated under reduced pressure. The crude obtained was purified by column chromatography and the desired compound was obtained as pale white crystals.

*4.8.2.3. Synthesis of substituted-phenyl analogs of carbazole (**2c-18c**)*

1-(9H-Carbazol-9-yl)-2-chloroethan-1-one (**1c**) (0.2g, 0.73 mmol), potassium carbonate (K_2CO_3) (0.2 mg, 1.45 mmol), potassium iodide (KI) (0.06 mg, 0.365 mmol) and corresponding phenyl amines (1.09 mmol) were taken in 10 mL Tetrahydrofuran (THF) and refluxed at 67°C for 12-15 hours. The crude material was subjected to flash column chromatography (SiO_2, EtOAc:hexane at 1:9 to 5:5 ratios) to afford different substituted-phenyl analogs of carbazole.

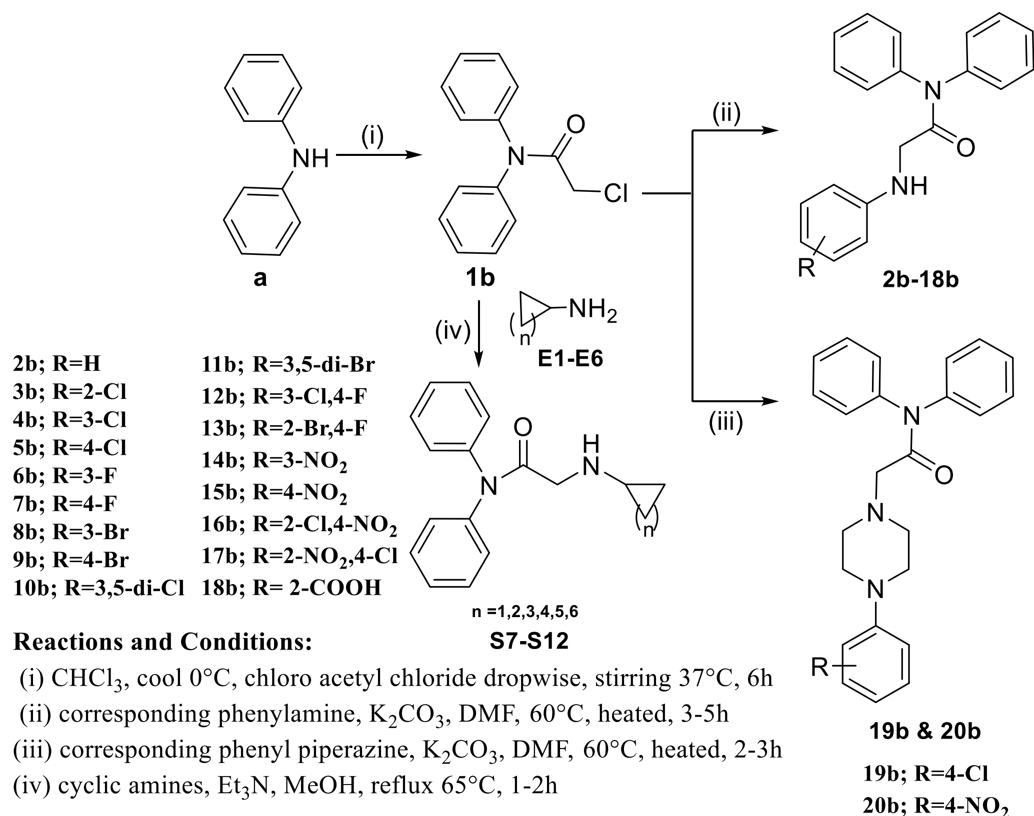
*4.8.2.4. Synthesis of substituted-phenyl piperazine analogs of carbazole (**19c & 20c**)*

1-(9H-Carbazol-9-yl)-2-chloroethan-1-one (**1c**) (0.2g, 0.73 mmol), potassium carbonate (K_2CO_3) (0.2 mg, 1.45 mmol), potassium iodide (KI) (0.06 mg, 0.365 mmol) and corresponding phenyl piperazines (1.09 mmol) were taken in 10 mL Tetrahydrofuran (THF) and refluxed at 67°C for 9-11 hours. The crude material was subjected to flash column chromatography (SiO_2, EtOAc:hexane at 1:9 to 5:5 ratios) to afford different substituted-phenyl piperazine analogs of carbazole.

*4.8.2.5. Synthesis of cycloalkane analogs of carbazole (**S13-S18**)*

1-(9H-carbazol-9-yl)-2-chloroethan-1-one **(1c)** (0.2 g, 0.73 mmol), triethylamine (0.11 mL, 0.82 mmol) and appropriate cyclic amine **(E1-E6)** (0.98 mmol) were taken in 10 mL methanol (MeOH) and refluxed at 65°C for 6-12 hours. The solvent was evaporated to get the crude product. The crude material was then subjected to flash column chromatography (SiO_2, EtOAc:hexane at 1:9 to 4:6 ratio) to afford different cycloalkane analogs of carbazole.

4.8.3. Synthesis of biphenyl derivatives



Scheme 3: Synthesis of biphenyl derivatives

*4.8.3.1. Synthesis of 2- Chloro-N,N-diphenyl-acetamide (**1b**)*

Diphenylamine (**a**) (5.0 g, 29.54 mmol) was taken in dichloromethane (DCM) (75 mL) and cooled to 0°C. Dropwise chloroacetyl chloride (3.5 mL, 44.31mmol) was added and the reaction mixture was stirred at 37°C for 6 hours. It was allowed to cool to room temperature and concentrated under reduced pressure. The crude material obtained was added with water (50 mL) and extracted with DCM (2×50 mL). The organic layer was dried over anhydrous Na_2SO_4, and the solvent was removed to get white solid.

*4.8.3.2. Synthesis of substituted-phenyl analogs of biphenyl scaffold (**2b-18b**)*

2- Chloro-N,N-diphenyl-acetamide (**1b**) (0.2g, 0.8mmol), appropriate phenyl amines (1.2mmol) and K_2CO_3 (0.8 mmol) were heated in 10ml DMF for 3-5 hours at 60°C. The completion of reaction was monitored by TLC. The reaction mixture was then poured into crushed ice. The solid obtained was filtered and washed with sodium bicarbonate

solution, followed by water. The crude solid was subjected to column chromatography (SiO_2, EtOAc:hexanes at 1:9-3:9 ratio to afford different substituted-phenyl analogs of biphenyl scaffold.

4.8.3.3. Synthesis of substituted-phenylpiperazine analogs of biphenyl scaffold ***(19b & 20b)***

2-Chloro-N,N-diphenyl-acetamide (**1b**) (0.2g, 0.8mmol), appropriate phenyl piperazines (1.2mmol) and K_2CO_3 (0.8 mmol) were heated in 10ml DMF for 2-3 hours at 60°C. The completion of reaction was monitored by TLC. The reaction mixture was then poured into crushed ice. The solid obtained was filtered and washed with sodium bicarbonate solution, followed by water. The crude solid was subjected to column chromatography (SiO_2, EtOAc:hexanes at 1:9-3:7 ratio to afford different substituted-phenyl piperazine analogs of biphenyl scaffold.

4.8.3.4. Synthesis of cycloalkane analogs of biphenyl scaffold ***(S7-S12)***

2- Chloro-N,N-diphenyl-acetamide **(1b)** (0.2 g, 0.8 mmol), triethylamine (0.11mL, 0.8 mmol) and appropriate cyclic amine **(E1-E6)** (0.97 mmol) were taken in 10mL methanol (MeOH) and refluxed at 65°C for 1-2 hours. The solvent was evaporated to get the crude product. The crude material was subjected to flash column chromatography (SiO_2, EtOAc:hexane at 1:9 to 3:7 ratios) to afford different cycloalkane analogs of biphenyl scaffold.

4.9. Biological Profiling

4.9.1. Antitubercular screening

The synthesised compounds were screened against *Mtb* H37Rv by microplate Alamar Blue assay (MABA) to determine their minimum inhibitory concentration (MIC). The MIC is the lowest drug concentration that prevented the growth of the *Mtb*. 200µL of deionised water was added to the outer walls of sterile 96 well plate to prevent vaporization of media used in the experiment. The wells received 100µL of

Middlebrook 7H9 broth, followed by the test compounds at concentrations ranging from 100 to 0.2µg/mL. 100µL of *Mtb* inoculum was then added to each wells, leaving the blank wells. The plates were then wrapped using parafilm and incubated at 37ºC for continuous five days. The Alamar blue reagent and 10% tween 80 were mixed at 1:1 ratio. From which, 25µL was added to the wells and reincubated for another 24 hours. The development of pink color in the well was interpreted as growth of the cells, and the blue color as absence of growth. A few wells appeared violet after 24 hours of incubation, but they invariably changed to pink after another day of incubation and thus were scored as growth. The experiment was performed thrice to validate the chance of error.

4.9.2. Antibacterial screening

The synthesised compounds were further evaluated against *Staphylococcus aureus* (ATCC 25323) and *Escherichia coli* (ATCC 35218) to understand their antibacterial efficacy. The experiment was performed using agar plate disc diffusion method. Fresh agar plates were prepared by spreading 20mL of Mueller Hinton Agar (MHA, Hi-Media) in the petri dishes. The 24-48 hold culture of *S. aureus* or *E. coli* was mixed with sterile physiological saline and the turbidity was adjusted to 0.5 in Mac-Farland scale *i.e.* ~10^6 colony forming units (CFU) per mL. The inoculum was then spreaded evenly on the agar plates using glass hockey stick. 20µL of test compounds or standards were impregnated to 'Whatman 1' paper discs of diameter of 6mm and were placed over the agar plate to determine their efficacy. Ciprofloxacin (5µg/disc) was used as positive control. The plates were then incubated for a period of 24 hours. The minimum concentration of test compounds that required to produce complete inhibition of *S. aureus* and *E. coli* was recorded as MIC. The experiment was repeated in triplicate to validate the results.

4.9.3. Blood Brain Barrier (BBB) permeability screening

The penetration of synthesised compounds into brain was assessed by parallel artificial membrane permeability assay (PAMPA). The experiment consisted of an acceptor and a donor plate. The acceptor plate was moisturised with buffer of pH 7.4 for 30 minutes. Then, it was removed and the wells were coated with 4µL of Porcine Brain Lipid (PBL) at 20mg/mL concentration in dodecane. The coating was allowed to settle and was followed by addition of 200µL of buffer to the wells. On other side, test compounds were prepared at a concentration of 25µg/mL. From which, 200µL was transferred to the wells of donor plate. Atenolol, Verapamil, Diazepam and Levofloxacin were the standard drugs used in this study for comparison and classification. The acceptor and donor plates were then coupled and incubated at room temperature for 18 hours. The plates were separated and absorbance of the compounds in both acceptor and donor plate was determined at their wavelengths using ELISA plate reader. The amount of drug that permeated or retained was determined from the absorbance measured, by using the following equation;

Pe (cm/s) = {-ln[l-CA(t)/Ceq]}/[A*(1/VD+ 1/VA)*t]

Where,

Pe = permeability in cm sec^{-1};

A = filter area (*i.e*. 0.3 cm^2);

VD = donor well volume (*i.e.* 0.3 mL);

VA = acceptor well volume (*i.e*. 0.2 mL);

t = incubation time (sec);

CA(t) = concentration in acceptor well at time t;

CD(t) = concentration in donor well at time t;

Ceq = [CD(t)*VD + CA(t)*VA]/(VD+VA).

The test compounds were then classified based on their penetrating nature, such as,

CNS+ = high BBB permeation compounds, *i.e.* Pe= > $4.0 * 10^{-6}$ cm s^{-1}

CNS- =low BBB permeation compounds, *i.e.* Pe= < $2.0 * 10^{-6}$ cm s^{-1}

CNS+/- =BBB permeation uncertain compounds, *i.e.* Pe =4.0 - $2.0 * 10^{-6}$ cm s^{-1}

4.9.4. **In-vitro** ***cytotoxicity screening***

The synthesized compounds were screened against African green monkey kidney epithelial cell line (VERO) by the MTT assay, to assess the cell cytotoxicity. Cells in T-25 flask were trypsinized and 5ml of Eagle's minimal essential medium (EMEM) was added to it. They were then centrifuged in a sterile falcon tube at 500 rpm for 5 minutes. The cells were then resuspended in 1mL of the media with a count of 1x 10^5 cells per mL. From which, 100µl was added to each well in 96-well plate. The plates were incubated at 37°C in 5% CO_2 for 24 hours, followed by addition of test compounds at different concentrations (1000 to 7.8µg/mL). The incubation was continued for another 72 hours. The sample solution was removed and washed with phosphate-buffered saline (pH 7.4). 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-tetrazolium bromide (MTT) reagent was freshly prepared at a concentration of 5mg/mL in phosphate buffered saline. From which, 20µL was added to the test wells and the incubation was continued for 4 hours. Then, DMSO (1mL) was added to solubilize the formazan crystals and absorbance was measured at 570nm in microplate reader. The relative growth of cells with respect to the control well was recorded as percentage cell viability by using the following formula and 50% cytotoxic concentration (CC_{50}) was then graphically determined.

Percentage Cell Viability= Absorption of sample/ absorption of control x 100

4.9.5. Type-2 NADH dehydrogenase (NDH-2) inhibition screening

NDH-2 activity inhibition screening was performed through NADH:menadione oxidoreduction assay. The study was performed at 37°C in 50mM Tris-Cl buffer (pH

8.0) containing 150mM NaCl, 1% DMSO and 1% octylglucoside. Activity was monitored by recording the absorbance change of NADH (340-380nm, ε=4.81 mM^{-1} cm^{-1}). 2-heptyl-4-hydroxyquinoline-N-oxide (HQNO) was used as positive control in all the experiments. NADH and menadione (MD) substrate concentrations were fixed at 200 and 50μM, respectively. Inhibitor concentration was fixed at 50μM. Enzyme (NDH-2) concentration used was typically 13.5$ngmL^{-1}$. Each reaction mix was pre-incubated with MD and inhibitors for 2 minutes and the reaction was initiated by adding NADH to the mixture. The activity was normalised against a control sample with no HQNO present in the assay mixture. The assay was performed in triplicate [109, 161].

4.9.6. Mycobacterial ATP synthase inhibition screening

ATP synthase inhibition was measured using inverted membrane vesicles (IMVs) from *Mycobacterium smegmatis* WT. IMVs were prepared according to the procedure reported by Koul *et al* [162] and the experiment was based on method described by Haagsma *et al* and Lu *et al* with slight modification [163, 164]. The ATP produced was quantified by glucose-6-phosphate dehydrogenase method. 1mg/mL of IMVs from *M. smegmatis* WT were suspended in 10 mM HEPES-KOH buffer of pH 7.5 containing 100mM KCl, 20mM KH_2PO_4, 5mM $MgCl_2$, 2mM ADP, 100μM P_1,P_5-di(adenosine-5′) pentaphosphate (Ap5A), 25.4mM glucose, 11.8UmL^{-1} hexokinase. N, N'-Dicyclohexyl carbodiimide (DCCD) was used as positive control in all the experiments. The compounds at different concentrations (1, 5, 15 and 100μM) and DCCD (100μM) were pre-incubated at 37°C with vigorous stirring for 30 minutes. The reaction was started with addition of 5mM succinate and incubated for 120 minutes at 37ºC. It was stopped with addition of 25mM EDTA. Samples were then boiled for 5 minutes and centrifuged at 10000g for 20 minutes to remove the denatured protein. The supernatants containing the synthesized glucose-6-phosphate were collected and oxidized by the addition of

2.5mM NADP and 3UmL^{-1} of glucose-6-phosphate dehydrogenase. The NADPH formed was measured at 340nm using spectrophotometer. The assay was performed in triplicate.