5. Experimental

The experimental work has been divided into three parts:

5.1 Molecular modeling studies
5.2 Chemical work
5.3 Biological work

5.1 Molecular modeling studies

5.1.1 Hardwares and softwares used in the molecular modeling

5.1.1.1 Hardwares

3D-QSAR (CoMFA/CoMSIA) studies were performed on PC with Linux operating system. The configuration was as follows:

Model	**DELL**
Processor	Optiplex 330, 2.53 GHz
Memory	2 GB
Data cache	3MB L2 cache
Operating system	Linux

Pharmacophore modeling, 3D-QSAR (atom-based) modeling, docking studies and virtual screening were performed on PC with Microsoft Windows operating system. The configuration was as follows:

Model	**DELL**
Processor	Intel(R)Core(TM)i7-3770CPU@3.40GHz
Memory	8 GB
Data cache	4 × 32 Kbytes
Operating system	Microsoft Windows

5.1.1.2 Softwares

- 3D-QSAR (CoMFA/CoMSIA) models were performed using QSAR module in SYBYL (version 7.0) procured from Tripos Inc. USA.[274]
- Docking studies were performed using GLIDE (version 5.5) procured from Schrödinger, LLC, New York, NY, 2009.[266]

- Pharmacophore mapping, 3D-QSAR (atom-based) modeling and virtual screening were performed using PHASE from Schrodinger, LLC New York, NY.[260,265]
- Physicochemical and pharmacokinetic properties were computed using Qikprop[256] and pkcsm[269] softwares.
- Applicability domain was determined by "Enalos KNIME nodes"[254] and "AD using standardization approach".[263]

5.1.2 Dataset, and selection of training and test set

Dataset used for the development of 3D-QSAR and pharmacophore models were selected from the literature having peripherally acting CB1 receptor antagonistic activity. Blocking of CB1 receptors in peripheral sites is possible through different ways such as designing of inverse agonists, antagonists, neutral antagonists and allosteric modulators. Those compounds in which the authors tried to increase the PSA or decrease the lipophilicity for designing of peripherally acting CB1 receptor antagonists were also considered for the studies. The selected dataset was based on the peripheral selectivity so that the CNS side effects could be minimized.

For performing 3D-QSAR and pharmacophore modeling, the dataset was divided into training and test set compounds. This segregation of compounds was done in such a manner that the test set compounds contained representative compounds of training set in terms of structural diversity and biological activity. The test set contained around 25 % to 33 % of total compounds which were used for external validation of the developed model.

5.1.3 Conversion of biological activities in terms of $p\mathrm{IC}_{50}/p\mathrm{K}_i$ (Negative logarithm of $\mathrm{IC}_{50}/\mathrm{K}_i$ in molar concentrations)

In all the computational studies, the biological activity in $\mathrm{IC}_{50}/\mathrm{K}_i$ (in nM) used as a dependent variable were converted to the corresponding $p\mathrm{IC}_{50}/p\mathrm{K}_i$ (in M) using the following equation 1:[251]

$$p\mathrm{IC}_{50}/p\mathrm{K}_i = -\log(\mathrm{IC}_{50}/\mathrm{K}_i) \qquad \text{...........(Eq. 1)}$$

5.1.4 3D-QSAR (CoMFA and CoMSIA) studies

5.1.4.1 Chemical structure building and energy minimization of the structures

3D-QSAR (CoMFA/CoMSIA) studies were carried out using the SYBYL 7.0 molecular modeling software from Tripos, Inc.[274] The 'Sketch Molecule' module of

SYBYL 7.0 software was used to construct the 3D structures of the molecules using standard geometry and bond lengths. Conjugate gradient and steepest descent methods were used for energy minimization of the structures using the TRIPOS force field and Gasteiger-Huckel charges until the root mean square deviation (RMSD) of 0.001 kcal/mol and 0.05 kcal/mol, respectively, were achieved. The energy minimization procedure was carried out by using a nonbonded interaction cutoff of 8.0 Å, having a distance-dependent dielectric function with dielectric constant of 1.0. Thus, the lowest energy conformers generated by using the above said two methods were subsequently used for the alignments. A conformational search for all the compounds was also carried out by using a simulated annealing technique with the Tripos force field in SYBYL. The molecules were heated to 700 ˚K followed by cooling to 300 ˚K and the time spent for annealing was 1000 fs. The time increment for dynamics calculations was 0.5 fs and the coupling time for temperature regulation was 2.0 fs. Ten consecutive cycles were performed. Further, a conjugate gradient method in SYBYL 7.0 software was used to minimize the lowest energy conformers using the Tripos force field until a root mean square deviation of 0.001 kcal mol^{-1}Å was achieved.[275]

5.1.4.2 Molecular alignment

To obtain the best 3D-QSAR model with high predictive accuracy, proper alignment of all the structures is essential because the results of CoMFA and CoMSIA studies are alignment sensitive. Thus, the molecular alignment of the 3D structures plays a crucial role in CoMFA and CoMSIA analysis.[276] Different types of molecular alignment rules were used for the development of 3D-QSAR models.

5.1.4.2.1 Atom-based alignment

This type of alignment was based on the atoms only in which certain atoms of the investigational compounds were used for RMS fitting on the corresponding atoms of the template.

5.1.4.2.2 Centroid-based alignment

In this type of alignment, centroids rather than exact superimposition of the atoms of the rings were used for RMS fitting to the template.

5.1.4.2.3 Centroid and atom-based alignment

This type of alignment involved a combination of both centroids and atoms for superimposition and RMS fitting to the template.

5.1.4.2.4 Data-based alignment

In this type of alignment, each analog was aligned to the template by rotation and translation so as to minimize RMSD between atoms and centroids in the template and the corresponding atoms/centroids in the analog, using the DATABASE ALIGN command in SYBYL.

5.1.4.3 CoMFA and CoMSIA interaction energy calculations

In the CoMFA analysis, the steric and electrostatic fields were calculated by applying regular space grid of 2.0 Å at each lattice intersection in all the three dimensions within the defined region. The van der Waals potentials and coulombic potentials were used to represent the steric and electrostatic fields respectively which were calculated using standard Tripos force fields. To hasten the analysis and reduce the amount of noise, the threshold value of column filtering was set to 2.0 kcal/mol. An sp^3 carbon atom was used as a probe atom with +1.0 charge and distance-dependent dielectric constant of 1.00. The steric and electrostatic fields of CoMFA analysis were truncated at +30.0 kcal/mol.[251,277,278] In the CoMSIA analysis, the interaction energy was calculated using five potential fields, namely the steric, electrostatic, hydrophobic, hydrogen bond acceptor and hydrogen bond donor fields at each lattice intersection of a regular space grid of 2.0 Å. The radius of the probe atom was 1.0 Å and +1.0 charge with +1.0 hydrophobicity, +1.0 hydrogen bond donor and +1.0 hydrogen bond acceptor values were used for the CoMSIA analysis. The contribution from all the descriptors was truncated at 0.3 kcal/mol.[251,279]

5.1.4.4 Partial least square (PLS) analysis

To develop our 3D-QSAR models, PLS analysis was used to linearly correlate biological activity ($p\mathrm{IC}_{50}$) as the dependent variable with CoMFA and CoMSIA descriptors as the independent variables. To assess the internal predictive accuracy of the models, a cross-validation was performed by using the leave-one-out (LOO) method. In this method, one compound was removed at a time from the dataset and its activity was predicted by the developed model from the rest of the compounds in the dataset. In PLS analysis, the minimum column filtering value used was 2.0 kcal/mol considered as the threshold column filtering value. The cross-validated correlation coefficient (r^2_{cv}) (Eq. 2) and optimum number of components (ONC) were obtained by this method. By using the ONC, the non-cross-validated correlation coefficient (r^2_{ncv}) and standard error of estimation (SEE) were calculated to evaluate the fitting quality of the models.[280-282] Evaluation of robustness and statistical confidence of the generated models was done by analysis of r^2_{cv}. The $r^2_{cv} > 0.3$ indicated that the probability of chance correlation was less than 5 % and the developed model was reliable one.

$$r^2_{cv} = 1 - \frac{\Sigma (Y_{\text{predicted}} - Y_{\text{actual}})^2}{\Sigma (Y_{\text{actual}} - Y_{mean})^2} \quad \text{..........(Eq. 2)}$$

Where,

$Y_{\text{predicted}}$ = Predicted value of the target property ($p\text{IC}_{50}$)
Y_{actual} = Actual value of the target property ($p\text{IC}_{50}$)
Y_{mean} = Mean value of the target property ($p\text{IC}_{50}$)

5.1.4.5 Predictive accuracy of CoMFA and CoMSIA models

The predictive accuracy of the derived CoMFA and CoMSIA models was calculated by using the test set compounds expressed as r^2_{pred} by using the following equation (Eq. 3):

$$r^2_{\text{pred}} = (\text{SD-PRESS})/\text{SD} \quad \text{............ (Eq. 3)}$$

Where,

SD is the sum of the squared deviations between the biological activities of the test set compounds and the mean activity of the training set compounds, and PRESS is the sum of the squared deviation between the actual and the predicted activities of the test set compounds.[283]

5.1.4.6 Validation parameters

a) Tropsha’s validation tests

According to Tropsha, a good model should pass the following criteria:[254,284]

- $r^2_{cv} > 0.5$
- $r^2_{pred} > 0.6$
- $[(r^2 - r^2_0)/r^2] < 0.1$ or $[(r^2 - r'^2_0)/r^2] < 0.1$
- $0.85 \le k \le 1.15$ or $0.85 \le k' \le 1.15$

Here, r^2 is the squared correlation coefficient between the observed and predicted values, r^2_0 is squared correlation coefficient between the observed and predicted values with intercept set to zero and r'^2_0 is squared correlation coefficient between the predicted and observed values of the test set compounds with intercept set to zero. *k* is the slope of the line obtained through the observed and predicted values of the test set compounds whereas *k*' is the slope of the line obtained through the predicted and observed values of the test set compounds.

b) Modified r^2 (r^2_m)

Modified r^2 (r^2_m), an essential parameter used to validate the external predictive accuracy of the developed model, was used to penalise the model containing large differences between the actual activity and the predicted activity for the test set compounds. The value of r^2_m is calculated by using equation (4):[285,286]

$$r^2_m = r^2 (1- \sqrt{|r^2- r^2_0|}) \quad \text{………………Eq. (4)}$$

where,

r^2 is squared correlation coefficient between observed and predicted values of the test set compounds and r^2_0 is squared correlation coefficient between the observed and predicted values of the test set compounds with intercept set to zero.

c) Applicability domain

Applicability domain is calculated by using both 'similarity measurement' and 'leverage'. The first method, similarity measurement is based on the Euclidean distances among all training and test set compounds for defining the domain of applicability of the models. The distance of a test compound to its nearest neighbour in the training set was compared to the predefined applicability domain (APD) threshold. If the distance is higher than APD, the prediction is considered unreliable. APD can be calculated by using Eq. (5)

$$APD = \langle d \rangle + Z\sigma \quad \text{…..Eq. (5)}$$

Where, $\langle d \rangle$ and σ are average and standard deviation of all distances and Z is the empirical cut-off value.

The second method is based on the calculation of the leverage h_i for each chemical, which can be calculated by using Eq. (6).

$$h_i = x_i^T (X^TX)x_i \quad (i = 1,...,n) \quad \text{…..Eq. (6)}$$

Where, x_i is the descriptor-row vector of the query compound and X is the $k \times n$ matrix of k model descriptor values for each one of the n training compounds. The superscript ‘T’ refers to the transpose of the matrix/vector. If the leverage value is greater than $3k/n$, then it is considered too large to be reliable, indicating that the predicted response is the result of substantial extrapolation of the model.[254,287,288]

5.1.5 Development of pharmacophore and 3D-QSAR (atom-based) models using PHASE module

5.1.5.1 Development of pharmacophore model

A set of pharmacophore features for the selected compounds was generated using create sites option. Site points were created for each conformer of the compounds under study. For creating pharmacophore sites, a default setting having aromatic ring (R), hydrophobic (H), positive (P), H-bond donor (D), and acceptor (A) features were used. Common pharmacophore hypothesis for the set of active ligands were generated using these features. A set of variants formed by a set of features was used to identify the common pharmacophore using a tree based partitioning algorithm with a criterion that the selected variants must match with all the active compounds. The generated common pharmacophores of all variants were scored for the active and inactive compounds to identify a set of hypotheses having the best alignment of all the actives. The common pharmacophore hypotheses so obtained were scored by setting the root mean square deviation (RMSD) value below 1.0 and vector score value to 0.5.[289] “Score hypothesis” was the last step in the pharmacophore development where the hypotheses were ranked to make rational choices among the generated hypotheses. The most appropriate hypothesis was selected for further exploration. All the generated pharmacophore hypotheses were ranked accordingly on the basis of their statistical parameters such as ‘survival score’ and ‘survival minus inactive’ (S-I).[290,291] Survival score for the active compounds is the weighed combination of the volume, site, vector, survival score and a term for the number of matches. The survival score and S-I correspond to ‘score active’ and ‘score inactive’ respectively. In addition to this, post hoc score was also used to validate the best pharmacophore hypothesis. The highest value of ‘score active’ of the pharmacophore model has the ability to identify active molecules whereas the highest value of ‘score inactive’ of the model has the ability to segregate the active and inactive molecules.[291] Survival score (S) is the final scoring function which can be represented with the following equation (7):

$$S = W_{\mathrm{site}}S_{\mathrm{site}} + W_{\mathrm{vec}}S_{\mathrm{vec}} + W_{\mathrm{vol}}S_{\mathrm{vol}} + W_{\mathrm{sel}}S_{\mathrm{sel}} + W^{m}_{rew} - W_{\mathrm{E}}\Delta E + W_{\mathrm{act}}A \quad \text{.....Eq. (7)}$$

Where, S represents the scores and W represent weights. S_{site}, S_{vec}, S_{vol} and S_{sel} denote site score, vector score, volume score and selectivity score respectively. S_{site} is the RMSD in the site point position, S_{vec} is the cosine of the angles formed by corresponding pairs of vector features in aligned structures and S_{vol} is based on overlap of van der Waals models of nonhydrogen atoms in each pair of structures. Default values of W_{site}, W_{vec}, W_{vol} were 1.0 whereas default value of W_{sel} was 0.0. W^{m}_{rew} represents the reward in which W^{m}_{rew} is user-adjustable (1.0 by default) where m denotes the number of actives that match the hypothesis minus one. $W_{\mathrm{E}}\Delta E$ denotes penalty for high-energy structures by subtracting a multiple of the relative energy from the final score and it penalizes a hypothesis for which the reference ligand activity is lower than the highest activity by adding a multiple of the reference ligand activity to the score denoted by $W_{\mathrm{act}}A$, where A denotes the activity.[292] Higher survival score indicates better mapping of the pharmacophore with the active ligands and the fitness score confirms the quality of the pharmacophore hypothesis that can be defined as how well the compounds could be mapped to a pharmacophore model which can be calculated by using the following equation (8):

$$Fitness = \frac{\Sigma_n [1 - \Sigma\,(d/t)^2]}{n} \qquad \text{...............Eq. (8)}$$

Where, n is the number of pharmacophore features, d is the displacement of the feature from the centre of the location constraint and t represents the radius of the location constraint sphere for the feature.[293,294] Based on 8 active ligands in the dataset, several 3-point, 4-point and 5-point pharmacophore hypotheses were generated. Each generated common pharmacophore hypothesis resulted into alignment of all the compounds. These alignments were further used to develop 3D-QSAR models.

5.1.5.2 Development of 3D-QSAR (atom-based) model in the PHASE module

A 3D-QSAR (atom-based) model was developed by using PHASE QSAR module of Schrodinger that employs a grid-based 3D-QSAR analysis. The dataset was divided into training and test sets in a ratio of 4:1 respectively. Atom-based alignment was utilised in the development of 3D-QSAR models using PLS factors. A maximum of n/5 PLS factors can be used where ‘n’ is the number of compounds in the training set. So, in the present work a maximum of 4 PLS factors were allowed. The developed 3D-QSAR models were validated using different parameters such as cross-validated coefficient, noncross-validated coefficient, F-value, predictive r^2, modified r^2 as were described earlier.

The statistical parameters used for the validation purpose such as sensitivity, specificity, accuracy, positive prediction value (PPV), negative prediction value (NPV) and Matthew’s correlation coefficient (MCC) were calculated by using the following equations (9-14).[295]

$$\text{Sensitivity} = TP / (TP + FN) \times 100\ \% \quad \text{……….Eq. (9)}$$

$$\text{Specificity} = TN / (TN + FP) \times 100\ \% \quad \text{……….Eq. (10)}$$

$$\text{Accuracy} = (TP + TN) / (TP + FP + TN + FN) \times 100\% \quad \text{……….Eq. (11)}$$

$$PPV = TP / (TP + FP) \quad \text{……….Eq. (12)}$$

$$NPV = TN / (TN + FN) \quad \text{……….Eq. (13)}$$

$$MCC = \frac{(TP)\ (TN) - (FP)\ (FN)}{\sqrt{(TP + FP)\ (TP + FN)\ (TN + FP)\ (TN + FN)}} \quad \text{……….Eq. (14)}$$

Where, TP, FN, TN and FP are true positives, false negatives, true negatives and false positives respectively.

5.1.6 Virtual screening

Virtual screening of Asinex database containing 435,214 diverse chemical compounds was performed with the aim of finding potential hits suitable for further development as selective CB1 receptor antagonists. Asinex database of known 3D-structures were downloaded from the official site[296] and optimized using OPLS 2005 force field in Ligprep, a standard option in Maestro 9.0.[261] The Asinex database was screened by using the following filters.

5.1.6.1 Using pharmacophore model - the first filter

The developed pharmacophore model was used as the first filter to screen 3D database to retrieve structures that fit the pharmacophore hypothesis. Only those compounds were selected at this stage which were fitting in all the features of the pharmacophore model. Rest of the compounds were discarded. In order to reduce the number of hits, other filters were also used such as docking, 3D-QSAR, Lipinski's rule of five etc.

5.1.6.2 Docking of the resulting compounds - the second filter

Docking is one of the methods to distinguish potential leads from inactive compounds by performing docking of the compounds into the active site of the receptor in order to rank the compounds by the nature of interactions they show with the receptor. The retrieved compounds from the previous stage were further filtered by using this second filter i.e. molecular docking studies using Glide 5.5 software[266] in order to decrease the number of hits obtained after the first filtration process. Docking studies were carried out by the following general procedure.

5.1.6.2.1 Structure of CB1 receptor

The crystal structure of the CB1 receptor was not known when virtual screening protocol was performed. So, homology model of CB1 receptor was used for the docking studies.[264] The crystal structure of CB1 receptor has been identified recently (PDB code: 5TGZ) which is available in Protein Data Bank (PDB, USA).[267, 297]

5.1.6.2.2 Protein preparation and receptor grid generation

Protein preparation was carried out using standard option 'protein preparation wizard' in GLIDE 5.5. For obtaining accurate docking studies, protein preparation procedure was performed to remove physically untenable steric clashes generally found in the crystallographically resolved proteins. This helps the native ligand to get favourable van der Waals interaction energies for a properly docked structure. Protonation states and hydrogen bonding patterns should be correct. Hydrogens should be added and minimization of the receptor ligand complex was performed in the final step.

Receptor grid was generated after the completion of protein preparation step. The ligand was excluded from the grid generation by highlighting the ligand so that the correct volume for the grid was calculated. Active site identification can be performed by standard option of "site tab" from the grid generation menu. A purple coloured enclosing box was generated which represented the volume of the protein. Thus, the obtained grid was used further for the docking of ligands into the active site of the receptor.

5.1.6.2.3 Docking of the ligands into the receptor grid

Well known compounds such as rimonabant, AM6545 and TM38837 were planted in the database as reference compounds. Compounds retrieved from the first stage of filtration were docked in the active site of CB1 receptors by using high-throughput virtual screening (HTVS) method and flexible docking mode in Glide with a limit of five retained poses per molecule. Top 25,000 poses were saved with fixing of van der Walls radii of the ligands to 0.8 and partial charge cutoff to 0.15.[268] Compounds so obtained were re-docked using standard precision (SP) method for obtaining higher precision. The process was repeated again by re-docking the compounds in the active site of CB1 receptors by using extra precision (XP) method. Visualization of hydrogen bond and van der Waals contacts (good, bad and ugly) of the ligands with the receptor could be seen by pose-viewer file. In Glide 5.5 software, the docking score was reported as G-score. Higher negative G-score suggested a better fit in the binding site.

5.1.6.3 Applying Lipinki's rule of five - the third filter

Christopher Lipinski's rule of five is very helpful to know about the properties and structural features of a molecule having a drug like properties. Lipinski's rule of five contains the following criteria:

- Molecular weight should be equal or less than 500 Da
- Lipophilicity (logP) should be equal or less than 5
- Number of hydrogen bond donors should be equal or less than 5
- Number of hydrogen bond acceptors should be equal or less than 10

Poor absorption or permeation of orally administered compounds was observed if a compound is having higher values than the listed ones. Thus, this rule is helpful in the understanding of pharmacokinetic properties of a new chemical entity in early preclinical development. This rule could help to save millions of dollars if the compound fails in the late preclinical and clinical stages.

The compounds resulting from the docking studies, the second filter, were further filtered out by using Lipinski's rule of five as the third filter. The successful compounds obtained from this process were submitted for the next step.

5.1.6.4 Predicting the activity by 3D-QSAR (atom-based) model - the fourth filter

The activity of the compounds was predicted by the developed 3D-QSAR model and those compounds having activity equal to or more than 7.5 only were considered for further processing and the rest were dropped.

5.1.6.5 Other filters

The compounds which survived through the above described filtering process were assessed for other properties such as polar surface area (PSA), CNS score and receptor-ligand interactions. Finally, those compounds which passed through all the filtration processes were selected for further studies.

5.2 Chemical work

All the solvents and reagents used in the synthesis were of analytical reagent grade, commercially procured from Sigma-Aldrich, Spectrochem, S. d. fine chemicals or Avra chemicals. Purification of all the solvents and reagents were carried out by using general laboratory techniques before their use for synthesis of the desired compounds.[298] The melting points (m.p.) of the compounds were determined by using Veego silicon oil bath-type melting point apparatus and are uncorrected. Thin layer chromatography (TLC) having silica gel pre-coated plates ($60F_{254}$, Merck, 0.25 mm thickness) were used to monitor the progress of the reactions which were visualized with ultraviolet light (254 nm) or iodine vapours. The practical yield of compounds reported here are un-optimized. The Infrared spectra (IR) expressed in wave numbers in cm^{-1} were obtained using BRUKER ALPHA-T (Germany) FT-IR spectrophotometer where potassium bromide discs were used in the region of 4000-450 cm^{-1}. ^1H-NMR (400 MHz) spectra and ^{13}C-NMR spectra were determined on Bruker Advance-II spectrometer in $CDCl_3$ or DMSO-d_6 solvents; the chemical shift has been expressed in parts per million (δ ppm) and coupling constant (J) in Hz. The mass spectra were recorded either on Thermo Fisher mass spectrometer having EI ion source or Shimadzu LCMS2020 with APCI & ESI probes for the compounds. Chromatographic separations were carried out on columns using silica gel (100–200) as the adsorbent.

5.2.1 4-Amino-2-mercaptopyrimidine-5-carbonitrile (5)

Method A: Freshly cut sodium (0.19 g, 8.26 mmol) was added into absolute ethanol (5 ml) in cold conditions. To this solution, thiourea (**4**) (0.62 g, 8.15 mmol) was added at room temperature. When thiourea got dissolved, Ethoxymethylenemalononitrile (**2**)

(1 g, 8.19 mmol) in ethanol (5 ml) was added using dropping funnel to the above stirred mixture over a period of 2 hrs and completion of the reaction was monitored by TLC. The solvent was evaporated, water (10 ml) added to the residue and 2M HCl was added to make the solution acidic. The precipitates so obtained were filtered to obtain the yellow colored solid compounds (**5**)[299] (0.92 g, 74 %), m.p. > 270 ˚C (Lit[299]: > 270 ˚C).

Anal:

TLC: R_f 0.15 (Chloroform: methanol, 19:1)
IR (KBr, cm^{-1}): 3443, 3370, 3042, 2959, 2223, 1646, 1565 and 1317
MS (ESI) (m/z): 153.1 $(M+1)^+$

5.2.2 Ethyl 4-amino-2-mercaptopyrimidine-5-carboxylate (6)

Reaction of thiourea (**4**) (0.45 g, 5.91 mmol) and ethyl 2-cyano-3-ethoxyacrylate (**3**) (1 g, 5.91 mmol) under a set of conditions described in **Method A**, afforded a white solid compound (**6**) (0.9 g, 76 %) m.p. > 270 ˚C (Lit[299]: > 270 ˚C).

Anal:

TLC: R_f 0.3 (Chloroform: methanol, 19:1)
IR (KBr, cm^{-1}): 3551, 3387, 3063, 2976, 1705, 1637, 1578, 1455, 1372 and 1064
MS (m/z): 199.7 $(M+1)^+$.

5.2.3 2-Chloro-1-(2-trifluoromethyl-10*H*-phenothiazin-10-yl)ethanone (12)

Method B: Commercially available 2-trifluoromethyl-10*H*-phenothiazine (**7**) (1 g, 3.75 mmol) was dissolved in dry toluene (8 ml) and triethylamine (1.12 g, 11.07 mmol) was added to it at room temperature and the solution was stirred for 10 minutes. Chloroacetyl chloride (**11**) (1.24 g, 10.98 mmol) in dry toluene (3 ml) was added drop by drop into the reaction mixture at 0-5 °C. The reaction mixture was refluxed for 5 to 6 hrs and completion of the reaction was checked by TLC. On completion of the reaction, the solvent was removed, water was added to the residue and the suspension extracted with ethyl acetate. Purification through silica gel column chromatography using pet ether : ethyl acetate resulted into the desired pure white solid compound (**12**)[300,301] (0.9 g, 70 %) m.p. 110-112 ˚C (Lit[300]: 110-111 ˚C).

Anal:

TLC: R_f 0.3 (*n*-Hexane: ethyl acetate, 16:4)
IR (KBr, cm^{-1}): 1690, 1608, 1467, 1417, 1330, 1033, 823 and 752.

5.2.4 2-Chloro-1-(2-chloro-10*H*-phenothiazin-10-yl)ethanone (13)

Reaction of 2-chloro-10*H*-phenothiazine (**8**) (1 g, 4.28 mmol), triethylamine (1.30 g, 12.85 mmol) and chloroacetyl chloride (**11**) (1.44 g, 12.75 mmol) in dry DCM (10 ml) under a set of conditions described in **Method B**, afforded white solid compound (**13**) (1.08 g, 82 %) m.p. 117-119 ˚C (Lit[300]: 118-119 ˚C).

Anal:

TLC: R_f 0.35 (*n*-Hexane: ethyl acetate, 16:4)

IR (KBr, cm^{-1}): 1682, 1579, 1458, 1336, 1240, 1029 and 758.

5.2.5 2-Chloro-1-(2-methoxy-10*H*-phenothiazin-10-yl)ethanone (14)

Reaction of 2-methoxy-10*H*-phenothiazine (**9**) (1 g, 4.08 mmol), triethylamine (1.24 g, 12.25 mmol) and chloroacetyl chloride (**11**) (1.38 g, 12.22 mmol) in dry DCM (10 ml) under a set of conditions described in **Method B**, afforded white solid compound (**14**) (1.04 g, 78 %) m.p. 120-122 ˚C (Lit[302]: 123-124 ˚C).

Anal:

TLC: R_f 0.32 (*n*-Hexane: ethyl acetate, 16:4)

IR (KBr, cm^{-1}): 1689, 1592, 1465, 1341, 1256, 1214, 1025 and 745.

5.2.6 2-Chloro-1-(10*H*-phenothiazin-10-yl)ethanone (15)

Reaction of 10*H*-phenothiazine (**10**) (1 g, 5.02 mmol), triethylamine (1.52 g, 15.02 mmol) and chloroacetyl chloride (**11**) (1.68 g, 14.88 mmol) in dry DCM (10 ml) under a set of conditions described in **Method B**, afforded white solid compound (**15**) (1.1 g, 80 %) m.p. 110-112 ˚C (Lit[300]: 114-115 ˚C).

Anal:

TLC: R_f 0.42 (*n*-Hexane: ethyl acetate, 16:4)

IR (KBr, cm^{-1}): 3067, 2951, 1693, 1458, 1399, 1337, 1242 and 762.

5.2.7 4-Amino-2-(2-(2-trifluoromethyl-10*H*-phenothiazin-10-yl)-2-oxoethylthio)-pyrimidine-5-carbonitrile (1)[296]

Method C: 4-Amino-2-mercaptopyrimidine-5-carbonitrile (**5**) (0.27 g, 1.77 mmol) was dissolved in dry DMF (2 ml) and stirred at room temperature. Anhydrous potassium carbonate (0.52 g, 3.77 mmol) was added to the above solution. 2-Chloro-1-(2-trifluoromethyl-10*H*-phenothiazin-10-yl)ethanone (**12**) (0.5 g, 1.45 mmol) dissolved in dry DMF (2 ml) was added dropwise to the above solution. The reaction mixture was stirred for 3 to 4 hrs and completion of the reaction checked by TLC. On addition of water to the reaction mixture, a white precipitate was obtained. Purification through silica gel column using pet ether: ethyl acetate (30 %) as eluent gave pure white solid of the desired product (**1**) (0.45 g, 67 %) m.p. 218-220 ˚C.

Anal:

TLC: R_f 0.6 (*n*-Hexane: ethyl acetate, 10:10)

IR (KBr) cm^{-1}: 3402, 3321, 2216 and 1684, 1631, 1573, 1542, 1375 and 1331.

^{1}H NMR: 8.25 (s, 1H), 7.64 (s, 1H), 7.54-7.52 (d, 1H, *J* = 7.8), 7.50-7.48 (d, 1H, *J* = 7.8), 7.40-7.36 (m, 2H), 7.32-7.28 (m, 1H), 7.26-7.23 (d, 1H, *J* = 9.04), 5.70 (bs, 2H), 4.03 (s, 2H).

^{13}C NMR: 172.88, 166.55, 161.22, 159.46, 138.12, 137.13, 131.35, 128.0, 127.79, 127.42, 127.19, 126.85, 124.67, 123.96, 123.0, 121.97, 114.91, 85.94, 34.02.

MS (ESI) m/z: 460.2 $(M+1)^+$.

Calcd for $C_{20}H_{12}F_3N_5OS_2$: C, 52.28; H, 2.63; N, 15.24; Found: C, 52.40; H, 2.76; N, 15.12 %.

5.2.8 4-Amino-2-(2-(2-chloro-10*H*-phenothiazin-10-yl)-2-oxoethylthio)pyrimidine-5-carbonitrile (18)

Reaction of 4-amino-2-mercaptopyrimidine-5-carbonitrile (**5**) (0.29 g, 1.91 mmol), anhydrous potassium carbonate (0.55 g, 3.99 mmol) and 2-chloro-1-(2-chloro-10*H*-phenothiazin-10-yl)ethanone (**13**) (0.5 g, 1.61 mmol) in dry DMF (4 ml) under a set of conditions described in **Method C**, afforded white solid compound (**18**) (0.49 g, 71 %) m.p. 222-224 ˚C.

Anal:

TLC: R_f 0.52 (*n*-Hexane: ethyl acetate, 10:10)

IR (KBr) cm^{-1}: 3460, 3332, 2224, 1645, 1574, 1534, 1462, 1402 and 1237.

^{1}H NMR: 8.24 (s, 1H), 7.91 (s, 1H), 7.58-7.54 (m, 2H), 7.51-7.49 (d, 2H), 7.43-7.39 (m, 1H), 7.35-7.31 (m, 1H), 5.74 (bs, 2H), 4.05 (dd, 2H).

MS (ESI) m/z: 426 $(M+1)^+$, 428 $(M+3)^+$.

Calcd for $C_{19}H_{12}ClN_5OS_2$: C, 53.58; H, 2.84; N, 16.44; Found: C, 53.72; H, 2.68; N, 16.30 %.

5.2.9 4-Amino-2-(2-(2-methoxy-10*H*-phenothiazin-10-yl)-2-oxoethylthio)-pyrimidine-5-carbonitrile (19)

Reaction of 4-amino-2-mercaptopyrimidine-5-carbonitrile (**5**) (0.29 g, 1.91 mmol), anhydrous potassium carbonate (0.55 g, 3.99 mmol) and 2-chloro-1-(2-methoxy-10*H*-phenothiazin-10-yl)ethanone (**14**) (0.5 g, 1.63 mmol) in dry DMF (4 ml) under a set of conditions described in **Method C**, afforded white solid compound (**19**) (0.48 g, 69 %) m.p. 215-217 ˚C.

Anal:

TLC: R_f 0.32 (*n*-Hexane: ethyl acetate, 10:10)

IR (KBr) cm^{-1}: 3337, 3179, 2219, 1664, 1575, 1537, 1465, 1353, 1245 and 1166.

^1H NMR: 8.26 (s, 1H), 7.70 (bs, 2H), 7.65-7.63 (d, 1H, *J* = 7.76 Hz) 7.51-7.49 (d, 1H, *J* = 7.76 Hz), 7.40-7.38 (d, 1H), 7.36-7.34 (d, 1H), 7.30 (s, 1H), 7.28-7.26 (m, 1H), 6.90-6.87 (dd, 1H, *J* = 8.7, 2.6 Hz), 4.18 (s, 2H), 3.80 (s, 3H).

^{13}C NMR: 173.02, 166.35, 161.23, 160.07, 159.99, 158.73, 139.25, 138.03, 128.23, 127.64, 127.25, 126.80, 115.18, 113.27, 113.02, 85.83, 55.46, 34.25.

MS (ESI) m/z: 421.12 (M^+).

Calcd for $C_{20}H_{15}N_5O_2S_2$: C, 56.99; H, 3.59; N, 16.62; Found: C, 56.80; H, 3.70; N, 16.72 %.

5.2.10 4-Amino-2-(2-oxo-2-(10*H*-phenothiazin-10-yl)ethylthio)pyrimidine-5-carbonitrile (20)

Reaction of 4-amino-2-mercaptopyrimidine-5-carbonitrile (**5**) (0.33 mg, 2.17 mmol), anhydrous potassium carbonate (0.62 g, 4.49 mmol) and 2-chloro-1-(10*H*-phenothiazin-10-yl)ethanone (**15**) (0.5 g, 1.81 mmol) in dry DMF (4 ml) under a set of conditions described in **Method C**, afforded white solid compound (**20**) (0.45 g, 63 %) m.p. 241-243 ˚C.

Anal:

TLC: R_f 0.46 (*n*-Hexane: ethyl acetate, 10:10)

IR (KBr) cm^{-1}: 3361, 3145, 2228, 1663, 1572, 1542, 1466, 1351, 1237, 1165 and 757.

^{1}H NMR: 8.21 (s, 1H), 7.66-7.64 (d, 2H, *J* = 7.8 Hz), 7.51-7.49 (d, 2H, *J* = 7.8 Hz), 7.48 (bs, 2H), 7.40-7.36 (m, 2H), 7.31-7.27 (m, 2H) and 4.12 (s, 2H).

MS (ESI) m/z: 414.4 (M+Na)$^+$.

Calcd for $C_{19}H_{13}N_5OS_2$: C, 58.29; H, 3.35; N, 17.89; Found: C, 58.42; H, 3.22; N, 17.78 %.

5.2.11 Ethyl 4-amino-2-(2-(2-trifluoromethyl-10*H*-phenothiazin-10-yl)-2-oxoethylthio)pyrimidine-5-carboxylate (21)

Reaction of ethyl 4-amino-2-mercaptopyrimidine-5-carboxylate (**6**) (0.36 g, 1.81 mmol), anhydrous potassium carbonate (0.52 g, 3.77 mmol) and 2-chloro-1-(2-(trifluoromethyl)-10*H*-phenothiazin-10-yl)ethanone (**12**) (0.5 g, 1.45 mmol) in dry DMF (4 ml) under a set of conditions described in **Method C**, afforded white solid compound (**21**) (0.47 g, 63 %) m.p. 148-151 ˚C.

Anal:

TLC: R$_f$ 0.72 (*n*-Hexane: ethyl acetate, 10:10)

IR (KBr) cm^{-1}: 3422, 3282, 1694, 1627, 1569, 1328 and 1117.

^{1}H NMR: 8.62 (s, 1H), 7.93 (s, 1H), 7.80 (bs, 2H), 7.59-7.55 (m, 2H), 7.51-7.47 (m, 2H), 7.42-7.38 (m, 1H), 7.33-7.29 (m, 1H), 4.35-4.29 (q, 2H), 4.08 (s, 2H), 1.38-1.34 (t, 3H).

MS (ESI) m/z: 506.2 (M$^+$).

Calcd for $C_{22}H_{17}F_3N_4O_3S_2$: C, 52.17; H, 3.38; N, 11.06; Found: C, 52.36; H, 3.26; N, 11.14 %.

5.2.12 Ethyl 4-amino-2-(2-(2-chloro-10*H*-phenothiazin-10-yl)-2-oxoethylthio)-pyrimidine-5-carboxylate (22)

Reaction of ethyl 4-amino-2-mercaptopyrimidine-5-carboxylate (**6**) (0.38 g, 1.91 mmol), anhydrous potassium carbonate (0.55 g, 3.99 mmol) and 2-chloro-1-(2-chloro-10*H*-phenothiazin-10-yl)ethanone (**13**) (0.5 g, 1.61 mmol) in dry DMF (4 ml) under a set of conditions described in **Method C**, afforded white solid compound (**22**) (0.53 g, 70 %) m.p. 156-158 ˚C.

Anal:

TLC: R$_f$ 0.58 (*n*-Hexane: ethyl acetate, 10:10)

IR (KBr) cm^{-1}: 3471, 3358, 1676, 1608, 1580, 1520, 1459, 1374, 1336, 1236, 1163 and 807.

^1H NMR: 8.62 (s, 1H), 7.79 (bs, 1H), 7.67-7.66 (d, 1H), 7.57-7.55 (dd, 1H, J = 7.8, 0.8 Hz), 7.48-7.46 (dd, 1H, J = 7.8, 1.36 Hz), 7.39-7.36 (m, 1H), 7.35 (s, 1H), 7.30-7.26 (m, 1H), 7.24-7.21 (d, 1H, J = 8.4 Hz), 5.78 (bs, 1H), 4.34-4.29 (q, 2H), 4.07 (s, 2H), 1.38 (t, 3H).

^{13}C NMR: 172.97, 166.63, 165.33, 161.15, 158.36, 158.31, 139.22, 137.61, 131.88, 131.56, 131.23, 129.07, 128.10, 127.64, 127.45, 127.24, 127.07, 100.89, 60.63, 34.04, 14.04.

MS (ESI) m/z: 494.1 $(M+Na)^+$, 495.8 $(M+2+Na)^+$.

Calcd for $C_{21}H_{17}ClN_4O_3S_2$: C, 53.33; H, 3.62; N, 11.85; Found: C, 53.66; H, 3.84; N, 11.96 %.

5.2.13 Ethyl 4-amino-2-(2-(2-methoxy-10*H*-phenothiazin-10-yl)-2-oxoethylthio)-pyrimidine-5-carboxylate (23)

Reaction of ethyl 4-amino-2-mercaptopyrimidine-5-carboxylate (**6**) (0.38 g, 1.91 mmol), anhydrous potassium carbonate (0.55 g, 3.99 mmol) and 2-chloro-1-(2-methoxy-10*H*-phenothiazin-10-yl)ethanone (**14**) (0.5 g, 1.63 mmol) in dry DMF (4 ml) under a set of conditions described in **Method C**, afforded white solid compound (**23**) (0.44 g, 57 %) m.p. 104-106 ˚C.

Anal:

TLC: R_f 0.39 (*n*-Hexane: ethyl acetate, 10:10)

IR (KBr) cm^{-1}: 3442, 3333, 1692, 1604, 1571, 1464, 1371, 1194 and 1024.

^1H NMR: 8.60 (s, 1H), 7.77 (bs, 1H), 7.58-7.56 (d, 1H), 7.47-7.45 (d, 1H, J = 7.6 Hz), 7.35 (s, 1H), 7.33-7.32 (m, 1H), 7.26-7.24 (d, 1H, J = 7.6 Hz), 7.24-7.21 (m, 1H), 6.84-6.81 (d, 1H), 5.93 (bs, 1H), 4.34-4.28 (q, 2H), 4.06 (dd, 2H), 3.81 (s, 3H), 1.37-1.33 (t, 3H).

^{13}C NMR: 173.97, 166.87, 166.22, 161.92, 159.30, 159.04, 139.75, 138.49, 128.51, 128.16, 127.18, 127.10, 113.59, 113.07, 101.34, 60.96, 55.73, 35.50, 14.25.

MS (ESI) m/z: 468.2 (M^+).

Calcd for $C_{22}H_{20}N_4O_4S_2$: C, 56.39; H, 4.30; N, 11.96; Found: C, 56.22; H, 4.44; N, 11.82 %.

5.2.14 Ethyl 4-amino-2-(2-oxo-2-(10*H*-phenothiazin-10-yl)ethylthio)pyrimidine-5-carboxylate (24)

Reaction of ethyl 4-amino-2-mercaptopyrimidine-5-carboxylate (**6**) (0.43 g, 2.16 mmol), anhydrous potassium carbonate (0.62 g, 4.49 mmol) and 2-chloro-1-(10*H*-phenothiazin-10-yl)ethanone (**15**) (0.5 g, 1.81 mmol) in dry DMF (4 ml) under a set of conditions described in **Method C**, afforded white solid compound (**24**) (0.53 g, 67 %) m.p. 202-204 ˚C.

Anal:

TLC: R_f 0.56 (*n*-Hexane: ethyl acetate, 10:10)

IR (KBr) cm^{-1}: 3399, 3278, 1704, 1669, 1632, 1573, 1461, 1335 and 761.

^1H NMR: 8.51 (s, 1H), 7.69-7.67 (d, 2H, *J* = 7.7 Hz), 7.55 (bs, 2H), 7.53-7.51 (d, 2H, *J* = 7.7 Hz), 7.41-7.37 (m, 2H), 7.32-7.28 (m, 2H), 4.32-4.27 (q, 2H), 4.17 (s, 2H), 1.36-1.32 (t, 3H).

MS (ESI) m/z: 438.4 (M^+).

Calcd for $C_{21}H_{17}N_3O_3S_2$: C, 59.56; H, 4.05; N, 9.92; Found: C, 59.72; H, 4.16; N, 9.84 %.

5.2.15 2-(5-(4-Pyridyl)-1,3,4-oxadiazol-2-ylthio)-1-(2-trifluoromethyl-10*H*-phenothiazin-10-yl)ethanone (25)

Reaction of 5-(pyridin-4-yl)-1,3,4-oxadiazole-2-thiol (**16**) (0.32 g, 1.79 mmol), anhydrous potassium carbonate (0.52 g, 3.77 mmol) and 2-chloro-1-(2-trifluoromethyl-10*H*-phenothiazin-10-yl)ethanone (**12**) (0.5 g, 1.45 mmol) in dry DMF (4 ml) under a set of conditions described in **Method C**, afforded white solid compound (**25**) (0.45 g, 64 %) m.p. 156-158 ˚C.

Anal:

TLC: R_f 0.24 (*n*-Hexane: ethyl acetate, 10:10)

IR (KBr) cm^{-1}: 1680, 1464, 1330 and 1169.

^1H NMR: 8.80-8.79 (d, 2H, *J* = 6.1 Hz), 7.93 (s, 1H), 7.83-7.82 (d, 2H, *J* = 6.1 Hz), 7.65-7.63 (d, 1H), 7.59-7.57 (d, 1H), 7.52-7.49 (d, 2H), 7.46-7.41 (m, 1H), 7.37-7.33 (m, 1H), 4.60 (bs, 1H), 4.25-4.22 (bs, 1H).

^{13}C NMR: 165.40, 164.92, 164.20, 150.90, 138.09, 137.08, 130.47, 129.84, 129.51, 128.63, 128.35, 128.20, 126.67, 124.92, 124.38, 124.1, 122.21, 120.0, 36.96.

MS (ESI) m/z: 486.6 (M^+).

Calcd for $C_{22}H_{13}F_3N_4O_2S_2$: C, 54.31; H, 2.69; N, 11.52; Found: C, 54.44; H, 2.82; N, 11.40 %.

5.2.16 1-(2-Chloro-10*H*-phenothiazin-10-yl)-2-(5-(4-pyridyl)-1,3,4-oxadiazol-2-ylthio)ethanone (26)

Reaction of 5-(pyridin-4-yl)-1,3,4-oxadiazole-2-thiol (**16**) (0.34 g, 1.89 mmol), anhydrous potassium carbonate (0.55 g, 3.99 mmol) and 2-chloro-1-(2-chloro-10*H*-phenothiazin-10-yl)ethanone (**13**) (0.5 g, 1.61 mmol) in dry DMF (4 ml) under a set of conditions described in **Method C**, afforded white solid compound (**26**) (0.54 g, 74 %) m.p. 194-196 ˚C.

Anal:

TLC: R_f 0.23 (*n*-Hexane: ethyl acetate, 10:10)

IR (KBr) cm^{-1}: 1679, 1576, 1461, 1334, 1235, 1166 and 700.

^{1}H NMR: 8.80-8.78 (d, 2H, J = 6.1 Hz), 7.83-7.82 (d, 2H, J = 6.1 Hz), 7.67 (s, 1H), 7.62-7.60 (d, 1H), 7.50-7.48 (d, 1H), 7.42-7.40 (m, 1H), 7.39-7.37 (d, 1H), 7.33- 7.29 (m, 1H), 7.27-7.24 (m, 1H), 4.53 (bs, 1H), 4.30 (bs, 1H).

MS (ESI) m/z: 452.2 $(M)^+$ and 454.2 $(M+2)^+$.

Calcd for $C_{21}H_{13}ClN_4O_2S_2$: C, 55.69; H, 2.89; N, 12.37; Found: C, 55.84; H, 2.76; N, 12.48 %.

5.2.17 1-(2-Methoxy-10*H*-phenothiazin-10-yl)-2-(5-(4-pyridyl)-1,3,4-oxadiazol-2-ylthio)ethanone (27)

Reaction of 5-(pyridin-4-yl)-1,3,4-oxadiazole-2-thiol (**16**) (0.34 g, 1.89 mmol), anhydrous potassium carbonate (0.55 g, 3.99 mmol) and 2-chloro-1-(2-methoxy-10*H*-phenothiazin-10-yl)ethanone (**14**) (0.5 g, 1.63 mmol) in dry DMF (4 ml) under a set of conditions described in **Method C**, afforded white solid compound (**27**) (0.48 g, 66 %) m.p. 166-168 ˚C.

Anal:

TLC: R_f 0.14 (*n*-Hexane: ethyl acetate, 10:10)

IR (KBr) cm^{-1}: 1671, 1596, 1450, 1345, 1021 and 746.

^{1}H NMR: 8.80-8.78 (d, 2H, *J* = 6.0 Hz), 7.83-7.82 (d, 2H, *J* = 6.0 Hz), 7.62-7.60 (d, 1H, *J* = 7.8 Hz), 7.48-7.46 (d, 1H, *J* = 7.8 Hz), 7.38-7.34 (m, 2H), 7.29-7.27 (m, 1H), 7.25-7.23 (d, 1H), 6.86-6.84 (d, 1H), 4.45 (bs, 2H), 3.83 (s, 3H).

MS (ESI) m/z: 448.6 (M^+).

Calcd for $C_{22}H_{16}N_4O_3S_2$: C, 58.91; H, 3.60; N, 12.49; Found: C, 58.78; H, 3.46; N, 12.62 %.

5.2.18 1-(10*H*-Phenothiazin-10-yl)-2-(5-(4-pyridyl)-1,3,4-oxadiazol-2-ylthio)-ethanone (28)

Reaction of 5-(pyridin-4-yl)-1,3,4-oxadiazole-2-thiol (**16**) (0.39 g, 2.18 mmol), anhydrous potassium carbonate (0.62 g, 4.49 mmol) and 2-chloro-1-(10*H*-phenothiazin-10-yl)ethanone (**15**) (0.5 g, 1.81 mmol) in dry DMF (4 ml) under a set of conditions described in **Method C**, afforded white solid compound (**28**) (0.54 g, 71 %) m.p. 212-214 ˚C.

Anal:

TLC: R_f 0.26 (*n*-Hexane: ethyl acetate, 10:10)

IR (KBr) cm^{-1}: 1667, 1462, 1350 and 757.

^{1}H NMR: 8.79-8.77 (d, 2H, *J* = 6.0 Hz), 7.82-7.81 (d, 2H, *J* = 6.0 Hz), 7.63-7.61 (d, 2H), 7.49-7.47 (d, 2H), 7.39-7.35 (m, 2H), 7.30-7.26 (m, 2H), 4.44 (bs, 2H).

MS (ESI) m/z: 418.6 (M^+).

Calcd for $C_{21}H_{14}N_4O_2S_2$: C, 60.27; H, 3.37; N, 13.39; Found: C, 60.39; H, 3.26; N, 13.54 %.

5.2.19 2-(4-Amino-5-(4-pyridyl)-4*H*-1,2,4-triazol-3-ylthio)-1-(2-trifluoromethyl-10*H*-phenothiazin-10-yl)ethanone (29)

Reaction of 4-amino-5-(4-pyridyl)-4*H*-1,2,4-triazole-3-thiol (**17**) (0.35 g, 1.81 mmol), anhydrous potassium carbonate (0.52 g, 3.77 mmol) and 2-chloro-1-(2-trifluoromethyl-10*H*-phenothiazin-10-yl)ethanone (**12**) (0.5 g, 1.45 mmol) in dry DMF (4 ml) under a set of conditions described in **Method C**, afforded white solid compound (**29**) (0.36 g, 49 %) m.p. 202-204 ˚C.

Anal:

TLC: R_f 0.36 (Chloroform : methanol, 19:01)

IR (KBr) cm^{-1}: 3321, 3111, 1670, 1606, 1464 and 1331.

^{1}H NMR: 8.70-8.68 (d, 2H, *J* = 5.6 Hz), 8.03-8.01 (d, 2H, *J* = 5.6 Hz), 8.01 (s, 1H), 7.82-7.80 (d, 1H), 7.75-7.73 (d, 1H, *J* = 8.3 Hz), 7.62-7.60 (d, 1H, *J* = 8.3 Hz), 7.58-7.56 (d, 1H), 7.47-7.44 (m, 1H), 7.39-7.35 (m, 1H), 6.22 (s, 2H), 4.54 (bs, 1H), 4.28 (bs, 1H).

MS (ESI) m/z: 500.89 (M^+).

Calcd for $C_{22}H_{15}F_3N_6OS_2$: C, 52.79; H, 3.02; N, 16.79; Found: 52.96; H, 3.16; N, 16.66 %.

5.2.20 2-(4-Amino-5-(4-pyridyl)-4*H*-1,2,4-triazol-3-ylthio)-1-(2-chloro-10*H*-phenothiazin-10-yl)ethanone (30)

Reaction of 4-amino-5-(4-pyridyl)-4*H*-1,2,4-triazole-3-thiol (**17**) (0.37 g, 1.91 mmol), anhydrous potassium carbonate (0.55 g, 3.99 mmol) and 2-chloro-1-(2-chloro-10*H*-phenothiazin-10-yl)ethanone (**13**) (0.5 g, 1.61 mmol) in dry DMF under a set of conditions described in **Method C**, afforded white solid compound (**30**) (0.45 g, 60 %) m.p. 225-227 ˚C.

Anal:

TLC: R_f 0.31 (Chloroform : methanol, 19:01)

IR (KBr) cm^{-1}: 3320, 3106, 1666, 1602, 1459, 1402, 1335, 1230, 1167 and 760.

^{1}H NMR: 8.69-8.68 (d, 2H, *J* = 4.8 Hz), 8.07-8.06 (d, 2H, *J* = 4.8 Hz), 7.71 (s, 1H), 7.71-7.69 (d, 1H), 7.52-7.50 (d, 1H), 7.47-7.45 (d, 1H), 7.42-7.38 (m, 1H), 7.34-7.32 (m, 1H), 7.30-7.28 (d, 1H), 6.12 (s, 2H), 4.45 (bs, 1H), 4.24 (bs, 1H).

MS (ESI) m/z: 465.9 $(M)^+$ and 467.6 $(M+2)^+$.

Calcd for $C_{21}H_{15}ClN_6OS_2$: C, 54.01; H, 3.24; N, 18.00; Found: C, 54.16; H, 3.10; N, 18.12 %.

5.2.21 2-(4-Amino-5-(4-pyridyl)-4*H*-1,2,4-triazol-3-ylthio)-1-(2-methoxy-10*H*-phenothiazin-10-yl)ethanone (31)

Reaction of 4-amino-5-(4-pyridyl)-4*H*-1,2,4-triazole-3-thiol (**17**) (0.37 g, 1.91 mmol), anhydrous potassium carbonate (0.55 g, 3.99 mmol) and 2-chloro-1-(2-methoxy-10*H*-phenothiazin-10-yl)ethanone (**14**) (0.5 g, 1.63 mmol) in dry DMF (4 ml) under a set of conditions described in **Method C**, afforded white solid compound (**31**) (0.46 g, 61 %) m.p. 188-190 ˚C.

Anal:

TLC: R_f 0.35 (Chloroform: methanol, 19:01)

IR (KBr) cm^{-1}: 3318, 3207, 1654, 1601, 1462, 1360 and 1213.

^{1}H NMR: 8.70-8.68 (d, 2H, *J* = 6.1 Hz), 8.03-8.01 (d, 2H, *J* = 6.1 Hz), 7.68-7.65 (d, 1H), 7.52-7.50 (d, 1H), 7.42-7.36 (m, 3H), 7.31-7.30 (m, 1H), 6.93-6.90 (d, 1H, *J* = 8.7 Hz), 6.22 (s, 2H), 4.40 (bs, 2H), 3.81 (s, 3H).

MS (ESI) m/z: 462.1 (M^+).

Calcd for $C_{22}H_{18}N_6O_2S_2$: C, 57.13; H, 3.92; N, 18.17; Found: C, 57.36; H, 3.76; N, 18.30 %.

5.2.22 2-(4-Amino-5-(4-pyridyl)-4*H*-1,2,4-triazol-3-ylthio)-1-(10*H*-phenothiazin-10-yl)ethanone (32)

Reaction of 4-amino-5-(4-pyridyl)-4*H*-1,2,4-triazole-3-thiol (**17**) (0.42 g, 2.17 mmol), anhydrous potassium carbonate (0.62 g, 4.49 mmol) and 2-chloro-1-(10*H*-phenothiazin-10-yl)ethanone (**15**) (0.5 g, 1.81 mmol) in dry DMF (4 ml) under a set of conditions described in **Method C**, afforded white solid compound (**32**) (0.46 g, 59 %) m.p. 232-234 ˚C.

Anal:

TLC: R_f 0.33 (Chloroform: methanol, 19:01)

IR (KBr) cm^{-1}: 3308, 3202, 1652, 1605, 1458 and 1368.

^{1}H NMR: 8.69-8.68 (d, 2H, *J* = 6.1 Hz), 8.03-8.02 (d, 2H, *J* = 6.1 Hz), 7.73-7.71 (d, 2H), 7.54-7.52 (d, 2H), 7.42-7.38 (m, 2H), 7.33-7.29 (m, 2H), 6.20 (s, 2H), 4.37 (bs, 2H).

MS (ESI) m/z: 432.80 (M^+).

Calcd for $C_{21}H_{16}N_6OS_2$: C, 58.31; H, 3.73; N, 19.43; Found: C, 58.48; H, 3.84; N, 19.30 %.

5.2.23 10-(3-Chloropropyl)-2-trifluoromethyl-10*H*-phenothiazine (34)

Method D: Commercially available 2-trifluoromethyl-10*H*-phenothiazine (**7**) (1 g, 3.75 mmol) was dissolved in dry THF (5 ml) and a suspension of NaH (0.13 g, 5.42 mmol) in dry DMSO (10 ml) and THF (5 ml) was added to it. The reaction mixture was stirred in the presence of nitrogen at 0˚C for 30 min. A solution of 1-bromo-3-chloropropane

(**33**) (1.74 g, 11.08 mmol) in dry DMSO (5 ml) was added to the above suspension. The reaction mixture was then stirred at room temp under nitrogen for 4 hrs and completion of the reaction was checked by TLC. On completion of the reaction, the reaction mixture was poured into 50 ml of ice water and extracted with DCM (3 X 75 ml) yielding the desired white solid compounds (**34**)[303](0.87 g, 68%) m.p. 72-74˚C (Lit[304]: 72˚C).

Anal:

TLC:	R_f 0.33 (Chloroform: methanol, 19:01)
IR (KBr) cm^{-1}:	2877, 1600, 1425, 1242, 1108 and 750.
MS (ESI) m/z:	342.9 $(M+1)^+$

5.2.24 2-Chloro-10-(3-chloropropyl)-10*H*-phenothiazine (35)

Reaction of 2-chloro-10*H*-phenothiazine (**8**) (1 g, 4.28 mmol), NaH (0.15 g, 6.25 mmol) and 1-bromo-3-chloropropane (**33**) (2.03 g, 12.93 mmol) in dry THF and DMSO under a set of conditions described in **Method D**, afforded yellow colored liquid compound (**35**) (0.99 g, 75 %).

Anal:

TLC:	R_f 0.33 (Chloroform: methanol, 19:01)
IR (KBr) cm^{-1}:	2929, 1637, 1457, 1026 and 751.
MS (ESI) m/z:	301 $(M+1)^+$ and 303 $(M+3)^+$

5.2.25 10-(3-Chloropropyl)-2-methoxy-10*H*-phenothiazine (36)

Reaction of 2-methoxy-10*H*-phenothiazine (**9**) (1 g, 4.08 mmol), NaH (0.15 g, 6.25 mmol) and 1-bromo-3-chloropropane (**33**) (1.93 g, 12.29 mmol) in dry THF and DMSO under a set of conditions described in **Method D**, afforded yellow colored liquid compound (**36**) (0.86 g, 65 %).

Anal:

TLC:	R_f 0.33 (Chloroform: methanol, 19:01)
IR (KBr) cm^{-1}:	2938, 1580, 1457, 1267, 1121, 1028 and 759.
MS (ESI) m/z:	305.1 $(M+1)^+$.

5.2.26 10-(3-Chloropropyl)-10*H*-phenothiazine (37)

Reaction of 10*H*-phenothiazine (**10**) (1 g, 5.02 mmol), NaH (0.18 g,7.50 mmol) and 1-bromo-3-chloropropane (**33**) (2.36 g, 15.03 mmol) in dry THF and DMSO under a set of conditions described in **Method D**, afforded white solid compound (**37**) (1.08 g, 78 %) m.p. 60-62 ˚C (Lit[305]: 60 ˚C).

Anal:

TLC: R_f 0.33 (Chloroform: methanol, 19:01)

IR (KBr) cm^{-1}: 2956, 1590, 1457, 1332, 1248, 1194, 1037 and 756.

MS (ESI) m/z: 275.2 $(M+1)^+$.

5.2.27 4-Amino-2-(3-(2-trifluoromethyl-10*H*-phenothiazin-10-yl)propylthio)-pyrimidine-5-carbonitrile (38)

Reaction of 4-amino-2-mercaptopyrimidine-5-carbonitrile (**5**) (0.27 g, 1.77 mmol), anhydrous potassium carbonate (0.52 g, 3.77 mmol) and 10-(3-chloropropyl)-2-trifluoromethyl-10*H*-phenothiazine (**34**) (0.5 g, 1.45 mmol) in dry DMF (4 ml) under a set of conditions described in **Method C**, afforded white solid compound (**38**) (0.4 g, 60 %) m.p. 164-166 ˚C.

Anal:

TLC: R_f 0.25 (*n*-Hexane: ethyl acetate, 16:04)

IR (KBr) cm^{-1}: 3475, 3306, 2220, 1645, 1573, 1538, 1470, 1423, 1363, 1329, 1247 and 1107.

^{1}H NMR: 8.23 (s, 1H), 7.72 (bs, 2H), 7.31-7.29 (d, 1H), 7.21-7.19 (m, 2H), 7.16-7.13 (m, 2H), 7.06-7.04 (d, 1H, *J* = 7.92), 7.00-6.98 (m, 1H), 4.09-4.05 (t, 2H), 3.18-3.14 (t, 2H), 2.14-2.10 (m, 2H).

MS (ESI) m/z: 459.3 (M^+).

Calcd for $C_{21}H_{16}F_3N_5S_2$: C, 54.89; H, 3.51; N, 15.24; Found: C, 54.72; H, 3.42; N, 15.38 %.

5.2.28 4-Amino-2-(3-(2-chloro-10*H*-phenothiazin-10-yl)propylthio)pyrimidine-5-carbonitrile (39)

Reaction of 4-amino-2-mercaptopyrimidine-5-carbonitrile (**5**) (0.29 g, 1.91 mmol), anhydrous potassium carbonate (0.55 g, 3.99 mmol) and 2-chloro-10-(3-chloropropyl)-10*H*-phenothiazine (**35**) (0.5 g, 1.61 mmol) in dry DMF (4 ml) under a set of conditions described in **Method C**, afforded white solid compound (**39**) (0.33 g, 48 %) m.p. 138-140 ˚C.

Anal:

TLC: R_f 0.39 (*n*-Hexane: ethyl acetate, 14:06)

IR (KBr) cm^{-1}: 3444, 3343, 2218, 1631, 1572, 1534, 1456, 1366 and 1237.

^1H NMR: 8.23 (s, 1H), 7.70 (bs, 2H), 7.18-7.16 (m, 1H), 7.13-7.11 (d, 1H), 7.09-7.07 (d, 1H, *J* = 8.1 Hz), 7.02-7.00 (d, 1H), 6.97 (s, 1H), 6.97-6.95 (m, 1H), 6.94-6.91 (d, 1H, *J* = 8.1 Hz), 4.02-3.98 (t, 2H), 3.16-3.13 (t, 2H), 2.14-2.11 (m, 2H).

MS (ESI) m/z: 446.5 ($M+Na^+$), 448.5 $(M+2+Na)^+$.

Calcd for $C_{20}H_{16}ClN_5S_2$: C, 56.39; H, 3.79; N, 16.44; Found: C, 56.54; H, 3.66; N, 16.58 %.

5.2.29 4-Amino-2-(3-(2-methoxy-10*H*-phenothiazin-10-yl)propylthio)pyrimidine-5-carbonitrile (40)

Reaction of 4-amino-2-mercaptopyrimidine-5-carbonitrile (**5**) (0.29 g, 1.91 mmol), anhydrous potassium carbonate (0.55 g, 3.99 mmol) and 10-(3-chloropropyl)-2-methoxy-10*H*-phenothiazine (**36**) (0.5 g, 1.63 mmol) in dry DMF (4 ml) under a set of conditions described in **Method C**, afforded white solid compound (**40**) (0.28 g, 40 %) m.p. 158-160 °C.

Anal:

TLC: R_f 0.18 (*n*-Hexane: ethyl acetate, 16:04)

IR (KBr) cm^{-1}: 3414, 3340, 2217, 1655, 1569, 1463, 1373, 1156 and 1036.

^1H NMR: 8.12 (s, 1H), 7.09-7.04 (m, 2H), 6.98-6.96 (d, 1H, *J* = 8.0 Hz), 6.87-6.82 (m, 1H), 6.81-6.79 (d, 1H, *J* = 8.0 Hz), 6.44-6.41 (d, 1H), 6.41 (s, 1H), 5.44 (bs, 2H), 3.92-3.89 (t, 2H), 3.70 (s, 3H), 3.12-3.09 (t, 2H), 2.17-2.12 (m, 2H).

^{13}C NMR: 174.06, 161.30, 159.49, 159.22, 145.84, 144.24, 127.05, 126.79, 126.70, 124.76, 122.07, 115.33, 115.16, 114.94, 106.64, 102.86, 85.41, 54.91, 45.12, 27.29, 25.91.

MS (ESI) m/z: 420.7 (M^+).

Calcd for $C_{21}H_{19}N_5OS_2$: C, 59.83; H, 4.54; N, 16.61; Found: C, 59.97; H, 4.40; N, 16.76 %.

5.2.30 4-Amino-2-(3-(10*H*-phenothiazin-10-yl)propylthio)pyrimidine-5-carbonitrile (41)

Reaction of 4-amino-2-mercaptopyrimidine-5-carbonitrile (**5**) (0.33 g, 2.17 mmol), anhydrous potassium carbonate (0.62 g, 4.49 mmol) and 10-(3-chloropropyl)-10*H*-phenothiazine (**37**) (0.5 g, 1.81 mmol) in dry DMF (4 ml) under a set of conditions described in **Method C**, afforded white solid compound (**41**) (0.43 g, 61 %) m.p. 117-119 ˚C.

Anal:

TLC: R_f 0.12 (*n*-Hexane: ethyl acetate, 16:04)

IR (KBr) cm^{-1}: 3353, 3160, 2224, 1661, 1573, 1542, 1459, 1360, 1239 and 781.

^{1}H NMR: 8.19 (s, 1H), 7.18-7.13 (m, 4H), 6.96-6.92 (m, 2H), 6.90-6.88 (d, 2H), 5.33 (bs, 2H) 4.05-4.02 (t, 2H), 3.22-3.18 (t, 2H), 2.25-2.18 (m, 2H).

^{13}C NMR: 175.88, 161.46, 159.49, 145.28, 127.64, 127.33, 125.61, 122.69, 115.70, 115.06, 86.13, 45.63, 28.42, 26.56.

MS (ESI) m/z: 391.5 $(M)^+$.

Calcd for $C_{20}H_{17}N_5S_2$: C, 61.36; H, 4.38; N, 17.89; Found: C, 61.54; H, 4.23; N, 17.76 %.

5.2.31 Ethyl 4-amino-2-(3-(2-trifluoromethyl-10*H*-phenothiazin-10-yl)propylthio)-pyrimidine-5-carboxylate (42)

Reaction of ethyl 4-amino-2-mercaptopyrimidine-5-carboxylate (**6**) (0.36 g, 1.81 mmol), anhydrous potassium carbonate (0.52 g, 3.77 mmol) and 10-(3-chloropropyl)-2-(trifluoromethyl)-10*H*-phenothiazine (**34**) (0.5 g, 1.45 mmol) in dry DMF (4 ml) under a set of conditions described in **Method C**, afforded white solid compound (**42**) (0.49 g, 67 %) m.p. 118-120 ˚C.

Anal:

TLC: R_f 0.10 (*n*-Hexane: ethyl acetate, 18:02)

IR (KBr) cm^{-1}: 3422, 3282, 1697, 1472, 1421 and 1113.

^{1}H NMR: 8.60 (s, 1H), 7.76 (bs, 1H), 7.23-7.21 (d, 1H, J = 8.0 Hz), 7.17-7.13 (m, 3H), 7.04 (s, 1H), 6.98-6.94 (m, 1H), 6.92-6.90 (d, 1H, J = 8.0 Hz), 5.40 (bs, 1H), 4.36-4.30 (q, 2H), 4.06-4.03 (t, 2H), 3.23-3.20 (t, 2H), 2.27-2.20 (m, 2H), 1.39-1.36 (t, 3H).

MS (ESI) m/z: 506.68 (M^+).

Calcd for $C_{23}H_{21}F_3N_4O_2S_2$: C, 54.53; H, 4.18; N, 11.06; Found: C, 54.66; H, 4.02; N, 11.19 %.

5.2.32 Ethyl 4-amino-2-(3-(2-chloro-10*H*-phenothiazin-10-yl)propylthio)-pyrimidine-5-carboxylate (43)

Reaction of ethyl 4-amino-2-mercaptopyrimidine-5-carboxylate (**6**) (0.38 g, 1.91 mmol), anhydrous potassium carbonate (0.55 g, 3.99 mmol) and 2-chloro-10-(3-chloropropyl)-10*H*-phenothiazine (**35**) (0.5 g, 1.61 mmol) in dry DMF (4 ml) under a set of conditions described in **Method C**, afforded white solid compound (**43**) (0.37 g, 49 %) m.p. 120-122 ˚C.

Anal:

TLC:	R_f 0.15 (*n*-Hexane: ethyl acetate, 16:04)
IR (KBr) cm^{-1}:	3412, 3268, 1695, 1458, 1381, 1089 and 749.
^{1}H NMR:	8.40 (s, 1H), 7.54 (bs, 1H), 6.96-6.91 (m, 2H), 6.83-6.81 (d, 1H, *J* = 8.2 Hz), 6.74-6.70 (m, 1H), 6.68-6.63 (m, 3H), 5.25 (bs, 1H), 4.14-4.09 (q, 2H), 3.79-3.75 (t, 2H), 3.00-2.95 (t, 2H), 2.05-1.98 (m, 2H), 1.18-1.14 (t, 3H).
MS (ESI) m/z:	472.5 $(M)^+$, 474.5 $(M+2)^+$.

Calcd for $C_{22}H_{21}ClN_4O_2S_2$: C, 55.86; H, 4.47; N, 11.84; Found: C, 55.71; H, 4.60; N, 11.98 %.

5.2.33 Ethyl 4-amino-2-(3-(2-methoxy-10*H*-phenothiazin-10-yl)propylthio)-pyrimidine-5-carboxylate (44)

Reaction of ethyl 4-amino-2-mercaptopyrimidine-5-carboxylate (**6**) (0.38 g, 1.91 mmol), anhydrous potassium carbonate (0.55 g, 3.99 mmol) and 10-(3-chloropropyl)-2-methoxy-10*H*-phenothiazine (**36**) (0.5 g, 1.63 mmol) in dry DMF (4 ml) under a set of conditions described in **Method C**, afforded white solid compound (**44**) (0.41 g, 53 %) m.p. 106-108 ˚C.

Anal:

TLC:	R_f 0.36 (*n*-Hexane: ethyl acetate, 16:04)
IR (KBr) cm^{-1}:	3410, 3268, 1691, 1430, 1382 and 1102.
^{1}H NMR:	8.61 (s, 1H), 7.74 (bs, 1H), 7.15-7.11 (m, 2H), 7.04-7.02 (d, 1H, *J* = 8.1 Hz), 6.92-6.90 (m, 1H), 6.89-6.87 (d, 1H, *J* = 8.1 Hz), 6.49 (s, 1H), 6.49-6.47 (d, 1H), 5.45 (bs, 1H),

4.35-4.30 (q, 2H), 4.00-3.96 (t, 2H), 3.76 (s, 3H), 3.21-3.17 (t, 2H), 2.28-2.22 (m, 2H), 1.38-1.35 (t, 3H).

^{13}C NMR: 175.34, 166.35, 161.80, 159.72, 158.97, 146.55, 144.97, 127.69, 127.46, 127.15, 125.70, 122.56, 116.20, 115.57, 106.77, 103.49, 101.15, 60.95, 55.50, 46.02, 28.25, 26.75, 14.29.

MS (ESI) m/z: 468.5 $(M)^+$.

Calcd for $C_{23}H_{24}N_4O_3S_2$: C, 58.95; H, 5.16; N, 11.96; Found: C, 58.84; H, 5.29; N, 11.81 %.

5.2.34 Ethyl 4-amino-2-(3-(10*H*-phenothiazin-10-yl)propylthio)pyrimidine-5-carboxylate (45)

Reaction of ethyl 4-amino-2-mercaptopyrimidine-5-carboxylate (**6**) (0.43 g, 2.16 mmol), anhydrous potassium carbonate (0.62 g, 4.49 mmol) and 10-(3-chloropropyl)-10*H*-phenothiazine (**37**) (0.5 g, 1.81 mmol) in dry DMF (4 ml) under a set of conditions described in **Method C**, afforded white solid compound (**45**) (0.33 g, 42 %) m.p. 146-148 ˚C.

Anal:

TLC: R_f 0.35 (*n*-Hexane: ethyl acetate, 16:04)

IR (KBr) cm^{-1}: 3416, 3270, 1698, 1533, 1455, 1368 and 1053.

^{1}H NMR: 8.60 (s, 1H), 7.72 (bs, 1H), 7.16-7.12 (m, 4H), 6.93-6.88 (m, 4H), 5.36 (bs, 1H), 4.35-4.29 (q, 2H), 4.04-4.00 (t, 2H), 3.22-3.19 (t, 2H), 2.26-2.20 (m, 2H), 1.38-1.35 (t, 3H).

MS (ESI) m/z: 438.7 (M^+).

Calcd for $C_{22}H_{22}N_4O_2S_2$: C, 60.25; H, 5.06; N, 12.78; Found: C, 60.44; H, 5.22; N, 12.63 %.

5.3 Biological work

5.3.1 *In vitro* blood-brain permeability assay

CB1 receptor antagonists are believed to be potent anti-obesity agents. But, their clinical application is limited because of their associated psychotic side effects. To overcome the CNS side effects, the clinical candidates must have restricted entry into the brain to facilitate their peripheral distribution. Therefore, the current drug designing approach includes development of drug candidates with peripheral activity only as successful anti-obesity agents.

To assess the probable *in vivo* brain permeability of the test compounds, PAMPA assay for BBB was performed as reported by Di. et al.[272] The donor microplate [polyvinylidene fluoride (PVDF) memberane, diameter 25 mm, pore size 0.45 µm, (Millipore)] and the acceptor microplate were used for the experiment. The acceptor microplate was filled with 200 µl phosphate buffer saline (PBS) and ethanol in the ratio of 70:30 whereas the donor microplate filter surface was impregnated with porcine brain lipid (Avanti Polar Lipids) in dodecane (Sigma) (4 µl of 20 mg/ml). 200 µl of the test compound (100 µg/ml in PBS/ethanol) was added to the donor well. A sandwich was formed by placing the donor plate carefully on the acceptor plate, which was kept undisturbed for 120 min at 25°C. The donor plate was carefully detached after the incubation period and the concentration of test compound in the acceptor wells was evaluated using UV spectroscopy. At five different wavelengths, each sample was analysed in four wells, and at least in three independent runs. Assay validation was performed by using nine commercial quality standard drugs of known BBB permeability[306] in which the reported permeability values of the standards were compared with their experimental permeability values which gave a linear relationship, P_e(exp.)= 1.171P_e(bibl.) + 1.489 ($R^2 = 0.983$).[272,273] From this equation and considering the limit established by Di. et al.,[272] the range of permeability (P_e) was determined which suggested that compounds having P_e value less than 3.8 resulted in low BBB permeation.

5.3.2 Evaluation of hypophagic response of the test compounds

Male Wistar rats weighing 200-250 gm were used to assess the hypophagic response. Animals were housed individually in a plexi-glass cage in a temperature and humidity controlled room with a 12 hr light/dark cycle. Standard chow pellets and water were made available *ad libitum*. Animals were handled daily for a week before performing the actual experiment. The animal study protocol was approved by the Institutional Animal Ethics Committee (IAEC) of Faculty of Pharmacy, The Maharaja Sayajirao University of Baroda (Approval no. MSU/IAEC/2014-15/1401). The experiments were conducted in accordance to the guidelines of CPCSEA (Committee for the Purpose of Control and Supervision of Experiments on Animals), Ministry of

Environment & Forests, Govt. of India. We have tried our best to reduce the number of animals and their suffering.

The acute effect of the synthesized test compounds on feeding behaviour was assessed in 24 hr food-deprived Wistar rats. Before performing the experiment, the bedding material was removed from the cage. The animals were food deprived for 24 hr with free access to water. The fasted animals were administered with the test compounds (10 mg/kg, p.o.). After 30 min of administration, weighed amount of food pellets were served to the animals. Amount of food pellets and food spillage remaining after 2 hr were weighed to find out the amount of food consumed by the animals.

In another set of experiments, *in vivo* CB1 receptor antagonistic potential of the test compounds was assessed. WIN-55212-2, a selective CB1 receptor agonist is known to increase the food intake. To assess the *in vivo* CB1 receptor antagonistic activity of the test compounds, 24 hr fasted animals were given WIN-55212-2 (2 mg/kg, i.p.) 15 min prior to the administration of the test compound and the experiment was proceeded as described above.