CHAPTER IV
MATERIALS AND METHODS

4.1 CHEMICALS AND REAGENTS

All the chemicals & reagents were used in the research of AR grade and presented in Table 4.1.

<table>
<thead>
<tr>
<th>Name of the reagent</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Absolute ethanol</td>
<td>SD Fine Chemicals, Mumbai</td>
</tr>
<tr>
<td>Carbon tetrachloride</td>
<td>Universal Chemicals and Scientific Industries, Mumbai.</td>
</tr>
<tr>
<td>99% Hydrazine hydrate</td>
<td>Nice Chemicals Pvt. Ltd., Kerala.</td>
</tr>
<tr>
<td>Potassium hydroxide</td>
<td>Universal Chemicals and Scientific Industries, Mumbai.</td>
</tr>
<tr>
<td>Carbon disulphide</td>
<td>Nice Chemicals Pvt. Ltd., Kerala.</td>
</tr>
<tr>
<td>Diethyl ether</td>
<td>Nice Chemicals Pvt. Ltd., Kerala.</td>
</tr>
<tr>
<td>Hydrochloric acid</td>
<td>Universal Chemicals and Scientific Industries, Mumbai.</td>
</tr>
<tr>
<td>Sulphuric acid</td>
<td>Universal Chemicals and Scientific Industries, Mumbai.</td>
</tr>
<tr>
<td>Benzaldehyde</td>
<td>CDH Ltd., New Delhi.</td>
</tr>
<tr>
<td>2,4-dichloro benzaldehyde</td>
<td>CDH Ltd., New Delhi.</td>
</tr>
<tr>
<td>P-Dimethyl amino benzaldehyde</td>
<td>CDH Ltd., New Delhi.</td>
</tr>
<tr>
<td>P-Nitro benzaldehyde</td>
<td>CDH Ltd., New Delhi.</td>
</tr>
<tr>
<td>Furfuraldehyde</td>
<td>CDH Ltd., New Delhi.</td>
</tr>
</tbody>
</table>
Diethylamine  
Morpholine  
Piperidine  
Ethyl acetate  
n-Hexane  
Chloroform-d  
7H9GC broth  
Alamar blue dye solution

<table>
<thead>
<tr>
<th>Biological</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>SKMEL (Human malignant melanoma cell line)</td>
<td>NCCS, (National Centre for cell science) Pune, India.</td>
</tr>
<tr>
<td>MCF7 (Breast cancer cell line)</td>
<td></td>
</tr>
<tr>
<td>Hep2 (He La derivative)</td>
<td></td>
</tr>
<tr>
<td>Dalton’s lymphoma ascites (DLA) cells</td>
<td>Amala cancer research center, Thrissur, Kerala, India</td>
</tr>
<tr>
<td><em>Mycobacterium tuberculosis</em> H$_{37}$Rv</td>
<td>MTCC, Chandighar, India.</td>
</tr>
<tr>
<td>Swiss albino mice and Albino Wistar rats</td>
<td>K.M College of Pharmacy, Madurai, India</td>
</tr>
</tbody>
</table>
4.3 INSTRUMENTS

Table 4.3 shows details of the instruments used in this research work.

Table 4.3: Details of the instrument used

<table>
<thead>
<tr>
<th>Instrument Name</th>
<th>Make and Model</th>
</tr>
</thead>
<tbody>
<tr>
<td>Magnetic stirrer</td>
<td>Remi Equipments, India.</td>
</tr>
<tr>
<td>IR Spectrometer</td>
<td>FTIR – 4100 type A Jasco, Japan.</td>
</tr>
<tr>
<td>$^1\text{H}$ NMR Spectrometer</td>
<td>Bruker Avance 400 MHz, USA.</td>
</tr>
<tr>
<td>$^{13}\text{C}$ NMR Spectrometer</td>
<td>Bruker Avance 100 MHz, USA.</td>
</tr>
<tr>
<td>Electrospray ionization mass</td>
<td>TOF MSES Mass Spectroscopy, USA.</td>
</tr>
<tr>
<td>Spectrometer</td>
<td></td>
</tr>
<tr>
<td>COBAS MICROS OT 18</td>
<td>Roche, Switzerland.</td>
</tr>
<tr>
<td>COBAS MIRA PLUS – S auto analyzer</td>
<td>Roche, Switzerland.</td>
</tr>
<tr>
<td>Digital plethysmometer</td>
<td>Ugobasile, Italy</td>
</tr>
<tr>
<td>Elemental analyser</td>
<td>Perkin Elmer 2400 CHNS analyzer</td>
</tr>
</tbody>
</table>

4.4 COMPUTATIONAL PLATFORM AND SOFTWARE

All the computational procedures of the research work were conducted with Intel®Core(TM) 2 Duo processor with Microsoft Windows XP. Software used in this research work are presented in Table 4.4.

Table 4.4: Software used in the research work

<table>
<thead>
<tr>
<th>Software</th>
<th>Usage</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACD Lab Chemsketch 10.00</td>
<td>3-D drawing, optimizing and calculating various physicochemical descriptors of the proposed molecules.</td>
</tr>
</tbody>
</table>
4.5 *IN SILICO MOLECULAR MODIFICATION AND WET LAB SYNTHESIS*

*In silico* molecular modifications of 55 proposed derivatives of 1,2,4-triazole were done by using the various type software mentioned in Table 4.4.

ACD Lab Chemsketch 10.00 was employed for 3-D drawing, optimizing and calculating various physicochemical descriptors of the proposed molecules.

Molinspiration was used for calculating the log $P$ values, drug likeness and Lipinski’s rule of five.

PASS software was utilized for the forecast of pharmacological activities of proposed moiety.

Schrodinger (Maestro) Version 9.6 – QIKPROP was employed to predict the ADME properties of proposed moieties.

Schrodinger (Maestro) Version 9.6 - Glide XP was utilized for molecular docking of proposed molecules.
4.6 DOCKING METHODOLOGY

The steps involved in docking are as follows:

4.6.1 Ligand structure:

The chemical structure of each ligand was drawn using build module.

4.6.2 Ligand preparation:

To prepare the elevated quality of ligands, all-atom 3D structures for more numbers of drug-like moieties, initiated with the 3D structures in the SD Maestro format, LigPrep was used. LigPrep produced a single, low energy and 3D structure for each effectively processed selected structure.

4.6.3 Preparation of protein:

Initially, the co-crystallized ligand is prepared by adding hydrogens and correctly describing multiple bonds. The attached hydrogens in the proteins were normally removed except in peptide bond. Then the residues which are not participated in the salt bridges were neutralized by modifying them to tautomeric forms. After that the receptors were preprocessed before grid preparation and finally the proteins were optimized by adding hydrogens, any co-factors and any structural waters to the proteins. This leads to a series of restrained minimization on the protein-ligand complex.

4.6.4 Receptor Grid Generation:

It requires a “prepared” structure: every atom structure with suitable bond orders and formal charges. Glide searches for constructive communications among one or multiple ligand and a receptor site, regularly a protein. The structure and behavior of the receptor are presented on a grid utilizing a number of dissimilar sets of fields that grant increasingly more precise scoring the ligand poses. The options in all tabs of the Receptor Grid Generation panel allow defining the receptor structure by exclusive of any co-crystallized ligand that may be present, determine the location and size of the active site as it will be represented by receptor grids, and set up Glide constraints. A grid area that was generated around the binding site of the receptor.

4.6.5 Ligand Docking:

Glide searches for positive communications among one or other ligand moiety and a receptor molecule, generally a protein. All the ligands act as sole molecule, while
the receptor may comprise more than one molecule, e.g., a protein and a cofactor. Glide was run in rigid or flexible docking modes; the latter involuntarily generated conformations for each contributed ligand. The blend of position and direction of a ligand relation to the receptor, all along with its conformation in bendable docking, is called as a ligand pose. The ligand poses, which Glide creates, pass all the way through a sequence of ordered filters, which evaluate the ligand’s interface with the receptor. The first filters test the spatial fit of the ligand to the specified active site, and inspect the support of ligand-receptor interacted, while using a grid-based method following the empirical ChemScore role. Poses that conceded these initial screens entered the last stage of the algorithm, that involve in the evaluation and minimization of a grid rough calculation to the OPLS-AA non bonded ligand-receptor interacted energy. At last the scoring is then conceded out on the energy-minimized poses.

4.6.6 Glide Extra-Precision Mode (XP):

The extra-precision (XP) mode of Glide combines a powerful sampling procedure with the use of a traditional scoring function intended to identify ligand poses that would be accepted to have unfavourable energies, based on a well known ideology of physical chemistry.

4.6.7 Docking Procedure:

The computational modeling investigation relied upon the GLIDE (Grid-based Ligand Docking from Energetics) program (Glide, version 9.6, Schrodinger, LLC New York, 2008) for the docking assignment. Crystallographic structures of the target of interests (receptors/enzymes) were obtained from the PDB (protein data bank) and saved in a standard 3D co-ordinate format. Protein targets and their PDB ID are listed out in Table 4.5.

All the water molecules in the crystal structure were deleted, bond orders were assigned, hydrogen’s were added and the protein was then further refined for the docking studies by dealing out it using Schrodinger’s Protein preparation wizard. This procedure minimizes the protein to 0.30 Å RMSD using the OPLS-2001 force field. Ligands were prepared using build panel in maestro. Further the ligands were prepared for docking using LigPrep tool and were energy minimized using MMFF Force Field. The Glide Grid generation panel has been used to generate receptor grids for docking. Default SP (Standard Precision) docking protocol was used to dock the library ligands.
Table 4.5: Protein targets and their PDB ID

<table>
<thead>
<tr>
<th>No.</th>
<th>Targets</th>
<th>PDB ID</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Tyrosine protein kinase</td>
<td>3 KRR</td>
</tr>
<tr>
<td>2</td>
<td>Protein kinase - C</td>
<td>1 ZRZ</td>
</tr>
<tr>
<td>3</td>
<td>Heat Shock Protein</td>
<td>2 CCU</td>
</tr>
<tr>
<td>4</td>
<td>Tubulin</td>
<td>3 M 89</td>
</tr>
<tr>
<td>5</td>
<td><em>Mycobacterium tuberculosis</em> pantothenate synthetase</td>
<td>3 COZ</td>
</tr>
<tr>
<td>6</td>
<td>Glutamine synthetase from <em>Mycobacterium tuberculosis</em></td>
<td>1 HTO</td>
</tr>
<tr>
<td>7</td>
<td>Mitogen-activated protein kinase</td>
<td>3 CTQ</td>
</tr>
</tbody>
</table>

4.7 GENERAL SCHEME FOR THE SYNTHESIS OF SELECTED 1,2,4-TRIAZOLE DERIVATIVES

The derivatives selected with the help of these selection parameters were taken to wet lab synthesis by conventional method through a series of four steps. The general scheme of synthesis is shown in Figure 4.1.
4-amino 5-(4-hydroxy phenyl) 1,2,4-triazolin 3-thione

**Schiff’s base**
Step-1: Synthesis of 4-hydroxy benzoyl hydrazine from methyl 4-hydroxy benzoate and hydrazine hydrate.

Methyl-4- hydroxy benzoate 1.52g (0.01M) and 99% hydrazine hydrate 0.97ml (0.02M) were refluxed in absolute ethanol (50ml) for 18h. The above solution was subjected to distillation for the removal of ethanol. The resultant solution was kept in an ice bath with constant stirring and kept in the room temperature for 3-4h. The product was separated out by filtration, dried and recrystallized from ethanol. Yield and MP of the product obtained were estimated. The TLC plate established the purity of the compound. The solvent used was n-hexane: ethyl acetate (9:1).

Step-2: Synthesis of 4-amino-5-(4-hydroxy phenyl)-1,2,4-triazolin-3-thione through, formation of potassium dithiocarbazinate from 4-hydroxy benzoyl hydrazine.

13.7g (0.1M) 4-hydroxy benzoyl hydrazine was dissolved in 200ml of absolute alcohol containing 5.6g (0.1M) of potassium hydroxide at ambient temperature. 12.5ml of carbon disulphide was added in portion and the above solution was stirred for 16h at ambient temperature. 100ml of diethyl ether was transferred and stirred for further 3h.
The resultant product was separated and dried. Yield, Rf value and MP of the product were recorded.

10.3g hydrazine hydrate (0.1M, 99%) was gradually added to Potassium dithiocarbazine dissolved in 100ml of water with mixed, refluxed for 8h during which hydrogen sulphide gas evolved and the color was changed to deep green. Then cooled to 0-5°C, acidified with dilute hydrochloric acid to make pH 1. The resultant product was separated by filtration and re-crystallized from ethanol. Yield, melting point and Rf value of the product were recorded. In TLC, the solvent used was n-hexane: ethyl acetate (8:2).

\[
\text{4-hydroxy benzyol hydrazine} \quad \xrightarrow{\text{CS}_2 + \text{KOH}} \quad \text{Potassium dithiocarbazine}
\]

\[
\text{Potassium dithiocarbazine} \quad \xrightarrow{\text{NH}_2\text{NH}_2 \cdot \text{H}_2\text{O}} \quad \text{4-amino-5-(4-hydroxy phenyl)-1,2,4-triazolin 3-thione}
\]

**Step-3: Synthesis of various Schiff’s bases by reacting 4-amino-5-(4-hydroxyphenyl)-1,2,4-triazolin-3-thione with different aromatic aldehydes.**

2-3 drops of concentrated sulphuric acid were added to the solution of 2.08g (0.01M) 4-amino-5-(4-hydroxyphenyl)–1,2,4-triazolin-3-thione in 20ml of ethanol. 0.01M of different benzaldehyde derivative or furfuraldehyde was added and refluxed for 2-6h. Then cooled to 0°C and the product obtained was filtered, dried and re-crystallized from ethanol. Yield, MP and Rf value of the product were recorded. In TLC, the solvent used was n-hexane: ethyl acetate (8:2).
Step-4: Synthesis of various Mannich bases (final compounds) by treating above Schiff’s bases with different secondary amines in the presence of formaldehyde.

Appropriate secondary amine (0.01M) was gradually added to the solution of Schiff’s base (0.01M) in 12ml of dry ethanol. 38% formaldehyde solution (0.8ml, 0.015M) was transferred to it. The pH of the above mixture was maintained between 3 and 4 by using concentrated hydrochloric acid. Then the solution was stirred for one hour at ambient temperature and kept overnight at 0°C. The product obtained was filtered, dried and re-crystallized from ethanol. Yield, melting point and R<sub>f</sub> value of the product were recorded. In TLC, the solvent used was n-hexane: ethyl acetate (8:2).

Melting points of the compounds were recorded through SPAC-A Service (India) open capillary melting point apparatus (Laboratory device) and are uncorrected. Analytical thin layer chromatography was performed on Merck 20 cm × 20 cm silica gel 60-F<sub>254</sub> plates. Visualizing agent used in the TLC was UV chamber.

4.8 CHARACTERIZATION OF COMPOUNDS BY SPECTRAL STUDY

4.8.1 IR Spectrum

IR has a greater practical use in the field of organic chemistry and is associated with functional groups. It is widely used for the structural elucidation and identification
of functional groups of unknown organic compounds. All the IR spectra are measured using a FTIR-4100 type. The spectral resolution of the instrument is 0.25 cm\(^{-1}\), and the spectral data are stored in the database at the intervals of 0.5 cm\(^{-1}\) at 4000 –2000 cm\(^{-1}\) and of 0.25 cm\(^{-1}\) at 2000–400 cm\(^{-1}\).

4.8.2 NMR Studies:

\(^1\)H NMR experiments for all the Mannich bases synthesized were performed on a Bruker Avance 400 MHz NMR instrument. About 2-3 mg of ligands was dissolved in 0.5 ml of CDCl\(_3\) in a NMR tube. About 50 scans were performed for every spectrum. In all the cases Tetramethylsilane (TMS) was used as an internal standard. All the spectra obtained were corrected with equivalent to TMS. The software Mestrec 2.0 was used to evaluate the peak positions and their integrations.

\(^13\)C NMR experiments for all the Mannich bases synthesized were performed by using Bruker Avance 100 MHz NMR instrument. About 30-40 mg of ligands was dissolved in 0.5 ml of either DMSO-d\(_6\) or CDCl\(_3\) in a NMR tube. About 1000 scans were performed for all the experiments. All the spectra obtained were corrected with equivalent to the solvent signal.

4.8.3 Mass Spectroscopy

A mass spectrum of the compounds was recorded using with TOF MS ES Mass Spectroscopy using electron impact process where the electron was accelerating at 75 eV voltage and 8-10 nV was an ion accelerating voltage.

4.8.4 Elemental Analysis

A destructive method used to quantify the elemental components of a moiety. The elements found in the synthesized compounds were recorded on Perkin Elmer 2400 CHNS analyzer.

4.9 LD\(_{50}\) AND SUB ACUTE TOXICITY STUDY OF COMPOUNDS

The compounds were subjected to toxicity evaluation based on the guidance of the World Health Organization (WHO 2000) and the OECD 2001. Animal experiments were conducted with the approval from the institute animal ethical committee (IAEC/KMCP/139/2013-2014 and IAEC/KMCP/142/2013-14). Healthy young albino Wistar rats of both sexes having 180-200 g body weight and 8 -12 weeks old were
selected for the toxicity study. In case of in vivo anticancer evaluation, healthy adult male Swiss albino mice weighing 20 - 25g were utilized for the study, which were performed in accordance OECD 423. Animals were procured from the central animal house, KMCP, Madurai, Tamil Nadu.

4.9.1 Determination of LD_{50}

The toxicity evaluation was performed by Sita Sharan, et al., 2011 and Shirish Sadashiv, et al., 2011. The experimental animals were selected randomly and marked for the identification and kept for 5 days preceding to dosing to permit for adaptation to the laboratory conditions. A temperature of 25 ±2°C was maintained in the experimental room. Conventional laboratory diet with unlimited drinking water was provided for feeding. The test compounds were given using single dose by gavage with a stomach tube. Three animals of a single sex were used in each step. The starting dose was selected as 5mg.kg\textsuperscript{-1}bw. The number of animal deaths was noted after 24h. After that the 2nd dose 50 mg.kg\textsuperscript{-1}bw was attempted. After 24h the numbers of animal deaths were noted. Then the 3rd dose 300mg.kg\textsuperscript{-1}bw was treated and the animal death was noted. After the administration of the 3rd dose 300 mg.kg\textsuperscript{-1}bw, all the three animals had died within 24h of administration. From this, it was concluded that the LD_{50} lies between the doses of > 50 to 300mg.kg\textsuperscript{-1}bw. Now a dose, 100mg.kg\textsuperscript{-1}bw was given to another three animals and the animal death was noted. Here, one of the animals died within 24h of administration. Again the similar dose was given to the remaining two animals for the confirmation of LD_{50}.

4.9.2 Subacute toxicity study

Selected animals were separated into 6 groups of 6 each. They were kept under the environment of temperature 25 ±2°C and 12h dark-light cycle and they were given access to water and a commercial diet ad libitum. Each selected compound was prepared at the concentration of 10mg.kg\textsuperscript{-1}bw by using purified water and treated to each group of animals fasted overnight and the control group animals received water vehicle for fourteen days. After dosing, treated animals were observed at least once during the first 30min for the first 4h daily, thereafter, for a total of 14 days. Toxic manifestations such as body weight, signs of toxicity, and mortality was observed daily. Finally, all animals were performed to gross necropsy. Heparinised blood samples were used for the determination of complete blood count, RBC count, platelet count and red
cell indices. The serum from the non heparinised blood was carefully collected for blood chemistry and enzyme analysis. The liver and kidney were preserved in 10% neutral formalin for histopathological examination.

4.10 EVALUATION OF ANTICANCER ACTIVITY

4.10.1 Assessment of in vitro anticancer activity

Antitumor activities of selected compounds were evaluated against SKMEL (Human malignant melanoma cell line), MCF7 (Breast cancer cell line) and Hep2 (HeLa derivative) cell lines by the MTT assay method. The cell lines were procured from NCCS, (National centre for cell science) Pune, India. The study was performed in reference to the procedure of Mathan, et al., 2011.

The cell lines were cultured in Dulbecco’s modified eagle’s medium (DMEM) supplemented with 10% heat inactivated FBS and incubated at 37°C in a humidified atmosphere of 5% CO₂.

Cells were transferred into 96- well flat bottom plates at the strength of 1×10⁴ cells/ml and incubated at 37°C in a humidified incubator (5% CO₂) for 24h followed by exposure to various concentrations of tested compounds for 48h. Then 20µl of MTT (3-(4,5-dimethyl thiazol-2yl)-2,5- diphenyl tetrazolium bromide) reagent dissolved in PBS (phosphate buffered saline, pH 7.4) was transferred to individual well and stirred and incubated for 4h. Subsequently, the supernatant was removed; 150µl DMSO (dimethyl sulphoxide) was transferred to individual well for dissolving the MTT- formazan crystals. Finally absorbance was recorded at 570nm using a micro plate reader with DMSO as a blank for determining the cell growth inhibition which was calculated by

\[
\text{Growth inhibition} = 1 - \frac{OD \text{ of treated cells}}{OD \text{ of control cells}} \times 100
\]

The concentration required to inhibit the growth by 50% (IC₅₀) was determined from absorbance value.

Based on the in vitro antitumor activity, the compound selected was subjected to in vivo anticancer activity evaluation against cancer induced male Swiss albino mice.
4.10.2 Evaluation of *in vivo* anticancer activity

4.10.2.1 Animals

Details of animals were described in chapter 4.9. The selected animals were housed in ambient temperature (25 ±2 °C), relative humidity (55± 5%) and 12h light / dark cycle and fed with commercial diet and water *ad libitum*. The study was conducted in reference to the procedure of Mathan, *et al.*, 2013.

4.10.2.2 Tumor cell line

DLA cells were obtained from ACRC, Kerala, India. The cells maintained *in vivo* in Swiss albino mice by ip transplantation. While transferring the cancer cells to the different group of animals the DLA cells were aspirated from the peritoneal cavity of the mice using saline. The cell count was adjusted to the concentration of the total cells to 1 x 10^6 cells/ ml. The final diluted solution was given by ip injection and allows the tumor to grow in the mice and after one week the treatment was started.

4.10.2.3 Experimental design

A total number of 30 mice were divided into 5 groups of 6 animals each:

Group I : Control group.

Group II : DLA cells (1 x 10^6 cells / mouse) ip.

Group III : DLA cells (1 x 10^6 cells / mouse) ip + 5-fluorouracil. (20 mg/kg.bw).

Group IV : DLA cells (1 x 10^6 cells / mouse) ip + synthesized compound (2- (piperidin-1-ylmethyl) -5- (4-hydroxy phenyl) -4- [[(4-dimethyl amino) phenyl methylidene] amino]-1,2,4-triazolin-3-thione) ip (10 mg/kg.bw).

Group V : DLA cells (1 x 10^6 cells / mouse) ip + synthesized compound (2- (piperidin-1-ylmethyl) -5- (4-hydroxy phenyl) -4- [[(4-dimethyl amino) phenyl methylidene] amino]-1,2,4-triazolin-3-thione) ip (20 mg/kg.bw).

The treatment was given once daily for 14 days. At the end of the 14 days, all the animals were sacrificed by and the blood was withdrawn from each mouse by retro orbital puncture bleeding and used for the estimation of clinical parameters such as cancer cell count, hematological and biochemical parameters.
4.10.3 Derived parameters

4.10.3.1 Body weight

All the groups of mice were weighed, initial and 15th day of the study. The average body weight was calculated on the 15th day.

4.10.3.2 Percentage increase in life span (ILS)

Survival time of treated groups was compared with those of control using the formula

\[
\%\text{ILS} = \frac{\text{Life span of treated group}}{\text{Life span of control group}} - 1 \times 100
\]

4.10.3.3 Cancer cell count

From the peritoneal cavity of each mouse, ascetic fluid (0.1ml) was withdrawn by sterile syringe and diluted with 0.8ml of ice cold normal saline and 0.1ml of tryphan blue (0.1mg/ml) and using hemocytometer the total number of the living cells were calculated.

\[
\text{Cell counts} = \frac{\text{Number of cells} \times \text{dilution}}{\text{Area} \times \text{thickness of liquid film}}
\]

4.10.4 Estimation of haematological parameters

4.10.4.1 Counting of RBC (Dacie, 1977).

The blood was taken up to 0.5 marks in the pipette and filled up to 101mark with Hayem’s red cell dilution fluid and mixed thoroughly. The first 2-3 drops of diluted blood are blown out and 2-3 drops were allowed to place in Haemocytometer (Neubauer, Fein-optik. Germany). A cover slip was kept over the chamber so that the fluid runs under the slip, filling the chamber.

To count red blood cells, the Haemocytometer was placed under the microscope at high power 45X. The cells were calculated in the 4 fields. The average number of cells in each small square was counted. The total number of cells/mm³ was calculated.

4.10.4.2 Counting of WBC (Dacie, 1977)

The blood was taken up to 0.5 mark, diluted with white cell diluting fluid up to the mark 11 and stirred thoroughly, first 2-3 drops were discarded and the fluid was kept in the haemocytometer. A cover slip was kept over the chamber and the counting
was carried under the microscope at low power 10X. Then the total count of WBC was calculated from the number of cells/mm$^3$ of the areas of counting chamber.

4.10.4.3 Haemoglobin estimation (Dacie, 1977)

0.1N Hydrochloric acid was placed up to the mark 20 in the graduated tube. 20mm$^3$ of blood, i.e. 0.02ml was added through the Haemoglobinometer pipette. The pipettes were rinsed 2 to 3 times, stirred well and kept for 5min, till the solution becomes dark brownish color. The solution was diluted drop by drop, each time, mixing the solution with stirring rod until it matches the standard.

The results are read from the scale on the graduated tube by observing the graduation mark at the lower edge of the meniscus at the top of the liquid column. Final reading was not made within 10min after mixing the blood with 0.1N hydrochloric acid, 2% should be deducted from the result obtained.

4.10.4.4 Estimation of packed cell volume (PCV) (Lloyd, 1958)

The portion of whole blood volume was engaged by erythrocytes i.e. red blood cells. PCV has traditionally been determined by measuring the height of the red cell volume in a micro-hematocrit capillary filled with whole blood, after centrifugation. PCV is a directly determined, whereas the hematocrit (Hct) is the corresponding calculated value, which is determined by multiplying a RBC count with the mean corpuscular volume (MCV). For most practical purposes PCV and Hct are interchangeable, but typically PCV is slightly higher than the more accurate Hct due to plasma trapping (between the packed cells in a centrifuged capillary).

4.10.5 Biochemical parameters

4.10.5.1 Estimation of Aspartate Aminotransferase (AST) Activity

The serum AST was estimated by Reitman and Frankel (1957) using AST test kit (Span Diagnostics Ltd.).

**Reagents**

Reagent I : Buffered aspartate - α-KG substrate, pH 7.4
Reagent II : DNPH (2,4- Dinitrophenyl hydrazine) color reagent
Reagent III : Sodium hydroxide, 4N
Reagent IV : Working pyruvate standard, 2mM
Solution I: Dilute one ml of Reagent III up to 10ml with purified water.
**Procedure**

0.25 ml of Reagent I was transferred in three clean test tubes and incubated at 37°C for 5min. 0.05ml of serum was transferred in the test, 0.05ml Reagent IV was transferred in standard and 0.05ml distilled water was transferred in the blank. They were stirred well and incubated at 37°C for 60min. Then 0.25ml of Reagent II was transferred to all the tubes, stirred well and kept at ambient temperature for 20min. Then 2.5ml of Solution I was transferred to all the tubes stirred well and kept at ambient temperature for 10min. The absorbances of test, standard and blank were read at 505nm.

**4.10.5.2 Estimation of Alanine Aminotransferase (ALT) Activity**

The serum ALT was estimated by Reitman and Frankel (1957) using an ALT test kit (Span Diagnostics Ltd.).

**Reagents**

- **Reagent I**: Buffered alanine - α-KG substrate, pH 7.4
- **Reagent II**: DNPH (2,4- Dinitrophenyl hydrazine) color reagent
- **Reagent III**: Sodium hydroxide, 4N
- **Reagent IV**: Working Pyruvate Standard, 2 mM
- **Solution I**: Dilute one ml of Reagent III up to 10ml with purified water.

**Procedure**

0.25 ml of Reagent I was transferred in three clean test tubes and incubated at 37°C for 5min. 0.05ml of serum was transferred in the test, 0.05ml Reagent IV was transferred in the standard and 0.05ml distilled water was transferred in the blank. They were stirred well and incubated at 37°C for 30min. Thereafter, 0.25 ml of Reagent II was transferred to all the tubes, stirred well and kept at ambient temperature for 20min. Then 2.5ml of Solution I was transferred to all the tubes, stirred well and kept at ambient temperature for 10min. The absorbance of test, standard and blank were read at 505nm.

**4.10.5.3 Estimation of Alkaline Phosphatase (ALP) Activity**

ALP activity was estimated by Kind and King (1954) using an ALP test kit (Span Diagnostics Ltd.).

**Reagents**

- **Reagent I**: Buffered substrate, pH 10.0
Reagent II : Chromogen reagent
Reagent III : Phenol, 10mg

Working solution: One vial of reagent I was reconstituted with the buffered substrate of 2.2ml distilled water.

Procedure
All the test tubes were marked properly and 0.5ml of working buffered substrate was transferred in clean tubes, 1.5ml of purified water was transferred in all the tubes. They were stirred well and incubated at 37°C for 3min. 0.05ml of serum was transferred in test (T), 0.05ml of reagent III (Phenol) was transferred in standard (S) and 0.05ml of distilled water was transferred in blank (B) tubes. Above 3 solutions were stirred well and incubated at 37°C for 15min. 1ml of reagent II was transferred in all the tubes. 0.05 ml of serum was transferred in standard (S). All the solutions were stirred well and absorbance was read at 510nm. Serum ALP activity is expressed as KA units.

4.10.5.4 Estimation of Triglycerides (TG)
Triglycerides were estimated by Foster, et al., 1973.

Reagents
1. Isopropanol.
2. Alumina (activity grade 1 for chromatography) was washed with distilled water and dried in an oven overnight.
4. Sodium metaperiodate reagent.
5. Acetyl acetone reagent.

Procedure
To an aliquot of dried lipid extract, 4ml isopropanol was transferred, stirred well and 400mg washed alumina was transferred. This was placed in a mechanical rotor for 15min and then centrifuged. To 2ml supernatant, 0.6ml potassium hydroxide was transferred and incubated at 60-70°C for 15min, cooled and 1ml of sodium metaperiodate solution and 0.5ml acetyl acetone reagent were transferred. It was then stirred and incubated at 50°C for 30min. A series of standards of concentration of
8-40µg triolein were treated similarly along with a blank. Cooled and read at 405nm against blank.

### 4.10.5.5 Estimation of Total Cholesterol (TC)

Increment levels of serum cholesterol are seen in coronary artery diseases, diabetes mellitus, hypothyroidism, nephritic syndrome, obstructive jaundice, cirrhosis of liver and decreased serum cholesterol levels are found in acute hepatitis, hyperthyroidism, anaemias etc. The serum cholesterol is estimated by Henry, et al., 1974.

Free cholesterol gets oxidized in the presence of cholesterol oxidase to liberate cholest-4-en-3-one and H₂O₂. Liberated H₂O₂ by this reaction combines with phenol and 4- amino antipyrine in presence of peroxidase to form red colored quinonimine complex, the intensity of which is measured at 505nm (490-530nm).

\[
\text{Cholesterol Ester + H}_2\text{O} \xrightarrow{\text{cholesterol esterase}} \text{Cholesterol + Fatty acids}
\]

\[
\text{Cholesterol + O}_2 \xrightarrow{\text{cholesterol oxidase}} \text{Cholest-4-en-3-one + H}_2\text{O}_2
\]

\[
2\text{H}_2\text{O}_2 + \text{Phenol + 4-amino antipyrine} \xrightarrow{\text{peroxidase}} \text{Quinonimine + 4H}_2\text{O}
\]

**Procedure**

The samples were incubated at 37°C for 10min. Mix well and the absorbance was taken at 505nm using auto analyzer. The concentration of TC in the sample was calculated.

\[
\text{Cholesterol concentration (mg/dl)} = \frac{\text{Sample}}{\text{Standard}} \times 200
\]

### 4.11 EVALUATION OF IN VITRO ANTITUBERCULAR ACTIVITY

Antitubercular activity of selected compounds was evaluated by Resazurin microtiter assay (REMA) method by the procedure described by Reham, et al., 2013. *Mycobacterium tuberculosis* H₃₇Rv utilised for the evaluation was procured from MTCC, Chandigarh, India. Back view, flat bottom 96-well micro plates were utilised in the experiment. The initial dilutions of test compounds were prepared by using DMSO and subsequent two fold dilutions were prepared in the micro plates by using 0.1ml of 7H9GC broth. 100µl of 2000CFU/ml of test organism in 7H9GC broth was transferred to each well of 96 well micro titer plate containing test compounds. Three controls-medium only, test compound and medium and test organism and medium were prepared and all are incubated at 37°C for 7days. On the 7th day alamar blue dye solution (20µl
alamar blue solution and 12.5 ml of 20% Tween 80) was transferred to all the wells and the plates were again incubated at 37°C for 24h. Results were recorded at 365nm using a micro plate reader.

4.12 EVALUATION OF IN VIVO ANTI-INFLAMMATORY ACTIVITY

Anti-inflammatory activities of selected compounds were evaluated by carrageenan-induced paw edema method. Diclofenac sodium was used as standard. Healthy adult male Albino Wistar rats weighing 180 - 200g were utilized for the experiments. The selected animals were housed in ambient temperature (25 ±2°C), relative humidity (55± 5%) and 12h light / dark cycle and fed with commercial diet and water ad libitum. The study was conducted in reference to the procedure of Sanjay, et al., 2012. The standard and the test compounds were treated to the animals by ip one hour prior to carrageenan injection. A freshly prepared suspension of carrageenan (1%w/v, 0.1 ml) was injected into the planter region of the left hind paw of each rat.

Experimental Design
Rats were divided into 4 groups of 6 animals each
Group I : Saline 5ml.kg⁻¹body weight
Group II : Diclofenac sodium at the dose of 10mg.kg⁻¹bw
Group III : Test Compound 17 dissolved in 0.5ml of DMSO at the dose of 10mg.kg⁻¹bw
Group IV : Test Compound 18 dissolved in 0.5ml of DMSO at the dose of 10mg.kg⁻¹bw

Half-an-hour before the administration of Diclofenac sodium / test compounds to the animals in group I, II, III and IV an injection of 0.1ml of 1 % carrageenan in saline was given into the sub planter region in the right hind paw of each rat, (Lanhers, et al., 1991). The paw volume of the injected animal was measured using a plethysmograph (UgoBasile, Italy) before and every 1hr, 3hr and up to 6h after the injection. The percentage of inhibition was calculated.

\[
\% \text{ Anti – inflammatory activity} = \frac{V_c - V_t}{V_c} \times 100
\]

Where, Vt - mean increase in paw volume in rats treated with test compounds
Vc - mean increase in paw volume in control group of rats.
4.13 STATISTICAL ANALYSIS

Results are expressed as means ± Standard Error Mean (SEM) of 6 rats in each group. Statistical significance was calculated by ANOVA followed by Newmann Keul’s multiple range tests. P-values less than 0.05 were considered significant.
Results and Discussion