CHAPTER-IV

4. MATERIALS AND METHODS

4.1 Study area

Kokrajhar (89.46' E to 90.38' E longitudes and 26.19" N to 26.54" N latitudes) is one of the twenty-seven districts of Assam and head quarter of Bodoland Territorial Council, described as the gateway to the north-eastern region of India located on the north bank of the river Brahmaputra dominated by the Boro tribe. It is surrounded by Bhutan on the north, the Sonkosh river and Jalpaiguri district of West Bengal on the west and tracts of Brahmaputra valley (Dhubri and Goalpara district) on the south and Chirang & Bongaigaon district on the east. It covers a total area of 3,169.22 sq. km; 2001 census of India, put population of Boro as 9,30,404, whereas Brahma *et al.* 2001, stated that Boro population is as high as 52 lakhs.

4.2 Topography

The Kokrajhar district land surface is generally slope from north to the south. Phukan 1990, has divided the area into two distinct physiological zones- (a) North zone are in foothills of the Himalaya and (b) the plain area towards the south. Kokrajhar has alluvial soil type and may vary from sandy loam to silty clay loam, silty clay and clay. In northern part, it is mostly covered with light gravel that originates from alluvial soil of old mountain valley and towards the southern part, varying proportion of sand and clay were found ranging from poor sand stiff clay. The soil is silt in organic matter and acidic in nature (Bhowmick *et al*. 2015). The district is situated in sub-tropical climate with moderately hot summer and cold winter with high humidity. Generally, the months of December – February is the coldest months of the year with less than 11 ºC and temperature goes up to 32 ºC during summer season with heavy rainfall during the month of June- September ranging up to 747 mm (Kour & Sharma 2016).

4.3 Collection and identification:

An ethno-botanical survey was carried out between March 2014 and May 2017 to obtain relevant information about medicinal plants used in the treatment of liver disorder in Kokrajhar, Lower Assam, India, through a series of oral interviews with traditional healers as well as elderly people of the villages who still practice/have knowledge of the indigenous system of medicine. The oral interview was accomplished in their local dialect, with the help of questionnaires attached in '***Appendix A***' (Willcox *et al.* 2011). The traditional healers were requested to accompany to collect the plant specimens from adjoining forest areas. In some

cases when the experienced and familiar persons were unable to accompany in the exploration visit to the forest area, the fresh specimens were collected from the target location with details of their vernacular name, habit, habitat, height of the plant, fragrance, taste, flower, fruit nature, etc., and consequently shown to them to obtain accurate information (Sharma *et al.* 2012).

The voucher specimens were prepared for all collected plants and deposited to get identified at B.S.I. Shillong, Meghalaya, India prior to initiation of the said work.

4.4 Extract preparation of Sample:

The collected samples were washed properly with water and were oven dried at 40-45 ºC for 7-14 days. After that, they were crushed up and ground to get homogeneous fine powder by a grinder and stored at air tight container for further use (Oyedemi & Afolayan 2011). 50 g of dried root powder is extracted twice in 200 mL of ethanol (70%) and acetone for 48 hrs and filtered with whatmann's filter paper number 42. Filtrate was concentrated by evaporating in rota-evaporator until fully dried (extract) and store at 4 °C for further studies.

4.5 Phytochemical screening

The qualitative phytochemical screening of roots of *Morus indica*, *Averrhoa carambola* and *Phlogacanthus thyrsiflorus* by 70% ethanolic and acetonic solvents were accomplished. The following methods were adopted for the phytochemical screening:

A. Detection of phenol (Hussein *et al.* 2012):

In a test tube, 5 mL of each previous filtered extracts were taken and 1 mL of $FeCl_3$ (1%) and 1 mL $K_3[Fe(CN)_6]$ (1%) were added. The appearance of fresh radish blue color indicated the presence of polyphenols.

B. Detection of flavonoids (Hussein *et al.* 2012):

Two solutions A and B from both parts of the plant extracts were prepared. The solution A contains 5 mL of ethanolic extract previously prepared. The solution B consists of 5 mL of ethanolic solvent added to 5 mL of KOH (50%). Then two solutions A and B were mixed together. The presence of flavonoids is indicated by the appearance of yellow color.

C. Detection of tannins (Ganesan *et al.* 2006):

About 2 mL of the extract and a few drops of 1% lead acetate were added. A yellow precipitate formation indicates the presence of tannins.

D. Detection of resins (Hussein *et al.* 2012):

10 mL of each previous filtered extracts were taken and 20 mL of HCl 4% were added. The appearance of turbidity indicates the presence of resins in the extracts.

E. Detection of terpenoids (Ayoola *et al.* 2008):

To 0.5 g each of the extract was added 2 mL of Chloroform. And again 3 mL of concentrated H_2SO_4 was carefully added to form a layer. The presence of reddish brown on the interface indicated the presence of terpenoids.

F. Detection of alkaloids (Hussein *et al.* 2012):

0.2 g of the powder extract of root of the plant was dissolved in 10 mL of 1% HCl. Then, they were transferred to a water bath for few minutes. After, 1 mL of the filtrated extract was treated with 2-4 drops of Dragendorff's reagent. The presence of alkaloids is indicated by the appearance of an orange reddish precipitation.

G. Detection of glycosides (Ganesan *et al.* 2006):

The extract was hydrolysed with HCl for few hours on a water bath. To the hydrolysate, 1 mL of pyridine was added and a few drops of Na-Nitroprusside solution was added and then it was made alkaline with NaOH solution. Appearance of pink to red colour shows the presence of glycosides.

H. Detection of cardiac glycosides (Ganesan *et al.* 2006):

To 0.5 g of extract diluted to 5 mL in water was added 2 mL of Glacial acetic acid containing one drop of ferric chloride solution. This was under layered with 1 mL of conc. H_2SO_4. A brown ring at the interface indicated the presence of a deoxy-sugar characteristic of cardenolides. A violet ring may appear below the brown ring, while in the acetic acid layer a greenish ring may form just above the brown ring.

I. Detection of reducing sugar (Kabesh *et al.* 2015):

To 1 mL of extract, added 1 mL of Fehling's – A solution and 1 mL of Fehling's-B solution. Formation of red colour indicates the presence of sugar.

J. Detection of steroids (Kabesh *et al.* 2015):

To 1 mL of extract, mix with 1 mL of chloroform and concentrated H_2SO_4 sidewise. A red color presence at the lower chloroform layer indicates presence of steroids.

K. Detection of anthraquinones (Ganesan *et al.* 2006):

About 5 mL of extract solution was hydrolysed with dilute H_2SO_4 extracted with benzene. 1 mL of dilute ammonia was added to it. Rose pink coloration suggested the positive response for anthraquinones.

L. Detection of saponins (Sathi & Sengottuvel 2016):

About 0.5 mg of the extract was shaken with 5 mL of distilled water. Formation of frothing (appearance of creamy miss of small bubbles) shows the presence of saponins.

4.6 *In-vitro* antioxidant tests

4.6.1 Total phenolic content

The folin–ciocalteau method (Maheswari *et al.* 2011) was used for the determination of total phenolic content. Briefly, in a test tube 100 µL of the extract was taken, to which 1600 µL of doubled distilled water was added and then 100 µL of folin-ciocalteau reagent (0.25 N) were added and mixed properly. The above sample mixture was allowed to react for 3 minutes. After that, 150 µL of Na_2CO_3 (1 N) solution was added. The mixture was incubated at room temperature in the dark for 2 hrs. The absorbance was taken at 725 nm using a PC based double beam spectrophotometer (Systronics) by taking Gallic acid as standard and were expressed in milligram of gallic acid equivalent (GAE)/g of dried extract.

4.6.2 Total flavonoid content

Total flavonoid content was determined by aluminum chloride method (Hsish *et al.* 2016). From all the extracts, 0.1 mg/mL of extracts were prepared in double distilled water and were reacted with 1.5 mL ethanol (95%), 0.1 mL aluminum chloride hexahydrate (10%), 0.1 mL potassium acetate (1 M) and 2.8 mL of doubled distilled water for 40 minutes at room temperature. Finally, the absorbance of the above mixture was measured at 415 nm taking quercetin as standard.

4.6.3 Total reducing power assay

The reducing power assay was determined by the method adopted by Hsieh *et al.* 2016. An aliquot of 1 mL of sample was reacted with 0.5 mL of phosphate buffer (0.2 M) and 0.5 mL of potassium ferricyanide (1%). The above reaction mixture was then incubated at 50 ºC for 20 minutes. After the cooling, 0.5 mL of trichloroacetic acid (10%) was added. Now, 2 mL of distilled water was mixed with 2 mL of the above reaction liquid and 0.2 mL of iron (III) chloride (0.1%). Finally, the absorbance was measured at 700 nm. BHA was used as a positive control.

4.6.4 Total antioxidant capacity

Phosphomolybdate method was adopted for the determination of total antioxidant capacity (Shah *et al.* 2013). In brief, 0.3 mL of each extracts was added to 3 mL of phosphomolybdate reagent (0.6 M H_2SO_4, 0.028 M sodium phosphate, 0.004 M ammonium molybdate). This reaction mixture was incubated at 95 °C in water bath for 90 minutes. After cooling to room temperature, the absorbance was taken at 765 nm. Ascorbic acid served as standard. Results were expressed in mg of ascorbic acid equivalent (AAE)/g of dried extract.

4.6.5 DPPH radical scavenging assay

The antioxidant ability of root extracts were determined by the method of Shukla *et al*. 2016, by their capacities to neutralize radicals of DPPH (di (phenyl)- (2,4,6-trinitrophenyl) iminoazanium). The antioxidants in the sample scavenge the free radical and turn it into yellow in colour. A working solution of DPPH (0.004%) was prepared freshly in methanol. 1 mL of sample and standard dilution of various concentrations (10, 20, 40, 80 and 160 µg/mL) was added to 3 mL of DPPH working solution. After 30 minutes of incubation in dark at room temperature 25 °C ±2, change in colour from violet to yellow was recorded at 517 nm with UV-VIS Spectrophotometer (Systronic). Ascorbic acid was used as a positive control. 1 mL of methanol with 3 mL of working DPPH solution serves as control. The ability to scavenge DPPH radical activity was calculated by-

$$\% \text{ inhibition} = Ac - As / Ac \times 100$$

Where, Ac is the absorbance of the control and As is the absorbance of samples or standard. Lower values represent higher antioxidant ability.

4.6.6 ABTS radical cation scavenging activity

ABTS (2, 2 azobis, 3-ethyl benzothiozoline-6- sulphonic acid) radical cation scavenging activity was determined by Shah *et al*. 2013 methodology. Briefly, ABTS (7 mM) solution was allowed to react with potassium persulfate (2.45 mM) overnight in dark for generation of dark colored ABTS radicals. For the analysis, the ABTS solution was diluted with 50% ethanol to obtain initial absorbance of 0.7 ± 0.05 at 745 nm. For the determination 100 µL sample of different dilution was added to 1 mL of ABTS solution. The decrease in absorbance was measured at 745 nm after 1 minute and 6 minutes of mixing. The difference was calculated and compared with control. The BHT was taken as positive control. ABTS radical % inhibition was calculated by the formula:

$$\% \text{ inhibition} = Ac - As / Ac \times 100$$

4.6.7 Iron chelating capacity

For the evaluation of ferrous ion chelating potential of the extracts were done by the method adopted by Sasikumar and Kalaisezhiyen 2014. In a reaction mixture, 1 mL of various concentration of extracts (200-1000 µg/mL) and 2 mM $FeCl_2$ (0.05 mL) was taken. Control contains all the reagents except for the sample. The reaction was initiated after the addition of 5 mM Ferrozine (0.2 mL). Shaken vigorously and left in the room temperature for

10 minutes. The absorbance of both reaction mixture and control was taken at 562 nm. Lower the absorbance higher will be ferrous ion chelating potential. EDTA was taken as standard.

% inhibition = Ac – As/ Ac × 100

4.6.8 H_2O_2 radical scavenging assay

H_2O_2 radical scavenging activity of the extracts was measured by the method adopted by Narzary *et al.* 2016. 20 mM H_2O_2 solution was prepared by mixing 226 µL from 30% H_2O_2 in 99.8 mL of 0.1 mM phosphate buffer saline having pH 7.4. In a different sample/standard concentration (2, 4, 6, 8 and 10 µL/mL), 2 mL of 20 mM H_2O_2 solution was added and incubated in dark for 10 minutes. The absorbance of the scavenging activity was taken at 230 nm using UV-VIS double beam spectrophotometer (Systronics). Phosphate buffer saline was used as blank and BHA as positive control. The amount of H_2O_2 inhibited by the extract was calculated from the equation:

% inhibition = Ac – As/ Ac × 100

4.6.9 Ferric reducing antioxidant assay

Ferric reducing antioxidant power of was determination by the method of Song *et al.* 2010. The FRAP reagent is freshly prepared by mixing 300 mmol/L sodium acetate buffer (pH 3.6), 10 mmol/L TPTZ solution in 40 mmol/L HCl and 20 mmol/L iron (III) chloride solution in a ratio of 10:1:1 to generate FRAP reaction solution, which should be warmed to 37 °C in a water bath before use. After that, 100 µL of the diluted sample was mixed with 3 mL of the FRAP reaction solution. It was then incubated in dark for 4 minutes at room temperature and the absorbance of the reaction mixture and standard was recorded at 593 nm. The standard curve was constructed using $FeSO_4.7H_2O$ solution, and the results were expressed as µmol Fe (II)/g dry extract. All experiments were performed in triplicate.

4.7 Gas Chromatography coupled with Mass Spectrometry analysis

The analysis of hydro-alcoholic and acetonic extracts RoMi were performed at Sophisticated Instrumentation Facility, VIT, Vellore, using Perkin Elmer gas chromatography (Clarus 680) coupled with mass spectrometry (Clarus 600 EI) employed with fused silica column and packed with capillary column Elite- 5MS (5% biphenyl 95% dimethylpolysiloxane, 30 m × 0.25 mm ID × 250µm df). The components were separated using Helium as carrier gas at a constant flow of 1 mL/minute. The injector temperature was set at 260 °C during the chromatographic run. 1 µL of extract sample was injected into the instrument. The oven temperature was programmed at: 60 °C (2 minutes); then increased to

300 °C for 6 minutes (at the rate of 10 °C per minute). The mass detector conditions were: transfer line temperature 240 °C; ion source temperature 240 °C; and ionization mode electron impact at 70 eV, a scan time 0.2 sec and scan interval of 0.1 sec. 40 to 600 Da fragment size were scanned. The spectrums of the components were compared with database of spectrum of known components stored in GC-MS NIST (2008) library.

4.8 *In-vivo* animal model experiment

4.8.1 Experimental animals

Thirty male wistar albino rats weighing 210-250 g were used for the experiment. The animals were housed in standard cages (46 × 24 × 20 cm) (Tarson, India), with six animals per cage in a standard environmental condition (25 ± 3 ºC, 60 ± 1% relative humidity, and light control room with a 12-h dark-light cycle). They were allowed to acclimatize for a period of 1-2 weeks and were fed with standard pellet feed (Lipton India Ltd., Bangalore) and water *ad libitum* (Vuda *et al.* 2012; Maheshwari *et al.* 2011). All the experiments were performed in strict accordance with standard guidelines accepted internationally, and the ethical approval was obtained from Maharani Lakshmi Ammanni College Ethical Committee (1368/ac/10/CPCSEA), Bangalore, for the care and use of laboratory animals.

4.8.2 Acute toxicity study

OECD guidelines (test 423: Acute oral toxicity-Acute toxic class method; 2002) were followed to study the acute toxicity of the RoMi ethanolic extract (OECD Library, 2002). All the animals (12 male Wistar albino rats) were kept on fasting overnight prior to the experiment. The RoMi-EE was administered orally in an increasing dose of 250, 500, 1000 and 2000 mg/kg body weight (BW). The followed concentrations are determined as per OECD guideline. After feeding of various doses of formulation, all the rats were carefully observed for the development of clinical or toxicological symptoms at 30 minutes and then at 2, 4, 8, 24 and 48 hours. Finally, the rats were observed for the development of clinical or toxicological symptoms till 14 days.

4.8.3 Experimental design

To evaluate the hepatoprotective effect of the RoMi-EE, the rats were divided into five groups containing six rats each. 3 mL/kg of CCl_4 was administered orally once on the first day. RoMi-EE was dissolved in milipore water and two different concentrations 100 mg/kg & 200 mg/kg BW (Khan *et al.* 2012) of RoMi-EE were administered orally everyday

in morning with the help of 16g gavage needle while the control group was maintained on distilled water. Body weights were monitored throughout the experiment.

Group 1 was administered distilled water + dietary supplement served as positive control.

Group 2 was administered CCl_4 (on the first day) + dietary supplement served as negative control.

Group 3 was administered CCl_4 (on the first day) + 100 mg/kg (BW) of RoMi-EE orally + dietary supplement for 7 days.

Group 4 was administered CCl_4 (on the first day) + 200 mg/kg (BW) of RoMi-EE orally + dietary supplement for 7 days.

Group 5 was administered CCl_4 (on the first day) + 25 mg/kg (BW) of silymarin orally + dietary supplement for 7 days.

Twenty-four hours after the last treatment, all rats were weighed and then euthanized through intraperitoneal injection using Xylazine (30 mg/kg BW) and Ketamine (300 mg/kg BW) ratio (1:10) as proposed by Committee for the Purpose of Control and Supervision of Experiments in Animal (CPCSEA). The blood was collected by retro-orbital puncture in an EDTA-containing tube from all the experimental rats. The liver and kidneys of each rat were removed, weighed and perfused in the ice-cold phosphate buffer of pH 7.0. A portion of liver was preserved in 10% formaldehyde solution for hisptopathological evaluation and from the remaining portion, the homogenate was prepared by centrifuging at 1000 × g for 10 minutes at 4 ºC. The supernatant was collected after centrifugation and was used for *in-vivo* enzymatic assays (Khan *et al.* 2012).

4.8.4 Assessment of serum biochemical assays

The activities of serum aspartate transaminase (AST), alanine transaminase (ALT), alkaline phosphatase (ALP), gamma-glutamyl transpeptidase, total bilirubin, tri-glycerides, HDL-cholesterol, LDL-cholesterol and total cholesterol were estimated using standard AMP Diagnostic Kits (Graz, Austria). Liver damage was assessed by estimating the enzymatic activities of serum AST, ALT, and ALP, as well as serum TC, TG, and albumin level, using the corresponding commercial kits, respectively. Silymarin was procured from indigenous pharmacy store, Bangalore, India. The kidney damage was assessed by creatinine level. The results were expressed according to the manufacturer recommendation.

4.8.5 Assessment of levels of antioxidant enzymes

The liver tissues were homogenized (1%) in 10 mL volumes of 100 mM KH_2PO_4 buffer containing 1 mM ethylenediamine tetra-acetic acid (EDTA; pH 7.4) and centrifuged at

12,000 × g for 30 minutes at 4 ºC (Khan *et al*. 2012). The supernatant was collected and used for the assessment of antioxidant enzymes. Protein concentrations in the supernatants of liver tissue homogenates were determined using crystalline bovine serum albumin (BSA) as standard. All chemicals used in enzymatic analysis were purchased from Sigma-Aldrich.

4.8.5.1 Superoxide dismutase (SOD) assay

SOD assay was measured by the method of Misra and Fredovich 1972. A volume of 880 µL of 0.05 M carbonate buffer (pH 10.2) containing 0.1 mmol EDTA and 20 µL of 30 mmol epinephrine in 0.05% acetic acid were added to the tissue extract of 100 µL and changes in activity were measured at 480 nm for 4 minutes. Activity was expressed as the amount of enzyme that inhibits the oxidation of epinephrine by 50%, which is equivalent to one unit and is expressed in terms of units/mg protein.

4.8.5.2 Catalase assay

Catalase activity was determined using the method of Bears and Sizer 1952. The reaction solution of catalase activity contained 1 mL of 59 mmol/L H_2O_2 (dissolved in 50 mmol phosphate buffer, pH 7.0) and 0.1 mL of hepatic supernatant were added to 1.9 mL deionized water. Changes in the absorbance of the reaction solution at 240 nm were determined every 1 minute up to 3 minutes (using Kinetics spectrometer). One unit of catalase activity was defined as an absorbance change of 0.01 as units/minute.

4.8.5.3 Gluthathione peroxidase

GPx activity was measured at 37 °C by the method of Middha *et al.* 2011. The reaction mixture consisted of 500 µL of phosphate buffer, 100 µL of 0.01 M reduced glutathione (GSH), 100 µL of 1.5 mM NADPH and 100 µL of GR (0.24 U). 100 µL of tissue extract was added to the reaction mixture and incubated 37 ºC for 10 minutes. 50 µL of 12 mM t-butyl hydro-peroxide was added to 450 µL of tissue reaction mixture and measured at 340 nm for 180 seconds in a biospectrometer (Eppendorf, Model BL 192). A molar absorptivity of 6.22 × 103 M/cm was used to determine enzyme activity. One unit of activity is equal to µM NADPH oxidized/minute/mg protein.

4.8.5.4 Gluthathione assay

GSH was estimated using the method of Jollow *et al.* 1974. A total of 1.0 mL of homogenate was precipitated with 1.0 mL of 4% sulfosalicylic acid. Samples were kept at 4 °C for 1 hour and then centrifuged at 1200 × g for 20 minutes at 4° C. The total volume of 3.0 mL assay mixture contained 0.1 mL of a filtered aliquot, 2.7 mL of phosphate buffer (0.1

mol; pH 7.4) and 0.2 mL of DTNB (100 mmol). The yellow color that developed was read immediately at 412 nm on a SmartSpec™ Plus Spectrophotometer (Bio-Rad, Hercules, CA, USA). It was expressed as µmol GSH/g tissue.

4.8.5.5 Lipid-peroxidation

Malondialdehyde (MDA), a marker of LPx was assessed by the method of Okhawa *et al.* 1979, using 1, 1, 3, 3- tetramethoxy propane as standard. Briefly, 8.1% SDS was added to the tissue homogenate and incubated for 10 minutes at room temperature, followed by boiling with 20% acetic acid and 0.6% thiobarbituric acid (TBA) for 1 hour in a water bath. After cooling, butanol: pyridine solution (15: 1 v/v) was added and the mixture was centrifuged at 600 × g for 5 minutes. Absorbance of the upper colored layer was measured at 532 nm and the concentration of MDA was expressed in terms of nM /mg protein.

4.8.5.6 Total protein assay

The total protein assay was done by the method of Lowry *et al.* 1975, taking BSA as standard. In brief, to the supernatant of 100 µL, 500 µL of alkaline copper sulphate was added and allowed to incubate for 10 minutes at room temperature. After incubation, 60 µL of diluted Folin Catecholamine reagent (1:1) was added and incubated for another 30 minutes at room temperature. The absorbance was measured at 660 nm against the reagent blank.

4.9 Histopathological study

A small portion of the liver and kidney was excised and washed with normal saline and processed separately for histopathological observation. Initially, the liver tissues were fixed in 10% buffered neutral formalin for at least 48 h, dehydrated in gradual ethanol (50-100%), cleared in xylene, and embedded in paraffin. The 4 µm sections were prepared using microtome. Then liver sections were dewaxed in xylene, rehydrated in a series of different grades of alcohol and then washed with distilled water for 5 minutes. Sections were prepared and stained with hematoxylin (40 s) & eosin (20 s) dye (Maheshwari *et al.* 2011). The sections were examined in Olympus microscope at 40× magnification (Khan *et al.* 2012) for any histopathological changes, including cell necrosis, fatty changes and vacuolation.

4.10 Molecular docking with hepatoprotective biotarget.

4.10.1 Ligand selection

The molecules that were detected in GC-MS analysis from the root of *Morus indica* are selected for the docking study with the NFκB (Nuclear factor kappa B) and COX-2

(Cyclooxyginase-2) proteins which are responsible for the hepatotoxicity and inflammation of the liver. For the docking analysis, molecules having 160-500 Da were selected for the ligand docking.

4.10.2 Selection of target proteins

The proteins were selected from the literature data available on the mechanism of hepatoprotective and anti-inflammatory effect (Glushenko *et al.* 2015; Lamie *et al.* 2015). The proteins (1NFK & 3LN1) having resolution of 2.3 & 2.4 Å was downloaded from https://www.rcsb.org. Both the proteins 3LN1 and 1NFK are from the organism *Mus musculus*. In the 3LN1 protein, Celecoxib is bound to the COX-2 active site and 1NFK is the nuclear factor kappa-B P50 homodimer. The X-ray crystal structure of protein 1NFK is complexes with DNA molecule.

4.10.3 Ligand molecules

The compounds that were identified by the GC-MS analysis of ethanolic root extracts of *Morus indica* were used as ligand molecules for the docking analysis and were downloaded from https://pubchem.ncbi.nlm.nih.gov in SDF format.

4.10.4 Ligand preparation

The ligands were prepared using "LigPrep" software for ligand preparation from Maestro Schrödinger Suite (Maestro, 2015). 3D conformers were generated using tautomers and ionising state at pH 7.0 followed by the optimization process using the force field OPLS2003e. The Relative Mean Square Deviation 0.30 Å (RMSD) was used to select the best conformation. The charges of the molecule were obtained using the Macro Model software module default setting from Schrödinger package. The stereochemistry was performed with maximum of 32 stereoisomers (default) per ligand.

4.10.5 Preparation of receptor

The crystallized proteins (1NFK & 3LN1) were processed in the Maestro (Schrödinger) using the protein preparation wizard facility. The following preparations were completed stepwise (Maestro, 2015):

1. Protein structures were preprocessed using the OPLS3e force field.
2. The protein missing side chains were added using prime.
3. Geometry refinement and optimization of the protein were completed. In the process the hydrogen atoms were added to heavy atoms of the protein.

4. The minimization of the protein were carried out using optimized potentials for liquid simulations (OPLS) 2005 imposing an RMDS (root mean square deviation) of 0.3 Å for the heavy atom coverage.
5. If the standard ligand in the active site is not present, then active binding site of the protein were generated using Site Map module with reports up to 5 different binding site based on their site score. The best site score were selected for the study.
6. Finally the protein molecules of best site score were grid generated (with grid box of 40 × 40 × 40) with a space of 0.375 Å and grid centre of x, y, z coordinates 9.740, 64. 640 and 15.986 respectively using receptor grid generation from Glide, Maestro.

4.11 Molecular docking

The docking study was carried out using Glide, Ligand Docking of Maestro Schrödinger software suite 2018. The prepared LigPrep molecules and the grid generated proteins were incorporated into the workspace. The 3D images of the ligands (pre-processed), proteins (minimized) and all the ligand-protein binding interactions were saved in JPEG format. The docking study was conducted at Intel (R) Core (TM) i5, CPU (3.2 GHz) having Windows 10 operating system.

4.12 ΔG Binding affinity

Binding affinity of molecules with that of proteins was performed using Molecular Mechanics/Generalized Born Surface Area (MM-GBSA) module of Maestro Schrödinger.

4.13 Adsorption distribution metabolism & excretion (ADME) property of ligands

Physically significant descriptors and pharmaceutically relevant properties of all the lead compounds of both the fractions were analysed using 'Molsoft' prediction tool (Gunalan *et al.* 2014; Tamilvanan & Hopper 2013). Molecular weight, log P Octanol/water partition coefficient, H-bond donors, H-bond acceptors, Mol Log S and their positions according to Lipinski's rule of five (Lipinski *et al.* 1997; Lipinski 2000) were obtained using the tool. It also evaluates acceptability of analogues according to Lipinski's rule of five (Lipinski *et al.* 1997), which are essential for rational drug design (Tamilvanan & Hopper 2013).

4.14 Statistical analysis

Results of all the experiments were presented as mean ± SD of triplicate experiment (n= 3) and ± SD (n= 6) for animal model experiments. Relative significant differences among the means were determined by one-way ANOVA test ($p \leq 0.05$) and Tukey's multiple comparison tests using Origin pro 8.5 software.