3

MATERIALS AND METHODS

The methodology adopted in the present investigation was presented under four phases:-

PHASE I

- Ovicidal activity of plant extracts

PHASE II

- Repellent activity of plant extracts

PHASE III

- Phytochemical screening and GC-MS analysis

PHASE IV

- *In silico* analysis of phytochemical compounds

PHASE I

3.1 Studies on the potency of selected botanicals

Laboratory culture of eggs

Hay infusion method was adopted for culturing mosquito eggs. Hay was taken, cut into small pieces and boiled in 5 litre of water for 20 minutes. After cooling, this water was poured into buckets and kept in different areas where mosquitoes were abundant. After one or two days eggs were laid by female mosquitoes in clusters forming an egg raft. The egg rafts were collected, maintained in the laboratory and was allowed to hatch for the ovicidal bioassay studies.

Collections of test materials

Leaves and flowers of the selected five plants namely *Calotropis gigantea* (L.) R. Br., *Thevetia peruviana* (Pers,) Merr, *Tagetes erecta* L., *Lantana camara* L.

var. aculeate (L.) Moldenke and *Bauhinia acuminata* L. were collected (BSI/SRC/5/23/2013-14/Tech.883) (Plate I & II) from the natural habitat of Coimbatore locale of 11^{0}1'N 76^{0}56'S Longitude (Table 1).

Table 1

Details of plant species used for the study and their collection area

Plant species	Family	Plant type / Shrub/ Herb / Tree	Vernacular name (Tamil)	Collection area
Calotropis gigantea	Asclepiadaceae	Shrub	Erukku	Kavundanpalayam
Thevetia peruviana	Apocyanaceae	Shrub	Ponnarali, Thiruvachipoo	Maruthamalai
Tagetes erecta	Asteraceae	Shrub	Sendu malli, Sevanthi	Saibaba Colony
Lantana camara	Verbenaceae	Shrub	Unnichedi	Sennanur
Bauhinia acuminata	Caesalpinaceae	Tree	Atti, Tataki	Saibaba Colony

Preparation of leaf powder

Fresh leaves were collected, washed in water and air dried under shade for 2 to 3 weeks. Dried leaves were powdered using an electric pulverizer. Fine powder was obtained by sieving.

Preparation of flower powder

Fresh flowers were collected washed in water and shade dried at room temperature. After drying for 2 to 3 weeks, the flowers were ground in an electric pulverizer to get the fine powder.

PLATE I

TEST LEAVES

C. gigantea

T. peruviana

T. erecta

L. camara

B. acuminata

PLATE – II

TEST FLOWERS

C. gigantea

T. peruviana

T. erecta

L. camara

B. acuminata

Preparation of leaf and flower extracts

10 g of each of the leaf powder or the flower powder was weighed using an electronic balance (Denver XS-210) and made into packets using Zerohaze filter paper (A Grade, SD's). These powders were subjected to extraction with 500 ml of the solvents for 8h using a Soxhlet apparatus (Plate IIIa) (Harbourne, 1973 and Vogel, 1978). Petroleum ether (60 – 80^{0}C) extraction was followed by chloroform and ethanol extraction, so that the powders were subjected to extraction with solvents in the order of increasing polarity.

The leaf and flower extracts thus obtained were concentrated by distillation and dried by evaporation in a water bath at 40^{0}C. The residue thus obtained was stored in tightly closed glass vials in the refrigerator for further bioassays.

OVICIDAL ACTIVITY OF PLANT EXTRACTS

Ovicidal bioassay studies

A pilot study was carried out to assess the effectiveness of leaf and flower extracts of selected five plants which may cause the mortality of the eggs of the mosquito, *C. quinquefasciatus*. Effective doses were determined first and then detailed investigation was carried out. For both leaf and flower extracts of *C. gigantea, T. peruviana, T. erecta, L. camara* and *B. acuminata* concentrations ranging from 20 - 100 ppm exhibited 100% egg hatchability at 48h itself. Therefore, the higher concentrations ranging from 100 - 300 ppm were used for the bioassay studies (Plate IIIb) using the extract of five plants.

Experimental design

The experiment was designed according to the Completely Randomized Design (CRD). The experimental setup consisted of five treatments each with three replications for leaf and flower extracts. Ovicidal activity was assessed by the slightly modified method of Su and Mulla, 1998. The egg rafts of *C. quinquefasciatus* were collected from the laboratory of Zoology Department, Avinashilingam Institute for Home Science and Higher Education for Women, Coimbatore. The different leaf and flower extracts were diluted in the appropriate solvent to achieve various concentrations ranging from 100 – 300 ppm.

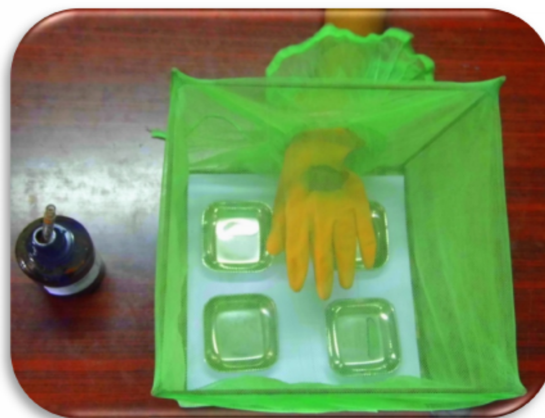
Plate - III

a) Soxlet apparatus

(i) Leaf extracts

(ii) Flower extracts

b) Experimental setup



c) Experimental setup for repellent activity

Twenty eggs were introduced into the beakers for the bioassay studies. Controls with three replications were also maintained simultaneously. 250 ml beakers each containing 200 ml of water were used for the experiment.

The following parameters were observed to assess the effective doses:

- Egg hatchability at 48 h, 72 h and 96 h of treatment
- Total egg hatchability
- Total egg mortality

Test for ovicidal activity

Each experiment was replicated three times along with appropriate control. The hatch rates were assessed 48 h after treatment by the following formula.

$$\text{\% of egg mortality} = \frac{\text{Mortality in the treatment} - \text{mortality in the control}}{100 - \text{mortality in the control}} \times 100$$

Statistical analysis

The data on bioassay studies were also subjected to One Way Analysis of Variance (ANOVA) as described by Panse and Sukahtme (1985). The egg mortality data were subjected to probit analysis for finding out LC_{50}, LC_{70} and LC_{90} values, regression equation, Chi-square values and 95 percent Upper Confidence Limit (UCL) and Lower Confidence Limit (LCL) (Finney, 1971).

PHASE II

3.2 REPELLENT ACTIVITY OF PLANT EXTRACTS

Repellent activity of the leaves and flowers of *Calotropis gigantea, Thevetia peruviana, Tagetes erecta, Lantana camara* and *Bauhinia acuminata* were tested.

Laboratory culture of adult mosquitoes

Larvae obtained from laboratory colony were fed with dog biscuits and yeast powder in the ratio 3:1. Adult mosquitoes were reared in wooden cages (30 x 30 x 30 cm) and daily provided with sponge pieces soaked with 10% sucrose solution

for a period of 3-4 days after emergence. Mosquitoes were held at $(28\pm2)^{0}C$, 70%−85% relative humidity (RH), with a photo period of 14h light, 10h dark. Three day old blood starved *C. quinquefasciatus* mosquitoes were used for repellent bioassay studies.

Bioassay studies

A pilot study was carried out to assess the repellent efficacy of leaf and flower extracts of five selected plants against the three day old blood starved *C. quinquefasciatus* mosquito. Effective doses were determined first and then detailed investigation was carried out. Concentrations of 1.0, 2.5 and 5.0 mg/cm^2 were used for determining the repellent efficacy of both the leaf and flower extracts of *C. gigantea, T. peruviana, T. erecta, L. camara* and *B. acuminata*.

Experimental design

The experimental setup consisted of three treatments each with three replications for leaf and flower extracts. Simultaneously control was also maintained. The repellent study was followed by the method of WHO (1996). Hundred three day old blood starved female, *C. quinquefasciatus* were kept in a net cage (45 cm × 30 cm × 45 cm). The arms had no contact with lotions, perfumes or perfumed soaps on the day of the assay. Dorsal side of the right arm was treated with extracts, left arm was kept as control and the remaining area was covered by rubber gloves. The crude extract was applied at 1.0, 2.5 and 5.0 mg/cm^2, separately in the exposed area of the forearm. The control and treated arms were introduced simultaneously into the mosquito cage, and gently tapping the sides of the experimental cages, the mosquitoes were activated (Plate IIIc). The test was conducted at each concentration by inserting the treated and control arms into the same cage for one full minute for every five minutes. The mosquitoes that landed on the hand were recorded and then shaken off before imbibing any blood and making out a 5 min protection from mosquito bite

Test for repellent activity

The percentage of repellency was calculated by the following formula.

$$\% \text{ Repellency} = [(Ta - Tb) / Ta] \times 100$$

Where Ta is the number of mosquitoes in the control group and Tb is the number of mosquitoes in the treated group.

Statistical analysis

The data on bioassay studies were also subjected to statistical analysis. Standard deviation was calculated for the data which was obtained from the test for repellency against *C. quinquefasciatus* mosquito. Each value ($\chi \pm SD$) represents average of three replications.

PHASE III

3.3 PHYTOCHEMICAL SCREENING

Qualitative analysis

Preliminary phytochemical screening of leaf and flower extract of five selected plants was carried out using the standard procedures of Raman (2006).

Test for Alkaloids

- **Mayer's test**

A fraction of extract was treated with Mayer's test reagent (1.36 g of mercuric chloride and 5 g of potassium iodide in 100 ml of water) and observed for the formation of cream coloured precipitate.

- **Wagner's test**

A fraction of extract was treated with Wagner's reagent (1.27 g of iodine and 2 g of potassium iodide in 100 ml water) and observed for the formation of reddish brown colour precipitate.

- **Hager's test**

A few ml of extract was treated with Hager's reagent (saturated aqueous solution of picric acid) and observed for the formation of prominent yellow precipitate.

Test for Tannins

- **Acetic Acid Test**

The extract was treated with acetic acid solutions and observed for the formation of red colour solution.

- **Dilute HNO_3 Test**

The extract was treated with dil. HNO_3. The extract turns from reddish to yellow colour which indicates the presence of tannins.

Test for Phenols

- **Ferric chloride test**

The fraction of extract was treated with 5% ferric chloride and observed for the formation of deep blue or black colour

- **Liebermann's test**

The extract was heated with sodium nitrite, added H_2SO_4 solution diluted with water and excess of dilute NaOH was added and observed for the formation of deep red or green or blue colour.

Test for Flavonoids

- **NaOH test**

A small amount of extract was treated with aqueous NaOH and HCl, observed for the formation of yellow orange colour.

- **H_2SO_4 test**

A fraction of the extract was treated with concentrated H_2SO_4 and observed for the formation of orange colour.

Test for Sterols

- **Liebermann-Burchard test**

Extract (1ml) was treated with chloroform, acetic anhydride and drops of H_2SO_4 was added and observed for the formation of dark pink or red colour.

Test for Terpenoids

- **Liebermann-Burchard test**

Extract (1ml) was treated with chloroform, acetic anhydride and drops of H_2SO_4 was added and observed for the formation of dark green colour.

Test for Saponins

- **Foam Test**

The extract or dry powder was vigourosly shaken with water and observed for the formation of persistent foam.

Test for Anthraquinones

- **Borntrager's test**

About 50 mg of powdered extract was heated with 10% ferric chloride solution and 1ml concentrated HCl. The extract was cooled, filtered and the filtrate was shaken with diethyl ether. The ether extract was further extracted with strong ammonia and observed for the formation of pink or deep red colouration of aqueous layer.

Test for Proteins

- **Ninhydrin test (Aqueous)**

The extract was treated with aqueous ninhydrin and observed for the presence of blue colour, indicating the presence of amino acid or purple colour indicating the presence of protein.

- **Ninhydrin (Acetone)**

Ninhydrin was dissolved in acetone and the extract was treated with ninhydrin and observed for the formation of purple colour.

- **Biuret test**

The extract was heated in distilled water and filtered. The filtrate was treated with 2% copper sulphate solution, 95% ethanol and potassium hydroxide and observed for the formation of pink ethanolic layer.

- **Test for Quinones**

A small amount of extract was treated with concentrated HCl and observed for the formation of yellow colour precipitate.

GC-MS ANALYSIS

Mass experiments were performed on GC (T8000 Top CE) combined with Mass Spectrometer (Md 800 FIS ONS). Sample was dissolved in methanol and

introduced into the column TR-5-MS capillary standard non-polar by splitles injection system. Ultra high purity helium was introduced as the buffered collision gas with flow rate of 1.0 ml/min. The source temperature for ionization was set at 250^0C. All the experiments were performed on the positive ion mode.

PHASE IV

3.4 *IN SILICO* STUDIES

BIOLOGICAL ACTIVITY PREDICTION

Biological activity has been predicted using PASS server. Computer program PASS is the product of ideas originated more than 25 years ago within the framework of the National Registration System of New Chemical Compounds organized in the USSR in 1972 (Burov *et al*., 1990). The PASS software product, which predicts more than 300 pharmacological effects and biochemical mechanisms on the basis of the structural formula of a substance, may be efficiently used to find new targets (mechanisms) for some ligands and conversely to reveal new ligands for some biological targets (Lagunin *et al*., 2000). The biological activity spectrum presents actions of all compounds, despite the difference in essential conditions of its experimental determination. "The biological activity spectrum" is defined as the "intrinsic" property of a compound depending only on its structure and physico-chemical characteristics.

Pa and Pi are the estimates of probability to be active and inactive respectively. If $Pa > 0.7$ the substance is very likely to exhibit the activity in experiment, but the chance of the substance being the analogue of a known pharmaceutical agent is also high. If $0.5 < Pa<0.7$ the substance is likely to exhibit the activity in experiment, but the probability is less and the substance is unlike known pharmaceutical agent.

MOLECULAR DOCKING STUDIES

Target protein retrieval and preparation

The PDB is a key resource in areas of structural biology, such as structural genomics. Most major scientific journals and some funding agencies, such as the NIH in the USA, now require scientists to submit their structure data to the PDB. If

the contents of the PDB are thought of as primary data, then there are hundreds of derived databases that categorize the data differently. SCOP and CATH categorize structures according to type of structure and assumed evolutionary relations; GO categorize structures based on genes.

Three dimensional structure of *C. quinquefasciatus* odorant binding protein (PDB id: 2L2C) was obtained from PDB databank (Fig 1a). Search was made against resolution of the structure. Experimental design and methods were followed from the wetlab. Protein was prepared with parameter of assigning bond orders, adding hydrogen, creating zero order bonds to metals, creating disulfide bonds and deleting 5A^{o} distance water from the protein (Fig 1b & c).

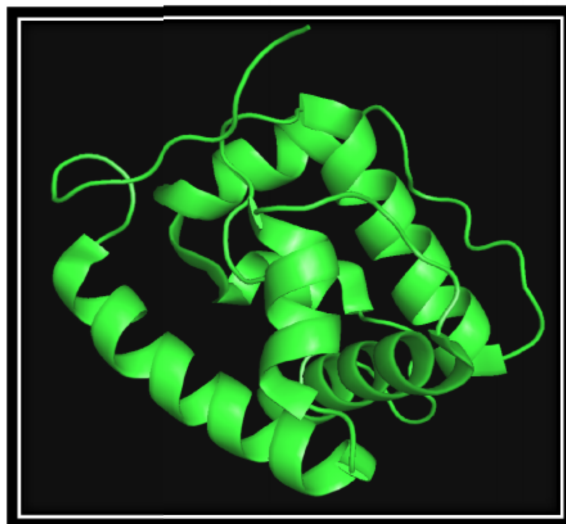
Maestro is Schrodinger's powerful, unified, multi-platform Graphical User Interface (GUI). It is designed to simplify modeling tasks, such as molecule building and data analysis, and also to facilitate the set up and submission of jobs to Schrödinger's computational programs. The main Maestro features include:-

- project-based data management facility
- scripting language for automating large or repetitive tasks
- wide range of useful display options
- comprehensive molecular builder surfacing and entry plotting facilities

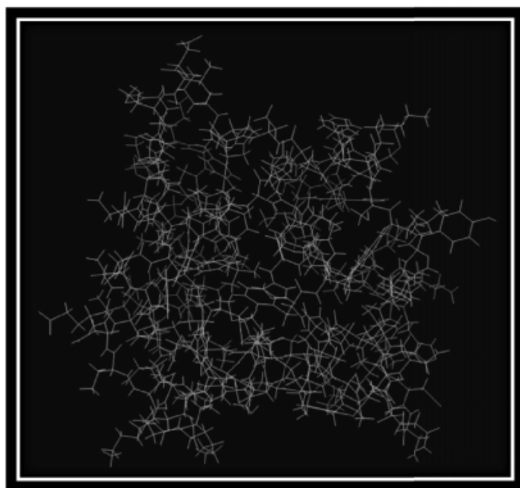
The preparation of a protein involves a number of steps, which are outlined below. The procedure assumes that the initial protein structure is in a PDB-format file, includes a co-crystallized ligand, and does not include explicit hydrogen. The result is refined and hydrogenated structures of the ligand and the ligand-receptor complex are suitable for use with other Schrodinger products. Mosquito odorant binding protein structure has been imported; water molecules have been deleted but water that bridge between the ligand and the protein are retained. If water is kept, hydrogen is added to them in the preparation process and the protein metal ions and cofactors were also adjusted. All serious errors in the protein are fixed. Structures that have missing residues near the active site were repaired. Bonds to metal ions were deleted and adjusted for the formal charges of the atoms that were attached to the metal. The orientation of misoriented groups (such as amide groups

Figure 1

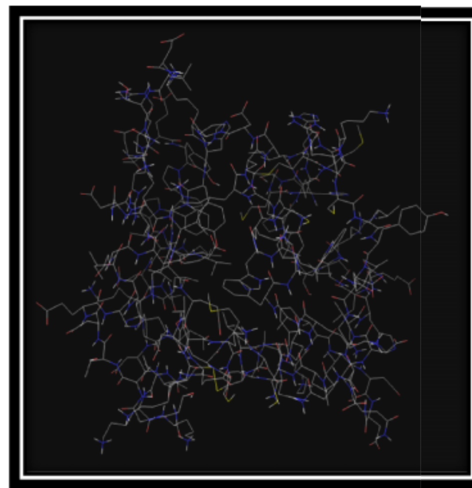
Odorant binding protein of *C. quinquefasciatus*



a) Three dimensional structure of (PDB id 2L2C)



b) Before preparation of the protein



c) After preparation of the protein

of Asn and Gln) were fixed. The ionization and tautomerization state of protein is adjusted and structure was refined.

Grid generation

Glide was used for receptor grid generation. Grid files represent physical properties of a volume of the receptor (specifically the active site) that are searched when attempting to dock a ligand. The prepared mosquito odorant binding protein is displayed in the Workspace. In the Receptor Grid Generation panel, the Receptor tab is displayed. In the Van der Waals radii scaling section default scaling factor value of 1.00 (no scaling) was chosen. The volume of grid was calculated. The entire complex was shown with several types of markers. The centre of the enclosing box is marked by green coordinate axes. The purple enclosing box represents the volume of the protein for which grids were calculated. The enclosing box was made small so that it will be consistent with the shape and character of the protein's active site and with the ligands that were expected to be docked.

Ligands retrieval and preparation

Ligand molecules were retrieved from PubChem database. The following compounds retrieved in 3D SDF format. All compounds were prepared using LigPrep module from Schrodinger. LigPrep is a robust collection of tools designed to prepare high quality, all-atom 3D structures for large numbers of drug-like molecules. The simplest use of LigPrep produces a single, low-energy, 3D structure with correct chiralities for each successfully processed input structure.

PubChem id	
CID_8343	CID_201783
CID_73170	CID_42608013
CID_5369951	CID_6549
CID_5281243	CID_5281515
CID_5280899	CID_6987
CID_56840852	CID_441849
CID_3037101	CID_441873

In the LigPrep process compounds were processed, unwanted structures were eliminated and optimized. The generation of ionization states and tautomers can be carried out with tools that are part of LigPrep. A separate product, Epik was

used to perform the structural adjustments during a LigPrep run. Epik more rigorously adjusts the tautomerization and ionization states than separate ionizer and tautomerized treatments. Finally Hydrogen atoms were added and charged groups were neutrilized for all compounds.

Molecular docking of target protein with ligands

Glide searches for favourable interactions between one or more ligand molecules and a receptor molecule, usually a protein. Each ligand must be a single molecule, while the receptor may include more than one molecule, e.g., a protein and a cofactor. Glide can be run in rigid or flexible docking modes; the latter automatically generates conformations for each input ligand. The combination of position and orientation of a ligand relative to the receptor, along with its conformation in flexible docking, is referred to as a ligand pose. The ligand poses that glide generates pass through a series of hierarchical filters that evaluate the ligand's interaction with the receptor. The initial filters test the spatial fit of the ligand to the defined active site and examine the complementarity of ligand-receptor interactions using a grid-based method patterned after the empirical ChemScore function.

Poses that pass these initial screens enter the final stage of the algorithm, which involves evaluation and minimization of a grid approximation to the OPLS-AA non-bonded ligand-receptor interaction energy. Final scoring is then carried out on the energy-minimized poses. By default, Schrodinger's proprietary glide score multi-ligand scoring function is used to score the poses. If glide score was selected as the scoring function, a composite Emodel score is then used to rank the poses of each ligand and to select the poses to be reported to the user. Emodel combines glide score, the non-bonded interaction energy, and for flexible docking, the excess internal energy of the generated ligand conformation.

Glide uses a hierarchical series of filters to search for possible locations of the ligand in the active site region of the receptor. The shape and properties of the receptor are represented on a grid by several different sets of fields that provide progressively more accurate scoring of the ligand poses. Conformational flexibility

is handled in glide by an extensive conformational search, augmented by a heuristic screen that rapidly eliminates unsuitable conformations, such as conformations that have long range internal hydrogen bonds. Each rotamer group is attached to the core by a rotatable bond, but does not contain additional rotatable bonds. The core is what remains when each terminus of the ligand is severed at the "last" rotatable bond.

Glide ligand docking jobs require a set of previously calculated receptor grids and one or more ligand structures. Preparation of the ligands before docking is strongly recommended. If a correct Lewis structure cannot be generated for a ligand, it is skipped by the docking job. Glide also automatically skips ligands containing unparametrized elements, such as arsenic or atom types not supported by the OPLS force fields, such as explicit lone pair "atoms."

All ligands were docked against mosquito odorant binding protein. Ligands were searched, perfect orientation and binding against target protein for inhibiting the function of the protein. When the ligand binds with protein, the conformation of the protein structure will change so the function of the protein will alter automatically.

Examining glide data

Glide results are examined with an emphasis on visual rather than numerical appraisal. The first set of exercises use the Project Table to display the results of the SP Glide docking job, examine individual ligand poses and their contacts with the input receptor structure.

The second set of exercises uses the Glide XP Visualizer panel to display information on the terms in the Glide XP scoring function that contribute to the ligand binding. The entire docked complex was visualized by using XP visualizers.

MOLECULAR DYNAMIC SIMULATION OF DOCKED COMPLEX

Molecular dynamics (MD) is a computer simulation of physical movements of atoms and molecules. The atoms and molecules are allowed to interact for a period of time, giving a view of the motion of the atoms. In the most common version, the

trajectories of molecules and atoms are determined by numerically solving the Newton's equations of motion for a system of interacting particles, where forces between the particles and potential energy are defined by molecular mechanics force fields.

Molecular and stochastic dynamics

Obtaining convergence in dynamics simulations has traditionally been problematic, because of the slow frequency at which systems undergoing these processes, cross barriers between the various minima on the potential energy surface. Stochastic dynamics will search conformational space more efficiently than does regular molecular dynamics, but neither method gives frequent crossing of barriers much larger than 3 kcal/mol (13 KJ/mol). A much higher rate of convergence can be obtained by the use of the mixed-mode, Monte Carlo / Stochastic Dynamics (MCSD) procedure. This procedure was used for simulations of acyclic systems because in it generation of the canonical ensemble is desired and no time dependent information is required. For cyclic systems, the Jumping Between Wells (JBW) method can be used. However, even with these methods, it is important to test that converged results have been obtained.

Dynamics using MacroModel

MacroModel is a general purpose, force-field-based molecular modeling program with applicability to a wide range of chemical systems. MacroModel provides researchers with multiple advanced methods to understand the chemical structure, energetics, and dynamics. A large selection of force fields is available in MacroModel, including the latest technical advances introduced into OPLS_2005, a force field that Schrodinger is actively developing. Numerous minimization methods are available, enabling geometry optimizations for a broad selection of structural classes. A wide range of methods is available for conformational searching, allowing efficient sampling of the potential energy surface for low-energy structures for systems ranging from small molecules to entire proteins. Additional advanced features include molecular dynamics simulations, free energy perturbation simulations and pure and mixed methods to ensemble sampling.

Best docked complex were taken for molecular dynamics. Dynamics is performed using following parameter such as keeping the constant temperature at 300K and in the integration step at 1.0ps and MD simulations for complex structures were run. MD simulation with position restraints was carried out for a period of 100ps in order to allow the accommodation of the water molecules in the system. Finally, Root Mean Square Deviation (RMSD) was calculated for checking the stability of target protein with their native motion. The entire coordinate file was saved every 0ps upto 100ps and the result was analyzed by Scatter Plot.