Chapter III.

Materials and Methods

3.1. Identification of PLA\textsubscript{2} inhibitor molecules in balloon vine

3.1.1. Preparation of plant extract

Arial parts of *Cardiospermum halicacabum* were collected and minced finely. Ten grams of the material was boiled with 100 ml distilled water till the volume was reduced to half. The solution was filtered and the filtrate concentrated to dryness by spin-drying.

3.1.2. Binding assay for PLA\textsubscript{2} inhibitors

Surface Plasmon Resonance analysis was conducted at GE Healthcare, Bangalore, using BIACORE T-100 machine. PLA\textsubscript{2} was immobilized on a sensor chip CM-5 using amine coupling method. The extracted phytochemicals were dissolved in HBS-EP buffer at pH 7.40 with 5mM CaCl\textsubscript{2}. HBS-EP buffer consisted of 0.01M HEPES, 0.15M NaCl, 3 mM EDTA and 0.005% surfactant polyethylene sorbitan. The solution was run over the chip at a flow rate of 30 µl/minute. The sensogram obtained was analyzed for PLA\textsubscript{2} inhibitors. Similarly, analyses of berberine and biotransformed berberine were carried out. Immobilization level achieved was 5043.8 RU. The running buffer was HBS-EP buffer at pH 7.4 with 5mM CaCl\textsubscript{2}. The regeneration solution was 5mM NaOH. This solution was used to regenerate the chip surface after each run. Berberine and biotransformed berberine were initially dissolved in Sodium acetate buffer.
at pH 5.0 and further diluted to the required concentrations using running buffer. Concentrations ranging from 10 to 50 µg/ml were used.

Surface Plasmon Resonance (SPR) is a real time, label free optical detection method for studying the interaction of soluble analyte with immobilized ligand. The information obtained is both qualitative and quantitative, and it is possible to obtain kinetic parameters of the interaction. This new technology has been used to study a diverse set of interaction partners of biological interest, such as protein-protein, protein lipid, protein nucleic acid or protein and low molecular mass molecules such as drugs, inhibitors and substrates. This is possible due to the reason that SPR is independent of the chemical nature of the sample. The principal interest in the use of SPR in biomedical research is that it provides a platform for monitoring molecular interactions and defining the characteristics of proteins in terms of their specificities of interaction with other molecules. The association and dissociation rate constants and their binding affinity is also called as equilibrium dissociation constant.

SPR biosensors use a highly specialized optical technique to monitor changes in refractive index in the vicinity of a surface. The SPR phenomenon occurs when, in conditions of total internal reflection, polarized light strikes a conducting gold layer at the interphase (sensor surface) between a solid support phase and a liquid phase, two media of different refractive indices: the glass of the sensor surface (high RI) and a buffer (low RI). The experimental procedure involves immobilizing one reactant on a surface and monitoring its interaction with a second component in solution. Essentially, SPR detects the changes in mass in the aqueous layer close to the sensor chip by measuring changes in refractive index.
A BIACORE instrument consists of an SPR detector, sensor chip and liquid handling system. The sensor chip consists of glass coated with a thin layer of gold. This layer is coated with dextran, which forms a hydrophilic environment for the attached molecules, preserving them in a non denatured state. When the analyte binds to the target molecule on the chip, the mass increases and when it dissociates, the mass falls. This produces changes in the refractive index close to the surface which are detected as changes in SPR signals expressed in resonance units (RU). A sensorgram is obtained by monitoring changes in SPR signals as a function of time (Glaser, 1999).

3.2. Phytochemical investigations
Investigation was conducted to detect berberine in this plant. This was reported previously (Lewis and Lewis, 2003). Berberine is known to possess anti inflammatory property (Kuo, Chi and Liu, 2002). Ten grams of minced aerial parts of this plant were suspended in 100 ml of a mixture of ethanol, water and acetic acid in the ratio 8:1:1. It was refluxed for two hours. The extract so obtained was filtered and kept undisturbed for a week for the precipitation of chlorophyll and other plant pigments. The clear yellow solution obtained was treated with Dragendorff reagent (Potassium Bismuth Iodide). The dark brown precipitate was collected and boiled with Sodium Carbonate solution. This solution was extracted with Diethyl ether. The ether extract was dried using anhydrous magnesium sulphate and ESI-MS carried out.

3.3. In silico molecular docking studies
Molecular docking was carried out using berberine and different types of PLA$_2$ available in the PDB. The coordinates of protein was retrieved from Protein Data Bank Accelrys Discovery Studio 2.0 (DS2) software package.
was used for the modeling and docking studies (Accelrys, San Diego, CA). Active site of the protein which constitutes residues D49 and H48 were identified using the tool available in DS2 and was verified using available literature (Berg et al., 1995). Water molecules were removed. Hydrogen atoms were added (Brooks et al., 1983). CHARMM Force field was applied and a geometric optimization of the structure was carried out. The structure of berberine was built, energy minimized and prepared for docking studies using DS2 Fragment Builder. MMFF (Halgren, 1996). Force field was applied and the structure was optimized. The prepared ligand was docked in the active site of protein using the CDOCKER protocol (Wu et al., 2003), which docks ligands into an active site using a CHARMM-based MD simulation scheme. In this method, random ligand conformations generated from a high-temperature MD are first translated into the binding site. The binding poses are searched using random rigid-body rotations, followed by simulated annealing. A final minimization with full force field potential is used to refine the ligand poses. Standard docking parameters in which site sphere and target temperature in the high temperature MD used to generate random starting conformations was set to 15 Å and 10,000K respectively. CDOCKER protocol calculates the CHARMM energy (interaction energy plus ligand strain) and the interaction energy. The poses are sorted by CHARMM energy and the top scoring, most negative poses (favorable to binding) are retained. Modeling and docking studies were also performed in order to understand the molecular level mechanism of inhibition and the reason for the difference in PLA₂ inhibitory activities of berberine and its biotransformed derivatives. Porcine pancreatic PLA₂ used for enzyme inhibition and SPR studies was taken as the model enzyme for the docking studies. This is similar in three dimensional structure to human PLA₂ (Berg
et al., 1995). Similarly docking studies were carried out using the various metabolites of berberine formed in the human body. The PDB coordinates of human PLA₂ were used for the purpose of docking in this case. The details of molecular docking techniques are discussed in appendix III.

3.4. Identification of the PLA₂ binding compound by crystallography

Preformed crystals of Russell Viper venom PLA₂ were obtained from Dept. of Biophysics, All India Institute of Medical Sciences, New Delhi. It is structurally similar to human synovial fluid PLA₂ and can therefore be used as a model for drug development programmes involving human PLA₂ (Nagendra, 2004). They were grown in Sodium acetate buffer of strength 25 mM of pH 6.2 and containing 5mM CaCl₂ in, 15% PEG 8000 and 0.2 M ammonium sulphate. The method was hanging drop and vapor diffusion. The rectangular crystals measuring up to 0.3 x 0.2 x 0.2 mm were obtained within three weeks.

The phytochemical mixture obtained from spin drying was dissolved in the mother liquor. Crystals of PLA₂ were carefully suspended in the solution for 48 hrs. The resulting crystal was mounted and subjected to X-ray diffraction. The diffraction was carried out 291K in a MAR Research Image Plate Reader Model No: 345. The X-ray generator was Rigaku RU300 model. The wave length of X-rays was set at 1.54 Å using graphite monochromator. The crystal diffracted up to 1.93 Å. The output of the diffraction was reduced and indexed using DENZO and SCALE PACK software. It was converted to MTZ file and was further processed using CCP4 Package. Details of X-ray crystallographic data collection are given in appendix-I.
3.5. Co-crystallization studies

Crystals of PLA$_2$ incorporating the compound berberine and dihydroxy-berberine which is the biotransformed derivative of berberine were grown and their structure determined by crystallography. Porcine pancreatic PLA$_2$ was used for the co-crystallization experiments. This is readily available and structurally similar to human PLA$_2$ (Berg et al., 1995). The overall RMS deviation between porcine pancreatic PLA$_2$ and snake venom PLA$_2$ is only 0.9 Å. Details of crystal growth methods are given in appendix- II.

3.5.1. Chemicals and Equipments

Lyophilized porcine pancreatic PLA$_2$ was obtained from Fluka Chemicals, USA. Berberine chloride was obtained from Fluka chemicals Switzerland, and 2-methyl 2, 4-pentandiol was from Sigma Chemicals, USA. Other reagents were from Merck India Ltd, Mumbai. HPLC grade water produced by Millipore system was used for preparing solutions. Cell culture plates with 24 wells were used for crystallization. Cover slips siliconized with 1% Dichlorodimethyl silane in CCl$_4$ were used.

3.5.2. Preparation of complex crystals

Appropriate amounts of PLA$_2$ and berberine were dissolved in tris maleate buffer to get 1:1 enzyme to berberine ratio and a protein concentration of 60 mg/ml. The strength of buffer used was 0.05 M at pH 7.2 in 5 mM CaCl$_2$. The well solution contained the above buffer and CaCl$_2$, but without protein and ligand. 2-Methylpentan 2, 4-diol was added in the well solution so as to get a concentration of 16 %. The well solution was 0.5 ml. Three microliters of the protein-ligand solution was placed on the siliconized cover slip and 3µl of well solution added to it. The well was covered with the cover slip and sealed with silicone grease. The set up was kept at a constant temperature of 21°C. Beautiful diamond shaped crystals were obtained in 10 days. The experiment
was repeated with biotransformed berberine and similar crystals were obtained.

3.5.3 Data collection
The X-ray diffraction data was collected using a MAR-RESEARCH image plate reader of model No 345, at Department of crystallography and Biophysics, University of Madras. The X-ray generator was of Rigaku RU300 model, with graphite monochromator. The wavelength of X-rays was 1.54 Å and data was collected at 100 K. A total of 120 frames were collected, each with one minute exposure to X-rays. The image plate to crystal distance was set at 150 mm. The data was processed using DENZO and SCALEPACK to produce an MTZ file which was subsequently analyzed using CCP4 software package.

3.6. Biotransformation of Berberine
Berberine chloride was obtained from Fluka Chemicals, USA. Other reagents were of L.R. Grade from Merck India Ltd. *Rhizopus oryzae* was obtained from The Institute of Microbial Technology, Chandigarh.

A special defined media for the growth of fungi was prepared, the composition of which was as shown in the Table 3.1.

<table>
<thead>
<tr>
<th>Components</th>
<th>g/l</th>
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<tbody>
<tr>
<td>KH₂PO₄</td>
<td>1 g</td>
</tr>
<tr>
<td>NaCl</td>
<td>1 g</td>
</tr>
<tr>
<td>(NH₄)₂SO₄</td>
<td>2 g</td>
</tr>
<tr>
<td>Sucrose</td>
<td>40 g</td>
</tr>
<tr>
<td>Distilled water</td>
<td>1 L</td>
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</tbody>
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**Table 3.1. The constituents of the defined fungal broth**
The above media was prepared and 1g/l of berberine chloride was added to it. The pH was adjusted to 7. This was poured into the cleaned reaction vessel of Sartorius fermenter and autoclaved at 120°C for 15 minutes in an electrical autoclave. It was inoculated with 10 ml of *Rhizopus oryzae* cultured in peptone broth. The rotator was set at 200 rpm at room temperature. The replenishment of media was done every alternate day with 10 ml concentrated and autoclaved media. The fermentation was continued for ten days with periodic aeration.

3.6.1. Recovery of berberine and its derivatives from the broth

The broth was filtered through Whatman No: 1 filter paper and berberine and its derivatives purified by ion exchange chromatography. CM-Sephadex was allowed to swell in water and a column was filled with it. The filtered broth was allowed to percolate through this column at a flow rate of 3 ml/minute. After passing of the broth, the column was washed several times with distilled water. Finally, berberine and its derivatives were eluted out using 2 N HCl. This solution was freeze dried to get crystals of bioconverted berberine. It was subjected to the following analysis:

3.6.2. FTIR analysis: This was conducted using Schimadzu instrument of model 8400S, and equipped with IR solution software, using KBr as matrix. Analysis range was between 400 and 4000/cm. The biotransformed berberine was dried under IR lamp to remove moisture and ground with KBr and pressed to form a pellet and exposed to IR beam. A sample of pure berberine was also analyzed for comparison.

Infra Red (IR) spectroscopy is related to the vibrational energy of molecules. If IR radiation of an appropriate frequency is passed through the sample, it is possible for the molecule to undergo a transition to a higher vibrational energy level by absorption of radiation. A vibrational transition
from the ground state to the first excited state due to absorption of IR radiation is called a fundamental absorption and the frequency associated with this is called fundamental frequency. Though other transitions are possible, they occur much less frequently. An IR spectrum consists of a plot of absorbance versus frequency or wave number.

FTIR (Fourier Transform Infra Red) Spectroscopy is an improved version of IR spectroscopy. This uses a Michelson interferometer which enables the scanning of the complete IR spectrum within a short time. FTIR is essentially an interference based technique rather than one based on absorption. Each molecule gives a characteristic FTIR spectrum which reflects its chemical structure. FTIR thus allows the detection of chemical groups in an organic compound. In principle, any chemical bond of a given type might be expected to have a fundamental absorption. Practically, the chemical environment of the bond has an effect on the precise frequency of absorption, since this may alter the electron density of the bond. Studies of FTIR spectra from a large number of molecules of known chemical structure suggest that absorbance of IR radiation near particular frequencies are characteristic for specific chemical groups. These group frequencies allow the determination of aspects of molecular structure from their IR spectrum alone (David, 2000).

3.6.3. H\textsuperscript{1}-NMR: This was conducted at the National NMR facility, Indian Institute of Science, Bangalore. The analysis was conducted in a 400 MHz instrument of AMX-400 make, with IRIX-6.5 software. The reference standard was Trimethylsilane and the spectrum range was 0 – 12 ppm. The solvent used was Deutero-chloroform. A sample of pure berberine was analyzed for comparison.
NMR is a spectroscopic technique that enables visualization of nuclei within an organic molecule. However not all atomic nuclei can give rise to an NMR signal, only nuclei with values of $I$ (the spin quantum number) other than zero are “NMR active”. The spin number of a nucleus is controlled by the number of protons and neutrons within the nucleus; the nuclear spin varies from element to element and also varies among isotopes of a given element. A nucleus with a spin quantum number $I$ may take on $2I+1$ energy levels when it is placed in an applied magnetic field of strength $H$. The amount of energy separating these levels increases with increase in $H$; however the amount of energy separating adjacent levels is constant for a given value of $H$. The specific amount of energy separating adjacent levels, $\Delta E$ is given by

$$\Delta E = (H\gamma h) / (2I)$$

Where $\gamma$ is the magnetogyric ratio for a given isotope, $H$ is the strength of the applied magnetic field and $h$ is the Plank’s constant. The creation of an NMR spectrum for a drug molecule is related to this difference in energy ($\Delta E$) between adjacent energy levels. In the NMR experiment, a nucleus is energetically excited from one energy level to a higher level. Since the exact value for $\Delta E$ is related to the molecular environment of the nucleus being excited, there now exists a way of relating the value of $\Delta E$ to the molecular structure: this enables the molecular structure to be determined.

Nuclear magnetic resonance (NMR) is based on the fact that a number of important nuclei (eg: $^1$H, $^2$H, $^{13}$C, $^{19}$F, $^{23}$Na, $^{31}$P, $^{35}$Cl) show the atomic property called magnetic momentum where nuclear spin quantum number $I$ is larger than zero (for $^1$H, $^{13}$C, $^{19}$F and $^{31}$P, $I =1/2$). When such a nucleus (or an unpaired electron) is put into a strong magnetic field, the axes of the rotating atom will describe a precessional movement, like that of a spinning
The precessional frequency $\omega_0$ is proportional to the applied magnetic field $H_0$:

$$\omega_0 = \gamma H_0$$

where $\gamma$ is the magnetogyric ratio, which is different for each nucleus or isotope. Since the spin quantum number of the nucleus can be either $+1/2$ or $-1/2$, there are two populations of nuclei in any given sample, one with a higher energy than the other. These populations are not equal: the lower energy population is slightly more abundant. The sample is then irradiated with the appropriate radiofrequency. It is at certain frequency, the atoms population with the lower energy will absorb the energy of the radiofrequency and be promoted to the higher energy level and will be in resonance with irradiating frequency. The energy absorption can be measured with a radio receiver and can be displaced in the form of a spectrum of absorption versus the irradiating frequency. The information content of this spectrum derives from the fact that each nucleus of a molecule and each proton will have a slightly different resonance frequency, depending on its environment. In other words, its magnetic momentum will be “shielded” differently in different functional groups. This makes it easy to distinguish, for example, the protons on a -C-CH group from an -O-CH$_3$ group or an N-CH$_3$ group, aliphatic or aromatic protons, carboxylic acid or aldehyde protons, and so on because they absorb at different frequencies. The only drawback to NMR is its low sensitivity.

Fourier-transform (pulsed) proton NMR techniques allow an even more sophisticated assignment of resonances to specific protons. If the single high-frequency pulse is replaced by two pulses of variable pulse separation, the introduction of a second time parameter yields a two-dimensional NMR spectrum with two frequency axes. Resonances on the diagonal are the
normal one-dimensional spectrum, but off-diagonal resonances show the mutual interaction of protons through several bonds. This allows the assignment of all protons even in very large molecules such as proteins (David, 2000).

3.6.4. ESI-MS: This was conducted at Molecular Biophysics Unit, Indian Institute of Science, Bangalore. The instrument was Bruker Daltonics make, Dionex Ultimate 3000 model. The sample, dissolved in methanol was used for injection. A sample of pure berberine was analyzed for comparison.

Mass spectrometry is an analytical tool for measuring the molecular mass of a substance. A mass spectrometer can be divided into three fundamental parts, the ionization source, the analyzer and the detector. The sample is introduced in the ionization source of the instrument. It gets ionized and the ions are extracted into the analyzer region. The ions get separated according to their mass to charge (m/z) ratio. The separated ions are detected by the detector and this signal is sent to the data system where m/z ratios are stored together with their relative abundance to generate the m/z spectrum. Electro spray ionization (ESI) is well suited for the analysis of molecules ranging from 100 to \(10^5\) Daltons. The sample is dissolved in a volatile solvent and pumped through a narrow stainless steel capillary at a flow rate of \(1\mu l/minute\). A high voltage of 3000 to 4000 V is applied to the tip of the capillary. This causes the sample to get dispersed into an aerosol of highly charged droplets. This process is aided by nitrogen gas flowing around the capillary, called nebulising gas. The charged droplets diminish in size by solvent evaporation, assisted by a warm flow of nitrogen, known as drying gas. The charged sample ions then move to the high vacuum area of mass spectrometer where analysis takes place (David, 2000).
3.6.5. Kinetic studies of PLA$_2$ by pH stat method

This was conducted to assess the inhibitory activity of berberine and its biotransformed derivatives towards PLA$_2$.

The study of an enzymatic reaction is known as enzyme kinetics. This is a powerful tool for characterizing enzyme activity. The application of simple mathematical models to enzyme activity under varying laboratory conditions and in the presence of competing substrates or enzyme inhibitors made it possible to deduce the probable physiological conditions and regulatory mechanisms of various enzymes.

In many enzymatic reactions, the rate of a reaction and how the reaction changes in response to different conditions reveal the path followed by the reactants and are therefore indicative of the reaction mechanism. Kinetic data combined with detailed information about the structure of the enzyme provide some of the most powerful clues to the biological function of the enzyme and may suggest ways to modify it for therapeutic or industrial purposes.

Enzymes catalyze a tremendous variety of reactions using different mechanisms. Yet, all enzymes can be analyzed such that their reaction rates as well as their overall efficiency can be quantified.

The Michaelis-Menten equation given below describes the rate of an enzymatic reaction as a function of substrate concentration:

$$V_o = V_{max}[S] / K_m + [S].$$

Where $v_o$ is the initial velocity, $V_{max}$ is the maximum velocity and $S$ is the concentration of substrate in the assay mixture. $K_m$ is the Michaelis constant. This is the basic equation of enzyme kinetics. It describes a rectangular hyperbola, which is the progress curve of an enzyme. (A plot of initial velocity versus the substrate concentration).
The Michaelis constant $K_m$ has a simple operational definition. It is that substrate concentration at which the reaction velocity is half maximal. If the enzyme has a small value of $K_m$, it achieves maximum catalytic efficiency at lower substrate concentrations itself. The $K_m$ is unique for every enzyme substrate pair. Different substrates that react with a given enzyme do so with different $K_m$ values. Also, different enzymes that act on a particular substrate have different $K_m$ values. The substrate with the lowest $K_m$ has the highest affinity for the enzyme. The best substrate is the one which has the highest $V_{max}/K_m$ ratio.

$V_{max}$ or the maximal velocity of the enzymatic reaction occurs at a high substrate concentration, when the enzyme is fully saturated. $V_{max}$ is not a constant, but depends on the concentration of the enzyme in the assay system. At very high substrate concentrations, initial velocity $v_o$ asymptotically approaches $V_{max}$.

Practically, it is difficult to determine $V_{max}$ and $K_m$ values from the rectangular hyperbolic progress curves. To overcome this, a reciprocal of Michaelis Menten equation, formulated by Hans Lineweaver and Dean Burk given below is used:

$$\frac{1}{v_o} = \frac{K_m}{V_{max}} \cdot \frac{1}{S} + \frac{1}{V_{max}}$$

The plot of $1/v_o$ versus $1/s$ describes a straight line and is called Lineweaver-Burk plot or Double reciprocal plot. The slope of this line is $K_m/V_{max}$. The intercept on $1/v_o$ axis is $1/V_{max}$ and the extrapolated intercept on $1/S$ axis is $-1/K_m$ (Lineweaver and Burk, 1938).

Many substances alter the activity of an enzyme by combining with it in a way that influences the binding of the substrate. Substances that reduce the activity of an enzyme are known as inhibitors. Inhibitors are mainly three types:
1. **Competitive inhibitors:** A substance that competes directly with a normal substrate for the substrate binding site of the enzyme is known as competitive inhibitor. Competitive inhibitors increase $K_m$ value leaving $V_{max}$ unaltered, in the Lineweaver Burk plot.

2. **Uncompetitive inhibitors:** The inhibitor binds directly to the enzyme-substrate complex, but not to the free enzyme. The uncompetitive inhibitor distorts the active site, rendering the enzyme catalytically inactive. Both $V_{max}$ and $K_m$ values are decreased in the case of uncompetitive inhibition.

3. **Noncompetitive inhibitors:** In this case, the inhibitor binds to the free enzyme as well as to the enzyme substrate complex. If the inhibitor binds free enzyme and enzyme substrate complex with equal affinity, the $K_m$ value is unaltered and $V_{max}$ suffers a decrease. Otherwise, $K_m$ may either increase or decrease (Voet and Voet, 2006).

The pH stat assays are used to monitor the progress of chemical or enzymatic reaction in which protons are liberated or taken up. This is achieved by measuring the quantity of acid or base that needs to be added at various times to keep the pH constant. This technique was developed by Knaffe Lenz in 1923 for estimating esterase enzymes.

The pH stat method of determination of reaction rates complements spectrophotometric methods. It requires a change in proton binding sites during the reaction, rather than a change in chromophoric character. It can be used to study kinetics in non buffered solution and in stirred suspension.

The technique is readily used to study turbid cellular extracts and immobilized cells or enzymes. A pH stat is a type of autotitrator that can be used to maintain a constant pH in a non buffered solution during a reaction that involves the production or consumption of protons. This is achieved by the addition of a solution of acid or base of known concentration. The
amount of titrant added to maintain the pH constant is recorded as a function of time to provide a progress curve for the reaction which may be subjected to kinetic analysis (Brocklehurst, 2002).

Assay of PLA$_2$ is based on volumetric measurement of acid produced by the hydrolysis of phospholipid by PLA$_2$. (Lombrado and Dennis, 1985). A suspension of phospholipid such as Soya lecithin is prepared in NaCl and CaCl$_2$ solution. A fixed amount of PLA$_2$ is added to it continuously monitoring the pH using a pH meter. 0.01 N KOH is added from a burette to keep pH constant at 8.90 for 10 minutes. The volume of KOH added gives the measure of enzyme activity. Soya lecithin is known to hydrolyze nonenzymatically also. So, a blank experiment is conducted without the addition of PLA$_2$ and this volume is subtracted from the earlier value obtained. From the titre value, the activity of enzyme in terms of IU/mg is calculated using the equation.

\[
\text{Enzyme Activity} = (\text{Vol of KOH} - \text{Vol of Blank}) \times \text{Normality of KOH} \times 10^3 / \mu g\ \text{of enzyme in the solution} \times \text{Time (In minutes)}.
\]

The experiment is repeated using different concentrations of soya lecithin and activity calculated in each case. The Lineweaver Burk plot is drawn and $K_m$ and $V_{max}$ are determined (Lineweaver and Burk, 1938). The experiment is repeated with the enzyme solution equilibrated with a definite amount of berberine and bioconverted berberine and the fall in activity and hence, alteration of $K_m$ and $V_{max}$ are determined.

**A. Preparation of Enzyme inhibitor complex**

Appropriate amounts of PLA$_2$ and berberine were dissolved in distilled water to get a 1:5 molar ratio of enzyme to inhibitor. The solution was kept for equilibration for three hours at 4°C. This was repeated with biotransformed berberine also.
B. Preparation of substrate (soya lecithin)
Appropriate amount of soya lecithin were weighed out to obtain 0.016, 0.032, 0.048 and 0.064 Molar solutions. It was moistened with 10 ml ethanol to facilitate the formation of suspension and stirred with 200 ml aqueous solution of 0.2 M NaCl and 0.005 M CaCl₂.

C. The Assay procedure
20 ml of the substrate solution was pipetted out in to a 100 ml beaker and its pH was noted. It was made up to 8.9 by the addition of 0.5 N KOH. 0.01 N KOH was added from a burette to keep the pH at 8.9 for 10 minutes. Burette readings were noted at two minute intervals. This served as the blank. Then 30 µl of native enzyme solution was added. The volume of KOH required to maintain pH at 8.90 is noted for 10 minutes at 2 minute intervals. Blank was subtracted from the noted value. This experiment was repeated with enzyme – berberine complex and also with enzyme – biotransformed berberine complex and also with different concentration of the substrate from 0.016 M, 0.032 M, 0.048 M and 0.064 M and result tabulated. The strength of enzyme in terms of IU/mg is calculated. Lineweaver Burk plot was drawn to calculate \( K_m \) and \( V_{max} \).

3.7. Structure comparison studies
The three dimensional structure of the native Russell viper venom PLA₂ was compared with that of PLA₂ complexed with berberine. Similarly, native porcine pancreatic PLA₂ was compared with that of the structures of complexes obtained by co crystallization using Discovery studio 2.0 software. The overall RMS deviation and any significant deviation among the side chains were noted.