3. MATERIALS AND METHODS

3. Materials and Methods

3.1. Preliminary Screening

Plant materials were either collected from BITS Pilani, Pilani campus or procured from commercial plant material suppliers (at Nashik and Chandigarh). All the plant materials were authenticated by a qualified botanist, (Dr A. S. Sandhu, Department of Natural Products, NIPER, S.A.S. Nagar). A voucher specimen has been deposited at the Herbarium of the Natural Product, Laboratory of Natural Drugs, Department of Pharmacy, BITS Pilani (Pilani Campus). Plant materials were subjected to shade drying, powdered and passed through sieves (# no BSS 10). Powdered materials were subjected to sequential extraction (hexane followed by methanol) using the continuous hot extraction technique (40°C and 60°C for hexane and methanol, respectively for 24 h), Cold maceration (RT, 72 h) and Ultrasonic assisted extraction (≈ 25-30°C, 1 h). The obtained extracts were filtered and concentrated *in vacuo* (Heidolph rotavapor).

3.2. PL inhibition assay and enzyme kinetics

Porcine PL (Type II), *p*-nitrophenyl butyrate and orlistat were procured from Sigma-Aldrich (St. Louis, MO, USA). Sodium chloride (molecular biology grade) and Tris buffer were procured from Sisco Research Laboratories (Mumbai, India). All other chemicals and solvents were of analytical grade. PL inhibition assay was performed as per the standardized protocol [1,2]. The enzyme solutions were prepared immediately before use and used afresh.

3.2.1. PL inhibition assay

Briefly, crude porcine PL (5 mg/mL) was suspended in Tris-HCl buffer (pH-7.4). The mixture was subjected to vigorous shaking, followed by centrifugation (3000 *g*, 10 min) and the supernatant was collected. Stock solutions of the orlistat/natural products and synthesized analogues were prepared in DMSO. Linear concentrations (0.78–2000 μg/mL and 39.06–5000 μg/mL) were used as stock solutions for orlistat/ echitamine/synthesized analogues and extracts, respectively. The 875 μL of Tris-HCl buffer and 100 μL of PL solution were pre-incubated (5 min) with 20 μL various concentrations of test solutions at 37 °C. Further, 5 μL of the substrate (4-nitrophenyl butyrate, 10mM in acetonitrile) was added and the amount of released *p*-nitrophenol was measured at 405 nm [BioTek EPOCH microplate spectrophotometer (VT, USA)]. The percentage inhibition was calculated using the formula 3.1

$$\% \text{ inhibition} = [1-(A_T/A_E)] \times 100 \ldots\ldots\ldots\ldots \text{Formula 3.1}$$

Where A_E is the absorbance of enzyme control (without inhibitor), and A_T is the difference between the absorbance of the test sample, with and without substrate. IC_{50} values were calculated from the slope of the linear regression curve (% inhibition *Vs.* concentration).

3.2.2. PL inhibition kinetics

Enzyme inhibition kinetics was performed for the most active analogues for determining their nature of inhibition. The procedure for the kinetics study was performed as per the standardized protocol [1,2]. Briefly, the PL inhibition assay protocol was repeated at four different concentrations of substrate (25, 50, 100 and 200 μM) using increasing concentrations of the inhibitor. The absorbance of the final reaction mixture was taken in kinetic mode (16 readings for 30 minutes) and the respective concentration of 4-nitrophenol was calculated from its calibration curve. The velocity of the reaction was then calculated as the slope of the graph for concentration *Vs.* time. A double reciprocal Lineweaver-Burk plot with reciprocals of velocity and substrate concentration (on y-axis and x-axis, respectively) was plotted to understand the nature of inhibition [3]. The inhibition constant, K_i, was calculated using the Cheng-Prusoff equation [4].

3.3. Fluorescence quenching studies

Effects of the most active analogues on the fluorescence quenching of the PL were evaluated by the previously reported methods with the necessary modification [5,6]. Standard stock solutions (2 mg/mL) of the most active analogues were prepared by dissolution in DMSO. A standard solution of PL (5 mg/mL) was prepared in pH 7.4 Tris-HCl buffer. Different concentrations of inhibitors (0.15625 – 100 μg/mL) were mixed along with 10 μM of PL and their fluorescence intensity was recorded. For the titration studies, an excitation wavelength of 290 nm and an emission wavelength in the range of 305 to 500 nm was selected, by using a cuvette of 1.0 cm path length. The slit widths of excitation and emission were set as 1.5 nm. The fluorescence spectra were recorded at three temperatures 298, 304 and 310 K by a spectrofluorometer (Fluorolog-3 fluorescence spectrophotometer, Horiba Jobin Yvon Inc., France) and a linear Stern-Volmer plot was used for the identification of quenching mechanism of the inhibitors.

3.4. Molecular Docking and Dynamics

3.4.1. Molecular Docking

Molecular docking studies were performed by using Molegro Virtual Docker 6.0 [7]. Prior to docking, the structures of the analogues were constructed in ChemDraw Professional 15.0 (PerkinElmer, USA) and were subjected to energy minimization using the Molecular

Mechanics 2 (MM2) force field in the Chem3D module (PerkinElmer, USA). The PDB structure of human PL (2.46 Å resolution structure of the pancreatic lipase colipase complex inhibited by a C11 alkyl phosphonate [methoxyundecyl phosphinic acid, MUP]; PDB ID: 1LPB) was retrieved from the RCSB PDB Data bank [8]. Receptor Grid file was generated by using prepared protein (Chain B) as an input file for docking simulation. The grid of active site was procreated at centroid of 7.86, 24.91, 55.11 on x, y, z-axis, respectively. The validation of the grid was performed by redocking the co-crystallised ligand (MUP, subjected to energy minimization) into the active site of PL. The redocked pose was deviated from the co-crystallised pose by an RMSD of 1.492 Å (**Fig. 3.1**). The energy minimized analogues were docked into the active site of 1LPB using validated grid parameters [1,2]. The obtained docked poses of the analogues were analysed for their MolDock scores (Kcal/mol). The 2D interpretation of the analogues interactions was constructed by BIOVIA Discovery Studio 4.5 visualizer (Dassault Systemes Biovia, Accelrys, USA).

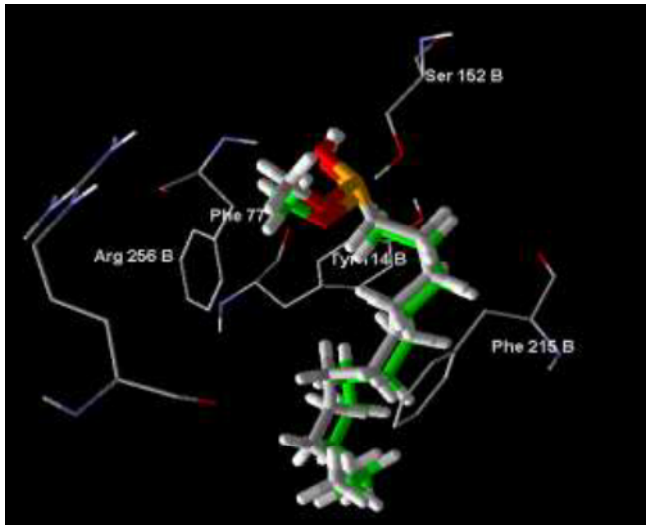

Fig. 3.1. Superimposition of the re-docked pose of MUP (Grey) with the co-crystallised pose (Green).

3.4.2. Molecular Dynamics

Molecular dynamics (MD) simulations were conducted in a High-performance computing facility of BITS Pilani, Pilani campus by a standardized protocol [1,2]. The MD simulations were performed on the docked analogue–PL complexes using GROMACS-5.1.4 packages [9] with a CHARMM27 force field [10]. The topology of analogues were obtained from an online tool provided by the Swiss Institute of Bioinformatics [11]. Bound structures were solvated using a TIP3P water model in a cubical box. Five sodium ions were added to neutralize the system. Steepest Descent algorithm was used for the energy minimisation of the system while the system was stabilised by using a canonical

NVT and NPT ensembles. A 20 ns standard MD simulation was performed, and trajectories were stored. The analyses were performed using GROMACS inbuilt analysis tools.

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