# CHAPTER 4

## 4. Materials and Methods

### 4.1 Botanical source

Leaves of *Aloe vera* (NIP-NPM-CD-251), *Azadirachta indica* (NIP-NPM-CD-229) and unripe fruits of *Aegle marmelos* (NIP-NPM-CD-242) were collected from the BITS Pilani (Pilani campus). Roots of *Berberis aristata* (NIP-NPM-CD-228) were collected from Mandi, Himachal Pradesh. Fruits of *Emblica officinalis* (NIP-NPM-CD-252), fruits of *Piper nigrum* (NIP-NPM-CD-233), bark of *Cinnamomum zeylanicum* (NIP-NPM-CD-253), leaves of *Thea sinensis* (NIP-NPM-CD-243) and rhizome of *Zingiber officinale* (NIP-NPM-CD-254) were bought from local market of Pilani. Few plants were also obtained from the local market of Chandigarh such as leaves of *Gymnema sylvestre* (NIP-NPM-CD-227), leaves of *Ocimum gratissimum* (NIP-NPM-CD-234), leaves of *Clerodendrum serratum* (NIP-NPM-CD-255), fruits of *Piper chaba* (NIP-NPM-CD-226), *Piper longum* (NIP-NPM-CD-241), bark of *Sphaeranthus indicus* (NIP-NPM-CD-256), bark of *Symplocos racemosa* (NIP-NPM-CD-257) and bark of *Terminalia arjuna* (NIP-NPM-CD-258). All the plant materials were authenticated by Dr. A.S. Sandhu, Department of Natural Products, National Institute of Pharmaceutical Education and Research (NIPER), S.A.S Nagar, Mohali. The voucher specimens have been deposited in the herbarium of Natural Drug Laboratory of Department of Pharmacy, BITS Pilani, Pilani campus.

### 4.2 Chemicals and reagents

Hexane, chloroform, ethyl acetate, methanol (Commercial grade) and acetonitrile, methanol, ortho-phosphoric acid, formic acid (Analytical grade) were purchased from (Merck Millipore, Massachusetts, USA). Silica grade # 60-120, 100-200 and 200-400 was also purchased from (Merck Millipore, Massachusetts, USA). Porcine Pancreatic Lipase (Type II), 4-nitrophenyl butyrate, and Sirius Red were procured from Sigma-Aldrich (St Louis, MO, USA). Gymnemagenin was purchased from Natural Remedies (Bangalore, India). Molecular biology grade Tris(hydroxymethyl) aminomethane (Tris) buffer and sodium chloride were obtained from SRL Pvt. Ltd. (New Delhi, India). Molecular biology grade dimethyl sulphoxide (DMSO), L-cysteine, formaldehyde, hematoxylin, eosin Y, xylene and potassium dihydrogen phosphate ($KH_2PO_4$) were procured from HiMedia Pvt. Ltd. (New Delhi, India). Casein was purchased from Clarion Pvt. Ltd. (India), while, corn starch, cellulose, sucrose, DL-methionine, cholic acid and DPX mount were obtained from Central Drug House fine chemicals, (New Delhi, India). Soybean oil, mineral and vitamin mix were obtained from the local market of Pilani, while lard

was obtained from local market of Karol bagh (New Delhi, India). Triglyceride, cholesterol and HDL kits were procured from Spinreact (S.A.U., Spain). All other chemicals and solvents were of analytical grade.

### 4.3 Preparation of Extracts

Fresh plant materials were collected (from BITS Pilani, Pilani campus and Mandi, Himachal Pradesh) and shade dried in Natural Drug Laboratory (Room no:3117). Once dried all these and other already dried plant materials were processed and passed through BSS no. 10 # to achieve desired particle size. The powdered materials were divided into three parts (30 g each) and were subjected to three different extraction techniques namely soxhlation (SX, 24 h, 40-60°C) or decoction (DC,1h), ultrasonication (SO, 1 h, ≈ 25-30°C), and maceration (MC, 72 h, RT). In case of soxhlation hexane and methanol were the choice of solvents, while for decoction water was used. Ultrasonication and maceration were carried out using all the three solvents. The obtained extracts were filtered (using cotton) and subjected for concentration using rotary evaporator [1].

The obtained extracts were coded in the following manner. For example, *Aloe vera* leaf powder (AV) when subjected to soxhlation (SX) using hexane (H) and methanol (M), it was coded as AVH-SX and AVM-SX. Similarly, when subjected to decoction using water (W) it was coded as AVW-DC. Thus, when ultrasonication (SO) technique was applied to the plant material using hexane, methanol and water, it was coded as AVH-SO, AVM-SO and AVW-SO. For maceration (MC) technique using hexane, methanol and water, it was coded as AVH-MC, AVM-MC and AVW-MC. Such codes were applied to all the extracts with respect to the solvent and the extraction techniques used.

### 4.4 Pancreatic Lipase Assay and Enzyme Kinetics

PL inhibition assay was performed as per the procedure standardized in our laboratory. Briefly, 50 mg of porcine PL was suspended in 10 ml of Tris-HCl buffer (containing 2.5 mmol of Tris and 2.5 mmol of NaCl, adjusted to pH 7.4 with HCl). The solution was shaken vigorously for 15 min using orbital shaker. Further the solution was then subjected to centrifugation (4000 rpm, 18 °C for 10 min). The supernatant was collected and used afresh as the enzyme solution.

Stock solutions of the extracts and phytochemicals were prepared in DMSO at linear concentrations ranging from 0.039 - 5 mg/ml and 0.0079 - 1mg/ml, respectively. The final 1000 µl reaction mixture comprised of pre-incubated mixture (5 min at 37 °C) of 875 µl of buffer, 100 µl of enzyme and 20 µl of extract/ phytochemicals of various stock concentrations, followed by

addition of 5 µl of the substrate (4-nitrophenyl butyrate, 10 mM in acetonitrile). The amount of DMSO

determined using microplate spectrophotometric reader (EPOCH, BioTek, Vermont, USA) after 5 min at 405 nm **(Figure 17)**. The assay was performed in triplicate and the percentage inhibition was calculated using the formula

$$\% \, Inhibition = \left[\frac{A_E - A_T}{A_E}\right] \times 100 \qquad \text{....(1)}$$

where $A_E$ is the absorbance of enzyme control (without inhibitor), and $A_T$ is the difference between the absorbance of test sample, with and without substrate. The $IC_{50}$ values of the extracts and phytochemicals were calculated byplotting linear regression curve [1].

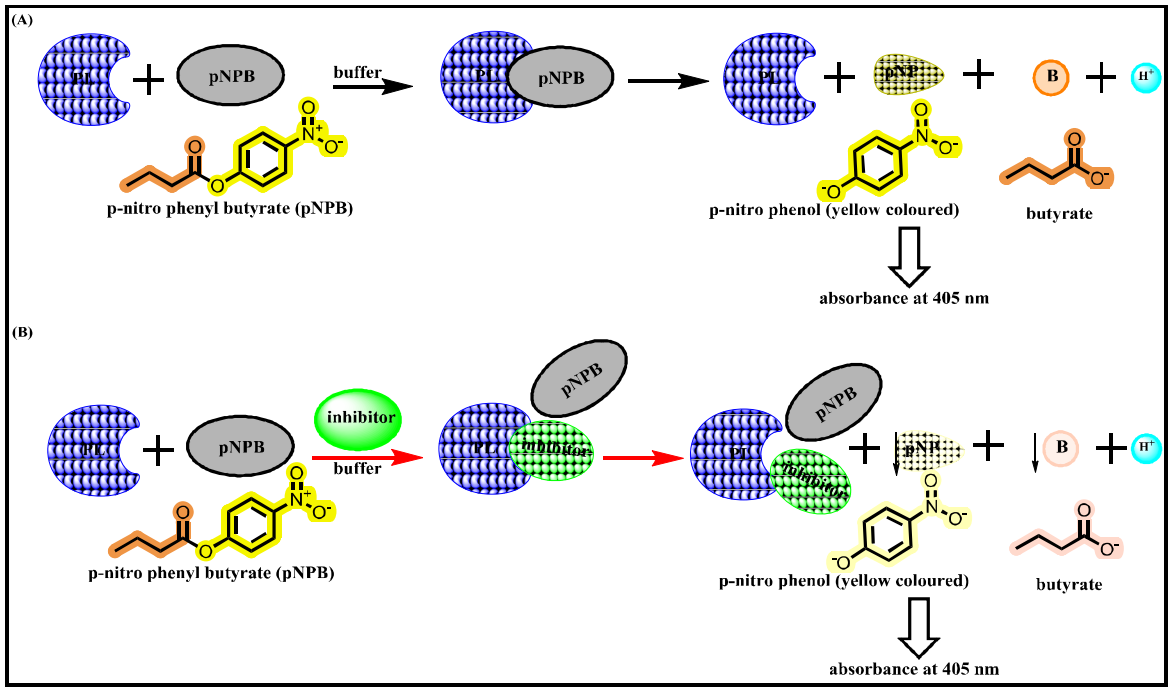


**Figure 17:**Enzymatic hydrolysis of p-nitrophenyl butyrate (p-NPB) in absence and presence of inhibitor. (A) PL catalyzed hydrolysis of p-NPB into p-nitrophenol (p-NB) (B) inhibition of PL catalyzed hydrolysis p-NPB; Low intensity of p-NP and butyrate indicates the presence of strong PL-inhibitor

To study the inhibition mode against the PL, enzyme inhibition kinetics analysis of the phytochemicals/Orlistat was performed. The type of inhibition was deduced from the Lineweaver–Burk (LB) plot. The plot was constructed by performing an *in vitro* PL inhibition

assay, using various concentrations of the phytochemicals/Orlistat that were evaluated against the different substrate concentrations (25, 50, 100 and 200 µM) [2]. Inhibition constant ($K_i$) values were deduced from the Cheng-Prusoff equation [3].

**4.5 Combination studies of extracts**

The extracts that exhibited $IC_{50} \leq 20$ µg/ml were considered for combination studies. The nature of the interaction of Extract 1 with Extract 2 was assessed by two different methods namely Combination Index (CI) and isobologram method, by using a function of the concentration of these extracts/phytochemicals for PL inhibition. The $IC_{50}$ values of Extract 1 and Extract 2 were obtained from the dose–dependent inhibition curve. Concentration of Extract 1 (0, $IC_{50}/8$, $IC_{50}/4$, $IC_{50}/2$ and $IC_{50}$) and Extract 2 (0, $IC_{50}/8$, $IC_{50}/4$, $IC_{50}/2$ and $IC_{50}$) were mixed in ratio of $IC_{50,EXT\ 1}/\ IC_{50,EXT\ 2}$. The above CI method was used to determine the combined inhibition effect on PL (**Figure 18**).

Data analysis was performed using the CompuSyn software to make a qualitative assessment of the effects (synergy, addition, or antagonism) of the composition [4-6]**.**

$$CI = \frac{C_{EXT\ 1,50}}{IC_{50,EXT\ 1}} + \frac{C_{EXT\ 2,50}}{IC_{50,EXT\ 2}} \qquad \ldots . (2)$$

Where, $C_{EXT\ 1,50}$ and $C_{EXT\ 2,50}$ are the $IC_{50}$ value for Extract 1 and Extract 2 respectively in the combination, while $IC_{50,EXT\ 1}$ and $IC_{50,EXT\ 2}$ are the $IC_{50}$ of the individual extracts. If combination shows CI<1, synergistic effect was considered, while for additive effect CI = 1 and for antagonistic effect CI > 1 [7].

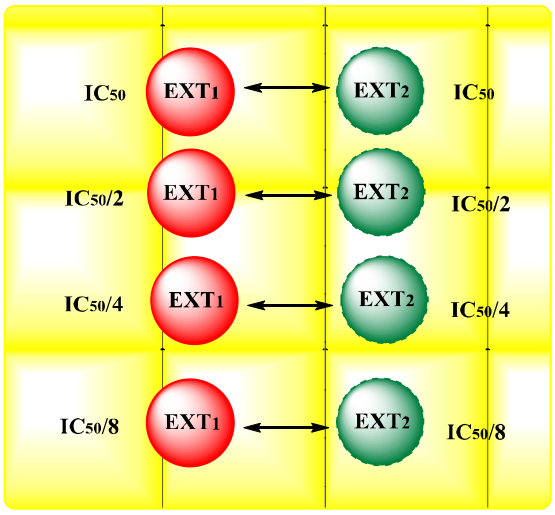


**Figure 18:** Strategy used for the combination experiments

Further, these results were used to determine the isobologram plot. In this method, the $IC_{50}$ values of the Extract 1/Extract 2 were plotted on the *y* and *x*-axis in a two-coordinate plot, corresponding to (0, $IC_{50,EXT\ 1}$) and (0, $IC_{50,EXT\ 2}$), respectively. The line connecting these two points denotes the line of additivity. The concentrations of the two extracts used in the combination to achieve similar effect denoted as ($C_{EXT\ 1}$, $C_{EXT\ 2}$) were marked as (*y*, *x*) coordinate in the same plot. When ($C_{EXT\ 1}$, $C_{EXT\ 2}$) were located below the line of additivity, it was said to be synergistic combination, while those above the line were considered antagonistic. Similar strategy as depicted in **Figure 18** was used for combination experiments for bio-active markers [8,9].

### 4.6 Animal Studies

Based on *in vitro* PL inhibition data, the two most potent compositions were further selected for *in vivo* experiments. Thirteen groups were considered, and each group included 6 animals (**Figure 19**). The activity of each composition was evaluated at 2 dose levels (low & high) [10]. The dose of extracts and their compositions were decided based on the reported literature and amount of bio-active markers present [11-16]. Male Swiss albino mice (22-25g) were purchased from the Central Animal Facility (CAF) of Birla Institute of Technology and Science, Pilani, (BITS, Pilani), Pilani campus, India (CPCSEA Reg number: 417/PO/ReBI/2001/CPCSEA). High fat diet (HFD) mice model was developed for 3 months using a diet composed of 25 % carbohydrates, 23% proteins and 47% fats (**Table 4**) [17]. All the experimental procedures were in compliance with the Institutional Animal Ethics Committee (IAEC) of BITS Pilani (Ref: IAEC/RES/24/17/Rev-1/28/37).

**Table 4**: HFD Composition for development of obese mice model

| Sl no | Ingredients | g/100g |
|---|---|---|
| **1.** | Casein | 20 |
| **2.** | Corn starch | 10 |
| **3.** | Cellulose | 5 |
| **4.** | Sucrose | 10 |
| **5.** | Lard | 40 |
| **6.** | Soybean oil | 7 |
| **7.** | DL- methionine | 3 |
| **8.** | Mineral and Vitamin mix | 5 |
| **9.** | Sodium chloride | 3 |

# CHAPTER 4

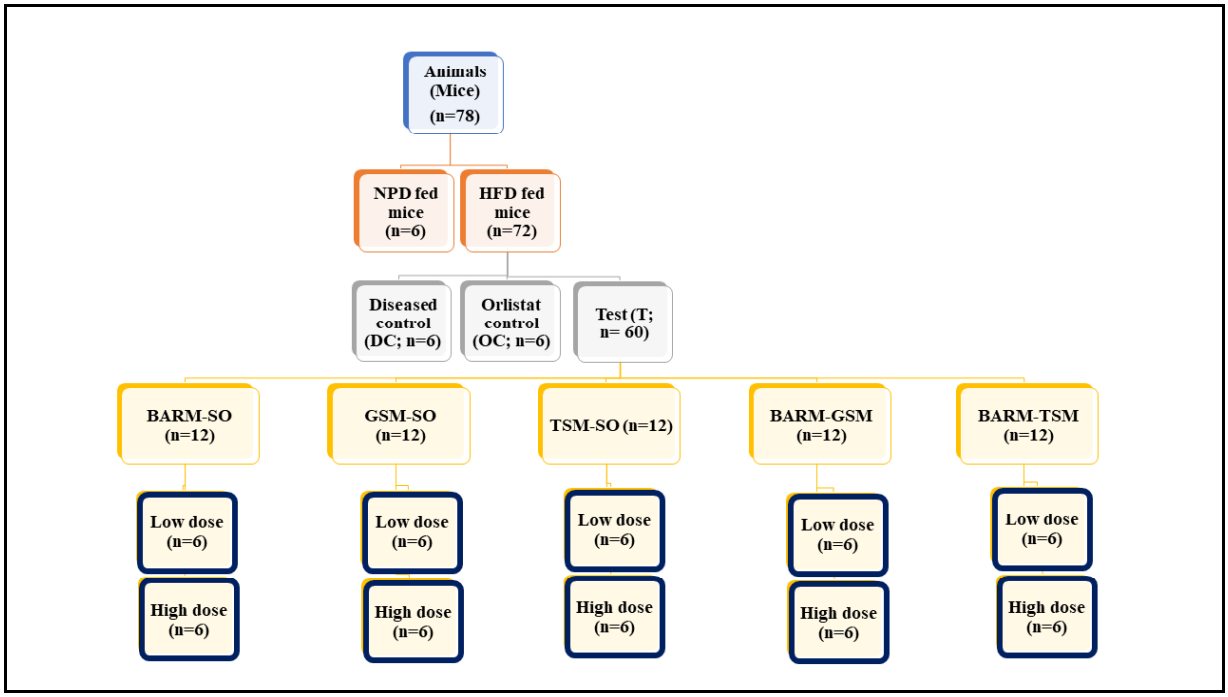


**Figure 19:** Animal groups for efficacy study of compositions

The total duration of the study was 4 months, in which animals were fed with HFD for initial 3 months for the model development followed by 1 month of treatment. Orlistat, Extracts (BARM-SO, TSM-SO, GSM-SO) and their combinations were suspended in water with 0.1 % CMC. Suspension dose of the vehicle, orlistat, extracts and their combinations were administered through oral route regularly in the evening. Body weight was monitered once in every week till 4 months from the commencement of the study. Blood was collected from retro-orbital plexus after every 15 days. Total cholesterol (TC), TG, high density lipoproteins (HDL) in serum were measured after every 15 days using commercially available diagnostic kits (Spinreact S.A.U., Spain). Low density lipoproteins (LDL) and very low-density lipoproteins (VLDL) were calculated using TC, TG and HDL data. Fecal matter was collected from each group and fecal TG were measured. At the end of the study the animals were sacrificed, liver and adipose tissues deposited in form of visceral fat, mesenteric fat, epididymal fat and retroperitoneal fat were collected and weighed (**Figure 20**).

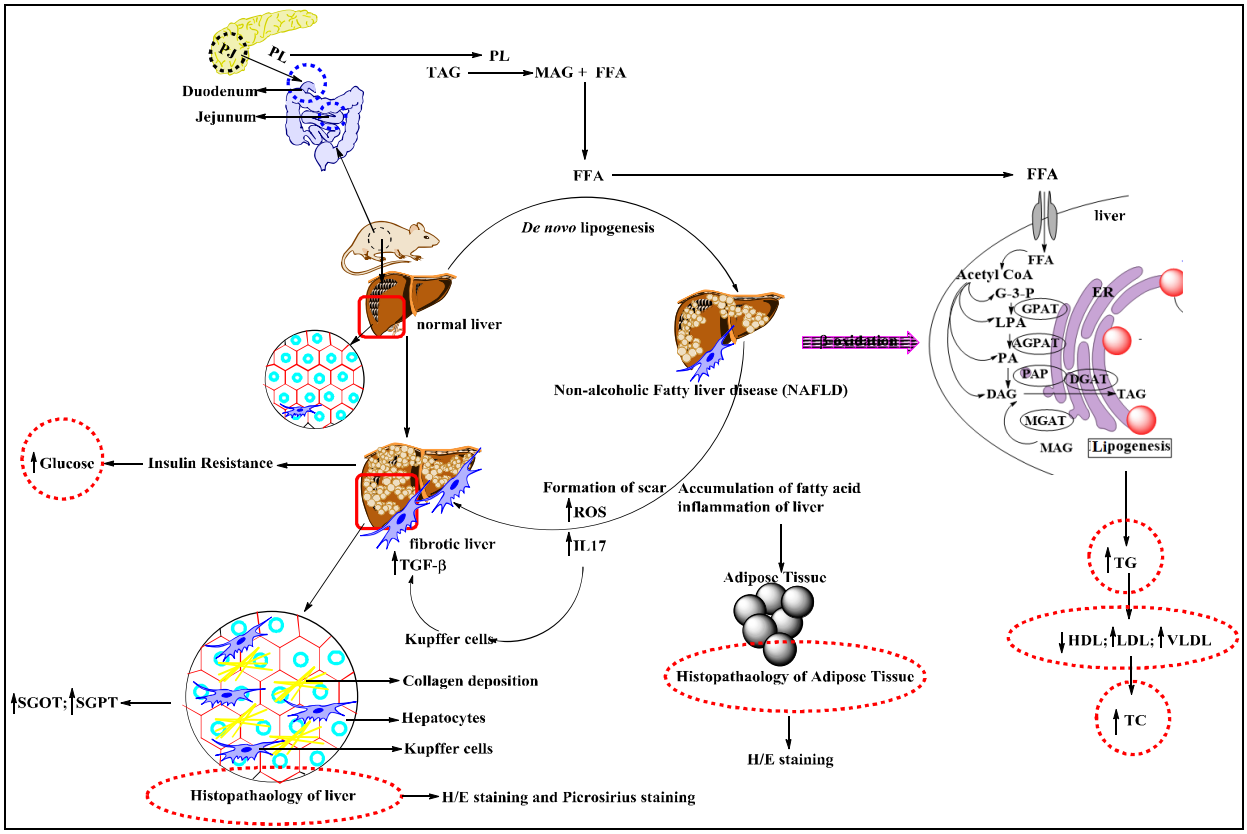


**Figure 20:** Mechanism of development of obesity using HFD

### 4.6.1 Quantification of fecal TG

Since the inhibition of PL is characterized by the excretion of feces rich in TG, the feces of the mice were collected daily during the long-term treatment period, and TG were quantified at the end of every week. The procedure for quantification of fecal TG was performed as per the literature report with minor modifications [18]. Briefly, 1 g of feces was taken in a separatory funnel and subjected to vigorous shaking in 0.15 M NaCl. To this suspension, chloroform/methanol (4:1, *v/v*) was added and the shaking was continued, and the mixture was allowed to separate. The lower organic phase was then collected, filtered and dried *in vacuo*. The obtained TG were then dissolved in 1 ml ethanol, and the quantity of TG was estimated using commercial kit (Spinreact S.A.U., Spain).

### 4.6.2 Histopathological studies

For histological examination, the liver and adipose tissue were fixed in 10% buffered formalin followed by embedding in paraffin. Tissue sections of 5 µm were cut and the sections were stained with Hematoxylin and Eosin (H & E) to assess hepatocytes' ballooning, and neutrophil

infiltration in liver, while the size of adipocytes and hypertrophy formed were assessed in the section [19,20]. Liver tissue sections of 5 µm were cut and the sections were stained with Picrosirius red to determine the amount of collagen deposition in liver [19].

### 4.6.3 Statistical analysis

All the data were represented as mean ± SEM, and the differences were analyzed using two-way analysis of variance (ANOVA) followed by *post-hoc* analysis of Tukey's multiple comparison test to determine significant differences between the groups. Statistical calculations were performed using GraphPad Prism (*v* 5.0). A level of $P < 0.05$ was considered to be statistically significant.

## 4.7 Development of HPLC methods and their validation

**4.7.1 Method 1:** The analysis was performed on Dionex UltiMate 3000 UHPLC (Thermoscientific) with Diode array detector installed with Chromeleon software. The column used was reverse phase $C_{18}$ column (Hypersil Gold, Thermoscientific) of dimension 250 × 4.6 mm with 5µm particle size. The wavelength was set at 210 nm, 272 nm and 434 nm. Total run time was set as 25 min using a gradient mobile phase using different ratios of Acetonitrile: $KH_2PO_4$ buffer {25:75 % *v/v* (0-20 min), and 45:55 % *v/v* (20-25 min)}. The total injection volume for analysis was 20 µl and the flow rate was 1.5 ml/min. The column and sample temperature were set at 27°C and 15°C, respectively.

**4.7.2 Method 2:** The analysis was performed on Dionex UltiMate 3000 UHPLC (Thermoscientific) with Diode Array detector installed with Chromeleon software. The column used was reverse phase $C_{18}$ column (Hypersil Gold, Thermoscientific) of dimension 250 × 4.6 mm with 5µm particle size. The wavelength and the total run time were set at 272 nm and 15 min, respectively using an isocratic mobile phase using 25:75 % *v/v* (Acetonitrile: water with 0.1% ortho-phosphoric acid). The total injection volume for analysis was 10 µl and the flow rate was 1.0 ml/min. The column and sample temperature were set at 35°C and 15°C, respectively.

**4.7.3 Method 3:** The analysis was performed on Dionex UltiMate 3000 UHPLC (Thermoscientific) with Diode Array detector installed with Chromeleon software. The column used was reverse phase $C_{18}$ column manufactured by Waters of dimension 250 × 4 mm with 4µm particle size. The wavelength was set at 342 nm and 271 nm. Total run time was set as 20 min using a gradient mobile phase using different ratios of methanol: water such as 50:50 % *v/v* (0-5 min), 60:40 % *v/v* (5-10 min), 70:30 % *v/v* (10-15 min) and 50:50 % *v/v* (15-20 min). The total

injection volume for analysis was 20 µl and the flow rate was 1.0 ml/min. The column and sample temperature were set at 30 °C and 15 °C, respectively.

**4.7.4 Validation:** The validation of the developed methods was performed as per the US FDA guidelines for Instrumental Precision, Specificity, Sensitivity, Linearity, Precision, Accuracy, and Robustness [21]. Instrumental suitability was evaluated by scanning analytes for seven times (n = 7) at their respective wavelength. The selectivity of the developed method was established by analyzing the sample solutions containing respective analytes (bioactive markers) in relation to interferences from other compounds of the combination of two plants. Signal: noise (S/N) ratio was calculated by considering 0.5 min of noise before and after the signals using the following equation

$$\frac{S}{N} = \frac{2H}{h} \quad \ldots(3)$$

where, H = height of signal and h = height of noise. The sensitivity of the developed method was established by determining the Limit of Detection (LOD) and Limit of Quantification (LOQ) by using the following equation:

$$LOD = \frac{conc.\ of std}{S/N} \times 3.3 \quad \ldots(4)$$

$$LOQ = \frac{conc.\ of std}{S/N} \times 10 \quad \ldots(5)$$

The linearity was evaluated at different concentration levels of standard by application of 20 µl of the respective stocks. The experiment was performed seven times (n =7), and the calibration curves were obtained by plotting peak area (Y-axis) *Vs*. concentration of analyte spiked (X-axis). Precision and accuracy were evaluated Intra-Day (n = 3) and Inter-Day (n = 3) at three quality control (QC) levels using low, medium and high concentrations of the calibration curves and the obtained peak areas were used to calculate % RSD (Relative Standard Deviation) and % biasness. The robustness of the method was evaluated by implementing small changes in column temperature, flowrate and change in pH of mobile phase. Each experiment was carried out thrice (n =3) and % RSD was calculated [22].

### 4.8 Chromatographic analysis of bio-active markers and *in vitro* stability studies

Stability in simulated gastric fluid (SGF) is one of the major concerns that may alter the *in vivo* parameters of the extracts/phytochemicals. The stability studies of compositions in SGF were carried out as per a previously reported method[23]. In brief, SGF was prepared by dissolving 2 g of NaCl and 3.2 g of pepsin in 80 ml of 1M HCl that was finally made up to 1000 ml with adjustment of final pH to 1.2. Extract (1ml of 100 µg/ml) was added to 9 ml of simulated fluid and the intactness of the phytochemicals was evaluated (2h). After 2h, aliquot of 1ml of suspension was added to 3 ml of acetonitrile for the precipitation of protein. The sample was centrifuged; supernatant was collected and analyzed using their respective HPLC method (1-3). After identification of stable phytochemicals in SGF they were isolated and characterized from their respective plant extract and used for further studies. Quantification of these phytochemicals in their respective composition was performed using validated HPLC methods (1-3).

### 4.9 Interaction between PL and isolated bio-active markers using fluorescence spectroscopy

Fluorolog-3 Horiba Yvon fluorescence spectrophotometer with slit width of 1.5 nm was used for fluorescence measurement. The fluorescence intensity of PL was measured using $\lambda_{ex}$ of 295 nm and ranges of $\lambda_{em}$ (300-500 nm) using a cuvette of 1.0 cm pathlength, in presence or absence of bio-active markers. PL (5 mg/ml) was prepared in 2.5 mmol of Tris buffer (Sigma Aldrich Pvt Ltd) and 2.5 mmol of NaCl. Different concentrations of bio-active markers (0.15625- 20 µg/ml) were mixed along with 10 µM of PL and their fluorescence intensity was recorded. Further, the fluorescence intensity of these solution was recorded at different temperatures (298, 303, 310K) to analyze the quenching mechanism of these inhibitors. Linear Stern-Volmer plot indicated the quenching mechanism of the inhibitors. It was calculated using the following equation:

$$\frac{F}{F_0} = 1 + K_{sv}[Q] = 1 + k_q \tau_0 [Q] \quad \ldots (6)$$

where $F_o$ and F are the fluorescence intensities in the absence and presence of the quencher, $k_q$ is the bimolecular quenching rate constant, [Q] is the concentration of the quencher and $K_{sv}$ is the Stern-Volmer quenching constant. In both cases, the bimolecular quenching constant $k_q$ were calculated by the ratio between $K_{SV}$ and $\tau_0$. Since porcine PL sequence is 87% homologue to human PL and all the tryptophan residues are conserved, the $\tau_0$ was considered in this work to be

equal to that of human PL (1.59 ns). Number of binding sites were calculated using the following equation:

$$\log\frac{(F_0 - F)}{F} = \log K + n[Q] \quad \ldots(7)$$

where [Q] is the analytical quencher concentration, K is binding constant, $F_0$ is the fluorescence intensity of the protein in the absence of bioactive marker and $F$ is the protein fluorescence at a given bioactive marker concentration.

The type of binding force between bioactive markers and PL were determined by calculating the main thermodynamic parameters. Because the temperature fluctuation was small throughout the experiment and the enthalpy change ($\Delta H^\circ$) was affected by temperature, $\Delta H^\circ$ was unchanged. The values of $\Delta H^\circ$, entropy change ($\Delta S^\circ$) and free energy change ($\Delta G^\circ$) were obtained according to the following formulas

$$\frac{\Delta H^\circ}{2.303RT} + \frac{\Delta S^\circ}{2.303RT} = \log K + n[Q] \quad \ldots(8)$$

$$\Delta H^\circ + T\Delta S^\circ = \Delta G^\circ \quad \ldots(9)$$

In the above formula, R represents a gas constant (8.314 J/mol/K), and K denotes a binding constant [3,24].

### 4.10 *In silico* studies

Molecular docking studies of the bioa-ctive markers were performed using Molegro Virtual Docker 6.0 (CLC bio). Prior to the docking, the structures of the bio-active markers and Orlistat were drawn using Chemdraw 2D module. The energy minimized structures of the bio-active markers and Orlistat were obtained using MM2 force field in Chemdraw 3D module of ChemBioOffice v12 (PerkinElmer, USA), and were docked into the preprocessed crystal structure of human PL (PDB ID: 1LPB) retrieved from RCSB PDB Data bank [25,26]. The validation was performed by redocking the co-crystallised ligand (subjected to energy minimization) into the active site of PL. The redocked pose was deviated from the co-crystallised pose by an RMSD of 0.99 Å. The grid resolution was 0.30 Å and radius 20 Å. The coordinates were X: 7.86; Y: 24.91 and Z: 55.11, respectively. The obtained docked poses of the bio-active markers and Orlistat were analysed for their MolDock scores, while the various interactions

exhibited by the bio-active markers and Orlistat with the active site were visualised in Discovery Studio Visualizer (Dassault Systemes Biovia, USA).

Molecular dynamics (MD) simulation of bio-active markers and Orlistat in complex with PL was performed using Desmond package (Schoringer LLC). OPLS3e force field was applied during the MD run, and the topology of the bio-active markers and Orlistat was generated. Prior to the initiation of MD, stabilization of the system to 310 K for 50 ns for bio-active markers (100 ns for orlistat), using canonical NPT and NVT ensembles were performed. Discovery Studio 4.5 visualizer (Accelrys, USA) was used to depict graphical representations of the complex.

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