CHAPTER 10

10. Conclusion & Future Perspectives

Obesity is one of the major global issue due to which the entire world is being affected. It is a metabolic syndrome that give rise to various serious co-morbidities such as IR, diabetes mellitus, hypertension, dyslipidemia, and atherosclerosis. As a result, it is referred to as 'slow poison'. There are various anti-obesity agents available in the market that act *via* various mechanisms such as digestive enzyme inhibition (orlistat, cetlistat) or suppressing appetite (fenfluramine) or suppressing the absorption of nor-epinephrine and serotonin (sibutramine), etc. Despite their potent anti-obesity action, many of these drugs have been withdrawn from the market by USFDA (e.g., sibutramine and fenfluramine) due to their cardiotoxicity. Orlistat, a potent PL inhibitor, has been used clinically over the past two decades for treatment of obesity. However, a long-term exposure to this drug has resulted in severe adverse events including hepatotoxicity and acute pancreatitis, highlighting the urge to develop safer and effective anti-obesity drugs/therapeutics.

Natural Products have served as a vast reservoir of biologically active chemical entities, representing an effective source in the treatment of various diseases and disorders, while maintaining tolerable side effects. Traditional systems of medicine have also contributed to the drug discovery program. The *Ayurvedic* literature *Sarangdhar Samhita*, has highlighted the concept of polyherbalism to achieve greater efficacy. The efficacy of polyherbal compositions and their reduced adverse effects have prompted many researchers to develop numerous such compositions for the treatment of various disorders. The present thesis work revolved around the preclinical treatment of obesity using the concept of polyherbalism. A set of 17 commonly available Indian medicinal plants were selected, extracted using three different techniques, and each extract was screened for their PL inhibitory effect. Total 153 extracts were screened. Out of 153 extracts screened for PL inhibition; 9 extracts showed potent activity ($\leq$ 20 μg/ml). It included TAM-SO, CZW-SO, OGM-SO, GSM-SO, AVH-SX, BARM-SO, AMM-SO, PLM-SO and TSM-SO. Further, the active extracts were combined (2 extracts at a time) to obtain four best compositions including BARM-GSM, BARM-TSM, GSM-TSM and AMM-PLM. Synergistic interactions between the extracts were understood using combination index (CI) and isobologram methods. To determine the CI value, CompuSyn software was used. All the compositions were found to be synergistic apart from the compositions of AMM-PLM that showed additive effect. *In vivo* studies in Swiss albino mice revealed that BARM-GSM and BARM-TSM were successful in

reduction of HFD induced obesity. Presence of TG in feces confirmed that these polyherbal compositions exhibited anti-obesity potential *via* PL inhibition. Histopathological studies concluded that the above compositions were effective in dose dependent manner, where in the high dose significantly reduced the size and number of adipocytes similar to the normal control. Moreover, the histopathology data also signified the reduction in collagen content, neutrophil infiltration and hepatic ballooning that was caused due to excess exposure of HFD.

Further, the compositions namely BARM-GSM and BARM-TSM were subjected to SGF. The results suggested the stability of the major phytochemicals of the above compositions. From the *in vitro* results in SGF media, the identification of berberine and palmatine and a hydrolysis product gymnemagenin in SGF treated BARM-GSM was observed. However, presence of ECG and EGCG along with berberine and palmatine were identified after SGF digestion of BARM-TSM. From *in vitro* findings, berberine, palmatine, gymnemagenin, ECG and EGCG and their combinations (berberine-gymnemagenin, palmatine-gymnemagenin, berberine-ECG, palmatine-ECG, berberine-EGCG, and palmatine-ECG) exhibited moderate to potent PL inhibition. In case of GSM-TSM quantification of ECG and EGCG was done. Gymnemagenin-ECG and gymnemagenin-EGCG were also found to exhibit synergistic effect.

Interaction of above bio-active markers with PL were confirmed using mechanistic studies including enzyme kinetics, fluorescence and *in silico* studies. Thus, from the results of enzyme kinetics and fluorescence studies, it could be postulated that berberine and gymnemagenin behaved as non-competitive inhibitor and there is a high chance of their binding at the site II of PL (nearer to the active site). From the molecular docking and dynamic studies ECG and EGCG were found to bind to the active site as they behaved as competitive inhibitor (evident from enzyme kinetic studies). Palmatine showed mixed inhibition of PL and was found to have unstable interactions with the site I and II of PL as confirmed by molecular dynamics studies.

The fluorescence studies also revealed involvement of both static and dynamic quenching during complex formation between these bio-active markers and PL. The PL complex with these bio-active markers showed enthalpy driven exothermic process that is due to the formation of van der Waals force and hydrogen bonding. Thus, it can be concluded that ECG and EGCG formed more stable complex as compared to berberine, palmatine and gymnemagenin.

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AMM-PLM showed additive effect on PL inhibition. Bio-active markers in AMM-PLM such as alloimperatorin, piperine and pellitorine were identified in the composition. Alloimperatorin showed synergy when combined with piperine. Antagonistic effect was observed when alloimperatorin was combined with pellitorine. Mechanistic studies such as enzyme kinetics revealed that alloimperatorin and piperine showed non-competitive inhibition, while uncompetitive inhibition was observed in case of pellitorine.

10.1 Specific conclusion

10.1.1 Chapter 1

- Obesity is a metabolic disorder associated with other diseases such as diabetes mellitus, atherosclerosis, dyslipidemia and high blood pressure
- Obesity is defined in terms of BMI (if BMI is greater than equal to 30, individual in considered obese)
- Waist-to-Hip Ratio is used to measure abdominal obesity (if WHR is in between 0.85 – 0.95, abdominal obesity is observed)
- Adipose tissue and liver are the two organs involved in lipid metabolism; adipose tissue deposits fat, while liver maintains homeostasis
- Other factors involved in obesity are polymorphism in genes, disbalance in hormones and other environmental factors (stress & craving for western diets)
- There are various peripheral targets for treatment of obesity, of which PL is the safest target

10.1.2 Chapter 2

- Comprehensive literature review was performed on the following topics such structure of PL, role of NPs in drug discovery, different NPs used as lead molecules in treatment of obesity and its associated diseases, different classes of NPs/extracts and patented compositions inhibiting PL
- The review highlighted that there are many potential gaps existing in research regarding the systematic approach in the development of PL inhibitory compositions against obesity

10.1.3 Chapter 3

- On considering the gaps in existing research aim and objectives were proposed

10.1.4 Chapter 4

- The materials including chemicals, instruments, software's, and animals used in the studies were detailed
- The method of extraction, PL inhibition assay, development of composition, *in vivo*, chromatgraphic anlysis, fluorescence, kinetics and *in silico* studies were mentioned
- New HPLC methods (1-3) were developed and validated using USFDA guidelines

10.1.5 Chapter 5

- One hundred and fifty-three extracts were screened for preliminary PL inhibition
- Out of one hundred fifty-three extracts, nine extracts showed potent PL inhibition ($IC_{50} \leq$ 20 µg/ml) namely, TAM-SO (1.64 µg/ml), CZW-SO (1.89 µg/ml), OGM-SO (7.76 µg/ml), GSM-SO (7.91 µg/ml), AVH-SX (9.57 µg/ml), BARM-SO (11.10 µg/ml), AMM-SO (13.02 µg/ml), PLM-SO (14.10 µg/ml) and TSM-SO (20.00 µg/ml)
- Advantages of ultrasonication and soxhlation over maceration were discussed in detail

10.1.6 Chapter 6

- Using the nine potent extract combination studies were performed; ${}^{9}C_{2}$ (36) compositions were further screened for PL inhibition
- Out of 36 compositions, 4 compositions (BARM-GSM; 80.35% inhibition, BARM-TSM; 70.86% inhibition, GSM-TSM; 60.21% inhibition) and AMM-PLM (59.77% inhibition) exhibited synergistic activity for PL inhibition
- cIC_{50} of BARM-GSM (5.05 ± 1.05 µg/ml) and BARM-TSM (10.63 ± 0.59 µg/ml) were lower compared to GSM-TSM (11.49 ± 0.45 µg/ml) and AMM-PLM (13.55 ± 0.21µg/ml), hence the former compositions (BARM-GSM & BARM-TSM) were further evaluated using HFD induced obesity in Swiss albino mice
- Total duration of the study was 4 months (3 months for model development followed by 1 month of treatment)
- Dose of the extracts GSM-SO and TSM-SO were considered from previous literatures, while for BARM-SO dose was considered based on amount of bioactive marker
- BARM-GSM and BARM-TSM dose (High and low) were based on the ratio of two extracts at which they produced synergy (1.5:1 and 1:2, respectively)

- Both high dose of BARM-GSM (650 mg/kg) and BARM-TSM (750 mg/kg) showed synergistic effect in *in vivo* studies, wherein body weight and lipid profile were reduced when compared with DC group
- Dose-dependent significant changes such as reduction of collagen deposition, hepatic ballooning and neutrophil infiltration were observed in the treatment group with BARM-GSM & BARM-TSM (high dose) as compared to DC group
- Dose-dependent significant reduction in the size of the inflamed and hypertrophic adipocytes compared to the DC group, indicated the effectiveness of BARM-GSM & BARM-TSM in obesity treatment
- Significant presence of fecal TG in stool samples collected from mice treated with BARM-GSM & BARM-TSM confirmed the anti-obesity effect through PL inhibition

10.1.7 Chapter 7

- Detailed literature for the plants namely *B. aristata*, *G. sylvester*, *T. sinesis*, *A. marmelos* and *P. longum* and their phytochemcials was discussed
- Isolation of sustained phytochemicals in SGF media using column chromatography, prep-TLC and semi prep-HPLC was performed
- Structures of isolated phytochemicals were confirmed using NMR spectroscopy and Mass spectrophotometery and comparision with inhouse standards

10.1.8 Chapter 8

- HPLC methods (1-3) were developed for all four compositions namely BARM-GSM, BARM-TSM, GSM-TSM and AMM-PLM
- Phytochemicals that sustained in SGF media for BARM-GSM and BARM-TSM were identified using Method 1 and 2
- Berberine, palmatine, gymnemagenin, ECG, EGCG, were identified in the BARM-GSM and BARM-TSM
- Using HPLC method 3, alloimperatorin, piperine and pellitorine were identified from AMM-PLM
- The above HPLC methods (1-3) were validated as per USFDA guidelines using these bio-active markers

- The validated methods were used to quantify these isolated bio-active markers in the compositions.
- In BARM-GSM, the %yield of palmatine and berberine were found to be 10.18 and 18.63 %, respectively
- After SGF digestion of BARM-GSM, the amount of gymnemagenin formed was 0.22% w/w, berberine (88.08%) and palmatine (87.14%) were found to be stable in SGF media
- BARM-TSM contained 2.5% ECG, 5.7% EGCG, 18.3% of berberine and 8.2% of palmatine.
- ECG (76.96 %), EGCG (82.96 %), berberine (86.88 %) and palmatine (83.66 %) in BARM-TSM were found to be stable in SGF media
- GSM-TSM revealed that it contained 3.25% of ECG and 4.13 % of EGCG.
- AMM-PLM contained 5.65 % w/w of alloimperatorin, 6.73% w/w of piperine and 4.34% w/w of pellitorine

10.1.9 Chapter 9

- PL inhibition assay of the phytochemicals were assessed and berberine (IC_{50} 28.84 ± 0.45 μM), palmatine (IC_{50} 37.16 ± 1.21μM), gymnemagenin (IC_{50} 7.04 ± 0.32μM), ECG (IC_{50} 12.75 ± 0.26 μM), EGCG (IC_{50} 5.84 ± 0.16 μM), alloimperatorin (IC_{50} 27.75 ± 0.67 μM), piperine (IC_{50} 20.08 ± 0.60 μM) and pellitorine (IC_{50} 18.64 ± 0.78 μM) were found to be bio-active markers
- Further these bio-active markers from different extracts were subjected to combination studies; ten combinations (gymnemagenin- berberine, gymnemagenin- palmatine, ECG-berberine, ECG- palmatine, EGCG- berberine, EGCG- palmatine, gymnemagenin-ECG, gymnemagenin-EGCG, alloimperatorin-piperine and alloimperatorin-pellitorine were evaluated using PL inhibition assay
- All the above combinations exhibited synergistic effect except alloimperatorin-pellitorine combination that showed antagonistic effect
- Enzyme kinetics, fluorescence studies and molecular simulation and dynamics were performed for the bio-active markers and orlistat
- Kinetics studies revealed berberine, gymnemagenin, alloimperatorin and piperine exhibited non-competitive inhibition; ECG, EGCG and orlistat showed competitive

inhibition; Palmatine showed mixed competitive inhibition, while pellitorine behaved as uncompetitive inhibitor

- From the molecular docking and dynamic studies, it was concluded that ECG and EGCG possessed highest binding affinity with the active site (site I) of PL, while berberine and gymnemagenin had affinity towards the putative site II amongst bioactive markers.
- ECG and EGCG interacted with Phe 77, Try 114, Ser 152, Phe 215, and Arg 256 (configuration of Site I)
- Gymnemagenin formed a strong alkyl bond with Trp 252 that is present on lid domain of the active site, while berberine forms strong conventional hydrogen bond with Arg 256 (residue near to Trp 252)
- The fluorescence studies revealed static and dynamic quenching were involved during complex formation between these bio-active markers and PL
- The PL complex with these bio-active markers showed enthalpy driven exothermic process that was due to the formation of van der Waals force and hydrogen bonding

10.2. Future Perspectives

The thesis work focuses mainly on *in vitro, in silico* and *in vivo* studies for development of anti-obesity compositions. Following are the future prospects of the current work.

- The study evaluates the extract-based compositions using HFD mice model. Further anti-obesity potential of the bio-active markers identified from the compositions can be evaluated using animal models. This study can further stimulate investigation of other commonly used antiobesity plants and their bio-active secondary metabolites
- Clinical trials can be performed for final validation
- After the clinical trials, these compositions can be transformed into different formulations such as tablets, capsules, etc. to commercialize these in the form of phytopharmaceuticals or nutraceuticals.
- The phytochemicals in the compositions were identified using HPLC. Sensitive hyphenated techniques such as LC-MS/MS can be used for in-depth understanding and identification of bio-transformed secondary metabolites.
- *In silico* interactions of bio-active markers and PL were performed using the active site. The conceptof active site is true for those bio-active markers that behaved as competitive

inhibitors. In case of non-competitive inhibitors, the concept of allosteric binding was implied. To identify such allosteric sites, detail studies using sophisticated techniques such as X-ray crystallography and others need to be used for PL. Thus, further investigation on effect of binding of bio-active markers to two or more sites on PL for synergy effect is required.