Abstract

Metabolic syndrome refers to biochemical process that involves obesity, blood pressure elevation, increased triglyceride, cholesterol and glucose levels with decreased HDL levels. Obesity can be defined as a body state with excessive accumulation of body fat. In modern days, obesity has greatly increased due to dietary problems and lack of physical activity. Obesity may lead to other degenerative diseases such as diabetes mellitus, hypertension, coronary heart disease, hyperlipidemia, fatty liver, atherosclerosis, tumor and cancer. Currently marketed drugs for obesity have major addiction problems and other side effects which call for the discovery of newer and safer anti-obesity therapeutics.

Natural products (NPs) especially those containing fibers, polyphenols, sterols, and alkaloids play a safe and effective role in the treatment of obesity. NPs such as saponins, alkaloids, vitamins etc from natural origins exhibit lipid lowering action. In general, NPs with potential action in treatment of obesity act as a general body cleanser, regulate metabolism, dissolve fat in the body, help to eliminate craving of food, stimulate glandular secretions etc. A promising future of NPs with no intrinsic side effects on long term treatment has fostered rapid development in these multifunctional agents for prevention and treatment of obesity. Hence, polyherbal anti-obesity compositions have gained a lot of attention. These compositions include addition of two or more phytochemcials or extracts. However, the major challenge that remains is in understanding of the mechanisms behind their anti-obesity properties and lack of systematic approach in their development.

In order to address these gaps in the exsisting stratergy for development of anti-obesity compositions, a pool of 17 plants was selected based on their previous reports for anti-obesity potential. They were collected/procured, authenticated, shade dried, pulverized, and sieved through BSS no 10#. Extract of the plant materials were prepared by various extraction methods (soxhlation, decoction, ultrasonication and maceration) and using different solvents such as hexane, methanol and water. These prepared 153 extracts were dried using rotary evaporator and screened for *in vitro* pancreatic lipase (PL) inhibition. From the *in vitro* results, plant extracts with potent PL inhibition (IC_{50} less than 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 and 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 were selected.

These active extracts were used in the development of the compositions using two extracts at a time. CompuSyn and isobologram approach were used to determine the synergistic mechanism. $^{9}C_2$ (36) combinations were screened for *in vitro* PL inhibition. Only 4 compositions namely BARM-GSM {BARM-SO & GSM-SO}, BARM-TSM {BARM-SO & TSM-SO}, GSM-TSM {TSM-SO & GSM-SO} and AMM-PLM {AMM-SO & PLM-SO} showed synergistic mechanism. cIC_{50} of BARM-GSM (5.05 ± 1.05 µg/ml) and BARM-TSM (10.63 ± 0.59 µg/ml) were lesser compared to GSM-TSM (11.49 ± 0.45 µg/ml) and AMM-PLM (13.55 ± 0.21µg/ml). Therefore, the best two compositions namely BARM-GSM and BARM-TSM were selected for *in vivo* experiments. The animals (male Swiss albino mice) were divided into 13 groups (n=6). All the 12 groups were fed with HFD for three months to induce obesity. After the development of obesity, the treatment was started and continued for next one month until the animals recovered to their normal conditions. HFD was continued during the treatment period.

In vivo studies disclosed that these compositions were successful in reduction of obesity. Presence of Triglyceride (TG) in feces confirmed that these polyherbal compositions exhibited anti-obesity potential *via* PL inhibition. Significant changes have been observed in fecal TG levels when BARM-GSM-HD and BARM-TSM-HD treated groups were compared to DC and OC groups. Significant reduction in body weight, decrease in serum total cholesterol and TG levels was observed in the treatment groups when compared to the disease control (DC) group. Increase in HDL levels signified the recovery of obese animals to their normal conditions. Histopathological identification of adipocytes showed that the treatment is effective as the size of the tissue reduced as compared to DC group. Moreover, the histopathology data of liver showed the reduction in collagen, neutrophil content and hepatic ballooning that was caused due to excess deposition of fat after exposure to HFD. Significant changes ($P<0.05$) were observed such as reduction of hepatic ballooning and neutrophil infiltration in the treatment groups (BARM-GSM-HD & BARM-TSM – HD) when compared with OC group.

Furthermore, chromatographic analysis of these compositions was performed. Isolation of phytochemicals from respective extracts of the active compositions was carried out using various techniques such as column chromatography, preparative thin layer chromatography (prep-TLC) and semi-preparative HPLC. Desired phytochemicals to be isolated were chosen based on their stability in *in vitro* simulated gastric fluid. The phytochemicals were identified using HPLC (R_t and λ_{max}) and characterized using mass, NMR spectroscopy and comparison with laboratory

standards. The phytochemicals were identified to be berberine, palmatine, gymnemagenin, ECG, EGCG, alloimperatorin, piperine and pellitorine.

The compositions BARM-GSM and BARM-TSM were exposed to simulated gastric media (pH =1.2). Berberine, palmatine, ECG, EGCG, and gymnemagenin were identified in SGF treated BARM-GSM and BARM-TSM. Gymnemagenin was not observed in the HPLC chromatogram of the composition BARM-GSM and GSM-TSM as gymnemic acids (the major compounds) in GSM-SO convert into their genin form only after exposure to low pH. Alloimperatorin, piperine and pellitorine were identified in the composition AMM-PLM. Quantitaive analysis of all the phytochemicals were done using newly developed HPLC methods. Validation of these methods were performed using USFDA guidelines.

Using HPLC method 1, phytochemicals of BARM-GSM and GSM-TSM were quantified. In BARM-GSM, the % yield of palmatine and berberine were found to be 10.18 and 18.63 % respectively, while GSM-TSM revealed that it contained 3.25% of ECG and 4.13 % of EGCG. Significant changes were observed when the BARM-GSM was exposed to SGF media of pH 1.2. Berberine and palmatine were found to be stable in the media, while a peak at 9.417 min was detected [52]. Using the previous reported literature, R_t and λ_{max}, the peak was understood to be of gymnemagenin. This peak was further confirmed by comparison with standard gymnemagenin. Presence of gymnemagenin confirmed the digestive instability of gymnemic acid in BARM-GSM in low pH. The amount of berberine and palmatine sustained in SGF media were recovered to be 88.08% and 87.14%, respectively. The % yield of gymnemagenin formed after *in vitro* digestion was found to be 0.22% w/w. Using HPLC method 2 was used to validate the bio-active markers of BARM-TSM. The composition contained 2.5% ECG, 5.7% EGCG, 18.3% of berberine and 8.2% of palmatine. When BARM-TSM was exposed to SGF medium of pH 1.2, ECG (76.96 %) exhibited some level of digestive instability as compared to EGCG (82.96 %), berberine (86.88 %) and palmatine (83.66 %). HPLC method 3 validated the bio-active markers of AMM-PLM. AMM-PLM contained 5.65 % w/w of alloimperatorin, 6.73% w/w of piperine and 4.34% w/w of pellitorine.

Further PL inhibition assay of the above phytochemicals were performed. The IC_{50} of berberine, palmatine, gymnemagenin, ECG, EGCG, alloimperatorin, piperine and pellitorine were found to be 28.84 ± 0.45 μM, 37.16 ± 1.21μM, 7.04 ± 0.32μM, 12.75 ± 0.26 μM, 5.84 ± 0.16 μM, 27.75 ±

0.67 µM, 20.08 ± 0.60 µM and 18.64 ± 0.78 µM, respectively. These phytochemicals were referred as bio-active markers as they exhibited moderate to potent PL inhibition.

After the confirmation of the presence of the bio-active markers before and after the digestion, synergistic combination amongst these bio-active markers were developed using CI and isobologram methods. All the combinations (CI < 1) were found to be synergistic except in case of alloimperatorin-pellitorine for which the CI value was found to be 1.07 at (½ IC_{50} alloimperatorin + ½ IC_{50} pellitorine).

Further, the interactions of these bioactive markers with PL were confirmed using different mechanistic studies. The enzyme kinetic studies revealed that berberine (K_i = 18.55 µM), gymnemagenin (K_i = 3.34 µM), alloimperatorin (K_i = 13.16 µM) and piperine (K_i = 9.86 µM) behaved as non-competitive inhibitor, whereas ECG (K_i =6.09 µM), EGCG (K_i =2.77 µM) and Orlistat (K_i = 0.87) possessed competitive inhibition. Pellitorine (K_i = 8.83 µM) behaved as uncompetitive inhibitor and palmatine (K_i = 23.91 µM) showed mixed inhibition. The fluorescence studies suggested that all bio-active markers formed complex with PL in a spontaneous manner with enthalpy driven process being exothermic in nature. Thus, from the results of enzyme kinetics and molecular docking studies, it could be postulated that berberine (-117.55 Kcal/mol) and gymnemagenin (-126.057 Kcal/mol) behaved as non-competitive inhibitor, there was a high chance of their binding at the putative site II of PL (nearer to the active site). ECG (-142.54 Kcal/mol) and EGCG (-146.23 Kcal/mol) interacted with the active site as they behaved as competitive inhibitor. From the molecular dynamic studies that were carried out for 50 ns, it can be concluded that all the ECG and EGCG showed stable interaction with the amino acids in the active site (site I), while berberine and gymnemagenin showed stable interaction with the amino acid near to the lid-domain of PL (site II). The stability of palmatine was unachieved as it behaved as mixed inhibitor i.e. it showed both competitive and non-competitive mechanisms. The MolDock score of orlistat was found to be -147.442 and had major interactions with the amino acid residues of the active sites such as Phe 77, Ser 152, Leu153, Tyr 114, Arg 256 and Trp 252. Orlistat was found to form a stable complex with the enzyme for 100 ns

In conclusion, the thesis resulted in development of the compositions namely BARM-GSM and BARM-TSM that were efficient in the treatment of HFD induced preclinical obesity.