Effect of *Macrotyloma uniflorum* on antiobesity in rats fed with a high fat diet

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1. Introduction

Obesity is a universal health burden develops from an inequity between food consumption and energy disbursement which causes excessive deposition of fat in adipose tissue, liver tissue, muscle, pancreatic islets and other organs involved in metabolism results in dyslipidaemia, glucose intolerance, coronary heart disease, diabetes, hypertension, non-alcoholic fatty liver disease and cancer (Isabelle et al., 2017). Globally, 600 million people are obese and 19 billion adults are overweight (Jian Bing et al., 2016). Fat absorption process is mediated by pancreatic lipase (PL) and militarization of fat stored in adipose tissues is mediated by triglyceride lipase (TGL) (Rudolf et al., 2012). Lifestyle modification and high energy diet have increase the incidence of obesity (Hasani et al., 2013). There are several antiobesity drugs are available, however, they have perilous side effects and hence medicinal plant including crude extracts and isolated compound from plant can be used to induce weight loss and prevent diet induced obesity (Matson and Fallon, 2012). The potential of natural products against obesity is still largely unexplored and can be an excellent alternative for the safe and effective antiobesity drugs from natural origin.

*Macrotyloma uniflorum* traditionally used as an antiobesity natural food supplement’s in India. It belongs to the family Fabaceae and has been used in ethnomedicine for treating haemorrhoids, tumours, bronchitis, cardiopathy, nephrolithiasis, urolithiasis, splenomegaly, strangury, hiccough, ophthalmopathy, verminosis, kidney stones, inflammation, and liver-related abnormalities (Bigoniya et al., 2014). As an effort to evaluate scientifically on the ethanolic extract of *M. uniflorum* leaves (EEMUL), ethanolic extract of *M. uniflorum* seeds (EEMUS), and ethanolic extract of *M. uniflorum* seeds and leaves combination (EESLC) against obesity. The preliminary investigation was carried out on in vitro inhibitory activity of fractions against PL. The potent PL inhibitor fractions was further characterised by in vivo anti-obesity including food intake, body weight, blood serum lipid profile and hepatoprotective potential on high fat diet (HFD) induced male albino Wistar rats.

2. Materials and methods

2.1. Plant collection and extraction

*M. uniflorum* leaves and seeds were collected from organic cultivation field and authenticated by Rapinat Herbarium, Trichy, Tamil Nadu, India (voucher number VB001) for future reference. The extraction was performed by standard procedure (Sasidharan et al., 2011) using coarse powder of *M. uniflorum* seed, leaves and mixture. 100 g of each coarse powder suspended separately in 300 mL of ethanol. The extract was filter through 420-µl stainless steel filter and excess solvent was removed by rotary evaporator and recovered yields were 22.4% ± 1.25 (leaves), 19.5% ± 2.07 (seed) and 56.8% ± 1.65 (leaf and seed) and all the filtrate was stored at 4°C until further use.

2.2. In vitro pancreatic lipase inhibition assay

An assay of PL activity was performed by standard method, described by Moreno et al. (2003), with some modification. Briefly, different concentrations (250, 500, 1000, and 2000 mg/mL) of EEMUS, EEMUL and EESLC added separately to test tubes contain Tris-HCl buffer and incubated for 3 min at 37°C and then 0.5 mL aliquot of porcine PL (250 mg/mL, type II, Sigma Chemical Co.) was added to each test tube to initiate the reactions. After 30 min of incubation, all the test tube was immersed in boiling water for 2 min to stop the reaction and then cooled. The free fatty
Acid concentration was determined according to manufacture protocol, using commercial kit (NEFA kit, Randox, China).

2.3 Animals

Adult healthy male albino Wistar rats were bred and reared in the Central Animal House, Department of Animal Science, SASTRA University. Weight matched animals (180–200 g) were selected and kept in natural conditions (12 h light/12 h dark). The animals were allowed free access to water and a standard pellet diet (Amrut Animal Feed Laboratory, Pranav Agro Industries Ltd., Bangalore, India). The standard pellet diet contained 21.1% protein, 5.1% fat, 60.0% carbohydrate, 3.9% fibre, 7.9% minerals, and 2.0% vitamins and HFD consist 34.1% of beef tallow along with standard pellet diet. Handling and experimental protocol ethical committee approval was obtained from SASTRA University Institutional Animal Ethics Committee (Reg. No. 376/SASTRA/IAEC/RPP).

2.3.1 Experimental design

EESLC showed potent PL inhibitor when compared to EEMUL and EEMUS. Therefore, EESLC further analysed on In vivo studies. Initially, before the study, all test animals were fed a standard diet. The animals were then divided into six groupings with six rats in each group as follows.

- Group 1 fed the standard pellet diet for 12 weeks;
- Group 2 fed with the HFD for 12 weeks;
- Group 3 fed with the HDF for the first 8 weeks, and then received EESLC (200 mg/kg BW) along with the HFD for the next 4 weeks;
- Group 4 fed with HDF for the first 8 weeks and then received EESLC (400 mg/kg BW) along with the HFD for next 4 weeks;
- Group 5 fed with HDF for the first 8 weeks and then received EESLC (600 mg/kg BW) along with the HFD for the next 4 weeks;
- Group 6 fed with HDF for the first 8 weeks and then received orlistat (10 mg/kg BW) along with the HFD for the next 4 weeks.

After the experimental period, all the rats were anesthetized intramuscularly with ketamine. Blood samples were collected from both control and experimental groups for biochemical analysis.

2.3.2 Biochemical estimations

Aspartate aminotransferase (AST), alanine aminotransferase (ALT) and Alkaline phosphatase (ALP) Gamma-glutamyl transpeptidase (GGT), Triacylglycerol (TG) and total cholesterol (TC) estimation kits were purchased from Sigma-Aldrich, USA. The biochemical parameters were analysed according to manufacturer instruction using auto-analyser (Indiko model-Thermo Fisher scientific, USA). Low-density lipoprotein cholesterol (LDL-C) and very low-density lipoprotein cholesterol (VLDL-C) levels were calculated as prescribed previously (So young et al., 2015; Mauro et al., 2006).

2.4 Statistical analysis

Results are expressed as mean ± S.D. Statistically, all the data were compared using one-way analysis of variance (ANOVA) followed by Duncan’s multiple range test (DMRT) by the SPSS software version 11.5 and p-values <0.05 considered as statistically significant.

2.5 High performance thin layer chromatography (HPTLC)

HPTLC was carried out according to a previous report (Misra et al., 2008). Briefly, 2 μl of EESLC was loaded on 5 mm band length in the 3 × 10 Silica gel 60F254 TLC plate using a Hamilton syringe and CAMAG LINOMAT 5 instrument (Mumbai, India). After the saturation of solvent vapour the sample loaded plate was kept in the TLC twin with mobile phase (ethyl acetate-methanol-water (10:1.35:1)) up to 90 mm. The developed plate was dried in hot air in order to evaporate the solvents and then kept in a photo-documentation chamber (CAMAG REPROSTAR 3) to capture the images in white light, UV 254 nm, and UV 366 nm. After derivatization, the plate was fixed in the scanner stage (CAMAG TLC SCANNER 3) and was scanned at 500 nm. The peak table, peak display, and peak densitogram was recorded. Furthermore, the identified bioactive compounds in EESLC were characterised by In silico docking, in order to understand the inhibitory mechanisms.

2.6 Molecular docking analysis

The structure of rat pancreatic lipase – RPL (PDB code – 1BU8) and Human Pancreatic lipase – HPL (PDB code – 2PPL) were retrieved from the protein data bank (www.rcsb.org) and adipose triglyceride lipase (ATGL) was predicted by homology modelling MODELLER (Natalie et al., 2010) using ATGL sequence retrieved from NCBI. The refined protein and its possible conformation sites were obtained using a procheck analysis with Ramachandran plot (Laskowsky et al., 1993). The structure of identified compound from HPTLC analysis of study subjects were retrieved from PubChem (www.pubchem.ncbi.nlm.nih.gov). Docking analysis was performed using Discovery Studio 2.5 (Accelrys Inc., 2010). Docking calculations were performed with program LibDock16 implemented in Discovery studio 2.5. The binding affinities are expressed as docking scores by logarithm of the dissociation constant (lgKd).

3 Results

3.1 Pancreatic lipase inhibition

The efficacy of EEMUS, EEMUL, and EESLC on PL activity was depicted in Fig. 1. EESLC showed dose dependent activity on PL inhibition. The standard drug, orlistat, showed 61.1% inhibition and EESLC showed 58.9% inhibition at the concentration of 50 μg/ml. EEMUS and EEMUL showed 47.8% and 46.2% inhibition, respectively. The activity of EESLC was analogous to that of the positive control orlistat and EESLC showed the significant inhibitory activity with an IC_{50} value of 30 μg/ml. This indicated that EESLC had prominent PL inhibitory potential compared to EEMUS and EEMUL. Hence EESLC were further investigated In-vivo anti-obesity effect in animal model using HFD-induced rats.

3.2 Effects of EESLC on reduction of body weight and obesity

The optimized dose was 400 mg/kg BW was finalized as the optimal dose for the present study. Changes in water intake, food intake and final body weight in both control and experimental rats were shown in Table 1. HFD-induced obesity rat final body weight (297.72 ± 17.15 g), food intake (29.91 ± 1.14 g), and water intake (27.63 ± 2.35 mL) were significantly elevated when compared to normal control (NC) rats (213.70 ± 8.87 g, 20.96 ± 1.11 g and 24.91 ± 2.11 mL, respectively). Supplementation of EESLC at 400 mg/kg BW for 4 weeks caused a substantial reduction in final BW (216.45 ± 8.15 g), whereas, EESLC fed group food intake slightly decreased (22.12 ± 1.28), when compared to HFD control (29.91 ± 1.14 g), but significantly increased in food intake on normal control group (20.96 ± 1.11 g). The obese status was recorded in HFD (BMI – 7.38 ± 0.31) control group rat. Interestingly, our
Results showed similar BMI on Orlistat treated (BMI 4.35 ± 0.143) and EESLC (400 mg/kg) treated (BMI 4.36 ± 0.136) rats.

3.3. Effects of EESLC on high fat-diet induced obesity

As mentioned in Table 2, it has been observed that rat fed on high fat with EESLC supplement consecutively for 28 days, resulted decreased level of serum lipid profile. The significant (p < 0.01) increased level of serum TG, TC, LDL-C and VLDL-C were observed in HFD-fed group and a significant (p < 0.01) decrease in serum HDL-C levels when compared to the NC group rats receiving normal fed. The HFD group that was administered EESLC showed a significant (p < 0.01) decrease in lipid metabolic parameters including TC, TG, LDL-C, and VLDL-C compared to HFD control group. Oral administration of orlistat for 4 weeks along with a HFD caused a noteworthy decrease in TG, TC, LDL, and VLDL levels and an upsurge in HDL compared to the HFD control group. However, no significant (p > 0.05) variance in serum lipid profile between the NC group and EESLC group was observed.

3.4. Effects of EESLC on liver enzymes

As shown in Table 3, the EESLC with fed exhibits a hepatoprotective effects indicated by the decreased level of liver enzymes AST, ALT, ALP, and GGT. The level of AST, ALT, ALP, and GGT were significantly increased (96.72 ± 2.06, 42.90 ± 3.79, 104.45 ± 6.47 and 34.48 ± 0.29 IU/L) in the HFD control group compared to NC group. The liver enzyme level significantly decreased AST, ALT, ALP, and GGT in the EESLC-fed group compared to HFD control group. However, no significant (p > 0.05) variance in serum lipid profile between the NC group and EESLC group was observed.
(82.69 ± 5.32 IU/L), ALT (35.74 ± 1.78 IU/L), ALP (90.31 ± 4.02 IU/L) and GGT (23.86 ± 0.24 IU/L) in all EESLC treated HFD rats.

3.5. HPTLC analysis of EESLC

The EESLC loaded TLC plate was visualized under UV light at 254 nm and visible light, without derivatization. Fig. 2 represents the presence of bioactive compounds gallic acid and kaempferol in EESLC on TLC plate photograph. The qualitative and quantitative presence of gallic acid and kaempferol was confirmed by HPTLC co-chromatography and overlain absorption spectra with quantitative reference standard, when scanned at 365 nm (Misra et al., 2008). The densitometric chromatogram of HPTLC fingerprint of the EESLC is shown in Fig. 3a–c. The peaks resolving at Rf 0.13 and 0.72 in test solution were found to be superimposing with those of respective standards. On the basis of calibration curve linear range of gallic acid and kaempferol in EESLC were found to be 1.17–300 μg/ml and 1.95–500 μg/ml respectively. The Regression equation ($Y = 757,131X / C0^{17,232}$ and $Y = 351,194X + 15,589$) and correlation coefficient was found not less than 0.999, which revealed a good linearity response of bioactive principles in EESLC.

3.6. Molecular docking analysis

To elucidate the affinity and mode of binding of Orlistat, kaempferol and gallic acid (ligands) with the PL enzymes (RPL - 1BU8 and HPL - 2PPL) and adipose triglyceride lipase (ATGL protein), docking analysis were performed. The details of libdoc scores and energy...
values of ligand poses with high binding energy are summarized in Table 4. Structural interaction differences in ligands with nucleotide binding sites are shown in Fig. 4a–h. The Orlistat showed high binding energy and LibDock scores with RPL and HPL (−72.384 and −76.713 kcal/mol), when compared to kaempferol and gallic acid interactions. In RPL and HPL β-sheet side chain of ILE536 (RPL), ASP767 and MET655 (HPL) residues are interact with Orlistat (Fig. 4a & b) and none of the residues of ATGL showed interactions. Kaempferol and gallic acid showed higher binding efficiency than that of orlistat. RPL active sites – LYS232, LYS329, ASN240 and LEU213, HPL active sites – PRO813, GLN814, MET534, ILE536 and ATGL active sites – GLY803 and GLN766 are interact with Kaempferol and showed binding energy −40.387, −15.477 and −40.387 respectively. Gallic acid interact with active sites residues of RPL 

Table 4

| Ligand   | Protein | Residues | Poses | Absolute energy (kcal/mol) | Libdock score | H-bond Length (Å) |
|----------|---------|----------|-------|----------------------------|---------------|-------------------|
| Orlistat | RPL -1BU8 | ILE 536 | 98    | −72.384                    | 149.593       | 1.643             |
|          | HPL     | ASP 767  | 91    | −76.713                    | 146.018       | 1.904             |
|          | ATGL    | NIR      | NIR   | NIR                        | N/A           | N/A               |
| Kaempferol | RPL -1BU8 | LYS 232 | 5     | −40.387                    | 101.973       | 1.938             |
|          | HPL     | PRO 813  | 25    | −15.477                    | 64.604        | 2.389             |
|          | ATGL    | GLY 803  | 30    | −40.387                    | 108.824       | 2.248             |
| Gallic acid | RPL -1BU8 | VAL 805 | 23    | −15.477                    | 85.759        | 1.879             |
|          | HPL     | GLN 814  | 29    | −40.387                    | 81.546        | 2.389             |
|          | ATGL    | VAL 805  | 20    | −15.477                    | 84.912        | 1.858             |

Fig. 4. Docking conformation of a. Orlistat with binding pocket of RPL, b. Kaempferol with binding pocket of RPL, c. Kaempferol with binding pocket of HPL, d. Kaempferol with binding pocket of ATGL e. Gallic acid with binding pocket of RPL f. Gallic acid with binding pocket of HPL.
4. Discussion

In this study we found that combination of M. uniflorum leaves and seeds decrease the obesity of rats fed with high fat diet. PL is an important enzyme for triacylglycerol absorption and hydrolyses of triacylglycerol to form a monoaoclylglycerol and free fatty acid. Substrates for the lipase enzyme are long-chain triacylglycerols (Kim et al., 2016). In fact, currently two anti-obesity drugs such as orlistat, as PL inhibitor and sibutramine, as appetite suppressant were approved by US FDA (Bujjirao and Kumar, 2013). However, administration of these drugs can cause adverse side effects, like steatorrhea, faecal incontinence and flatulence, hence its limited in use (Shin et al., 2003; Shi and Burn, 2004; Zhang et al., 2008).

HFD-fed cause the development of obesity by acceleration of body weight with expanded adipose mass (Buettner et al., 2006). Many previous studies describing the obesity with the consumption of HFD in albino rats (Schrauwen and Westerterp, 2000; Hariri and Thibault, 2010). Current findings are coinciding with those of earlier experiments, where rats fed with HFD gained additional weight than those fed a normal diet (Kumar et al., 2013). In HFD and NC groups Body weight and daily food intake showed no statistical correlation. The HFD-fed groups constantly up taking the analogous amounts of food which providing higher calorie value diet. As a result, the calorie content of the diet increases the total body weight, resulting obese state in HFD groups. Administration of EESLC to rats fed a HFD resulted in a remarkable reduction in body weight compared to the HFD-group rats (Mukherjee, 2003).

The hypercholesterolemic effect may be ascribed to an increased dietary cholesterol intake (John Griffin and Alice Lichtenstein, 2013) and subsequently increased rate of intestinal cholesterol absorption (García Otín et al., 2007). Saturated fatty acids and monounsaturated fatty acids are rich in animal fat; hence the serum LDL cholesterol and serum triacylglycerols level are increased (Lopez Alvarenga et al., 2010). Therefore, the lowering of serum lipid profiles can be used as additional key factor for antiobesity. Current study revealed that the administration of a EESLC cause the elevated level of lipid profile in plasma of HFD fed rats. Furthermore, instigation of intestinal fat absorption, lipolysis, and gastric lipases cause the modification of the lipid profile in HFD-fed group. Increasing TC and LDL-C level in blood plasma are prone to developing coronary heart disease (Walkowiak et al., 2013) whereas increase HDL-C in blood plasma can cause transitory of additional cholesterol to the liver for the elimination of bile (Yoshizaki et al., 2014). The bioactive principles of EESLC may cause decreasing the absorption of fat and cholesterol through inhibition of PL activity. Our finding agreed with a previous report by Han et al. (2003).

Liver markers such as AST, ALT, ALP, and GGT were found in elevated concentrations in the hepatic cells of EESLC treated rats. In the present study, HFD rats showed significant decreases level of hepatic enzymes, which exhibit hepatoprotective and consistent with the observations of previous report (Abbas and Hussein, 2013). The potential outcome of the present study due to the presence of quantitative bioactive principles such as kaempferol and gallic acid in the EESLC. The standard drug, orlistat, binds to the serine amino acid on active sites of PL. Therefore, the substrate lipids are unable to bind with the enzyme and are not metabolized into simpler forms monoglycerols and free fatty acids. In addition, there is no absorption of fat, which results in the reduction of obesity (Zhi et al., 1995; Guerciolini, 1997).

Molecular docking assessment reveals high binding potential of kaempferol and gallic acid towards the active sites of PL and ATGL enzymes. Kaempferol and gallic acid in HPL of C-terminal domain active sites predicted with hydrophobic interactions by hydrogen bond at inner core residues PRQ813, GLN814, MET534 and ILE536. PL activity requires co-lipase as co factor to which C-terminal domain binds (Ganesh Kumar et al., 2015). Kaempferol and gallic acid interacts and inhibit the docking lid from accomplishing PL protein optimal conformation. The compound binding to these catalytic interaction with other nearby charged residues (LYS232 and LYS329) are expected to alter the stability and PL inhibition by docking simulations (Benarous and Abderrahman, 2011). Cumulatively, the computational and experimental data presented here propose that these high content of kaempferol and gallic acid in EESLC have definite effect on lipid metabolism by inhibition of PL through reducing the intestinal absorption of dietary fats.

5. Conclusion

Current study, exhibit the ameliorative potential of EESLC on HFD-fed obesity in albino Wistar rats. Through animal model and docking analysis, we confirmed that EESLC was an appropriate and potential anti-obesity nutraceutical candidate.

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