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Pre-clinical evolutionary study of *Clerodendrum phlomidis* as an anti-obesity agent against high fat diet induced C57BL/6J mice

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ABSTRACT

Objective: Anti-obesity activity of alcoholic and methanolic extracts of roots of *Clerodendrum phlomidis* was evaluated against high fat diet (HFD) induced obesity in C57BL/6J female mice. **Methods:** Obesity was induced by feeding high fat diet for 13 weeks to C57BL/6J female mice and one group was kept on normal chow diet in order to evaluate the effect of *Clerodendrum phlomidis* on food intake, body weight changes, digestive enzyme activity, lipid metabolism, theromogenesis, adiposities diameter and histology of fat pad. **Results:** Among these two extracts methanolic extract of *Clerodendrum phlomidis* (MECP) have shown strong anti-obesity effect compare to alcoholic extract of *Clerodendrum phlomidis* (AECP). LD_{50} value was found to be more than 2000 mg/kg. **Conclusions:** MECP have shown more promising effects than AECP may be because of its multiple mechanisms. Anti-obesity activity produced by MECP is because of inhibition of pancreatic lipase activity which delays the intestinal absorption of dietary fat. Inhibition of pancreatic lipase activity was confirmed by in-vitro studies. MECP also contains β-sitosterol in abundant amount which was confirmed by HPTLC analysis. Moreover flavonoid content in the plant has anorexic property. By this study we concluded that MECP is beneficial for the suppression of obesity and associated complications like T2DM.

1. Introduction

Obesity has turned up as one of the major health concerns in the 21st century and is one of the leading causes of preventable death [1]. Thermodynamically speaking, imbalance between energy intake (feeding) and energy expenditure (physical activity) leads to obesity [2]. Development of obesity is, however, more complicated than that; sedentary life style, genetic factors, medical illness, microbiological aspects, social factors and neurobiological mechanisms are also involved[3, 4].

The medical problems caused by obesity begin at the head and end at the toes and involve almost every organ in-between, and is known to be strong risk factor for type-II diabetes associated with insulin resistance.

A large study of literature indicates that substantial progress has been made concerning our knowledge of bioactive components in plant foods and their links to obesity. For the present research protocol we have chosen Clerodendrum phlomides to evaluate its anti-obesity and related complications like T2DM etc. As per the earlier literatures flavonoids, saponines, sitosterols and tannins shown anti-obesity effect by various mechanisms and the selected plant has shown the presence of triterpenoids, flavonoids and saponins content in its extract [5]. Moreover traditional Indian medicine system also claims for its anti-obesity activity with this back ground we have selected these plants for its phytochemical analysis and screening its anti-obesity activity [6]. It has been reported that the roots of plant are used for inflammation, swelling of the body, chronic enlargement of the spleen or any glandular enlargement in the abdomen and various urinary disorder [7]. The plant also reported to be used as bitter tonic, antidote, analgesic, anti-asthmatic, inflammatory disease and in rheumatism [8, 9]. The root barks are used in cough, asthma, cold, oedema and nervous disorders [10]. Whole plant has been reported for its anti-diabetic activity [11].

In general, C57BL/6J (ob/ob) models have been used for investigation of human obesity and metabolic syndrome [12]. The aim of the present study was to determine the potential

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of Clerodendrum phlomides as anti-obesity and a lipid-lowering product and its mechanism of action by using C57BL/6J (ob/ob) female mice model.

2. Material and methods

2.1. Plant authentication

Plant samples of the *C. phlomidis* (L) were collected in July 2007 from amargardh-bichari Rajkot, India, and verified by Prof. (Dr.) H.B. Singh, Head, Raw Materials Herbarium and Museum, NISCAIR, New Delhi, India. Duplicate herbariums were also retained in the Department of Pharmacognosy of Shree H. N. Shukla Institute of Pharmaceutical Education & research, Rajkot, for the future reference, the voucher No. of specimen is HNSIPER/herb/04.

2.2. Materials

Remi research centrifuge (R-24), Soxhlet extractor, OLYMPUS iNEA 5X, 10X/0.2; India, and 100X/1.25 oil India, HPTLC (CAMAG, Switzerland), Shimadzu UV-visible Spectrophotometer (UV1800), micrtone, Open field model was fabricated based on earlier standard literature, Stat Fax autoanalyser (2000), Afcoset digital balance (ER-180A), 250 μ pone nylon mesh, micro-pipette etc.

2.3. Chemicals

Sibutramine & Orlistat were gifted by the Ranbaxy Laboratory Ltd, Devas, MP India; Bovine Serum Albumin, J. Mitra & Co. Pvt. Ltd., New Delhi, India; Collagenase Type-,1 Fluka, biochemia, product of Switzerland; Methylene blue, Vanshi Chemicals Pvt. Ltd, Mumbai-400064, India; Oil red O; Trypsin, Alna Biotech Pvt Ltd., Chandigarh, India, biochemical kits of Span diagnostics. Triolein and pancreatic lipase were purchased from Sigma Chemical, India. Taurocholic acid, 0.1M N-tris (hydroxymethyl) methyl-2-aminoethanesulfonic acid (TES), chitin-chitosan etc. All the chemicals used in the study are of AR grade.

2.4. Preparation of Extract

The dried roots of *C. phlomidis* were collected & powdered by using the pulverizer. The powdered drug was then passed through sieves # 40 and used for extraction process.

Successive solvent extraction was carried out; a dried material is extracted with different solvents, starting from solvent of low polarity first with 95% and then 90%. After extraction by one solvent, material is removed from thimble, dried and recharged, extracted with solvent of successively high polarity. Successive solvent extraction was done by using methanol [13].

The extract was filtered and concentrated by using rotary flash vacuum evaporator (ROTEVA, EQUITRON, Mumbai, India). The extract was dried in vacuum drier and stored below 10℃.

2.5. Toxicity Study

Procedure: Acute toxicity studies were performed according to OECD-423 guidelines category IV substance (acute toxic class method). Albino mice (n=3) of either sex selected by random sampling technique were employed in this study. The animals were fasted for 4 hrs with free access to water only. The plant extracts of *C. phlomidis* were administered orally with an initial dose of 1000 mg/kg body weight. The mortality was observed for three days. If mortality was observed in 2/3 or 3/3 of animals, then the dose administered was considered as a toxic dose. However, if the mortality was observed only one mouse out of three animals then the same dose was repeated again to confirm the toxic effect. If mortality was not observed, the procedure was then repeated with higher dose [14].

2.6. Phytochemical Investigation

Phytochemical investigation of AECP and MECP was carried out by the method prescribed by Kokate, 1994 results of phytochemical investigation is given in Table 2. [13]

2.7. Estimation of Total Flavonoid Content

Flavonoid concentration was determined by method prescribed by Chang et al., 2002 [15]. The temperature of the surrounding atmosphere was 25 ± 0.5 oC. Known volume of methanolic extract was diluted with 80 % aqueous ethanol (0.9 ml). Aliquot of 0.5 ml was added to test tube containing 0.1 ml of 10% aluminum nitrate, 0.1 ml 1 M aqueous potassium acetate and 4.3 ml of 80 % ethanol. After 40 min at room temperature the absorbance was determine at 415 nm with UV spectrophotometer. Total flavonoid content was calculated according to a standard curve established with Quercetin given in Table 3.

2.8. Estimation of Total Saponin Glycosides

a.Total saponin content of both extracts was carried out by method prescribed by Rajpal, 2002 [16]. Five gram of sample extracted with 90% v/v methanol 25 ml refluxed for half an hour.b.Residue was extracted two or more times by taking 25 ml methanol and combined with the methanolic extract and distiled off the solvent.c.Soft extract was treated left after distillation of methanol with Pet. Ether 60-80, 25 ml by refluxing for half an hour. solvent was cooled and removed by decantation and discarded.d.Treat the residue left with 25 ml ethyl acetate and reflux the contents for half an hr., cool and decant off the solvent and discard.e.Residue was dissolved in the left in 25 ml of 90 % v/v methanol. Filtered and concentrate to 5 ml.f.The above was added drop by drop with constant stirring to 25 ml solvent ether to precipitate the saponin. Precipitate was filtered the or if the filtrate is settled in the beaker decant off the solvent ether and dry the residue to constant weight. Total Saponin content is given in Table 3.

2.9. Quantitation of β-sitosterol in MECP & AECP by

HPTLC

The HPTLC pattern for MECP and AECP were recorded using HPTLC (CAMAG, Switzerland) using pre-coated silica gel plates (E. MERCK KGaA) at following specific criteria.

2.10 .Animals

Five months old forty five C57BL/6J female mice weighing around 20 to 24 g, were purchased from National Institute of Nutrition, Hyderabad (India). Animals were housed in a standard controlled animal care facility in cages (5 mice/cage). The animals were maintained in a temperature-controlled room (22-25℃, 45% humidity) on a 12:12-h dark-light cycle. The animals were maintained under standard nutritional and environmental conditions throughout the experiment. All the experiments were carried out between 9:00-16:00 hours at ambient temperature. Nations CPCSEA guidelines were strictly followed and all the studies were approved by the Institutional animal ethical committee (IAEC), (Ref: IAEC/HNSIPER/RJK/05/2009) Shree H. N. Shukla Institute of Pharmaceutical Education and Research. Rajkot- Gujarat, India.

2.11. Induction of Experimental obesity

Animals were divided in to 9 groups each group having 5 animals, first group (lean mice) had free access to standard pelleted chow which provided 76.8% of energy as carbohydrates, 19.2% as protein, and 4.3% as fat. Remaining 40 mice were fed with a high fat diet providing 60% of energy as fat, 20% as protein and 20% as carbohydrates. Experimental obesity and other metabolic changes were induced by dietary manipulation (by proving HFD) for 13 weeks to remaining 8 groups. After 13 weeks mice were found to be obese, change in body weight during this period was recorded (Data not shown). Five mice in each group were given plant extracts at various doses or vehicle alone (distilled water). All drugs including extracts and standard Sibutramine (10 mg/kg) are soluble in water so DW was used as media to dissolve drugs and animals were administrated with 100 mg/kg, 200 mg/kg and 400 mg/kg/day of plant extracts (MECP and AECP) by oral gavages for 4 weeks. Refer Table 1 [17]. All the drug and extract concentration were prepared freshly just before administration. All the extracts including standard were given by oral gavages by p.o. route. Mice in first group (lean mice, kept on standard regular diet) were given an equal volume of distilled water (DW). Body weight and food intake of all groups were monitored weekly throughout the treatment period. At the end of 4 weeks of treatment, behavioral study was performed in all groups and mice from each group including control were sacrificed to check the serum biochemistry like SGOT, SGPT, LDL, VLDL, HDL, glucose level etc.

2.12. Assessment of food consumption behavior in Mice

In the earlier study carried out by Kaur and Kulkarni 2002, 10 g of sweeten chow was provided to the group of 5 mice of Laca strain and food consumption was observed [18]. In the present we found that C57BL/6J mice consumes food more rapidly so here we have provided 20 g of sweetened chow instead of 10 g to the group of 5 mice. This modification was made by doing a pilot study as C57BL/6J mice consume fatty food more rapidly than Laca strain mice.

Food intake studies were carried out on days 1, 7, 14, 21 and 28. Mice were deprived of food 1 h prior to experimentation. The test feed for the feeding experiments was standard mice chow modified for palatability by adding 10% of sucrose. On experimental days, 30 min after last drug administration, 20 g of sweetened chow was presented to groups of mice in glass petri dishes and food intake was recorded at 0.5, 1 and 2 h time intervals. Nearest to 0.1 g with correction for spillage and the amount of food consumed/20 ± 3 g body weight was calculated. All the feeding experiments and biochemical studies were carried out on the same day on all animals by considering their initial body weights and biochemical readings as a zero point.

2.13. Body weight

The body weight of mice (g) was recorded for every week for 28 days in each group just before dosing by using precision balance of 10 mg sensitivity.

2.14. Body temperature

The body temperature of mice was recorded on 29th day of study using rectal telethermometer, before and after drug administration at 1 and 2 hrs. After measuring the body weight, each animal was placed in specially designed restricter to measure rectal temperature. A Yellow Spring Instrument telethermometer with a series 500 probe was used. The probe was lubricated using petroleum jelly prior to use and was inserted between 1.0 and 1.3 cm into the rectum and held in position for 10 seconds before temperature was determined. Measurements were made once for each animal and were conducted during a 2-hour period before 4-hours of light offset.

2.15. Biochemical parameters

2.15.1 Preparation of serum

On 29th day of study, after the last of test drug administration animals were anaesthetized under light ether anesthesia and blood for plasma preparation was collected by retro orbital puncture, using 10 μl x 20mm (L) x 0.8 mm (2R) glass capillary into sterile EDTA-coated tube (3 mg/ml). Blood was kept in wet ice for 30 min, centrifuged for 5 min at 4000 rpm at 4o C (REMIMAK, India) and plasma was aspirated out for the analysis of lipid profile and glucose. The plasma was stored in the refrigerator for the analysis of biochemical parameters. All analyses on plasma were completed within 24 h of sample collection. Plasma samples were analyzed for glucose, Triglyceride and total cholesterol using biochemical kits of Span diagnostics Glucose: GOD/POD method [19]; Cholesterol: one step method of Wybenga and Pilleggi [20]; Triglycerides: GPO-PAP, end point method

[21] and HDL-C by Henry, 1974 method [22].

2.16. Organ weights

The mice were sacrificed by cervical dislocation on day 30 and then different organs including kidneys, liver, heart and body fat i.e. WAT (periovarian, perirenal, and mesenteric fat pad) were isolated, the organ to body weight (mg/gm) ratio was recorded. After weighing of fat content it was preserved for the further study. Adiposity index, a quantitative measure of total fat mass was also calculated using the previously determined equation (Gregoire, et. al., 2002).

Adiposity Index (%) = [$\sum$ (fat pad)/ Body weight X 100]

2.17. Isolation of fat pads and experimental procedure

Four regions of adipose tissue were carefully dissected: a.The periovarian fat, ovaries were taken out by gentle squeezing from the peripheral fat and then by horizontal cut from all sides fat was isolated; care has been taken that too much traction was avoided on ovaries and fat. b.The retroperitoneal, by first separating the perirenal fat and then dissecting the retroperitoneal pad. c.The mesenteric, all fat found along the mesentery starting at the lesser curvature of the stomach and ending at the sigmoid colon was considered mesenteric fat; obtained by cutting the intestine below the duodenal-jejunum junction and stripping the fat by gently pulling the intestinal loops apart.

2.17.1. Isolation, sizing and counting of fat cells

Selection of periovarian fat: Periovarian fat was selected because it a.It is located within the abdominal cavity.b.It is large enough that it provides enough tissue for subsequent biochemical experiments, whereas other fat depots such as the omental, mesenteric, or perirenal are too small in the size of rat being used to provide sufficient tissue for biochemistry. c.It is easy to dissect out, making it possible to sacrifice all animals within a shorter period of time and decreasing the possibility of a time effect on any measured variable.d. Periovarian making possible any potential comparison of results with the published literature.

2.17.2. Adipocyte Isolation

Periovarian adipocytes were isolated using trypsin and centrifuged with slight modifications [23] . The samples were provided with (5% CO_2 & 95% O_2), capped and incubated at 37 ℃ with shaking until digestion was complete (30-40 min).

2.17.3. Sizing of fat cells

Add 0.2 to 0.4 ml aliquots of the stirred suspension of stained cells were placed on a siliconized glass slide and examined with a Zeiss microscope equipped with a Polaroid camera attachment. The insertion of a micrometer disc in a focusing eyepiece placed in the phototube of the camera attachment produced a projected caliper scale. At a magnification of 200 X, the caliper scale was calibrated so that the unit marks had a constant interval of 7 μ. The free fat cells, floating on the surface of the medium, were recognized by the spherical shape, the stained nucleus with one or two nucleoli, and the stained cytoplasm; the latter features readily distinguished the fat cells from occasional droplets of floating lipid. One hundred cells one by one from the same population were brought in the caliper field with systematic motion of the stage control knobs. The cells were aligned on the caliper scale, the equatorial plane of the cell was brought into focus, and the fat cell diameter was determined with accuracy. The sizing and grouping of 100 fat cells was performed by one observer in approximately 15-20 min. From this, the mean diameter and the standard deviation about the mean could be rapidly calculated by the usual formulas (Fig. 1).

2.18. Histology of fat pad

The periovarian fat was selected for histological study. The periovarian fat of each group were excised and rinsed in 0.9 % saline blotted dry of saline and excess blood. They were fixed in 12% formalin for 24 hr. The tissues, after fixation, were washed in water to remove excess fixative. Washed tissues were then dehydrated through a graded series of ethyl alcohol, cleared with xylene and embedded in paraffin wax. Sections were cut at 3 μm with microtone blade and mounted on clean glass slide. The sections were routinely stained with haemotoxyllin and eosin. The stained slides were observed (200 X) in research microscope. Figure 11.

2.19.In-vitro Measurement of Pancreatic Lipase Activity

2.19.1. Measurement of pancreatic lipase activity

Lipase activity was determined by measuring the rate of release of oleic acid from triolein. A suspension of triolein (80 mg), lecithin (10 mg) and taurocholic acid (5 mg) in 9 ml of 0.1M N-tris (hydroxymethyl) methyl-2-aminoethanesulfonic acid (TES), pH 7.0,containing 0.1M NaCl was sonicated for 5 min. This sonicated substrate suspension (100 ml) was incubated with 50 ml (10 units) of pancreatic lipase and 100 ml of various concentrations of chitin-chitosan suspension for 30 min at 37 oC in a total volume of 250 ml. The amount of oleic acid produced was determined by the method of Zapf et. al. 1981 with a slight modification [24, 25]. The incubation mixtures were added to 3 ml aliquots of a 1:1 (v/v) mixture of chloroform and heptane containing 2% (v/v) methanol and extracted by shaking the tubes horizontally for 10 min in a shaker. The mixture was centrifuged at 2000 g for 10 min, and the upper aqueous phase was removed by suction. Copper reagent (1 ml) was then added to the lower organic phase. The tube was shaken for 10 min, the mixture was centrifuged at 2000 g for 10 min and 0.5 ml of the upper organic phase, which contained copper salts of the extracted free fatty acids (FFA), was treated with 0.5 ml of 0.1% (v/v) bathocuproine in chloroform containing 0.05% (w/v) 3-(2)-tert-butyl-4-hydroxy-anisol. The absorbance was then measured at 480 nm. In addition, pancreatic lipase activity was determined using gum arabic (acidic polymer) or Triton X-100 (neutral polymer) as emulsier; 45 mg gumarabic or 2.25 mg Triton X-100, instead of lecithin, were used and the enzyme activity assayed as described above. Lipase activity was expressed

as mmol oleic acid released per ml reaction mixture per 1 h.

2.20. Statistical Analysis

The results are expressed as mean ± SEM. Comparisons between the treatment groups and positive control; positive control and control were performed by one way analysis of variance (ANOVA) followed by Dunnett test. In all tests the criterion for statistical significance was $P < 0.05$ (95% level) and P<0.01. The analysis was performed by using Graphpad Prism IV.

3. Results

3.1. Extractive values

The extractive value for ethanol and methanol was found to be 2.41± 0.013 % and 3.58 ± 0.016 % respectively.

3.2.Total flavonoid content

Total flavonoid content was found to be 0.388 % w/w and 0.321 % w/w for methanolic and alcoholic and extract respectively.

3.3. Total Saponin content

Total Saponin content was found to be 0.121% w/w and 0.108 %w/w for methanolic & alcoholic respectively.

3.4. Acute toxicity study

The plant extracts of *Clerodendrum phlomidis* didn't show any mortality and toxicity on any of the organ even at highest dose of 2000 mg/kg body weight employed so LD50 value is expected to exceed 2000 mg/kg body weight. The present research study was carried out using three different doses (low, medium and high) alcoholic and methanolic extracts of *Clerodendrum phlomidis* as 100, 200 and 400 mg/kg body weight for anti-obesity.

--3.5. Quantitation of β -sitosterol

HPTLC study indicated presence of β-sitosterol in both MECP and AECP. β-sitosterol resolved at Rf 0.65, showing a single peak having absorption maxima at 366 nm. The chromatogram further resolved almost similar chemo profile for methanolic fractions of AECP and MECP. The method of chromatography using chloroform: methanol (8:0.6) as a mobile phase and 10% ethanolic H2SO4 as detecting agent have gave good resolution of β-sitosterol without any other compounds present in extracts of MECP and AECP. The calibration curve of MECP and AECP were linear with r2 = 0.991 in the concentration range. β-sitosterol was found more in MECP (0.78 % w/w) as compared with to that of AECP (0.61 % w/w), refer fig. 2, 3, and 4. β -sitosterol content in AECP and MECP was found to be 0.78 %w/w and 0.61 %w/w respectively.

Table 1.
Animal grouping and Diet High fat fed C57BL/6J mouse (DIO mouse)

Animal Groups 5 mice in each Group	Treatment
01	DW + Normal Standard diet
02	DW + HFD
03	AECP (100 mg/kg) + HFD
04	AECP (200 mg/kg) + HFD
05	AECP (400 mg/kg) + HFD
06	MECP (100 mg/kg) + HFD
07	MECP (200 mg/kg) + HFD
08	MECP (400 mg/kg) + HFD
09	Sibutramine 10 mg/kg + HFD

DW: Distilled Water; HFD : High Fat diet; MECP: Methanolic extract of Clerodendrum phlomidis; AECP: Alcoholic extract of Clerodendrum phlomidis

Table 2.
Phytochemical Investigation:

Phytoconstituents	Extracts	
	MECP	AECP
Steroids	+	+
Triterpenoids	+	+
Carbohydrates	+	+
Proteins	+	+
Glycosides	+	+
Flavonoids	+	+
Anthocynidine	+	–
Saponin	+	+
Alkaloids	–	–

+ indicates presence of constituents ; – indicates absence of constituents. MECP: Methanolic extract of Clerodendrum phlomidis;AECP: Alcoholic extract of Clerodendrum phlomidis

Table 3.

Secondary metabolites	MECP	AECP
Total flavonoid content	0.388 % w/w	0.321 % w/w
Total saponine content	0.121% w/w	0.108 %w/w

MECP: Methanolic extract of Clerodendrum phlomidis
AECP: Alcoholic extract of Clerodendrum phlomidis

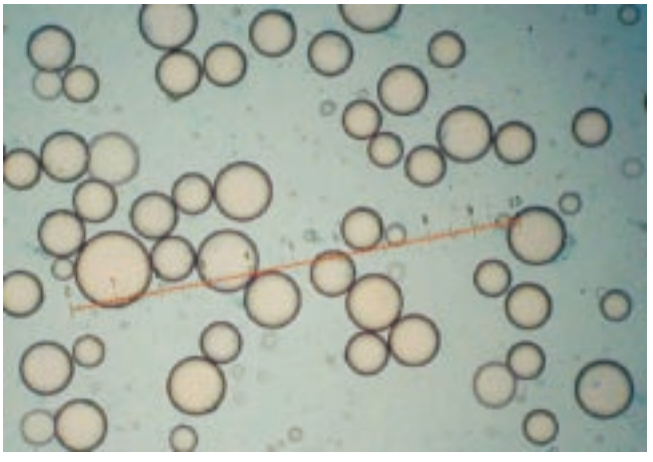
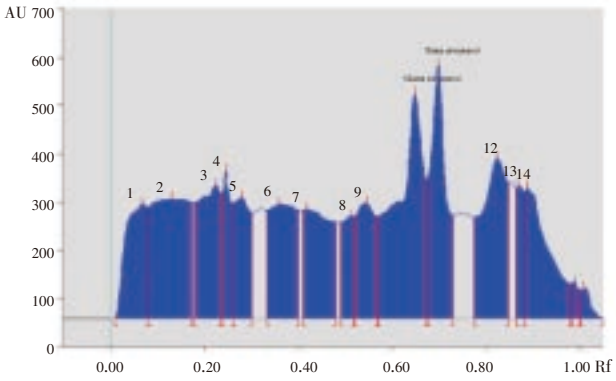
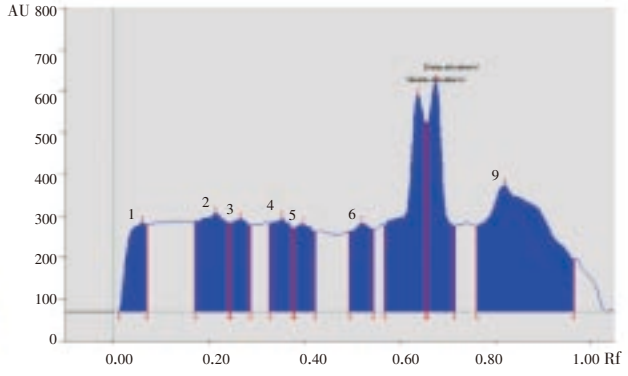


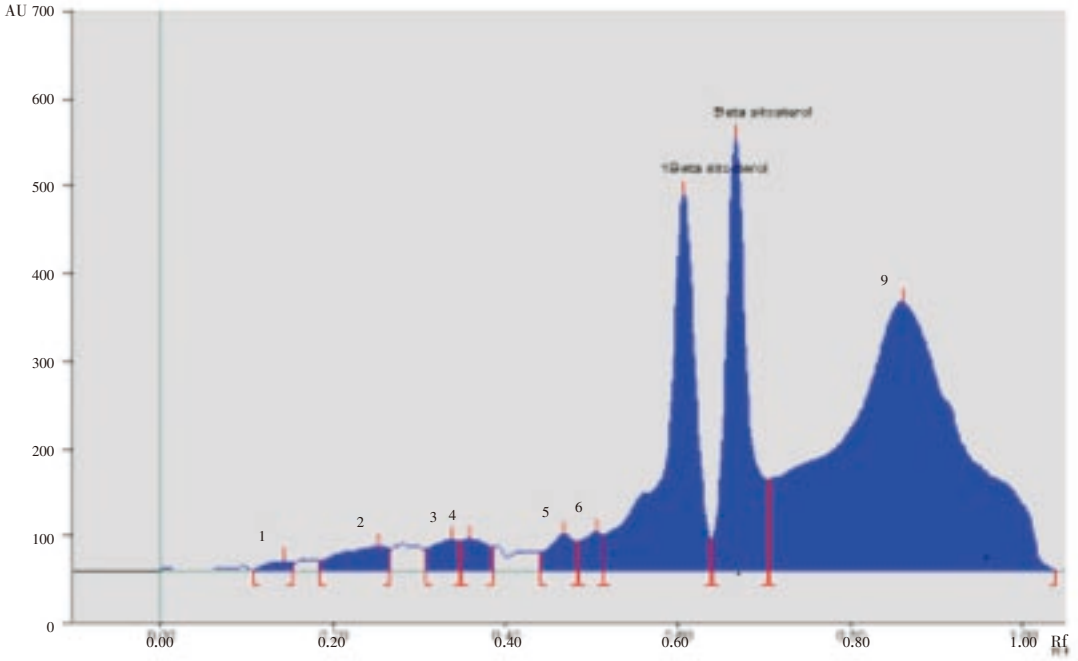
Figure 1. Basic microscopic determination of fat cell diameter. Stained fat cells, floating on surface of medium, are aligned on caliper scale, brought into focus, and transverse diameter was recorded in units. Magnification 200X. Free fat cells seen in this photograph are derived from prioverian fat pad of a C57BL/6J female mice.



Chromatogram:peak display of MECP



Chromatogram:peak display of AECP



Chromatogram:peak display of standard β-sitosterol

Figure 2. Quantitation of β-sitosterol:

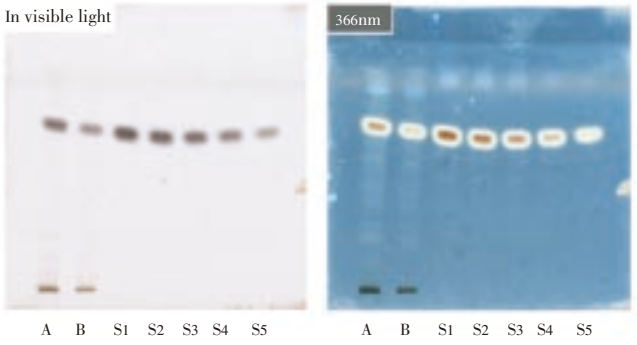


Figure 3. HPTLC Fingerprinting of MECP, AECP & β-sitosterol (After Derivatization)
A: Track Ist in 20 μl MECP, B: Track IInd in 20 μl MECP, C: Track IIIrd Standard β-sitosterol 10 μl, D: Track IVth Standard β-sitosterol 8 μl, E: Track Vth Standard β-sitosterol 6 μl F: Track VIth Standard β-sitosterol 4 μl, G: Track VIth Standard β-sitosterol 2 μl.

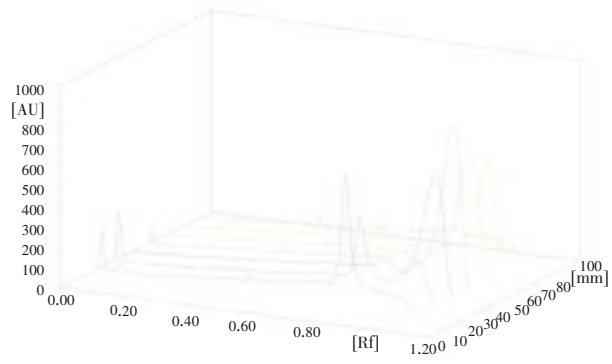


Figure 4. HPTLC chromatogram scanned at 366 nm.

3.6 Effect of AECP and MECP extract on pancreatic lipase activity

Effects of AECP and MECP on pancreatic lipase activity.

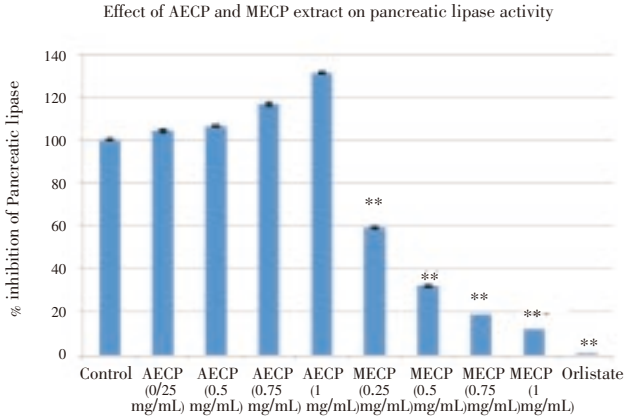


Figure 5. Effect of AECP and MECP extract on pancreatic lipase activity:
Effects of AECP and MECP on pancreatic lipase activity. Values are expressed as means ± SEM of five experiments.
*Comparison of test and disease control with normal control
Comparison test with Disease Control

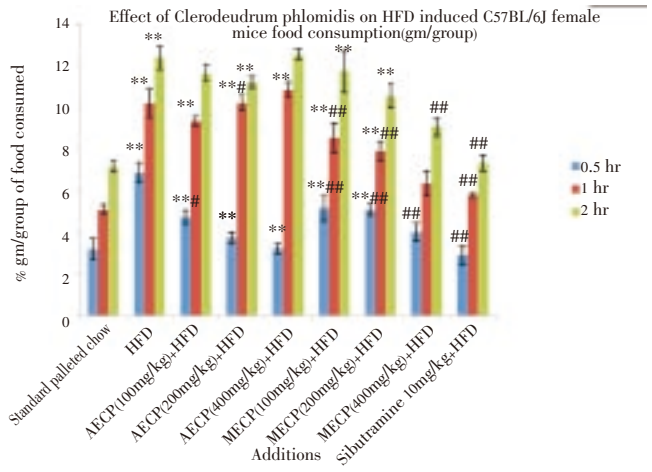


Figure 6. Effect of Clerodendrum phlomidis on HFD induced C57BL/6J female mice food consumption (gm / group).
Values are expressed as means ± SEM of five experiments.
*Comparison of test and disease control with normal control
Comparison test with Disease Control

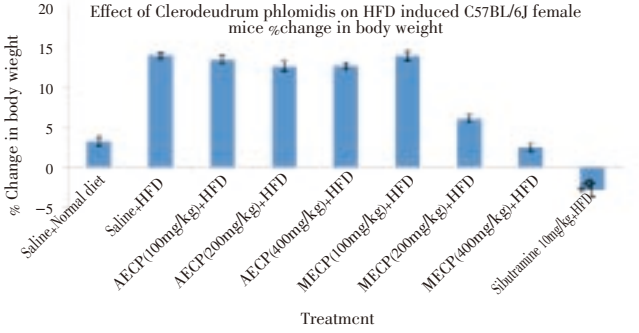


Figure 7. Effect of Clerodendrum phlomidis on HFD induced C57BL/6J female mice % change in body weight
Values are expressed as means ± SEM of five experiments.
*Comparison of test and disease control with normal control
Comparison test with Disease Control

Lipase activity was measured using porcine pancreatic lipase and triolein with lecithin as substrate. Inhibiting effect was shown as the lowering of pancreatic lipase activity (%) against the lipase activity of control (0 mg/mL of

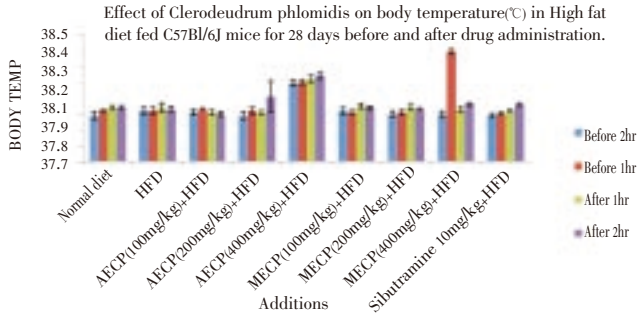


Figure 8. Effect of Clerodendrum phlomidis on body temperature (℃) in High fat diet fed C57BL/6J mice for 28 days before and after drug administration.
There wasn't any significant change found in the body temperature in any of the group of animals

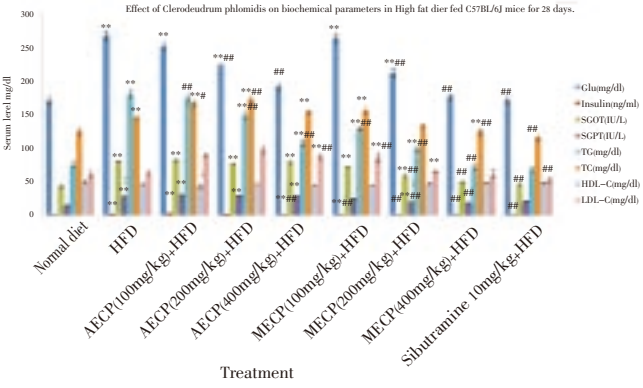


Figure 9. Effect of Clerodendrum phlomidis on biochemical parameters in High fat diet fed C57BL/6J mice for 28 days.
Values are expressed as means ± SEM of five experiments.
*Comparison of test and disease control with normal control
Comparison test with Disease Control

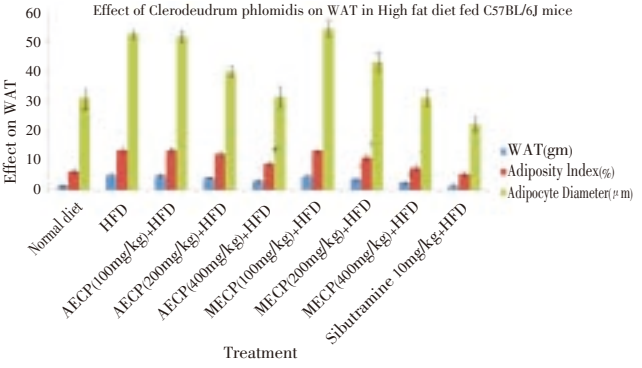


Figure 10. Effect of Clerodendrum phlomidis on WAT in High fat diet fed C57BL/6J mice.
Values are expressed as means ± SEM of five experiments.
*Comparison of test and disease control with normal control
Comparison test with Disease Control

additions). Treatments with MECP have shown significant $P<0.001$ reductions in pancreatic lipase activity in dose dependent manner and treatment with Orlistate also significantly $P<00.1$ reduced the pancreatic lipase at dose of 1 mg/ml. The mean difference is significant from control (0 mg/mL of additions).

3.7 Effect of Clerodendrum phlomidis on HFD induced C57BL/6J female mice food consumption (gm / group).

The HFD group animals showed significantly (P<0.001) increased food consumption as compared to the normal control group animals at 30 min, 1hr and 2 hr which was significantly P<0.001 decreased by the co-administration of AECP 400 mg/kg compared to normal control group. Treatment with MECP 400 mg/kg have shown significant (P<0.001) reduction in food consumption compared with plane HFD treated and other test groups. Standard sibutramine was most significant ($P<0.001$) in reduction in food intake compared to any plane HFD treated groups (disease control).

3.8 Effect of *Clerodendrum phlomidis* on HFD induced C57BL/6J female mice % change in body weight

Animals fed with HFD showed significant % increase in the body weight as compared to normal control animals. Treatment with MECP significantly $P<0.01$ reduced the body weight compared with disease control group in dose dependent manner but less than that of sibutramine treated group. Standard sibutramine significantly $P<0.001$ reduced body weight of normal and plain HFD treated groups.

3.9 Effect of Clerodendrum phlomidis on body temperature (℃) in High fat diet fed C57BL/6J mice for 28 days before and after drug administration.

There wasn't any significant change found in the body temperature in any of the group of animals; neither in test nor in standard compared with normal and plain HFD treated groups.

3.10 Effect of Clerodendrum phlomidis on biochemical parameters in High fat diet fed C57BL/6J mice for 28 days.

Feeding with HFD caused significant ($P<0.001$) increase in the glucose, insulin, SGOT, SGPT, TG, TC and LDL-C levels in HFD group animals as compared to normal control animals which was significantly ($P<0.001$) decreased by the co-administration of MECP 200 and 400 mg/kg compared to disease control and normal control group while MECP 100 mg/kg has not shown any protection on lipid profile. Sibutramine was the most significant in this regard.

3.11 Effect of Clerodendrum phlomidis on WAT in High fat diet fed C57BL/6J mice

Feeding with HFD caused significant ($P<0.001$) increase in WAT, adiposity index, and adiposity diameter compared with normal control animals which was significantly ($P<0.001$) decreased by the co-administration with MECP 200 and 400 mg/kg compared to disease control and normal control group. Sibutramine was the most significant in this regard.

4. Discussion

Obesity is a chronic metabolic disorder that characterized by enlarged fat mass and elevated lipid concentration in blood. Globally, more than 1.1 billion adults worldwide are overweight and 312 million of them are clinically obese.

Due to incomprehensible etiology, the treatment of obesity is difficult and challenging. Further, the cause of concern is the non-availability of drugs for its treatment and the short-term efficacy and limiting side effects of the available drugs [26].

Ayurvedic system of medicines is one of the oldest system of medicine having a history of more than 3500 years. Several prototype derived from these herbal medicines are in use for various kind of disease and disorders. It not only gives new molecule but also with newer mechanism of action, hence is called Gold mine.

A variety of natural products including crude extracts and isolated compounds from plants can induce body weight reduction and prevent diet-induced obesity. Therefore, they have been widely used in treating obesity [27, 28].

The present investigation was carried out to evaluate the anti-obesity and obesity associated complications like T2DM against ethanolic and methanolic extracts of *Clerodendrum phlomidis* by using high fat diet induced obesity in C57BL/6J female mice.

Clerodendrum phlomidis Linn. (Family: Verbenaceae) is a large bush or small tree, reported to possess potent spasmolytic and anti-diarrheal effect. The plant is commonly used as an anti-fungal and anti-pyretic agent [29]. The juice of leaves is used as an alterative and bitter tonic. The decoction of root is slightly aromatic and astringent is used as a demulcent in gonorrhea [30]. The plant has been found to possess hypoglycemic activity [31]. Rural people of Tamilnadu use fresh juice of the leaves of the plant to treat mental tension and mental disturbance. Methanolic extract of leaves of *Clerodendrum phlomidis* was found to have an effect on alteration in general behavioral profiles, including alertness, awareness, spontaneous activity, touch, pain and sound responses in mice and showed its characteristics as minor tranquilizer.

In our study initially pharmacognostic and phytochemical analysis was carried out data not shown here. In the phytochemical investigation it was clear that *C. phlomidis* contains triterpenoids, glycoside, carbohydrates, proteins, flavonoids, anthocynidine and saponins as shown in table 2. Some of the chemical constituents like saponines, flavonoids and some triterpenoids have reported for its anti-obesity effect in various plants [5]. Based on this phytochemical study and ethnobotanical claims this plant was selected for carrying out this study.

Obesity in C57BL/6J female mice was induced by feeding with high fat diet. The C57BL/6J mouse develops an obese phenotype only when allowed ad libitum access to a high-fat diet whereas on a low-fat diet, C57BL/6J mice remain

normal [32]. The obesity in the C57BL/6J mouse results from both adipocyte hypertrophy and hyperplasia. The fat gained in the C57BL/6J mouse is deposited selectively in the mesentery which indicates the central obesity.

The HFD C57BL/6J group animals showed significant increase in food consumption as compared to the normal control group animals at 30 min, 1hr and 2 hr which was significantly decreased by the co-administration of MECP 100, 200 and 400 mg/kg and by lone AECP 400 mg/kg. Standard sibutramine was most significant in this case as indicated in fig. 6.

C57BL/6J mice exhibits an increased weight gain per ingested energy unit (feed efficiency rate) and the ability of ingested energy to be metabolized (metabolic efficiency) is lower in high-fat diet fed C57BL/6J mice compared with control mice [33] the same have been observed in our study as shown in fig. 7.

It is well known that an increase in food ingestion results in the activation of both heat production and deposition of reserves, mainly fat [34]. Consumption of HFD results in overweight animals but also increased heat production through diet-induced thermogenesis [34], so measurement of rectal temperature recording was considered one of the parameter in this study. The drugs which demonstrating such changes indicating the thermogenic property of the drugs, but in our study we have not observed any significant changes in change in body temperature even in the standard Sibutramine as shown in fig. no. 8.

Feeding with HFD caused significant increase in the serum glucose, insulin, SGOT, SGPT, TG, TC, LDL-C and fall in HDL-C levels as compared to normal control animals which was significantly decreased by the administration AECP 400 mg/kg and MECP 200 and 400 mg/kg. Sibutramine was most significant in this case. Increase in the glucose as well as serum insulin level indicates obesity induced insulin resistance DM (T2DM) as publicized in fig. 9.

Several studies show that an increase in HDL cholesterol is associated with a decrease in cardiovascular risk which is a major complication of obesity associated dyslipidemia and most of the drugs that decrease total cholesterol also decrease HDL cholesterol [35, 36]. But in the present study the extract decreased the total cholesterol and LDL cholesterol and enhanced the HDL cholesterol significantly. This is an important advantage in treatment of hypercholesterolemia especially among Indians where low HDL cholesterol is the prevalent lipoprotein abnormality [37, 38]. Total cholesterol/ HDL cholesterol and LDL: HDL cholesterol ratios are also predictors of cardiovascular risks as per National Cholesterol Education Program Expert Panel [39]. In this study these ratios are markedly reduced by methanolic extract of *Clerodendrum phlomidis*.

LDL carries cholesterol from the liver to the peripheral cells and smooth muscle cells of the arteries, a rise in LDL may cause deposition of cholesterol in the arteries and aorta and hence is bad for health and a direct risk factor for coronary heart disease [40]. One species of LDL, Lipoprotein (a), which is associated with arthrosclerosis (and is localized in atherosclerotic lesions), contain a unique apoprotein, apo (a) and is similar in structure to plasminogen. Hence lipoprotein (a) competes with and inhibits the binding of plasminogen to its receptors on the endothelial cell. Plasminogen is normally the substrate for plasminogen activator. Plasminogen activator is secreted by and bound to endothelial cells, generating the fibrinolytic enzyme plasmin. The effect of this binding of lipoprotein (a) is that less plasmin is generated, fibrinolysis is inhibited and thrombosis promoted. LDL also activate platelets, constituting a further thrombogenic effect [41].

Administration of MECP 400 mg/kg lowered both TC and LDL cholesterol in experimental animals. The lowering of TC and LDL in serum by 17.74 ± 0.12 and 4.49 ± 0.06 % respectively after administration of MECP would reduce the incidence of obesity and associated cardiovascular complications.

The decrease of serum TG level is an important finding of this experiment. Recent studies shown that triglycerides are independently related to obesity induced cardiovascular complications and most of the antihypercholesterolemic drugs (can also be used to correct obesity associated dyslipidemia) do not decrease triglycerides levels, but MECP 400 mg/kg has lowered it significantly by 39.68 ± 0.2 % compared to HFD group [42, 43]. This effect might be related to presence of sitosterols in the extract. The presence of β-sitosterol and sterol compounds in *Clerodendrum phlomidis* have already been reported (Muthu, et al., 2010) also confirmed by phytochemical study in our laboratory [44]. Moreover from HPTLC analysis it was found that MECP contains 0.28 gm/gram of the extract so presence of this abundant amount of sterol may be one of the mechanism responsible for its anti-obesity activity (figure 2, 3 and 4). Stigmasterols and β-sitosterol are the plant sterol with a structure similar to that of cholesterol. Among that β-sitosterol compound having more comparable except for the substitution of an ethyl groups at C-24 of its side chain and it is cholesterol lowering agent [45]. β-sitosterol reduced absorption of cholesterol by 42% in a meal containing 500 mg of cholesterol [46]. Therefore, β-sitosterol may be a bioactive phytoconstituent in *Clerodendrum phlomidis* which may decrease the serum cholesterol by increasing the LDL receptor activity.

There was significant increase in the WAT, adiposity index and adipocyte diameter in the HFD group animals as compared to the normal control animals as indicated in fig. 10. These parameters were significantly decreased by administration of MECP 200 mg/kg & 400 mg/kg and AECP 400 mg/kg which exhibited the inhibitory activity of the extracts against adipocyte differentiation and proliferation. Moreover adipocyte diameter is clearly reflected high in HFD induced group compared to normal diet feed mice. Treatment with AECP (400 mg/kg), MECP (200 mg/kg) and MECP (400 mg/kg) along with HFD have shown smaller size of adipocytes than mice feed with plain HFD. Standard sibutramine have shown the best protection in this regard as reflected in fig. 11.

The possible mechanism of action behind this may be the inhibition of pancreatic lipase which is the most important enzyme of the human lipases for digesting fats responsible for the hydrolysis of 50-70% of total dietary fats. Verger, 1984

reported that dietary fat was hydrolyzed during digestion by pancreatic lipase [47]. The two main products formed by the hydrolysis of pancreatic lipase are fatty acids and 2-monoacylglycerols [48]. These lipolytic products are mixed with bile salts, dispersed as micelles and carried in this form to the site of fat absorption. Lipid absorption takes place in the apical part of the plasma membrane of epithelial cells or enterocytes lining the gut. Previously, in in-vitro study of pancreatic lipase activity we found that MECP inhibited the hydrolysis of triolein emulsified with phosphatidylcholine in a pancreatic lipase activity assay (Fig. 5). On the basis of these results, we designed in-vivo experiments and clarified that the increase in body and WAT weights induced by a high fat diet through inhibition of the intestinal absorption of dietary fat [49]. It has been reported in clinical studies that a pancreatic lipase inhibitor, orlistat prevented obesity and hyperlipidemia after treatment for 12 wk through inhibition of fat absorption [50-52]. It has been reported that various saponins isolated from foodstuffs have anti-obesity or hypolipidemic actions [53-56]. We found that the crude saponin fractions isolated from MECP strongly inhibited pancreatic lipase activity (figure 5). Therefore, it seems likely that the obesity, T2DM, dyslipidemia and other obesity associated complications which were reversed by MECP may be attributed in part to its crude saponin fractions. MECP have shown more potent action then AECP may be because of its β-sitosterol content which is high compared with AECP confirmed by HPTLC study (Fig. 2, 3, and 4). Another possible explanation is effect produced by the crude saponine fraction responsible for pancreatic lipase inhibitory activity, which is absent in AECP and present in MECP. Moreover crude flavonoid content of MECP may have anorexic activity which is responsible for decrease in food consumption [5].

Oral administration of extracts reduced the level of circulating lipids as well as the size of adiposite diameter, resulting in the decrease of body weights in C57BL/6J mice bearing close resemblance to human obesity. Extracts appear to show such activities by modulating the lipid metabolism through the decreased activity in lipogenesis, inhibition of pancreatic lipase activity as well as by decreasing food consumption. Among these two extracts MECP have shown more promising effects compared to AECP may be its multiple targets as mentioned above.

Conflict of interest statement

We declare that we have no conflict of interest.

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