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An *In vitro* Approach to Evaluate the Anti Adipogenesis Activity of *Passiflora edulis f. Flavicarpa* Degener Leaf Extracts in 3T3-L1 Cell Lines

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Abstract

Context: Obesity has been proved to be a complex multifactorial chronic disease in today's era accelerating the risk for Type 2 diabetes, coronary heart disease, and hypertension. The use of natural products in the management of obesity as well as reducing weight is being carried out by many researchers in the field of pharmacology.

Aim: The present study was carried out to determine the anti-adipogenic as well as anti-hyperlipidemic activities Extracts of *Passiflora edulis f. Flavicarpa* Degener leaves (EEPE) using 3T3-L1 Murine adipocyte cell lines.

Settings and Design: MTT assay was performed for cell viability as well as inhibition of the extract produced on the differentiation of pre-adipocytes; to study the anti-hyperlipidemic activity we used Oil red O staining method and pancreatic lipase inhibition activity.

Methods and Material: MTT assay was performed for cell viability studies on 3T3-L1 cell lines using the extract. The anti-hyperlipidemic activity of the EEPE in 3T3-L1 cell lines was evaluated by Inhibition of pancreatic lipase and lipid accumulation study using Oil Red O Staining wherein Orlistat and Simvastatin were used as the reference standard respectively.

Statistical analysis used: All the experiments were performed in triplicates and the results were expressed Mean ± SEM where n=3. A student's t-test was done and a p-value <0.05 was considered to be significant.

Results: In the course of cell viability determination, EEPE exhibited 42.06% at 150 µg concentration which was higher when compared to Simvastatin whose cell viability was 41.36% at 50 µg. The Lipid accumulation was substantially inhibited by test extract and simvastatin at 45.19% and 31.45% respectively whereas the % inhibition of the pancreatic lipase in matured 3T3-L1 adipocytes by the inhibition of breakdown of triglycerides was found to be 60.44% and 49.43% at 450 µg for EEPE and Orlistat respectively.

Conclusion: Thus, we conclude that EEPE has significant hypolipidemic effects on 3T3-L1 cell line, therefore, holds promise as an anti-hyperlipidemic herb.

Keywords: Antiadipogenic; Antihyperlipidemic; Murine adipocytes; Ethanolic extract of *Passiflora edulis f. Flavicarpa* Degener (EEPE)

Introduction

Obesity can be due to hyperplastic (increase in adipocyte number) and hypertrophic (an increase of adipocyte volume) too and confers to be a characteristic feature of obese and overweight individuals and is correlated with the degree of obesity along with increased risk of hyperlipidemia [1].

3T3-L1 cell lines derived from murine adipose tissue are well-established *in vitro* model for assessing adipocyte differentiation and pathological stages related to obesity. To examine the effect of plant extract on cell viability of 3T3-L1 pre-adipocytes, MTT cytotoxicity assay should be performed that measures mitochondrial activity. The screening strategies used in the development of anti-obesity drugs are by monitoring its lipase inhibition activity as well as by measuring the lipid accumulation in 3T3-L1 cell lines using Oil Red O staining quantification method. The principle of pancreatic lipase inhibition assay is that it inhibits the breakdown of triglycerides into fatty acids; thereby digestion and metabolism of lipids are inhibited and promotes anti-obesity and anti-hyperlipidemic activity [2]. The lipids being stored in the cells can be utilized for energy expenditure and for the synthesis of the lipid bilayer. Lipid reserves are a characteristic of adipocyte cell; increased lipid droplet accumulation within cells can be an indicator of metabolic deficiency or pathogenesis. For example, excessive accumulation of lipids in liver cells (steatosis) can lead to cellular dysfunction [3]. The fat droplet formed in 3T3-L1 cells and effect of plant extract inhibition on fat droplet formation is quantified by Oil Red O staining method.

Passion (*Passiflora edulis f. Flavicarpa* Degener) fruit plant, its juice, seed and peel of passion fruit shows antioxidant properties; the passion fruit peel extract and pulp could be used for lowering the blood glucose level and has anti-diabetic properties too [4]. These are less explored plants on account of its anti-hyperlipidemic activity. Since it possesses phytoconstituents such as phenols and flavonoids which are

purported to exhibit wide pharmacological activities [5]. However, there were no scientific, systematic investigations carried out with these plants for anti-hyperlipidemic activity. Out of our quest for knowledge and keeping in view of the therapeutic efficacy of herbal medicines, the plant *Passiflora edulis f. Flavicarpa* Degener has been selected to evaluate anti-hyperlipidemic activity using *in vitro* models.

Materials and Methods

Plant material and extraction

Fresh leaves of *Passiflora edulis f. Flavicarpa* Degener leaves were collected from the local areas of Kottayam district, Kerala during the months of September-October and was taxonomically identified and authenified by a botanist, Dr Jomy Augustine, HOD, Associate Professor, Botany Department, St. Thomas College, Palai. A voucher specimen (4751) was preserved at the Herbarium of Department of Pharmaceutical Sciences, Cheruvandoor.

Extraction of leaves of *Passiflora edulis f. Flavicarpa* Degener leaves were carried out using soxhlet apparatus. 779 g of shade-dried leaves were taken size reduced, extracted with 5.6 L ethanol solvent system in a round bottom flask and extraction was continued for 24 hr. The extract obtained was collected and the solvent was evaporated using a distillation unit and then the extract was concentrated by gentle heating. The concentrated extract was then weighed and stored in the refrigerator in a glass bottle till further use. The concentrate was thoroughly air dried to remove all traces of the solvent and the percentage yield was calculated.

Phytochemical screening of plants extract

Preliminary phytochemical screening was carried out using standard laboratory measures to evaluate the presence of alkaloids, flavonoids, tannins, saponins, quinone, and steroid/triterpenoids in the plant's ethanolic extract.

Cell lines and culture medium

3T3-L1 cell lines were obtained from National Centre for Cell Sciences (NCCS), Pune, India. Stock cultures of the cell lines were cultured in DMEM medium supplemented with 10% inactivated fetal bovine serum, Penicillin (100 IU/ml), Streptomycin (100 mg/ml), and Amphotericin B (5 mg/ml) in a CO_2 atmosphere at 37°C until confluent. The cells were dissociated with 0.2% trypsin, 0.02% EDTA in PBS solution. All the experiments were carried out in 96 well microtitre plates (Corning, USA).

In vitro cell viability of the plant extract in 3T3-L1 cells

The effect of plant extract on cell viability was performed by MTT assay. For testing the *in-vitro* cytotoxicity of the plant extract, an MTT assay was performed to assess the cell viability with concentrations of test extracts of the selected plants ranging from 50 µg to 450 µg. 100 ml of different test concentrations of test drugs were added on to the partial monolayer in microtitre plates. The plate was incubated for 24 hr at 37°C in a 5% CO_2 atmosphere. MTT reagent to a final concentration of 0.5 mg/mL of total volume was added to the wells and further incubated for 3 hours. The MTT reagent was replaced with 100 µl DMSO. Simvastatin was used as a positive control. The absorbance was read using an ELISA reader at 570 nm and 630 nm. The IC_{50} value was determined by using a linear regression equation. The percentage growth inhibition was calculated using the following formula:

In vitro anti-obesity studies of EEPE in 3T3-L1 cells: Pancreatic lipase Inhibition Assay

The pancreatic lipase inhibitory activity of given sample is determined using p-nitrophenyl palmitate (pNPP) as a substrate. The enzyme under the reaction conditions hydrolyses p-NPP to release p-nitrophenol, which is a coloured substance and can be monitored at 410 nm. The cells were exposed to various concentrations of the crude extracts (50, 150, 250, 350, 450 µg) and fractions were prepared in DMSO (50-450) ug/mL. Lipase (0.1 mg) was dissolved in Tris-buffer (50 mM, pH 8) and added to the cell supernatant. The mixture was stirred for 15 min and centrifuged at 2000 rpm for 10 min. The clear supernatant was recovered. Different concentrations of given sample (or, Orlistat) was mixed with 0.5 mL lipase solution. It was incubated for 30 min at 37°C. Then, 1 mL substrate p-NPP (3 mM in 2-propanol) was added to all the tubes. After incubating the mixture for 2 hr at 37°C, its absorbance was recorded at 410 nm against a blank. The control contained all constituents except a test sample. Orlistat was used as a positive control.

The per cent inhibition was calculated using the following formula:

$$\%\ Lipase\ Activity = 100 - \left[\frac{A_s - A_{Blank}}{A_C - A_{Blank}}\right]$$

Where, A_S and A_B are the absorbance of Sample and Control, respectively.

In vitro anti-adipogenic studies of EEPE in 3T3-L1 cells: Oil red O staining method

The test concentrations of plant extract for anti-adipogenic studies were determined based on the results obtained in cytotoxicity studies and test were carried out on 3T3-L1 cells and the effect of plant extracts on inhibition of fat droplet formation was determined by quantification of Oil Red O staining method.

Lipid (Oil red O) staining: 1000 µl cell suspension was seeded in a 6-well plate at required cell density (106 cells per well), without the test agent. The cells were allowed to grow for about 72 hr. A stock solution of 1000 mcg/mL was prepared by making up the sample extract and DMEM supplemented with 2% inactivated FBS concentration. The plate was incubated for 24 hr at 37°C in 5% CO_2 atmosphere, following which the spent medium was removed.

Cell Fixing was done on the removal of the cells from the media by gentle washing with PBS. 10% formalin was added to each well, incubated for 30 min to 1 hr. Cell Staining was performed based on the standard procedure [6]. The absorbance was read on an ELISA reader at 492 nm.

The Oil Red O intensity in treated samples was calculated relative to untreated samples using the following equation:

$$\%\ Intensity\ of\ Oil\ Red\ Staining = \frac{Absorbance\ of\ treatment}{Absorbance\ of\ control} \times 100$$

Results

Quantification of ethanolic extracts of *Passiflora edulis f. Flavicarpa* Degener leaves

The ethanolic extract after soxhletation and distillation was obtained and the percentage yield of the ethanolic extract of *Passiflora edulis f. Flavicarpa* Degener leaves was found to be 24.73% w/w.

Phytochemical screening of plant extract

The Ethanolic extract of *Passiflora edulis f. Flavicarpa* Degener leaves (EEPE) was subjected for qualitative chemical analysis for the identification of various phytochemicals and results of the chemicals tests for the extract was recorded and tabulated in the following Table 1.

Sl. No.	Test for phytochemical analysis	Observation
1	Carbohydrates	+ +
2	Flavonoids	+ +
3	Phenolics	+ +
4	Saponins	-
5	Glycosides	-
6	Triterpenes	+ +
7	Alkaloids	+ +

Table 1: Result of phytochemical screening of EEPE.

In vitro cell viability of EEPE in 3T3-L1 cells

The results of *in vitro* cell viability are presented in Table 2 and Figure 1 shows the photographs of cell lines when assessed for cell viability at 20X.

In vitro anti-obesity studies of EEPE in 3T3-L1 cells

The inhibitory effect of different concentrations of EEPE on pancreatic lipase enzyme is shown in Table 3.

In vitro anti-adipogenic studies of EEPE in 3T3-L1 cells

Effect of EEPE on Oil Red O staining in cultured 3T3-L1 cells is depicted in Figure 2 and the percentage inhibition is represented in Table 4.

	Absorbance (mean ± SEM)	Viability %
Blank	0.06	
Control group	0.5370 ± 0.0032	100.00 ± 0.003
Simvastatin (50 µg)	0.2573 ± 0.0065	*41.36 ± 0.006
EEPE 50 µg	0.3916 ± 0.0008	*69.53 ± 0.008
EEPE 150 µg	0.2606 ± 0.0151	*42.06 ± 0.01
EEPE 250 µg	0.1826 ± 0.0008	*25.71 ± 0.002
EEPE 350 µg	0.117 ± 0.0012	*11.94 ± 0.001
EEPE 450 µg	0.08 ± 0.0012	*4.19 ± 0.001

Table 2: MTT Cell viability readings; Data is shown as Mean ± SEM where n=3 and * shows p value <0.05.

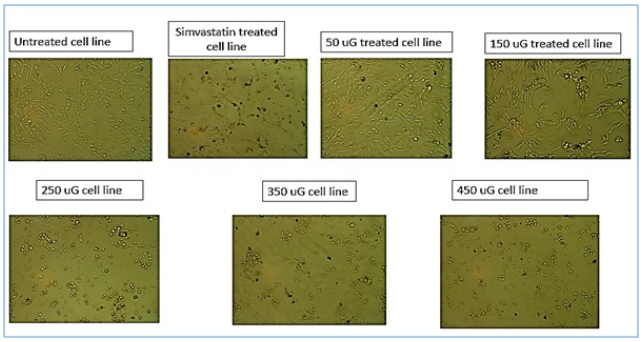

Figure 1: Photos for MTT cell viability assay at 20X

	Absorbance (mean ± SEM)	Lipase inhibition activity (%)
Blank	0.0013	
Control group	0.646 ± 0.0153	100.00 ± 0.01
Standard group (Orlistat-50 µg)	0.3273 ± 0.0014	*49.43 ± 0.001
EEPE 50 µg	0.5643 ± 0.0034	*12.66 ± 0.004
EEPE 150 µg	0.4833 ± 0.0010	*25.23 ± 0.001
EEPE 250 µg	0.42 ± 0.0048	*35.05 ± 0.002
EEPE 350 µg	0.326 ± 0.0067	*49.63 ± 0.001
EEPE 450 µg	0.2563 ± 0.0029	*60.44 ± 0.002

Table 3: *In vitro* pancreatic lipase inhibition values on 3T3-L1 cell lines. Data is shown as Mean ± SEM where n=3 and * shows p-value <0.05

	Control (Cell line)	Simvastatin (50 µg)	Test (130.56 µg)
Reading 1	0.798	0.265	0.375
Reading 2	0.816	0.243	0.368
Reading 3	0.809	0.254	0.352
Mean ± SEM	0.807 ± 0.0006	0.254 ± 0.0002	0.365 ± 0.0001
% Intensity	100	*31.44 ± 0.002	*45.19 ± 0.001

Table 4: Oil Red O staining values on 3T3-L1 cell line; Data is recorded for single concentrations only after MTT assay and shown as Mean ± SEM where n=3 and * shows p-value <0.05

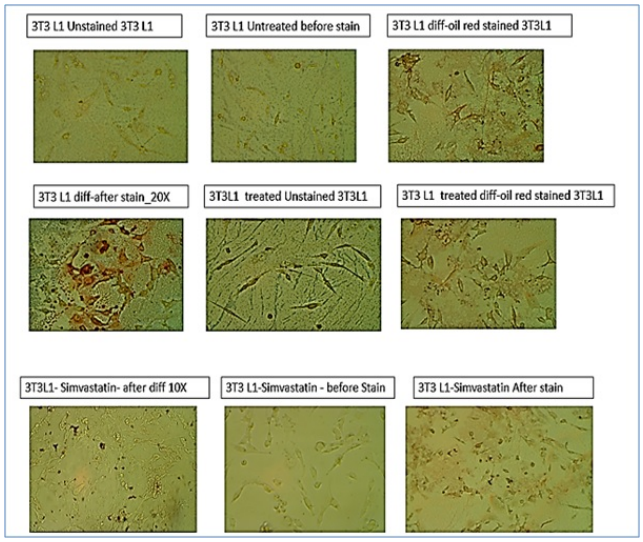

Figure 2: Photos of Oil red O Staining at 20X.

Discussion and Conclusion

The percentage yield obtained after the ethanolic extraction of dried leaves of *Passiflora edulis f. Flavicarpa* Degener leaves was found out to be 24.73% w/w. The ethanolic extract of *Passiflora edulis f. Flavicarpa* Degener leaves (EEPE) was subjected for qualitative chemical analysis for the identification of various phytoconstituents and the observations showed that the crude ethanolic extract of *Passiflora edulis f. Flavicarpa* Degener showed the presence of carbohydrates, alkaloids, flavonoids, phenolics, triterpenoids and steroids in the extract. The presence of flavonoids and phenolics attribute to anti- obesity effect by anti-lipogenic activities [7]. Flavonoids also influence lipolysis and anti-adipogenesis in lipid cells. Several flavonoids have exhibited lipolytic activity additively with epinephrine in primary rat adipocytes [8]. The effect of EEPE on cell viability of 3T3-L1 pre-adipocytes was assessed by MTT assay by measuring the mitochondrial activity in 3T3-L1 cells, when treated with different concentrations of test extracts. The cell viability percentage was found to be 69.53% and 42.06% at 50 µg and 150 µg respectively and the cell viability against Simvastatin was found to be 41.36% at 50 µg concentration. The cell viability was found to decrease due to the inhibition activity produced by the extract upon adipocytes [9].

At the cellular level, obesity was originally considered a hypertrophic disease. Hyperplasia, however, is correlated more strongly with obesity severity and is most marked in severely obese individuals. New fat cells constantly arise from a pre-existing population of undifferentiated progenitor cells or through the dedifferentiation of adipocytes to pre-adipocytes. In both cases, the generation of new fat cells plays a key role in the development of obesity [10]. Therefore the increase in concentration of EEPE to the cell line results in decreased number of cells after incubation of pre-adipocytes. This can attribute to the fact that there is decrease in the number of adipocytes (Anti-obesity effect as per the definition of obesity in cellular level) after EEPE treatment is due to its anti-adipogenic activity i.e, the extract inhibits the differentiation of pre-adipocytes, not due to cell cytotoxicity, which points out the fact that the ethanolic extract of *Passiflora edulis f. Flavicarpa* Degener leaves are hypolipidemic in nature.

The aim of pancreatic lipase activity assay was to know the ability of plant extract to inhibit lipid absorption into the body by inhibiting pancreatic lipase activity [11]. The two main products formed by the hydrolysis of pancreatic lipase are fatty acid and 2-monoacylglycerol. Pancreatic lipase activity is known to act by promoting absorption of monoglyceride and free fatty acid into the body, which is a known cause of obesity [2]. Due to inhibition of pancreatic lipase activity of EEPE extract prevents lipid accumulation into the body [7]. Orlistat was chosen as the standard drug of comparison, approved by FDA and available for the obesity treatment apart from centrally acting anti-obesity drugs. This extract acts through the pancreatic lipase inhibition [12]. The untreated cell lines showed 0% lipase inhibition activity; the Orlistat showed 49.43% lipase inhibition activity and the extract showed a dose dependent increase in the inhibition activity i.e, 12.66% lipase inhibition activity at 50 µg and 60.44% lipase inhibition activity was reported at 450 µg respectively.

The anti-hyperlipidemic activity was performed using Oil Red O staining method on 3T3-L1 cell lines. IC_{50} value from MTT Assay was selected for the concentration of test extract. Adipogenesis was substantially inhibited by the test extract of PEE (130.56 µg) and showed anti adipocyte accumulation and color intensity was found to be decreased to 45.20%. The control cell line did not show inhibition of fat accumulation while, Simvastatin (50 µg) showed anti adipocyte accumulation and the color intensity was found to decrease to 31.45%. These observations demonstrate that the test extract for anti-adipogenesis activity when compared with standard drug proved to be beneficial in the management of hyperlipidemia [13]. Thus the EEPE as well as Simvastatin were found to significantly reduce lipid accumulation in 3T3-L1 adipocytes, suggesting anti-obesity activity.

These observations from the data demonstrate that evaluation of anti-adipogenesis activity *in vitro,* ethanolic extract of *Passiflora edulis f. Flavicarpa* Degener (EEPE) leaves proved to be beneficial in the management of hyperlipidemia and obesity. Further investigations are in progress to screen the selected plant extract for anti-hyperlipidemic and anti-atherosclerotic activities *in vivo.*

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