ANTI-ADIPOGENIC EFFECT OF *ELATERIOSPERMUM TAPOS* ON 3T3-L1 CELLS

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Abstract

Obesity is defined as an excessive fat accumulation that causes metabolic syndrome such as diabetes, hypertension, and abnormal cholesterol levels. Adipocytes are a major part of the adipose tissue that grows the abnormal lipid droplets which leads to fat accumulation. In modern drugs, treating obesity without any side effects is challenging. Natural products such as plants and herbs are widely used to cure obesity. Natural products are safer because of their reduced toxicity and infrequent negative effects. This study focuses on examining the potential anti-adipogenesis effects of *Elateriospermum tapos* on 3T3-L1 cells. The extracts from both seed and shell of the fruit were extracted using hot, cold and ethanol extraction. The extracts were tested for the cytotoxicity on 3T3-L1 cells and on zebrafish embryo *in vivo*. Zebrafish study on heartbeat, heart rate and scoliosis formation, shell extract show less toxic compared to seed extract. This shell extracts showed a positive correlation determination of $R^2 = 0.96$. Therefore, shell extracts were used to indicate the maximum non-toxic dose (MNTD) of each extraction. The lowest MNTD was observed in ethanol extract at 5.2 ± 0.01 µg/mL, hot extract at 7.6 ± 0.25 µg/mL and cold extract at 8.1 ± 0.31 µg/mL. Oil Red O staining to determine the amount of lipid accumulation showed a significant ($P < 0.05$) decrease of 70% when compared to adipocytes when treated with ethanol shell extract, and a significant ($P < 0.05$) decrease of 60% by hot shell extract. With the reduction in lipid accumulation, lipolysis was measured by accounting the amount of glycerol produced. The highest production of glycerol was significant when treated with hot shell extract at 42 mg/L, followed by cold extract at 38 mg/L. This is concluded that the hot shell extract has potential to curb obesity by reducing lipid accumulation through lipolysis at a maximum non-toxic dosage.

Keywords**:** Anti-adipogenesis, *Elateriospermum tapos*, 3T3-LI cells

Introduction

Obesity is no longer a minor problem worldwide; it is now associated with several diseases, including cancer, coronary artery disease, hypertension, and type 2 diabetes (1). Obesity is defined as a BMI of 30 kg/m^2 or higher (2). In past decades, the prevalence of obesity has been plummeting and in accordance with the Malaysian National Health and Morbidity Survey (NHMS) 2019 (3), the percentage of overweight and obese was 21.8% and it increased from 2011 prevalence (16.6%) and 17.75 from 2015 prevalence.

Obesity is defined by an imbalance in energy expenditure and consumption, resulting in an increase in total fat mass in the body (4). Although reducing calories intake and increasing physical exercise is a good approach to reduce weight, the use of supplements and medicines is gaining popularity. Medicine-induced weight loss provides only short-term benefits in the treatment of obesity, and it is commonly associated with undesirable side effects such as rebound weight gain when the medication is stopped or other side effects such as kidney failure etc.

Here we propose that a variety of extraction solvents derived from natural such as plants and herbs can be widely used to help people lose weight. *Elateriospermum tapos (E. tapos)* belongs to the Euphorbiaceae family and is found in abundance in Peninsular Malaysia's Jengka Forest Reserve (5). Novel phytochemicals with anti-adipogenesis activities have been found in this fruit which is known as flavonoids.

The seed and shell parts of the *E. tapos* fruit were included in this investigation since they have been documented to contain flavonoids, tannin, alkaloids, oleic acid, linolenic

acid, saturated and unsaturated fatty acids (6). Flavonoids are polyphenolic substances that are added to the human diet (7) and have been shown to help in obesity treatment (8). As a result, the goal of this study is to see if *E. tapos* fruit extracts may inhibit adipogenesis in 3T3-L1 cells.

Materials and methods

Sample preparation (hot, cold aqueous and ethanol extraction)

A 70°C water bath was used to heat 50 g of dried seed and shell powders in 500 mL distilled water for 24 hours. The solution was left at room temperature in 500 mL distilled water for two days for cold extraction. The aqueous extracts were vacuum filtered through Whatman No. 1 paper, then freeze-dried. Later the powder was kept at -20°C for subsequent analysis. For the ethanol extraction, 50 g of dried powdered shell and seed samples were extracted for three days with a 70% ethanol solution at a 1:9 ratio. Before agitating with an orbital shaker, the mixtures were placed in a conical flask coated in aluminum foil. To get a clear solution, the mixtures were filtered through a Whatman No. 1 filter paper. The filtrate is then allowed to evaporate under reduced pressure at 40°C in a rotary vacuum evaporator. Before analysis, the crude extracts were weighed and kept at -20°C (2, 9).

Fish embryo acute toxicity test (FET) OECE guidelines No 236

Zebrafish embryos were obtained from Danio Assay Laboratories Sdn Bhd, Universiti Putra Malaysia. A single 24-hour post fertilization (hpf) embryo was added into a 96 well-plate and treated with the *E. tapos* extracts for four days. The development of each embryo was monitored in a 24-hour interval for 96 hours. Every 24-hours, the embryos were checked on the coagulation of fertilized eggs, the lack of somite formation, lack of detachment of the tailbud from the yolk sac, lack of heartbeat, and the hatching rate. After 96-hours, the IC_{50} and LC_{50} (log inhibitor versus response) with the 4 parameters were selected for the regression correlation graph.

Cell culture and differentiation

The mouse fibroblast cell line 3T3-L1 (ATCC® CL-173TM) from *Mus musculus* was employed in this work. The ATCC technique for differentiating of 3T3-L1 pre-adipocytes was followed. Seeded cells were maintained in Pre-adipocyte Expansion Medium (PEM) for 48 hours at 37°C in a 5% (v/v) CO_2 incubator until they reached 100% confluence (Day 2). The cells were cultured for 72 hours at 37°C in a 5% (v/v) CO_2 incubator after being replenished with an equal amount of Differentiation Medium (DM) Day-0. The medium was changed to Adipocyte Maintenance Medium (AMM) three days later (Day-3), and AMM was changed every 48-hours after that. After stimulation, the cells had fully differentiated by Day-10. Pre-adipocytes can be fully differentiated and used for bioassays between eight to 12 days after DM induction. Medium formulations are as described in Table 1.

Table 1: Description of medium formulation

Medium	Formulation
Pre-adipocyte expansion (PEM)	90% Dulbelcco's Modified Eagle's Medium (DMEM), and 10% Fetal Bovine Serum
Differentiation (DM)	90% DMEM, 10% FBS, 0.5 mM IBMX, 0.25 µM Dexamethasone, 1 µg/ml Insulin and 2 µM Rosiglitazone
Adipocyte Maintenance (AMM)	90% DMEM, 10% FBS and 1 µg/ml Insulin

Determination of maximum non-toxic dose (MNTD) by MTT Assay

3T3-L1 pre-adipocytes were seeded at a density of 2,500 cells per well in a 96-well plate. The cells were treated with *E. tapos* crude extracts (0-1000 g/mL) at 70% confluence. Cell viability was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, followed by a 48-hour incubation of the cells at 37°C in a humidified environment containing 5% (v/v) CO_2. The cells were rinsed twice with phosphate buffered saline (PBS) after 48 hours. Twenty microliters of MTT stock solution (5 mg/mL) were added to each well, and the cells were then incubated at 37°C for an additional 4 hours to solubilize the water-soluble purple formazan crystals (10). After an hour of incubation, the absorbance was measured using a microplate reader at a wavelength of 570 nm (M200 Tecan, USA). A graph showing percentage of toxicity against the $\log^{10}$ concentration of phytochemicals was plotted to determine the MNTD or 12 MNTD of the extracts.

Characterization of adipogenesis using oil red O (ORO) staining

The 3T3-L1 pre-adipocytes were seeded at a density of 5.0 x 10^5 cells per well in a 12-well plate, and the normal adipogenic induction protocol was followed. From MNTD Day-0 onwards, the procedure of normal adipogenic induction was followed, with the addition of phytochemicals. The cells were kept in the normal adipogenic induction regimen until Day-10. Oil Red O staining confirmed the mature adipocytes' differentiation (ORO). ORO labelling was used to evaluate intracellular lipid levels in differentiated adipocytes. The cells were washed three times with phosphate buffered saline (PBS) eight days (Day 10) after induction of differentiation. The cells were fixed in 10% formalin for one hour at room temperature. The cells were rinsed in PBS again after fixation, and then stained with a freshly diluted Oil Red O solution made up

of three parts of 0.5% Oil Red O in isopropyl alcohol and two parts of waters. After staining for 30 minutes, the cells were rinsed twice with water and examined under a phase contrast microscope. The optical density of Oil Red O staining was measured after it was dissolved in isopropyl alcohol for quantitative analysis. A microplate reader was used to read the absorbance at 520 nm wavelength with isopropyl alcohol used as a blank.

Measurement of glycerol production

The Glycerol Assay Kit (Sigma) was used to measure glycerol production according to the manufacturer's instructions. The supernatants were collected and added to a 96-well plate containing reagent, with absorbance at 570 nm recorded with a microplate reader.

Statistical analysis

All results are reported as standard error of means (SEM) of three independent experiments performed in triplicate. One-way analysis of variance (ANOVA) and Tukey's post-hoc test IBM SPSS statistics were used for statistical analysis (Version 22). Statistical significance was defined as a *P* value less than 0.05 ($P < 0.05$).

Results

Effect of E. tapos extracts against cytotoxicity of normal cell line (3T3-LI)

The effect of *E. tapos* shell extracts against the normal cell lines (3T3-LI) determined by MTT assay is shown in Table 2. As observed in Table 2, the IC_{50} of seed extracts were all lower compared to the shell extracts. The lowest IC_{50} for seed was by using the cold aqueous extract (28.6 ± 0.05 µg/mL), followed by ethanol (36.1 ± 0.02 µg/mL) and hot aqueous extract (45.8 ± 0.05 µg/mL). The hot aqueous extract of the *E. tapos* shell showed the lowest IC_{50} (49.8 ± 0.01 µg/mL) when compared to the other extractions. Followed by ethanol extract (51.4 ± 0.01 µg/mL) and cold aqueous extract (75.9 ± 0.01 µg/mL). To make sure that the extracts were not toxic to animals, another toxicity test was performed using zebrafish embryo to obtain the correlation on the concentration of *E. tapos* extracts to be used in this study.

Table 2: Cytotoxic effect of *E. tapos* extracts against normal cell line (3T3-LI). Data are expressed as mean ± SEM, n = 12, results are from three independent experiments

E.tapos	IC50 (µg/mL) ± SEM	
	Solvents	**µg/mL**
Seed	Hot Aqueous (HSD)	45.8 ± 0.05
	Cold Aqueous (CSD)	28.6 ± 0.05
	Ethanol (ESD)	36.1 ± 0.02
Shell	Hot Aqueous (HSL)	49.8 ± 0.01
	Cold Aqueous (CSL)	75.9 ± 0.01
	Ethanol (ESL)	51.4 ± 0.01

The LC_{50} of zebrafish embryo at 72 hpf

It is observed that the embryo treated with the IC_{50} of *E. tapos* seed has caused the embryo to coagulate, showing that the concentration of the extracts was toxic as shown in Table 3. This is, however, opposed to the *E. tapos* shells which demonstrated live embryos with an almost similar patterns in the heart rate (beats/min) when compared to the control (embryo media) and paracetamol. There was no scoliosis seen in the live larvae which display that there is no toxicity observed from the treated extracts.

Table 3: Cytotoxic effect of *E. tapos* extracts against zebra fish. Data are expressed as mean ± SEM, n = 12, results are from three independent experiments

	Plant extracts	**Heart rate (beats/ min)**	**Heart beat**	**Scoliosis**
E.tapos seed	Hot	1	1	-
	Cold	1	1	-
	Ethanol	1	1	-
E.tapos shell	Hot	182 ± 0.6	0	0
	Cold	136 ± 0.5	0	0
	Ethanol	188 ± 0.5	0	0
Control (embryo media)		186 ± 0.5	0	0
Paracetamol		188 ± 0.5	0	0

1 = absence of heart beat, dead or coagulated, severe scoliosis
0 = present of heart beat, healthy, no scoliosis

The correlation study between IC_{50} of 3T3-LI cell lines and LC_{50} Zebrafish embryo (72hpf)

The results from the IC_{50} of MTT assay on 3T3-L1 cell and LC_{50} of the zebrafish embryo to observe the correlation between both the outcome has been plotted in Figure 1. There is a positive correlation between both the IC_{50} and LC_{50} making the coefficient of determination, $R^2 = 0.9649$.

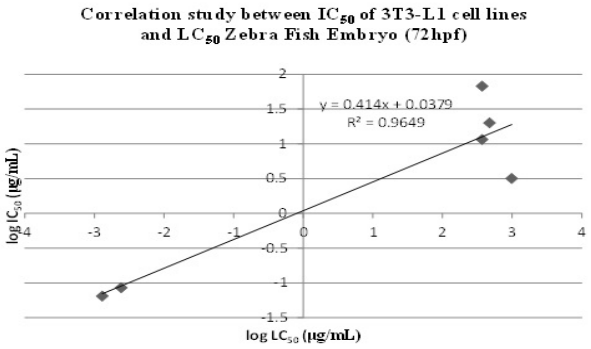

Figure 1: Above figure show the correlation between LC_{50}-72 hpf and IC_{50} of 3T3-LI cell lines and zebrafish embryo. The $R^2 = 0.9649$ represents the positive correlation between cytotoxicity and embryo toxicity

The maximum non-toxic dose (MNTD) of E.tapos shell extracts

With the correlation being positive for the shell extracts, MNTD was determined to rule out a direct cytotoxic effect of the extracts on 3T3-L1 pre-adipocytes. MNTD was selected as the graph crosses zero at both axis and the concentration µg/mL were as tabulated in Table 4.

Table 4: The maximum non-toxic dose of *E. tapos* extracts against normal cell line (3T3-LI). Data are expressed as mean ± SEM, n = 12, results are from three independent experiments

MNTD (µg/mL) ± SEM			
	E. tapos	**Solvents**	**µg/mL**
Plant extracts	Shell	Hot Aqueous (HSL)	7.6 ± 0.25
		Cold Aqueous (CSL)	8.1 ± 0.31
		Ethanol (ESL)	5.2 ± 0.01

The effect of E. tapos shell on lipid accumulation

To assess the impact of *E. tapos* shell extracts at their maximum non-toxic dose (MNTD) on pre-adipocyte differentiation and lipid accumulation, we performed Oil Red O staining and visualized cell morphology through light microscopy (Figure 2). The control group (adipocytes) exhibited cell differentiation and the presence of lipid droplets induced by the differentiation media over the course of 10 days. However, the treatment of three extracts (HSL, CSL, and ESL) inhibited differentiation of adipocytes and lipid droplets production, compared with control group. In particular, ESL inhibited more effective differentiation and lipid droplets among other extracts.

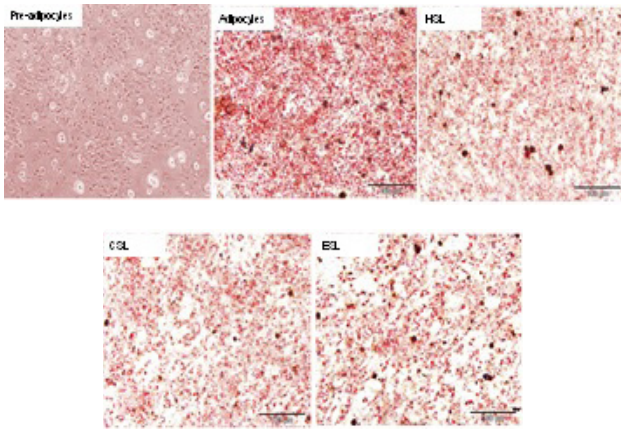

Figure 2: The effects of various extracts obtained from *E. tapos* shell were examined on differentiated 3T3-L1 cells. Adipocyte differentiation was induced by treating the cells with PEM media for two days, either with or without the presence of extracts. Afterward, the media was replaced with DM media and allowed to differentiate for three days. Throughout the differentiation process, the media was changed with AMM media every 48 hours until day-10. The cells were visually confirmed using light microscopy at a magnification of x 40. Subsequently, the cells were fixed and subjected to Oil Red O staining to visualize the lipid droplets in the differentiated cells, following treatment with different extracts: HSL (Hot shell extract), CSL (Cold shell extract), and ESL (Ethanol shell extract).

The pre-adipocytes (Figure 2) showed the normal architecture of the undifferentiated 3T3-L1 in cell lines with the nucleus and no lipid droplets. *E. tapos* treatment groups showed few massive lipid droplets. The treatment has been replaced the large fat droplets into small fat droplets. However, the *E. tapos* shell treated groups has reduced the fat accumulation in the cells.

After visualization, the cells where Oil Red O staining was dissolved with isopropyl alcohol for quantitative analysis, and the optical density was measured at 520 nm. As shown in Figure 3, the percentage of lipid accumulation in adipocyte has significantly ($P < 0.05$) increased when compared to pre-adipocytes. The cells were treated with different extractions HSL, CSL and ESL showed a reduction in lipid accumulation when compared to adipocytes (positive control). All treatment groups have suppressed the lipid accumulation however, HSL has a significant decrease when compared to the adipocytes. ESL has only 30% of lipid accumulation which was the lowest percentage of lipid compared to the other extracts.

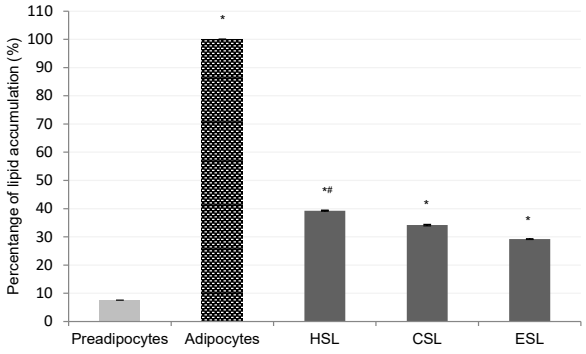

Figure 3: Effect of *E. tapos* on 3T3-LI cell lipid accumulation (%). The one-way ANOVA, post-hoc, and Tukey's test were used to analyse the data, which are expressed as mean and SEM. Significant differences are shown by different superscript letters * and # at $P < 0.05$

The effect of E. tapos shell extracts on glycerol production

All the treatment groups demonstrated a significant ($P < 0.05$) higher production of glycerol when compared to adipocytes (Figure 4). HSL showed an increased 2 folds significantly ($P < 0.05$) compared to the adipocytes. While CSL also presented an increase in glycerol production about 1.9 folds compared to the positive control ($P < 0.05$).

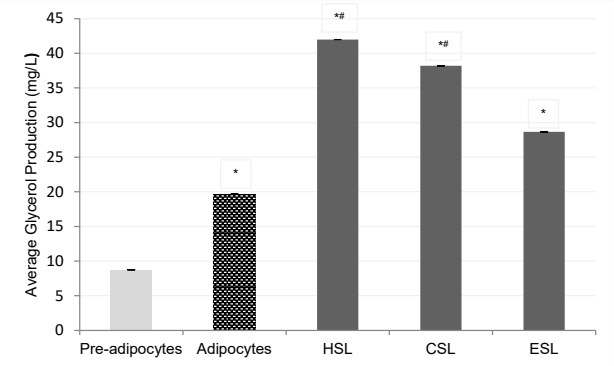

Figure 4: Effect of *E. tapos* on 3T3-LI cell glycerol synthesis. The one-way ANOVA, post-hoc, and Tukey's test were used to analyse the data, which are expressed as mean and SEM. Significant differences are shown by different superscript letters * and # at $P < 0.05$

Discussion

In the present study, we subjected to testing both the seed and shell part of the fruit *E. tapos*. Toxicity test was carried out for all the extracts (hot aqueous, cold aqueous, and ethanol) on 3T3-L1 cell line to obtain the IC_{50}. The toxicity was also measured on the animal model which is the *Danio rerio* or zebrafish embryo accounting for their LC_{50} to acquire the correlation between both cell and animal toxicity testing. The correlation of determination was at R^2 of 0.96 when compared with the results from shell extracts only. However, the seed extracts have shown toxicity on the zebrafish embryo and were excluded from the subsequent parameters.

The maximum non-toxic dosage (MNTD) was determined from treatment with 3T3-L1 cells and the concentration was used subsequently. Here our study shows that the shell extracts have significantly reduced the lipid accumulation in 3T3-L1 adipocytes. The hot aqueous shell extract has a significant reduction when compared to the adipocytes itself. The reduction in lipid accumulation is commonly due to high phenolic content and flavonoids in the extracts as suggested by previous research (11-13). As reported by Nor-Liyana et al. (13), the hot extraction has the highest total phenolic content (1298.60 ± 4.24 µg GAE 100 g^{-1}) and a total flavonoid content of (16685.58 ± 487.77 µg CE 100 g^{-1}). Moreover, with the presence of high flavonoid content, Poudel et al. (14) showed that flavonoid from *Triticum aestivum* increases the gene expression by activating the adipogenic factor that is associated with lipid metabolism.

Other experiments demonstrated that the effect of green tea extraction had been reduced the fat accumulation by down regulated gene expression (15). In our study showed that the *E. tapos* shell suppressed the lipid accumulation in 3T3-LI cells. With the reduction of lipid accumulation, the glycerol production was then looked upon. It has been long assumed that this glycerol is a by-product of lipolysis, released by cell lipases acting on triacylglycerol (TAG) stores (16), and/or lipoprotein-carried TAGs (also known by lipoprotein lipase) (17). Lipolysis is of interest to anti-adipogenesis study due to the ability to reduce lipid storage. The extracts that exhibited inhibitory activity towards the lipid accumulation were found to trigger the metabolism process to glycerol release. The increase in lipolysis indicates that they could have valuable anti-adipogenic activity. The detailed mechanism of accelerating lipolysis by shell extracts is still unclear and should be resolved in the future. Further study should be conducted such as animal study to understand the mechanism of lipolysis effect of *E. tapos* and also other mechanisms as well.

Conclusion

In conclusion, our study indicated that hot aqueous shell extract exhibited the most beneficial acting as anti-obesity treatments supported by the previous study in its pharmacological activities of *E. tapos*. Specifically, these extracts have potential to curb obesity with no measurable cytotoxic activity towards 3T3-L1 adipocytes *in vitro* and in zebrafish embryo *in vivo* while decreasing the lipid accumulation. In aggregate, our results demonstrate that hot, cold, and ethanol shell extracts have a potential as anti-obesity optimization leads.

Competing Interest

The authors state that they do not have any conflicts of interest.

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