CHEMICAL ENGINEERING TRANSACTIONS
VOL. 56, 2017
A publication of AIDIC
The Italian Association of Chemical Engineering
Online at www.aidic.it/cet
Guest Editors: Jiří Jaromír Klemeš, Peng Yen Liew, Wai Shin Ho, Jeng Shiun Lim

ISBN 978-88-95608-47-1; **ISSN** 2283-9216
DOI: 10.3303/CET1756187

Eugenia Polyantha Enhances Adipogenesis via CEBP-A and Adiponectin Overexpression in 3T3-L1

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Fat cells or adipocytes are important sites in glucose metabolism where triglycerides are synthesised from the excessive glucose in blood. These cells are produced from a complex process called adipogenesis where pre-adipocytes are differentiated by chemical inducer into mature adipocytes. Herbs and plants have shown tremendous effects on adipogenesis. *Eugenia polyantha* is a tropical plant that has been used in Asian culinary preparations as well as traditional medicine in treating diabetes, arthritis and cardiovascular diseases. In this study, we examined the effect of water extract of *Eugenia polyantha* on 3T3-L1 cells differentiation in the presence and absence of insulin. Result shows that water extract of *Eugenia polyantha* alone failed to enhance differentiation, but addition of 1 µg/mL of insulin significantly increased the formation of adipocytes. Ten days treatment with *Eugenia polyantha* significantly increased the triglyceride content as measured by Oil Red O assay and glucose utilisation in medium by dose dependent manner. Intracellular CEBP-α, adiponectin and GLUT4 proteins were also expressed significantly during the treatment of low concentration of *Eugenia polyantha*. This study provides the first evidence of *Eugenia polyantha* sensitising effect on 3T3-L1 adipogenesis process by enhancing the expression of CEBP-α, adiponectin and GLUT4 proteins.

1. Introduction

Metabolic syndromes such as diabetes mellitus, hyperlipidaemia, stroke and cardiovascular diseases are closely linked to overweight and obesity (Singla et al., 2010). Individuals that have BMI 30 and above are categorised as obese. Poor diet and lack of exercise limits the breakdown of carbohydrate in which the excessive amount of sugar will be converted into lipid via glycolysis process and stored in fat cells for energy backup. Excessive amount of lipid causes the cell to expand/ enlarge, thus affecting the body weight and other metabolism pathways (Greenberg and Obin, 2006). Adipocyte tissue is an endocrine organ and important tissue for energy homeostasis and metabolic control. It acts as an energy storage in the form of lipids/ triglycerides. These molecules will undergo lipolysis as a feedback process during certain condition when glucose in blood dropped below normal levels.

Adipogenesis is a systematic differentiation process of pre-adipocytes undergoing differentiation to be matured adipocytes and can be differentiated by the changes in genotype and phenotypes characteristics (Gregoire et al., 1998). Adipocyte has emerged as an experimental model in obesity and diabetes research area due to its important role in glucose metabolism and insulin signalling pathways. 3T3-L1 cells derived from 3T3-Swiss albino mouse can be differentiated through chemical cocktail induction which consists of IBMX, DEX and insulin mixture (Lowe et al., 2011). This complex and systematic process is primarily regulated by two proteins that down and up-regulates during the differentiation process; PPARs and CEBPs (Rosen and Spiegelman, 2000).

Eugenia polyantha (EP) or known as daun salam is a tropical tree and usually added in daily culinary for additional aromatic ingredient. EP is also applied as traditional medicine to treat diabetes, diarrhoea, arthritis and cardiovascular diseases. Chromatographic fingerprinting showed that the main phenolic phytochemicals of EP are catechin, gallic acid and rutin (Lelono and Tachibana, 2013). Previous studies have demonstrated that this plant contains abundance of phenolic compound and shown tremendous antioxidant activities as

Please cite this article as: Ismail H.F., Majid F.A.A., Hashim Z., 2017, Eugenia polyantha enhances adipogenesis via cebp-α and adiponectin overexpression in 3t3-l1, Chemical Engineering Transactions, 56, 1117-1122 DOI:10.3303/CET1756187

compared to others popular plants and herbs such as *Melicopelunu ankeda* (Tenggek Burung), *Polygonum minus* (Kesum) and *Murraya koenigii* (curry) (Othman et al., 2014). However, experimental studies on the pharmacological effect and mechanistic study of this ethnomedicinal plant are limited. In this study, we examined the effect of water extract of EP on 3T3-L1 cells differentiation in the presence and absence of insulin. The expression of adipocyte related proteins were quantified by immunoblotting.

2. Materials and methods

2.1 Materials

Mouse preadipocytes 3T3-L1 were purchased from American Type Culture Collection, ATCC, Manassas, USA and 1.1B4 were purchased from Public Health England, Salisbury, UK. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT powder) were purchased from Invitrogen, Carlsbad, CA, USA. Dulbecco's Modified Eagle's Medium (DMEM), Roswell Park Memorial Institute Medium (RPMI), foetal calf serum (FCS) and penicillin strep (PS) were purchased from Gibco.

2.2 Herbs and extraction

Eugenia polyantha (leaves) was supplied by NatureMedic Laboratories (Terengganu, Malaysia) and taxonomically confirmed by Prof Dr. Fadzilah Adibah Abdul Majid. Specimen was deposited at Tissue Culture Engineering Research Group, Universiti Teknologi Malaysia under vouchers number EP-TCERG. Raw materials were ground into coarse form and extracted with filtered water by ratio 1.5 : 10 for 3 h at 60 °C. Spray drying was carried out at Institute of Bioproduct Development (IBD), Universiti Teknologi Malaysia. Extracts were stored at 4 °C until future purposes.

2.3 Selected biomarkers identification and quantification

One hundred mg of EP were dissolved in 25 mL of methanol and vortexed for 5 min to ensure dissolution and filtered through a 0.45 µm nylon filter. Standard stock solutions of gallic acid were prepared by dissolving 1 mg of biomarkers in 1 mL of methanol and sonicated for 5 min. Quantification of gallic acid was determined by HPLC fingerprinting analysis (Waters 2690 Alliance Separation Module with LiChrospher® 100 RP-18 endcapped, Merck column cartridge (250 x 4.6 mm, 5 µm). The chromatogram was monitored at wavelength of 272 nm.

2.4 Cytotoxicity assay

3T3-L1 cells at concentration of 5 X 10^4 cells/mL were seeded in 96 well plate and incubated at 37 °C under a humidified atmosphere of 5 % CO_2 for overnight. Cells were treated with a series of concentration ranging from 5 µg/mL to 10,000 µg/mL for 24 h. Treated wells were observed using inverted microscope to confirm the absence of contaminations before proceeding to 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. 20 µL of MTT solution was added to each well containing treated cells. Plate was incubated for 3 - 4 h in dark. MTT formazan were dissolved in DMSO and analysed at wavelength of 570 nm. Samples were run in 6 replicates. Plates without treatments were used as a control.

2.5 Cells differentiation and treatment

Two-days post confluent 3T3-L1 cells were incubated (Day 0) with differentiation medium (DM1) containing 1.0 µM dexamethasone (DEX), 0.5 mM methylisobutylxanthine (IBMX) and 1.0 µg/mL insulin for 96 h. DM1 were replaced by DM2 (DMEM supplemented with 10 % FBS and 1.0 µg/mL insulin) and incubated for another 48 h. Differentiated adipocyte were maintained in complete DMEM (10 % FBS and 1 % PS) for another 96 h by replacing medium every 48 h interval. Successful differentiations were considered with 80 - 90 % of lipid formation. EP treatment was carried out from Day 0 to Day 6.

2.6 Oil Red O (ORO) staining for lipid formation quantification

3T3-L1 cells were differentiated according to the protocol mentioned above. Cells were stained with ORO solutions and images were captured using inverted microscope equipped with a digital camera and imager software. To quantify the amount of formed lipid, 300 µL isopropanol were added and dissolved ORO dyes were measured at 670 nm. Experiments were carried out in triplicates. Cells without treatment were set as control.

2.7 Glucose utilisation treatment

Pre-adipocytes were differentiated according the protocol mentioned above. To start the treatment, mature adipocytes were serum-starved for 3 h supplemented with 0.2 % bovine serum albumin (BSA). Cells were treated with extracts diluted in complete DMEM with additional of 1 µg/mL insulin and incubated for 48 hours.

Spent DMEM were collected, separated from debris via centrifugal and stored at -20 °C for further analysis. Cells were lysed according to the protocol provided by kits supplier; Roche Diagnostics GmbH, Mannheim, Germany (Cat. No. 04 719 964 001) and centrifuged for 10 min at 13,000 rpm. Supernatant was collected for adipogenesis-related protein detection via Western blot. Cells treated with insulin (1 µg/mL) was used as control.

2.8 SDS-PAGE and Western Blotting

Twenty to thirty milligram of protein were loaded into SDS-PAGE gel wells. Electrophoresis was initiated by voltage setup of 50 V for 15 to 20 min and continued for another 90 to 120 min with 100 V. Separated proteins were transferred to methanol-activated PVDF membrane by 100 V for 1 h. Transferred proteins were confirmed by Ponceau Red solution. Membrane were blocked in 5 % skim milk in PBST overnight at 4 °C. Primary antibody anti CEBP-α (1 : 2000), adiponepectin (1 : 2000), GLUT4 (1 : 1000) and β-actin (1 : 2000) were incubated overnight at 4 °C. After washed with PBST 3X, membrane were incubated with secondary antibody-AP conjugated for 1 h at RT. Finally, membrane were incubated with NBT/BCIP solution for 5 - 15 min. Developed band were scanned and quantified using ImageJ software.

2.9 Statistical analysis

All data were expressed in mean ± SEM. Statistical analysis were performed using SPSS program with one way ANOVA and Turkey test. Significant difference were considered as $p < 0.05$.

3. Results and discussion

3.1 HPLC fingerprinting of EP

Gallic acid in EP was identified and quantified using HPLC. Figure 1 illustrates the chromatogram of gallic acid presence in EP. Concentration of 16.86 µg/mg was quantified at 272 nm.

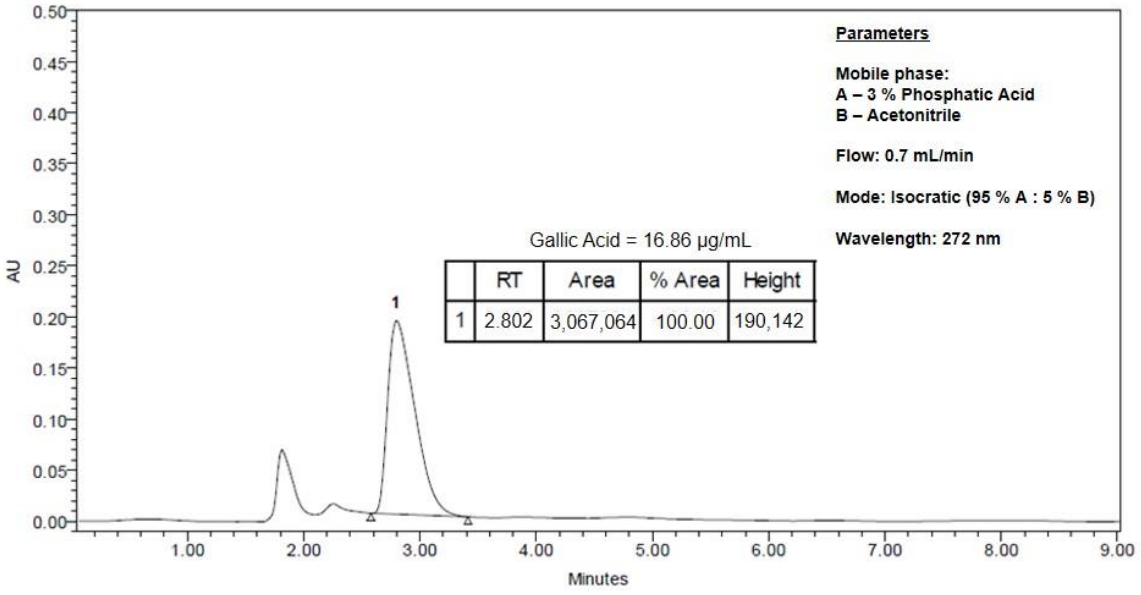

Figure 1: HPLC fingerprinting of gallic acid

3.2 MTT assay

MTT proliferation assay is a calorimetric method to measure the cytotoxicity effect of samples on metabolically active cells. It is a direct method to estimate the cell viability which is affected by external factors; in our case, plant extract (van Meerloo et al., 2011). The tetrazolium MTT dye is reduced into insoluble formazon which is purple colour and the intracellular localisation of the formazan can be quantified at 570 nm. The cytotoxicity effects of EP on 3T3-L1 cells proliferation were assessed via MTT assay. Figure 2 illustrates the dose-dependent effect on cells viability during 24 h treatment period. Significant decrease ($p < 0.05$) in cells viability

were observed starting from 500 µg/mL. The median inhibition IC50 value was 365 µg/mL. Hence the safe concentration for EP to be used on 3T3-L1 is 100 µg/mL and below. These values were used in further experiments.

3.3 EP effectively enhance glucose utilisation in medium

The ability of cells to utilise glucose is one the indicators of effective glucose transport from extracellular to intracellular part. Insulin signalling pathway plays an important role in the rate of glucose transport. Ineffective transportation of glucose resulted from insulin resistance is the main cause of diabetes mellitus type2 (TDM2). Glucose transport can be enhanced by increasing the sensitivity of insulin receptors (IRs). Several drugs have been developed to increase the glucose transport in cell, such as metformin and rosiglitazone. Glucose absorbed in fat cells mostly converted to lipid/ triglyceride via lipogenesis process. Herbs and plants such as ginseng, bitter melon and cinnamon (Hui et al., 2009) have shown the similar ability on enhancing glucose transport/ uptake. In our study, the glucose utilisation from media was analysed after 48 h of treatment with EP extracts. Significant ($p < 0.05$) increase on glucose utilisation was observed when treated with 10 µg/mL of EP (Figure 3). Slight increase of glucose utilisation was observed for 100 µg/mL and 1 µg/mL but not significant.

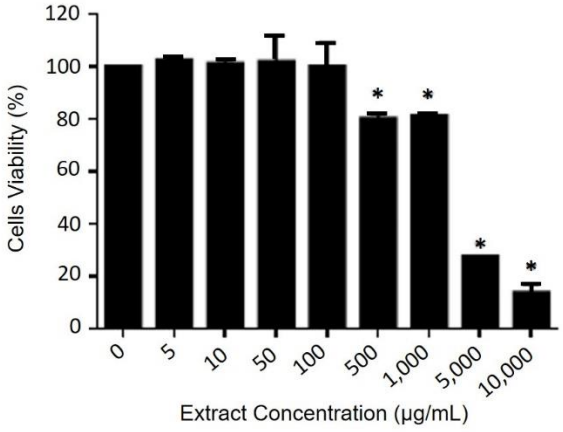

Figure 2: 3T3-L1 cells viability against EP water extract ranging from 5 µg/mL to 10,000 µg/mL

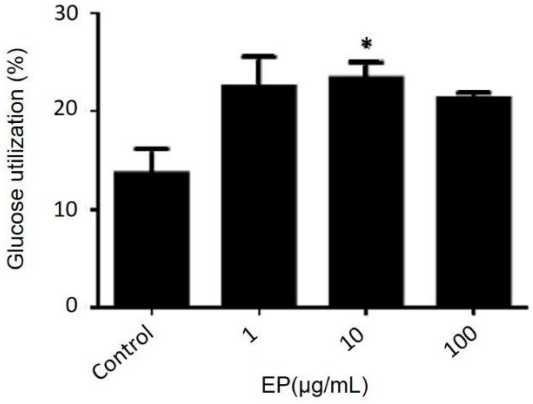

Figure 3: Glucose utilisation of adipocytes from media

3.4 EP enhance lipid accumulation in adipocytes

Accumulation of intracellular lipid droplets were measured via microscopic imaging acquisition and Oil Red O (ORO) staining. One of the major viable phenotypes for mature adipocytes is the formation of intracellular lipid droplets (Morrison and McGee, 2015). Figure 4 visualises the intracellular lipid formation in adipocytes after 6 days of treatment with differentiation cocktail, 1, 10 and 100 µg/mL of EP with and without the presence of 1 µg/mL insulin. Lipid accumulation was enhanced with increasing concentration of EP in the presence of insulin. EP alone failed to enhance lipid accumulation. In agreement to these results, the amount of lipid accumulated measured from ORO assay (Figure 5) showed significant ($p < 0.05$) increase of lipid amount for all EP concentrations. 100 µg/mL of EP produced the highest amount of lipid with 155 % as compared to group control (insulin only). For positive control (rosiglitazone) accumulated 183 % of lipid.

3.5 EP induced the expression of adipocytes related proteins

CEBP-α, adiponectin and GLUT4 are highly expressed proteins during 3T3-L1 differentiation. Immunoblotting analysis was performed to measure the effect of EP on the expression of these proteins. Results shown in Figure 6 demonstrate that low concentration (1 and 10 µg/mL) of EP enhanced the expression of CEBP-α, adiponectin and GLUT4. β-actin was used as internal control. Rosiglitazone was used as positive control. Compared to control, CEBP-α protein expression was 0.6 fold and 0.35 fold higher in 10 µg/mL and 1 µg/mL treatment. Adiponectin was expressed 0.5 fold and 0.27 fold higher for the same concentration. For GLUT4, 1 µg/mL of EP significantly enhanced the expression of this protein by 0.88 fold higher than control.

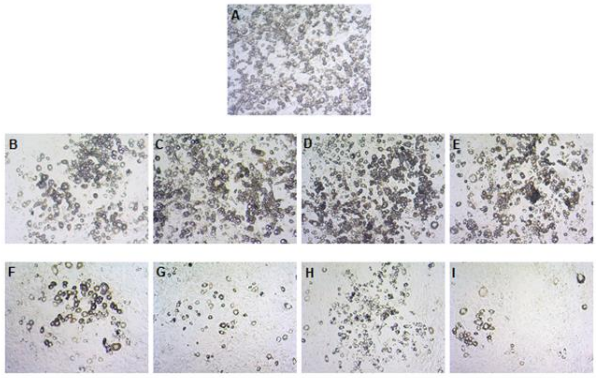

Figure 4: Microscopic images of dose dependent effect of lipid accumulation

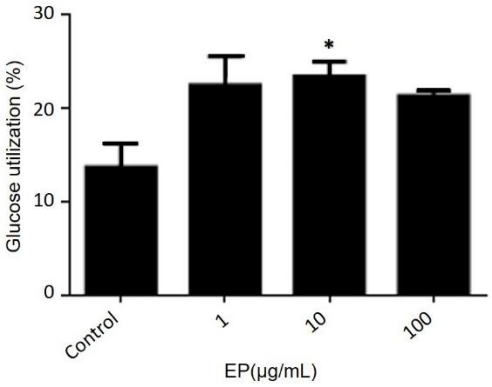

Figure 5: Lipid accumulation measured during 1, 10 and 100 µg/mL of EP treatment

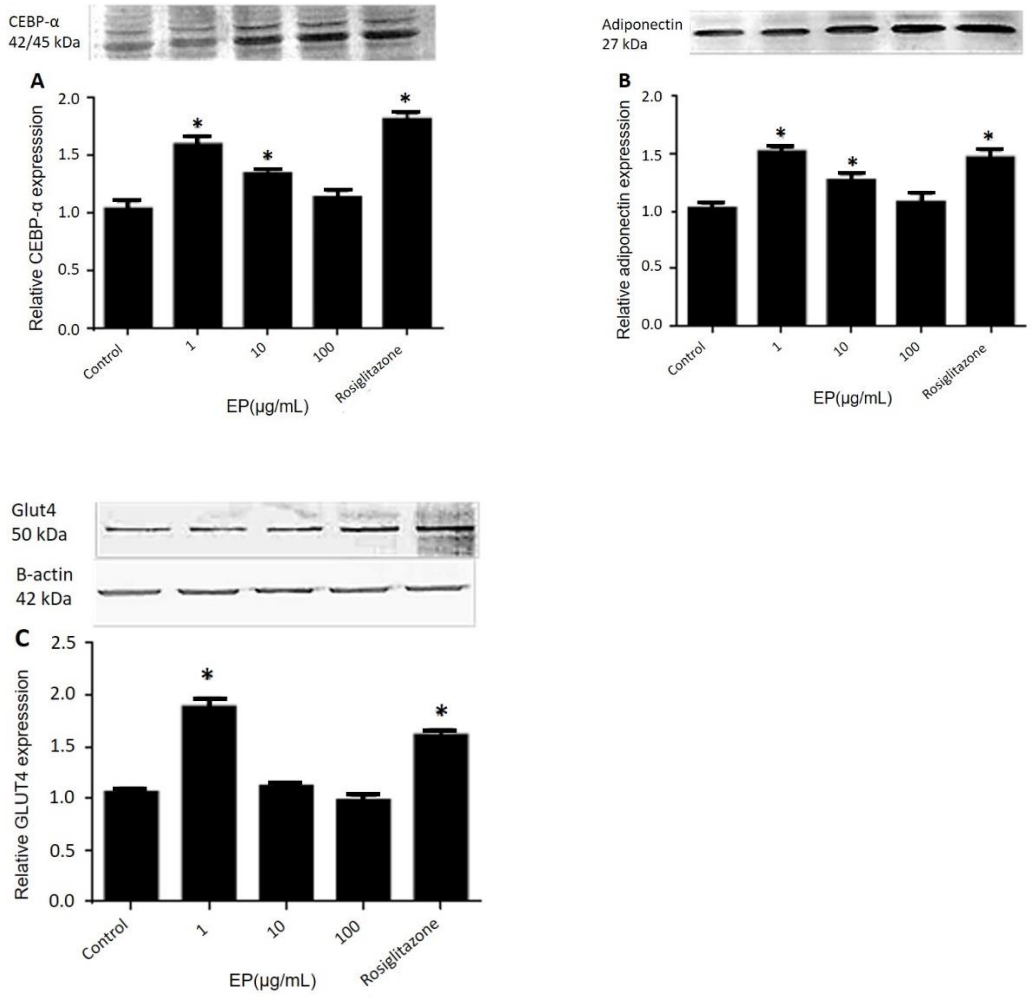

Figure 6: Effect of EP on the expression of adipogenesis-related proteins. A) Relative expression of CEBP-α, B) Relative expression of adiponectin and, C) relative expression of GLUT4

4. Conclusion

In summary, EP stimulated adipogenesis in 3T3-L1 cells with the presence of 1 µg/mL of insulin. Successful differentiation of adipocytes expressed significant amount of CEBP-α, adiponectin and GLUT4. Dose dependent effect on triglyceride content and glucose utilisation from medium was also quantified. This study provides evidence that EP has insulin sensitising effect but not insulin mimicking effect on adipocytes.

Acknowledgments

This study was sponsored by Universiti Teknologi Malaysia; Research University Grant (10H27).

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