The Inhibitory Effect of Betanin on Adipogenesis in 3T3-L1 Adipocytes

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Abstract Betanin, a natural pigment that presents ubiquitously in plants, has been reported to show biological effects. However, not much is known on the effectiveness of betanin in regulating fat accumulation. Therefore, the aim of this study is to explore the inhibitory effect of betanin on adipogenesis in 3T3-L1 adipocytes and its mechanism action. The results show betanin significantly inhibited oil red O-stained material (OROSM) and triglyceride levels in 3T3-L1 adipocytes, indicating betanin inhibited lipid accumulation in 3T3-L1 adipocytes. In addition, the peroxisome proliferator–activated receptor γ (PPARγ) expression was significantly inhibited in the betanin-treated adipocytes, implying that betanin suppressed the cellular PPARγexpression in 3T3-L1 adipocytes. Moreover, the suppression of lipid accumulation by betanin occurred by decreasing the gene expression of PPARγ, CCAAT-enhancer-binding protein α (C/EBPα) and sterol regulatory element binding protein 1c (SREBP-1c). Taken together, these findings suggest betanin may be a mediator of adipocyte accumulation, leading to the inhibition of lipogenesis in 3T3-L1 adipocytes and betanin is therefore potentially useful for designing new antiadipogenic agent.

Keywords: adipogenesis, betanin, gene expression, lipogenesis, lipid accumulation

1. Introduction

Overweight and obesity are defined by the World Health Organization (WHO) as abnormal or excessive fat accumulation that presents a risk to health. Obesity is a global medical issue that can cause metabolic syndrome, hypertension, atherosclerosis, diabetes, cancers and sleep disorders [1]. Further, a WHO survey in 2016 indicated that over 340 million children and adolescents aged 5-19 were overweight or obese. Moreover, most of the world's population lives in countries where overweight and obesity kills more people than underweight [2]. Consequently, emergence of metabolic syndrome incidence has led to an increased need for therapeutic and preventive strategies. Lifestyle changes such as improving eating habits and increasing physical activity, along with potential treatments that target specific molecules for regulation of metabolic pathways are recommended approaches to preventing obesity [3].

Many studies point to the health benefits of phytochemicals. Epidemiological evidence has shown inverse associations between intakes of the dietary phytochemicals present in natural sources and risk of developing chronic diseases. In recent years, therefore, many studies have focused on the biological effects of natural sources in preventing and treating overweight and obesity.

Betanin (betanin-5-*O*-β-glucoside) is a water soluble pigment belonging to the betalain family that presents ubiquitously in plants. Betanin is widely used a colorant in cosmetics, food products and pharmaceuticals [4,5]. Recently, the biological activities of betanin have been reported. For example, betanin showed antioxidant [6,7], anti-inflammatory [8], anti-carcinogenic [9], antihypertensive [10], and heptoprotective [11] properties. Apart from these, in the previous studies by the authors reported that the presence of bioactive compounds such as betanin, rutin, and kaempferol in Djulis may partly account for the anti-adipogenesis of Djulis [7]. Given that betanin has shown biological effects, it is also possible that fat accumulation may be regulated by betanin. However, not much is known about the antiadipogenic effect of betanin. Therefore, the aim of this study is to evaluate the anti-adipogenesis of betanin.

2. Material and Methods

2.1. Chemicals

Betanin and Oil red O solution were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Anti-PPARγ and anti-β-actin were purchased from Cell Signaling Technology (Danvers, MA, USA). All other chemicals were reagent grade.

2.2. Cell Viability Assay and Adipocytic Differentiation

3T3-L1 cells (BCRC Number: 60159) were purchased from Bioresources collection and Research Center (BCRC, Food Industry Research and Development Institute, Hsinchu, Taiwan.). Cell viability was determined by the MTT assay [12]. 3T3-L1 preadipocytes were planted into 6 well plates and maintained in DMEM supplemented with 10 % bovine calf serum at 37°C in a humidified 5% CO_2 incubator. Adipocytic differentiation was done as described by the method of Chyau et al. [7].

2.3. Oil Red O Staining

3T3-L1 cells were harvested 8 days after the initiation of differentiation. Then, the lipid accumulation of cells was measured using oil red O working solution. The determination of oil red O-stained material. (OROSM) was carried out by the method of Tzang and Liu [13].

2.4. Measurement of Triglyceride Content

3T3-L1 adipocytes were harvested 8 days after the initiation of differentiation. The cells were incubated with 10-50 μM of betanin for 72 h at 37°C in a humidified 5% CO_2 incubator. Cells were collected and lysed in lysis buffer (1% Triton X-100 in PBS) for 30 min on ice box. The intracellular triglyceride content was measured using a commercial triglyceride assay kit (Cayman Chemical, Ann Arbor, MI, USA). The protein concentration was determined with the Pierce[TM] BCA protein assay kit (Thermo Suientific, Rockford, IL, USA) using bovine serum albumin as a standard [7].

2.5. Western Blot Assay

To determine peroxisome proliferator-activated receptor γ (PPARγ) expression, western blot assay was done. 3T3-L1 adipocytes were harvested 8 days after the initiation with 10-50 μM of betanin for 24 h at 37°C in a humidified 5% CO_2 incubator. The cells were collected and lysed in ice-cold lysis buffer (20 mM Tris-HCl (pH7.2), 2 mM EDTA, 500 μM sodium orthovanadate, 1% Triton X-100, 0.1% SDS, 10 mM NaF, 10 μg/ml leupeptin and 1 mM PMSF). The protein concentration was estimated with the Pierce[TM] BCA protein assay kit (Thermo Suientific) using bovine serum albumin as a standard. Total protein was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using a 10% polyacrylamide, and blotted for 1.5 h at 100 V. The proteins in the gel were transferred to a PVDF membrane. Then, the membrane was blocked with 5%

BSA in PBST (0.1% v/v Tween-20 in PBS, pH7.2) for 1 h. β-actin and PPARγ of primary antibodies were used in this study. Goat anti-rabbit IgG-HRP and rabbit anti-rabbit IgG-HRP (Jackson, West Grove, Chester, PA, USA) were used as secondary antibodies, and ECL prime (Advansta Inc., Menlo Park, CA, USA) reagent was used for developing. Membranes were incubated with primary antibody at 4°C overnight and then with secondary antibody for 1 h. Membranes were washed in PBST for 10 min three times between each step. Blots were developed using the Western Bright ECL kit (Advansta Inc.), exposed to Gel Electrophoresis Documentation-Multi-Function Gel Image system (Topbio Co., Taipei, Taiwan). The relative expression of proteins was quantified densitometrically using the Image J software (Wayne Rasband, Madison, WI, USA) and calculated according to the reference band of β-actin [7].

2.6. Reverse Transcription-Polymerase Chain Reaction (RT-PCR) Assay

To determine the mRNA expression level of PPARγ, CCAAT-enhancer-binding proteinsα(C/EBPα) and sterol regulatory element-binding protein 1c (SREBP-1c) in the 3T3L1 adipocytes, total RNA was extracted using Total RNA Isolation kit (NA017-0100, GeneDirex, Lais Vegas, NV, USA) according to the manufacturer's instructions. RNA quantity and purity were checked by spectrophotometric analysis at 260 and 280 nm. One microgram of total RNA was reverse transcribed into cDNA using GScript First-Strand Synthesis Kit (MB305-0050, GeneDireX, USA). The lists of specific primers (Invitrogen, Carlsbad, CA, USA) were: GAPDH, Forward: 5'-GTATGACTCCACTCACGGCAAA-3' (N3541G11), Reverse: 5'-GGTCTCGCTCCTGGAAGATG-3' (N3541H01); PPAR-γ, Forward:5'-TTTTCAAGGGTGCCAGTTTCGA TCC-3' (N3541H02), Reverse:5'-AATCCTTGGCCCTCT GAGAT-3' (N3541H03); C/EBPα, Forward: 5'-CGCAAG AGCCGAGATAAAGC-3'(N3541G05), Reverse: 5'-CAC GGCTCAGCTGTTCCA-3' (N3541G06); Sterol regulatory element binding protein 1 (SREBP/lc), Forward: 5'-GGA GCCATGGATTGCACAT-3' (N3541H06), Reverse: 5'-GC TTCCAGAGAGGAGGCCAG-3' (N3541H07)

The primers were added at final concentration of 250 nM to a 20 μl reaction mixture containing 4 μl of 5× OmicsGreen qPCR Master Mix and 1 μl of DNA template (Stock conc. 1 μg/ml). The PCR (StepOnePlus™ Real-Time PCR System, Applied Biosystems™) conditions were denaturation at 90 °C for 15 s, annealing at 60-65 °C for 20 s, and elongation at 72 °C for 20 s in a cycle of 40 (OmicsGreen qPCR 5X Master Mix with ROX Dye, QE3931, Omics Bio, Estonia). The relative levels of gene expression were quantified using the ΔΔCt method, which results in a ratio of target gene expression relative to equally expressed housekeeping genes (GAPDH) [9,14].

2.7. Statistical Analysis

All data were expressed as means ± SD, and ANOVA was conducted by using the SPSS software (SPSS Inc., Chicago, IL, USA). Analysis of variance was performed by ANOVA procedures. Significant differences between means were determined by Duncan's multiple range tests or a Dennett's test at a level of $p < 0.05$.

3. Results and Discussion

3.1. Effect of Betanin on Cell Growth

To understand whether betanin affects the cytotoxicity of 3T3-L1 adipocytes under the test concentrations, the cell viability of 3T3-L1 was measured using MTT assay. None of the betanin levels in the range from 10-25 μM shows any cytotoxic effect on the survival of 3T3-L1 adipocytes. (Figure 1), indicating that betanin did not affect 3T3-L1 cell growth under the tested concentrations.
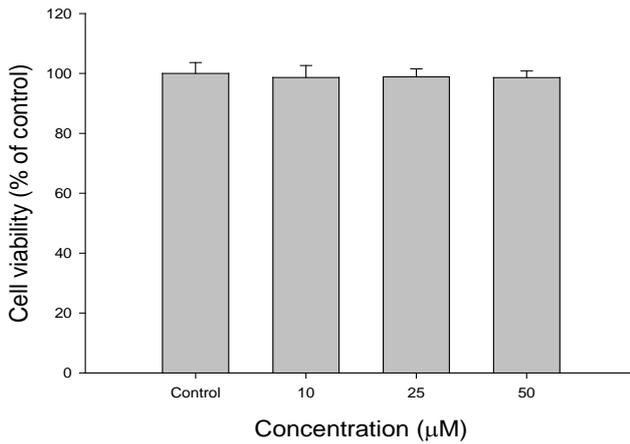

Figure 1. Effect of betanin on cell proliferation in 3T3-L1 adipocytes. 3T3-L1 adipocytes were harvested 8 days after the initiation of differentiation. The cells were treated with betanin for 72 h

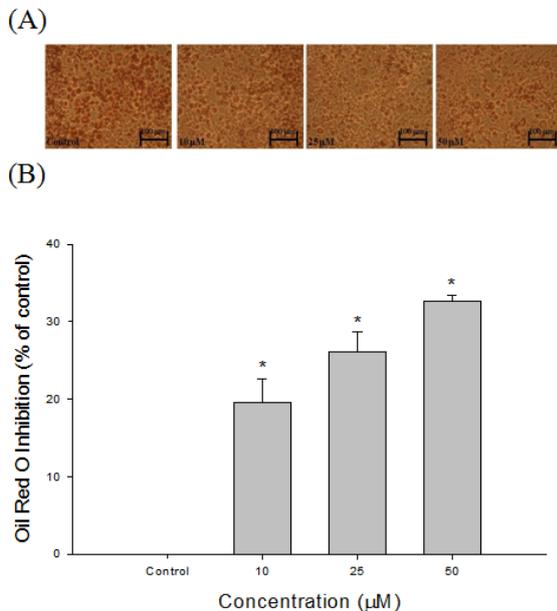## 3.2. Effect of Betanin on Llipid Accumulation

(A)

(B)

Figure 2. Effect of betanin on oil red O-stained material (OROSM) in 3T3-L1 adipocytes. 3T3-L1 adipocytes were harvested 8 days after the initiatintion of differentiation and were stained with oil red O (A). The cells were treated with betanin for 72 h (B)

Next, to understand the effect of betanin on lipid accumulation in 3T3-L1 adipocytes, the cells were allowed to differentiate into adipocytes for 8 days, and then the effect of betanin on lipid accumulation was measured. Figure 2 shows the effect of betanin on

adipogenesis. The microscopic images of oil red O-stained adipocytes are shown in Figure 2A, indicating that the number of lipid droplets in the differentiated cells treated with betanin decreased compared with the control. Quantitatively, the OROSM data showed that betanin levels at 10, 25 and 50 μM reduced lipid accumulation by 19.6, 26.1 and 32.6%, respectively (Figure 2B), revealing the number of lipid droplets in the differentiated cells was significantly reduced in a concentration-dependent manner when the cells were treated with betanin. Figure 3 shows the effect of betanin on the intracellular triglyceride levels in 3T3-L1 adipocytes. Betanin levels at 10, 25 and 50 μM reduced intracellular triglyceride levels in 3T3-L1 adipocytes by 10.5, 16.6 and 18.1%, respectively, indicating the intracellular triglyceride levels in 3T3-L1 adipocytes treated with betanin were decreased compared with the control.
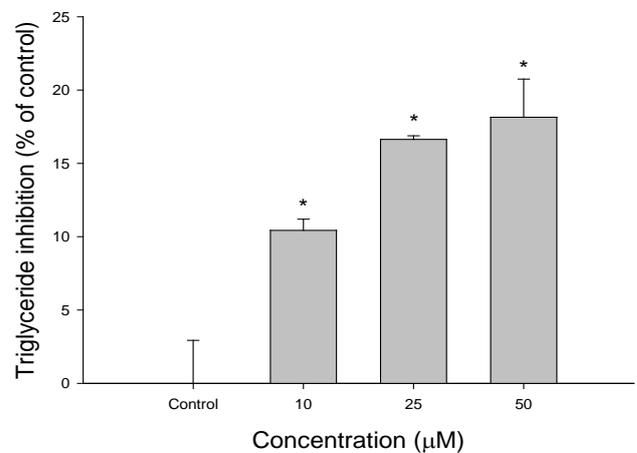

Figure 3. Effect of betanin on inhibition of intracellular triglyceride in 3T3-L1 adipocytes. 3T3-L1 adipocytes were harvested 8 days after the initiation of differentiation. The cells were treated with betanin for 72 h

3.3. Betanin Reduced Lipid Accumulation by Regulating Adipogenic Genes
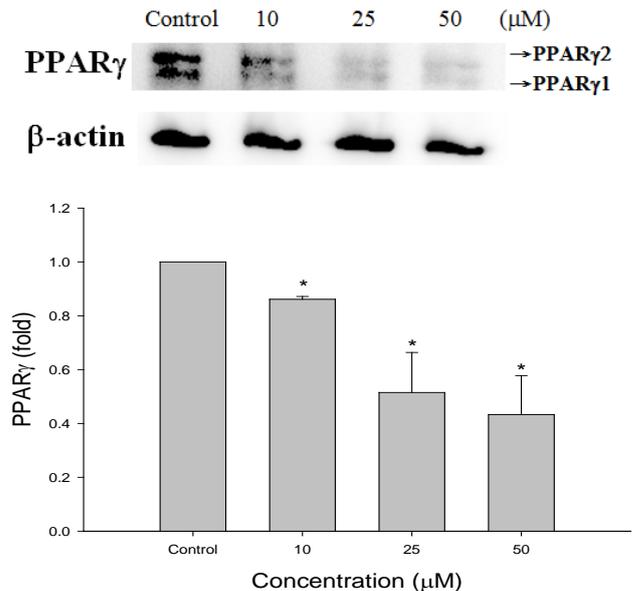

Figure 4. Effect of betanin on PPARγ expression in 3T3-L1 adipocytes. 3T3-L1 adipocytes were harvested 8 days after the initiation of differentiation. The cells were treated with betanin for 24 h
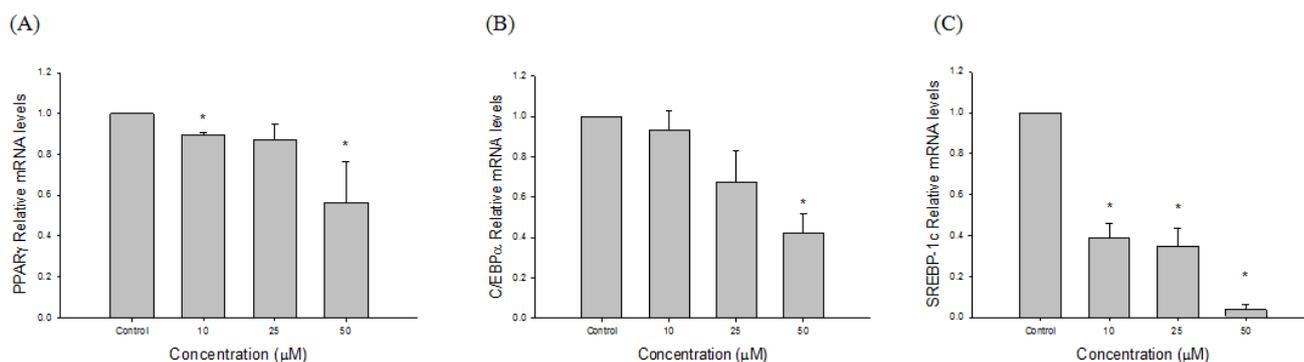
Figure 5. Effect of betanin on gene expression on PPARγ (A), C/EBPα (B), and SREBP-1c (C) in 3T3-L1 adipocytes. 3T3-L1 adipocytes were harvested 8 days after the initiation of differentiation. The cells were treated with betanin for 6 h

PPARγ, a transcription factor, plays an important role in the induction and maintenance of differentiated adipocyte phenotype [15]. Therefore, to understand whether betanin reduces lipid accumulation through regulating PPARγ, the PPARγ protein expression was determined using western blot analysis. As shown in Figure 4, in the betanin-treated adipocytes, the PPARγ protein expression was decreased in a concentration-dependent manner. In addition, the effect of betanin on the expression of adipogenic genes was determined by quantitation PCR analysis (Figure 5). As expected, in the betanin-treated adipocytes at different concentrations, PPARγ (Figure 5A), C/EBPα (Figure 5B) and SREBP-1c (Figure 5C) mRNA levels were significantly down-regulated. In addition, a decline in protein levels of PPARγ was observed due to the direct relationship between mRNA and protein levels (Figure 4). PPARγ is a potent inducer of adipogenesis, which can promote the transdifferentiation of myoblasts to adipocytes, particularly when coexpressed with C/EBPα [7,16,17]. In addition, C/EBPα is a key transcriptional factor in adipogenesis and its expression is activated by C/EBPβ, C/EBPδ and PPARγ in adipocytes [18]. This means PPARγ and C/EBPα emerge as master regulators of adipogenesis [19]. Furthermore, SREBP-1c, a key lipogenic transcriptional factor, directly activates the expression of more than 30 genes; SREBP-1c is dedicated to fatty acid biosynthesis and uptake of fatty acids, cholesterol and triglycerides [7,20,21]. Clearly, betanin suppressed the expression of adipogenic genes in the betanin-treated adipocytes; therefore, betanin was able to regulate the expression of adipogenic genes, such as PPARγ, C/EBPα and SREBP-1c, thereby inhibiting lipid accumulation in 3T3-L1 cells.

4. Conclusion

According to the results presented in this study, betanin inhibited lipid accumulation. The mechanism action may be attributed to down regulation of the gene expression of PPARγ, C/EBPα and SREBP-1c, leading to inhibition of adipose tissue formation. These findings offer new insight into prevention of adipogenesis. Supplementation with betanin could be a beneficial treatment to reduce adipogenesis. In addition, despite the mentioned benefits, betanin suffers from limited oral bioavailability (below 1%) due to incomplete oral absorption and low shelf life [4]. Therefore, scientific trials in vivo are needed to confirm the results.

Conflict of Interest

The authors declare no conflict of interest.

Acknowledgements

References

[1] Tzeng, T. F., Lu, H. J., Liou, S. S., Chang, C. J. and Liu, I. M. "Reduction of lipid accumulation in white adipose tissues by Cassia tora (Leguminosae) seed extract is associated with AMPK activation, *Food Chemistry*, 136(2). 1086-1094. Jan. 2013.

[2] WHO Media Centre. Obesity and overweight. Available online: https://www.who.int/ (Accessed on 16 Feb. 2018).

[3] Mubarak, A., Hodgson, J. M., Considine, M. J., Croft, K. D. and Matthews, V. B., "Supplementation of a high-fat diet with chlorogenic acid is associated with insulin resistance and hepatic lipid accumulation in mice," *Journal of Agricultural and Food Chemistry*, 61(18). 4371-4378. 2013.

[4] Amjadi, S., Ghorbani, M., Hamishehkar, H. and Roufegarinejad, L. "Improvement in the stability of betanin by liposomal nanocarriers: Its application in gummy candy as a food model," *Food Chemistry*, 256. 156-162. Aug. 2018.

[5] Esatbeyoglu, T., Wagner, A. E., Schini-Kerth, V. B. and Rimbach, G. "Betanin--a food colorant with biological activity," *Molecular Nutrition & Food Research*, 59(1). 36-47. Jan. 2015.

[6] Shaaruddin, S., Ghazali, H. M Mirhosseini, S. H. and Muhammad, K. "Stability of betanin in pitaya powder and confection as affected by resistant maltodextrin," *Lebensmittel-Wissenschaft & Technologie*, 84. 129-134. Oct. 2017.

[7] Chyau, C. C., Chu, C. C., Chen, S. Y. and Duh P. D., "The effects of Djulis (*Chenopodium formosanum*) and its bioactive compounds on adipogenesis in 3T3-L1 adipocytes," *Molecules*, 23(7), 1780; Jul. 2018.

[8] Sutariya, B. and Saraf, .M. "Betanin, isolated from fruits of Opuntia elatior Mill attenuates renal fibrosis in diabetic rats through regulating oxidative stress and TGF-β pathway," *Jouranl of Ethnopharmacology*, 198. 432-443.Feb. 2017.

[9] Farabegoli, F., Scarpa, E. S., Frati, A., Serafini, G., Papi, A., Spisni, E., Antonini, E., Benedetti, S. and Ninfali, P. "Betalains increase vitexin-2-O-xyloside cytotoxicity in CaCo-2 cancer cells," *Food Chemistry*, 218. 356-364. Mar. 2017.

[10] Chen, S. Y., Chu, C. C., Chyau, C. C, Fu, Z. H. and Duh P. D., "Effect of water extract of Djulis (*Chenopodium formosaneum*) and its bioactive compounds on alcohol-induced liver damage in rats," *International Journal of Food and Nutritional Science*, 5(1). 55-63. Jul. 2018.

[11] Chen, S. Y., Chu, C. C., Chyau, C. C., Yang, J. W. and Duh, P. D. "The antihypertensive effects of Djulis (*Chenopodium*

formosanum) and its bioactive compounds, in vitro and in vivo," *Food Bioscience*., (in submission) 2019.

[12] Hsu, C. L. and Yen, G. C. "Effects of capsaicin on induction of apoptosis and inhibition of adipogenesis in 3T3-L1 cells," *Journal of Agricultural and Food Chemistry,* 55(5). 1730-1736. Mar. 2007.

[13] Tzeng, T. F. and Liu, I. M. "6-gingerol prevents adipogenesis and the accumulation of cytoplasmic lipid droplets in 3T3-L1 cells," *Phytomedicine*, 20(6). 481-487. Apr. 2013.

[14] Noh, J. R., Kim, Y. H., Hwang, J. H., Gang, G. T., Yeo, S. H., Kim, K. S., Oh, W. K., Ly, S. Y., Lee, I. K. and Lee, C. H. "Scoparone inhibits adipocyte differentiation through down-regulation of peroxisome proliferators-activated receptor gamma in 3T3-L1 preadipocytes," *Food Chemistry*, 141(1) 723-730. Nov. 2013.

[15] Gregoire, F. M., Smas, C. M. and Sul, H. S. "Understanding adipocyte differentiation," *Physiological Reviews*, 78(3). 783-809. Jul. 1998.

[16] Rosen, E. D., Walkey, C. J., Puigserver, P. and Spiegelmam, B. M. "Transcriptional regulation of adipogensis," *Genes Development*, 14(11). 1293-1307. Jun. 2000.

[17] Hu, E., Tontonoz, P. and Spiegelman, B.M. Transdifferentiation of myoblasts by the adipogenic transcription factors PPAR gamma and C/EBP alpha. *Proceedings of the National Academy of Science of the united state of America*, *92*. 9856-9860. 1995.

[18] Fujimori, K. and Shibano, M. "Avicularin, a plant flavonoid, suppresses lipid accumulation through repression of "Reduction of lipid accumulation in white adipose tissues by Cassia tora (Leguminosae) seed extract is associated with AMPK activation, *Food Chemistry*, 136(2). 1086-1094. Jan. 2013.

[19] Lefterova, M. I. and Lazar, M. A. "New developments in adipogenesis," *Trends in Endocrinology & Metabolism*, 20(3) 107-114. Apr. 2009.

[20] Wu, C. H. Lin, M. C. Wang, H. C. Yang, M. Y. Jou, M. J. Wang, "Rutin inhibits oleic acid induced lipid accumulation via reducing lipogenesis and oxidative stress in hepatocarcinoma cells."*Journal of Food Science,* 76(2).65-72, Mar. 2011.

[21] Strable, M. S. and Ntambi, J. M. "Genetic control of de novo lipogenesis: role in diet-induced obesity." *Critical Reviews in Biochemistry and Molecular Biology*, 45(3). 199-214. Jun. 2010.