Anti-Obese Activity of Ivy Gourd (*Coccinia grandis* L. Voigt)

Ruthaiwan Bunkrongcheap

A Thesis Submitted in Fulfillment of the Requirements for the Degree of

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บทคัดย่อ

ตำลึง (*Coccinia grandis* L. Voigt) เป็นพืชในตระกูล Cucurbitaceae พบได้ทั่วไปทั้งในเขตร้อนและในเขตกึ่งร้อน แทบทุกส่วนของพืชชนิดนี้มีสรรพคุณทางยา รวมทั้งความสามารถลดความอ้วนของรากตำลึงซึ่งถูกระบุไว้ในตำราสมุนไพรไทย แต่ยังขาดหลักฐานทางวิทยาศาสตร์ที่สนับสนุนข้อกล่าวอ้างดังกล่าว ดังนั้น งานวิจัยนี้จึงมุ่งเน้นศึกษาศักยภาพของตำลึงในการลดความอ้วน โดยประเมินจากความสามารถยังยั้งการเปลี่ยนรูปเป็นเซลล์ไขมันของเซลล์เพาะเลี้ยงชนิด 3T3-L1 และความสามารถลดไขมันในหนูสายพันธุ์ C57BL/6J ซึ่งได้รับอาหารที่มีปริมาณไขมันสูง พบว่า เมื่อสกัด ราก ใบ และลำต้นของตำลึง ด้วยเอทานอล แล้วนำสารสกัดแต่ละชนิดมาทดสอบกับเซลล์ 3T3-L1 พบว่า สารสกัดจากส่วนรากไม่เป็นพิษต่อเซลล์ และสามารถยับยั้งการเปลี่ยนรูปของเซลล์ได้ดี เนื่องจากปริมาณไขมันในเซลล์ซึ่งติดสีย้อม oil red O ลดลงอย่างชัดเจน นอกจากนี้ ยังพบว่า สารสกัดดังกล่าวกดการแสดงออกของยีนที่เกี่ยวข้องกับการเปลี่ยนรูปเป็นเซลล์ไขมัน ได้แก่ peroxisome proliferator-activated receptor-γ (PPARγ), glucose transporter-4 (GLUT4) และ adiponectin และเมื่อแยกสารสกัดจากรากตำลึงออกเป็นส่วนๆ ตามลำดับของตัวทำละลายที่ใช้ คือ เฮกเซน เอทิลอะซิเตท คลอโรฟอร์ม และน้ำ พบว่า ส่วนที่ละลายในชั้นเฮกเซน (IGH) ยับยั้งการสะสมไขมันในเซลล์ 3T3-L1 โดยกดการแสดงออกของยีน CCAAT/enhancer-binding protein-α (C/EBPα), PPARγ, fatty acid binding protein-4 (FABP4), FAS, lipoprotein lipase (LPL), glucose transporter-4 (GLUT4) และ mesoderm-specific transcript (MEST)

เมื่อนำ IGH มาทดสอบในหนูสายพันธุ์ C57BL/6J ที่ถูกเหนี่ยวนำให้อ้วนด้วยการบริโภคอาหารที่มีไขมันสูงอย่างต่อเนื่อง พบว่า หนูกลุ่มทดลองซึ่งได้รับอาหารผสม IGH ในสัดส่วน 2% โดยน้ำหนัก ติดต่อกันนาน 4 สัปดาห์ มีระดับไตรกลีเซอไรด์ (TG) และกรดไขมันอิสระในเลือด รวมทั้งปริมาณ TG และคอเลสเตอรอล (TC) ในตับ ลดลง นอกจากนี้ ยังพบว่าการแสดงออกของยีน PPARγ1 ในเนื้อเยื่อไขมันของหนูเหล่านี้ลดลง แต่ขนาดของเนื้อเยื่อไม่เปลี่ยนแปลง ผลจากการวัดกิจกรรมของเอนไซม์ FAS, glucose-6-phophate dehydrogenase และ malic enzyme ในตับ ซึ่งพบว่า ลดลงอย่างมาก นั้น แสดงให้เห็นว่า IGH ยับยั้งกระบวนการสังเคราะห์ไขมันในตับ แล้วส่งผลให้ระดับไขมันในเลือดลดลง ประกอบกับ IGH ยัง

กระตุ้นการขับออกของไขมันผ่านทางระบบน้ำดีเพราะตรวจพบ TG และ TC ในมูลของหนูมีปริมาณเพิ่มขึ้น ผลการทดลองเหล่านี้แสดงให้เห็นว่า ในรากตำลึงมีสารออกฤทธิ์ที่มีศักยภาพในการป้องกันและ/หรือลดความอ้วนได้ ซึ่งจะต้องแยกออกมาให้บริสุทธิ์ และศึกษาทั้งในแง่ประสิทธิภาพและกลไกการออกฤทธิ์ เพื่อนำไปสู่การพัฒนาและประยุกต์ใช้ต่อไป

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ABSTRACT

Ivy gourd (*Coccinia grandis* L. Voigt) is a tropical and subtropical vine of the family Cucurbitaceae. Various parts of this plant have been traditionally used in the treatment of illness symptoms. An anti-obesity property of its root has also been claimed but still of lack of supporting evidence. In this study, the anti-adipogenic activity of ivy gourd in a murine 3T3-L cell line and in C57BL/6J mice fed high-fat diet were investigated. Ethanol extracts were prepared from the leaves, stems and roots. The root extract did not show any toxic effect to 3T3-L1 cells and significantly inhibited adipocyte differentiation in a dose-dependent manner, as assessed by oil red O staining of intracellular fat droplets whereas the stem and leaf extracts were ineffective. The expression of peroxisome proliferator-activated receptor-γ (PPARγ), glucose transporter-4 (GLUT4), and adiponectin genes was found to be decreased in response to the root extract treatment. When the root extract was subjected to sequential solvent partitioning in hexane, ethyl acetate, chloroform and water. Only the hexane-soluble fraction was active. Furthermore, the hexane fraction of root extract (IGH) also potently inhibited lipid accumulation in 3T3-L1 cells. The CCAAT/enhancer-binding protein-α (C/EBPα) and PPARγ transcription factors were down-regulated after IGH treatment. Consequently, the expression of their downstream target genes, including fatty acid binding protein-4 (FABP4), fatty acid synthase (FAS), lipoprotein lipase (LPL), and GLUT4 were decreased. It also suppressed mesoderm-specific transcript (MEST), a marker of adipocyte size.

In animal experiments, C57BL/6J mice were fed a high-fat diet supplemented with 2% (w/w) of IGH for 4 weeks. Daily consumption of IGH resulted in a significant decrease of serum triglycerides (TG) and free fatty acid concentrations, as well as hepatic TG and total cholesterol (TC) levels. Dietary IGH potently down-regulated peroxisome proliferator activated receptor-γ1 (PPARγ1)

gene expression in white adipose tissue without attenuating lipid accumulation in that tissue. An increased fecal excretion of TG and TC and decreased activity of lipogenesis-related enzymes including FAS, glucose-6-phosphate dehydrogenase, and malic enzyme in liver, were also observed upon the intake of IGH. These alterations could be a cause of lowered blood lipid levels seen among IGH given mice. The overall results from this study thus indicate that ivy gourd root would contain some ingredient(s) having anti-obesity property as demonstrated by inhibitory effect of both the extract and IGH in 3T3-L1 adipocyte differentiation and also blood and liver lipid lowering effects in a high-fat diet induced obese mouse model. They also suggest a potential application of this plant root in obesity prevention and/or management.

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Ruthaiwan Bunkrongcheap

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ABBREVIATIONS AND SYMBOLS

ABCG5	=	ATP-binding cassette transporter G5
ABCG8	=	ATP-binding cassette transporter G8
ACC	=	Acetyl-CoA carboxylase
ACL	=	ATP Citrate Lyase
Adipor1	=	Adiponectin receptor 1
ATCC	=	American Type Culture Collection
AOX	=	Acyl-CoA oxidase
BM	=	Basal medium
bp	=	Base pairs
CO_2	=	Carbon dioxide
CPT1α	=	Carnitine palmitoytransferase 1 alpha
C/EBPα	=	CCAAT/enhancer-binding proteins-alpha
C/EBPβ	=	CCAAT/enhancer-binding proteins-beta
C/EBPδ	=	CCAAT/enhancer-binding proteins-delta
cm	=	Centimeter
ζ	=	Zeta
CYP7A1	=	Cholesterol 7α-hydroxylase
cAMP	=	Cyclic adenosine monophosphate
CYP8B1	=	Cytochrome P450, family 8, subfamily b, polypeptide 1
°C	=	Degree Celsius
DEX	=	Dexamethasone
DMEM	=	Dulbecco's modified Eagle's medium
DMSO	=	Dimethyl sulfoxide
DNA	=	Deoxyribonucleic acid
DTT	=	Dithiothreitol
ECL	=	Enhanced chemiluminescence
ε	=	Epsilon
FABP4	=	Fatty acid-binding protein 4

ABBREVIATIONS AND SYMBOLS (continued)

FAS	=	Fatty acid synthase
FBS	=	Fetal bovine serum
g	=	Gram
GAPDH	=	Glyceraldehyde 3-phosphate dehydrogenase
GLUT4	=	Glucose transporter 4
GPAT	=	Glycerol phosphate acyltransferase
HMGR	=	HMG-CoA reductase
HSL	=	hormone sensitive lipase
h	=	Hour
IBMX	=	Isobutylmethylxanthine
IRS-1	=	Insulin receptor substrate 1
LDLR	=	Low-density lipoprotein recepter
min	=	Minute
mA	=	Milliampere
MEST	=	Mesoderm specific transcript
ml	=	Milliliter
mm	=	Millimeter
mM	=	Millimolar
M	=	Molar
MTT	=	1-(4, 5-Dimethylthiazol-2-yl)-3, 5- diphenyl-formazan
nm	=	Nanometer
O.D.	=	Optical density
PAGE	=	Polyacrylamide gel electrophoresis
PDK4	=	Pyruvate dehydrogenase kinase 4
pH	=	Potential of hydrogen ion
PPARγ	=	Peroxisome proliferators-activated receptor-γ
PS	=	Penicillin-streptomycin
PVDF	=	Polyvinylidene difluoride

ABBREVIATIONS AND SYMBOLS (continued)

sec	=	Second
SIRT1	=	sirtuin (silent mating type information regulation 2 homolog)-1
SDS	=	Sodium dodecyl sulfate
S.E.	=	Standard error
S.D.	=	Standard deviation
SREBP-1c	=	Sterol regulatory element binding protein-1c
RNA	=	Ribonucleic acid
rpm	=	Revolution per minute
RT-PCR	=	Reverse transcription-polymerase chain reaction
TBS-T	=	Tris-buffer saline-tween-20
TNFα	=	Tumor necrosis factor-α
µl	=	Microliter
V	=	Volt
v/v	=	Volume per volume
w/v	=	Weight per volume
w/w	=	Weight per weight
%	=	Percent

LIST OF PAPERS

This thesis is based on the following papers:

1. Ruthaiwan Bunkrongcheap, Nongporn Hutadilok-Towatana, Kusumarn Noipha, Chatchai Wattanapiromsakul, Masashi Inafuku and Hirosuke Oku. (2014). Ivy Gourd (*Coccinia grandis* L. Voigt) Root Suppresses Adipocyte Differentiation in 3T3-L1 Cells. Lipids in Health and Disease. 13:88.
2. Ruthaiwan Bunkrongcheap, Masashi Inafuku, Hirosuke Oku, Nongporn Hutadilok-Towatana, Chatchai Wattanapiromsakul and Decha Sermwittayawong. (2016). Lipid-Lowering Effects of Hexane Fraction of Ivy Gourd (*Coccinia grandis* L. Voigt) Root in Mice Fed a High-Fat Diet. Walailak Journal of Science and Technology. 13, xxx-xxx. (accepted)

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2. Ruthaiwan Bunkrongcheap, Masashi Inafuku, Hirosuke Oku, Nongporn Hutadilok-Towatana, Chatchai Wattanapiromsakul and Decha Sermwittayawong. (2016). Lipid-Lowering Effects of Hexane Fraction of Ivy Gourd (*Coccinia grandis* L. Voigt) Root in Mice Fed a High-Fat Diet. Walailak Journal of Science and Technology. 13, xxx-xxx. (accepted)

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Cc: Ruthaiwan Bunkrongcheap (ruthaiwan.b@hotmail.com); Masashi Inafuku (h098648@eve.u-ryukyu.ac.jp); Hirosuke Oku (okuhiros@comb.u-ryukyu.ac.jp); Chatchai Wattanapiromsakul (chatchai.w@psu.ac.th); Decha Sermwittayawong (decha.s@psu.ac.th)

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We have reached a decision regarding your submission to Walailak Journal of Science and Technology (WJST), "Lipid-Lowering Effects of Hexane Fraction of Ivy Gourd (Coccinia grandis L. Voigt) Root in Mice Fed a High-Fat Diet".

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CHAPTER 1

INTRODUCTION

Obesity is generally defined as a condition of abnormal or excessive body fat accumulation that may impair health. In adults, it is identified by a body mass index (BMI) of 30 kg/m^2 or higher (Seidell and Flegel, 1997). The growing prevalence of obesity is one of the biggest public health concerns worldwide. According to the news released from World Health Organization (WHO) in August 2014, the global epidemic of obesity has almost doubled since 1980 (http://www.who.int/mediacentre/factsheets/fs311/en/). Obesity is considered to be a serious health problem nowadays as it significantly increases risk of non-communicable diseases (NCDs) like type II diabetes, heart disease and stroke, osteoarthritis and some kinds of cancer (Kopelman, 2000). Causes of obesity are multifactorial involving complex interactions among behavioral, life style and environmental factors, and may include genetic predisposition among people (Seidell and Flegel, 1997). Basically, obesity is developed through a long-term and/or high degree of positive energy balance, *i.e.*, energy intake being greater than energy expenditure, in the body. Most of excess energy is then converted and stored as triglycerides (TG) in adipose tissues, leading to an increase in body fat mass and also body weight (Ogawa *et al.*, 2010). The TG storage during positive energy imbalance occurs particularly in white adipose tissue (WAT). WAT is the major type of body fat in mammals, located beneath skin as subcutaneous fat and around internal organs as visceral fat (Seale *et al.*, 2011). It is consisted mostly of adipocytes which are round cells containing a large unilocular lipid droplet filled with TG and cholesteryl ester. These adipose cells are capable of enlarging (hypertrophy) by accumulating more lipids and proliferating (hyperplasia) when their lipid storage capacity becomes exhausted (Otto and Lane, 2005). Adipocyte hypertrophy due to increased TG storage has been implicated to play a major role in body fat mass expansion during the development of obesity (Blüher, 2009). An increase in adipose cell number, however, becomes more apparent in severe obesity (Hausman *et al*., 2001).

The developmental origin of adipocytes remains to be established. It has been recently proposed based on the results from various adipose lineage tracing

studies that adipocytes might originate from multiple distinct lineages derived from a common embryonic mesenchymal stem cell precursor. Their early progenitors with the identity still under debate then undergo differentiation commitment giving rise to fibroblast-like preadipocytes (Sanchez-Gurmaches and Guertin, 2014). In response to adipogenic stimuli such as insulin, glucocorticoids, growth hormone and certain growth factors, these preadipocytes perform mitotic clonal expansion and produce a cascade of transcription factors, mainly belonging to peroxisome proliferators-activated receptor (PPAR) family and CCAAT/enhancer-binding proteins (C/EBPs), to induce adipocyte-specific gene expression for their further differentiation into mature adipocytes. Upon becoming fully differentiated cells, they secrete adipokines such as leptin, adiponectin, resistin, visfatin and tumor necrosis factor-α (TNFα), and express various adipogenic markers such as glucose transporter-4 (GLUT4), fatty acid-binding protein (FABP), fatty acid synthase (FAS) and acetyl CoA carboxylase (ACC), which play different roles to promote intracellular lipid accumulation, resulting in a profound increase in the cell size and subsequent expansion of fat mass (Moreno-Navarrete and Fernández-Real, 2012). An overview of the molecular events in adipocyte differentiation process (also known as “adipogenesis”) is illustrated in Figure 1.

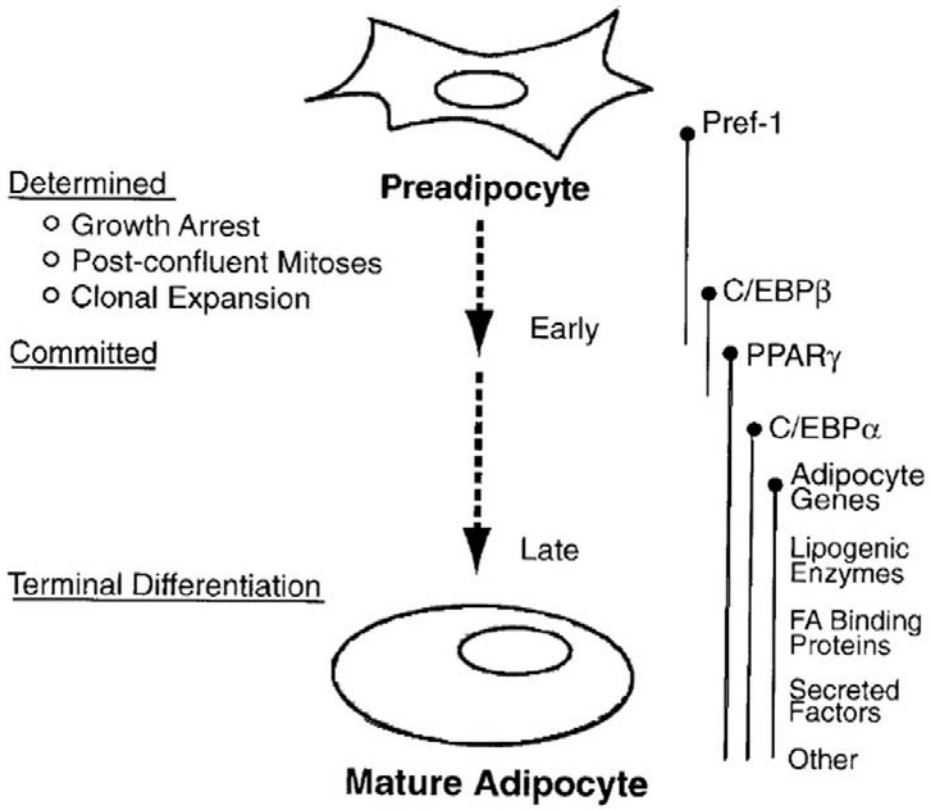

Figure 1 Scheme for development of mature adipocytes from preadipocytes. The duration of maximal expression of the different genes accompanying with the differentiation process is represented by the solid line. Preadipocyte factor-1 (Pref-1), C/EBPβ and C/EBPδ are expressed at the early differentiation stages. After commitment, PPARγ and C/EBPα are up-regulated and target many genes relevant to the function of mature adipocytes (adapted from Gregoire *et al*., 1998).

In obesity management, the use of plant-based products with significant anti-obesity efficacy is fast gaining acceptance due to public awareness of toxicity and deleterious side effects of synthetic drugs (Kang and Park 2012). In the hope of finding new drug candidates with high preventive and/or therapeutic potential but lack of toxicity for more effective and safe applications, a number of plants and their products have been investigated both *in vitro* and *in vivo* for anti-obesity related bioactivities over the past decade. Some examples of those found having anti-obesity activities are: tea catechins reduce body fat in both high-fat induced obese mice and healthy humans partly through the activation of hepatic lipid metabolism (Tokimitsu, 2004); hydroxycitric acid from Malabar tamarind (*Garcinia cambogia*) suppresses epididymal fat accumulation in developing Zucker obese rats (Saito *et al.*, 2005); the ethanol extract of lotus *(Nelumbo nucifera*) leaves demonstrates anti-obesity effects in high-fat diet induced obese mice by impairing dietary fat and carbohydrate absorption *via* digestive enzymes inhibition, and enhancing lipolysis in adipocytes and thermogenesis in skeletal muscle (Ono *et al.*, 2006); the water extract of hibiscus (*Hibucus subdariffa*) flowers inhibits 3T3-L1 adipocyte differentiation through the modulation of phosphatidylinositol 3'-kinase (PI3K)/Akt and extracellular-signal-regulated kinases (ERK) pathways (Kim *et al.*, 2007); bitter melon (*Momordica charantia*) exerts anti-adiposity effects in high-fat diet fed rats by decreasing adipocyte hypertrophy *via* down-regulation of lipogenic gene expressions (Huang *et al.*, 2008), and later its ethanol extract has been reported to decrease both fat accumulation and proliferation of 3T3-L1 preadipocytes by a G2/M arrest of cell cycle (Popovich and Zhang, 2010); grape (*Vitis vinifera*) skin ethanol extract inhibits TG accumulation in 3T3-L1 cells by suppressing various genes involved in PPARγ signaling (Jeong *et al.*, 2012); anthocyanin-rich blueberry and mulberry juice decrease body weight gain, serum lipids and fat accumulation in adipose tissue of mice fed a high-fat diet (Wu *et al.*, 2013); mulberry leaf ethanol extract reduces fat accumulation and protein level of C/EBPα, PPARγ, PPARγ coactivator-1α (PGC-1α), FAS and adiponectin in differentiated 3T3-L1 adipocytes (Yang *et al.*, 2014); hexane phase from *Peucedanum japonicum* Thunb. ethanol extract down-regulates lipogenic gene expressions in HepG2 cells, decreases TG level and cell size *via* increased lipolysis in 3T3-L1 adipocytes, and stimulates thermogenesis in C2C12 myotubes (Nugara *et al.*,

2014); and lately soy bean saponins inhibit adipogenesis by down-regulating PPARγ and C/EBPα in 3T3-L1 adipocytes (Yang *et al*., 2015).

Despite those extensive investigations, there are still unstudied plant species all over the world. Without any doubt, some of them would harbor the promising phytochemicals that may counteract obesity efficiently by inhibiting digestive enzymes, modulating adipogenesis and adipogenic factors, suppressing appetite, or any other means (Sahib *et al.,* 2012). In Thai traditional medicine, there are several plants that have been claimed to possess anti-obesity property. Among them is ivy gourd (*Coccinia grandis* L. Voigt), called in Thai as "Tum-Leung". It is a fast-growing climbing, perennial vine native to Asia, which is found abundantly in many tropical countries. Every part of ivy gourd exhibits pharmacological activities (Pekamwar *et al*., 2013). This medicinal plant has been used as a household remedy, especially in the Indian subcontinent, to alleviate various illness symptoms (Yadav *et al.*, 2010). Its leaf is best known for anti-diabetic property (Munasinghe *et al.*, 2011). The root, however, has been mentioned to exert weight-lowering effect (Bunyapraphatsara and Chokchaijaroenporn, 1999) whereas anti-obesity activity of the fruit has recently been demonstrated in animals (Ahmed and Manoj, 2012). As very limited information is available on anti-obesity potency, mechanism of action and the active constituents of ivy gourd, this study was then focused to evaluate anti-adipogenic activity of the plant by employing a murine preadipocyte 3T3-L1 cell line, and to examine anti-obesity effects in a high fat-diet induced obese C57BL/6J mouse model. The phytochemical analysis of ivy gourd was also attempted to identify its major anti-obesity component(s).

OBJECTIVES OF THE RESEARCH

1. To study the effects of an ethanol extract prepared from different parts of ivy gourd on lipid accumulation in 3T3-L1 adipocytes.
2. To fractionate the active extract(s) and study the effects of each fraction obtained on adipocyte differentiation of 3T3-L1 cell line.
3. To study the effects of active fraction(s) in C57BL/6J mice fed a high-fat diet**.**
4. To identify the major anti-obesity component(s) of ivy gourd.

BENEFITS GAINED FROM THE RESEARCH

It is expected that the resulting data could provide a line of scientific evidence to explain and support anti-obesity related effects of ivy gourd. In addition, the findings could raise a potential value of this plant and offer its possibility to be developed into a therapeutic agent for further applications.

CHAPTER 2

Effects of Ivy Gourd on Lipid Accumulation in 3T3-L1 Adipocytes

1. Introduction

Adipocyte differentiation or adipogenesis is the key step to obesity development. It is a complex cellular pathway involving sequential regulation of gene expression leading to a dramatic increase in many enzymes and proteins that promote lipid synthesis. As a result, phenotypic alteration of the precursor cells (preadipocytes) occurs. They transform from elongate fibroblastic to spherical in shape with filled lipid droplets. The molecular and cellular events associated with adipocyte differentiation have been extensively studied over the past decades (Rosen *et al.*, 2000). Most of the obtained knowledge comes from *in vitro* experiments using two kinds of cell line models, established preadipocyte cell lines which have undergone commitment to the adipose lineage and multipotent fibroblastic cell lines, able to commit to different lineages including adipose, bone and muscle lineage (Ntambi and Kim, 2000).

The 3T3-L1 cells are the most frequently used among available models of preadipocyte cell line (Figure 2). They are derived from 17-19 days old Swiss 3T3 mouse embryos (Green and Kehinde, 1976). These adipose clonal cells provide a consistent source of preadipocytes for study since they are homogeneous, have a long life span in culture, and exhibit morphology and biochemical features similar to *in vivo* adipocytes upon induction of differentiation. They also differentiate and develop into adipose tissue when transplanted subcutaneously into mice (Student *et al.*, 1980).

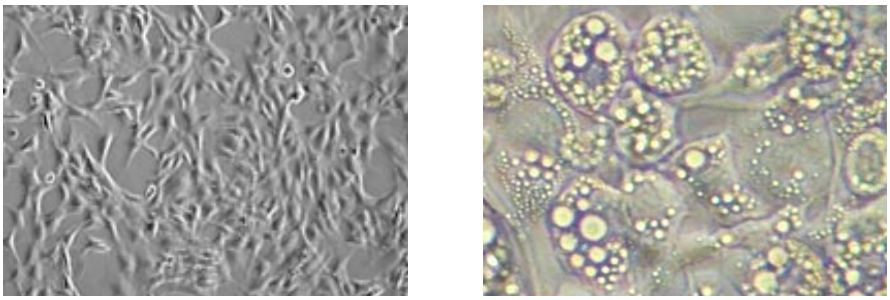

Figure 2 The 3T3-L1 cells (200x) before (left) and after (right) adipocyte differentiation

The course of 3T3-L1 adipocyte differentiation has been well-studied (Ntambi and Kim, 2000). In general, 3T3-L1 preadipocytes need post-confluence mitosis to unwind DNA, allowing important transcription factors get access to adipogenic genes (Cornelius *et al.*, 1994). Then, the post-confluence cells are differentiated synchronously upon exposure to a hormonal cocktail of differentiation inducers consisting of insulin which mediates adipogenic action through IGF-1 receptor signaling cascade (Tong and Hotamisligil, 2001), 3-isobutyl-1-methylxanthine, a phosphodiesterase inhibitor, elevates intracellular cAMP and dexamethasone (DEX) which stimulates glucocorticoid receptor leading to the expression of C/EBPδ and PPARγ, both are responsible for activation of adipogenic genes (Tong and Hotamisligil, 2001). In the presence of fetal bovine serum, growth hormone is also provided to promote cell differentiation. Maximal differentiation is achieved upon early hormonal induction for 48 h since early induction with DEX is required to trigger the process. This synthetic glucocorticoid has been found to inhibit adipogenesis in 3T3-L1 cells when added at late stages of differentiation (Pantoja *et al.*, 2008). The differentiated cells become mature adipocytes within 8 days after the induction by accumulating intracellular fat to increase in cell size and secrete adipocyte markers such as adipokines, leptin, resistin which play different roles in biochemical and physiological processes.

The molecular event associated with adipogenesis of 3T3-L1 cells is multi-step. It involves a cascade activation of transcription factors which coordinately regulate gene expression and leading to adipocyte development (Rosen *et al.*, 2000).

Of the different transcription factors playing role in molecular control of adipogenesis, PPARγ and C/EBPα are the most important ones.

PPARγ is a member of the PPAR family which belongs to the nuclear receptor superfamily. It appears to function as a dominant activator of fat cell differentiation through transactivator domain. Its effects are mediated *via* the formation of heterodimer with retinoid X receptor prior to binding of DNA to the peroxisome proliferator element in the promoter region of the target genes. This transcription factor, also known as a master regulator of adipogenesis, is necessary for both promotion and maintaining the differentiated state of the adipocytes (Rosen *et al*., 2000). There are two main isoforms of PPARγ, namely PPARγ1 and PPARγ2, that differ only in their N-terminal-30 amino acids (Werman *et al*., 1997). Both are derived from the same gene by alternating promoter usage and differential mRNA splicing (Zhu *et al*., 1995) and are expressed in adipose tissue. Although PPARγ2 protein level is lower in the liver, it is more important than PPARγ1 in adipogenesis (Vidal-Puig *et al*., 1996). Expression of both PPARγ genes is down-regulated by fasting and insulin-deficient diabetes (Vidal-Puig *et al*., 1996). Sterol regulatory element binding protein-1c (SREBP-1c) is a transcription factor that can activate PPARγ transcription (Tong and Hotamisligil, 2001).

The CCAAT/enhancer binding protein transcription factors (C/EBPs) are a family of transcription factors that all contain highly conserved, basic leucine zipper (bZIP) transcription factors, which have a C-terminal leucine zipper domain for dimerization and a basic domain for binding to DNA. They consist of 6 isoforms α, β, γ, δ, ζ (CHOP-10), and ε. All of them except the ε form are involved in adipogenesis (Ramji and Foka, 2002). In the early phase of adipocyte differentiation, C/EBPβ and C/EBPδ are up-regulated (Yeh *et al*., 1995). In turn, these changes increase the transcription of PPARγ and C/EBPα (Rosen *et al*., 2002). PPARγ and C/EBPα are major regulators of adipogenesis that promote the transcription of terminal adipocyte differentiation marker genes such as fatty acid binding protein-4 (*FABP4*), fatty acid synthase (*FAS*), leptin, adiponectin, glucose transporter-4 (*GLUT4*), insulin receptor and others (Rosen and MacDougald, 2006) (Figure 3).

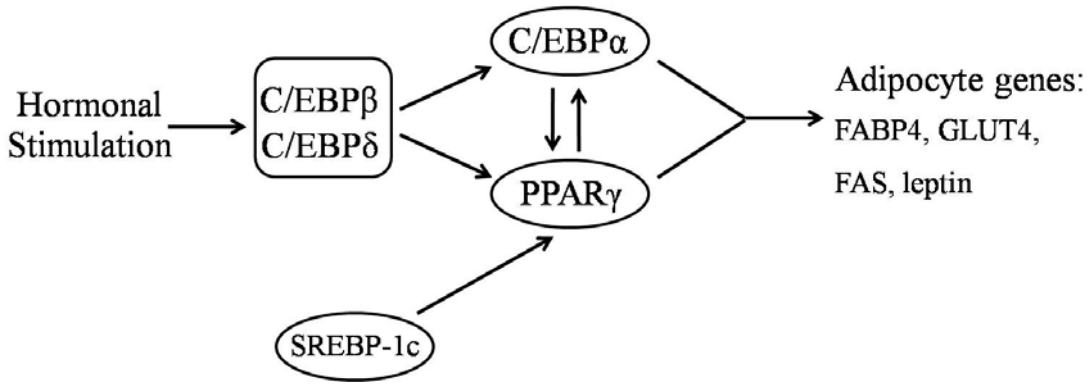

Figure 3 Transcription factors and adipogenic genes involved in 3T3-L1 adipocyte differentiation (Tong and Hotamisligil, 2001).

The availability of 3T3-L1 cell line as well as its established adipogenic properties has allowed investigators to explore anti-adipogenic potential of various pharmacological compounds either synthetic or natural-derived substances. So far, various kinds of plant ingredients have been found to potently inhibit adipogenesis in 3T3-L1 adipocytes through modulation of key adipogenic genes and transcription factors. Some examples of most recent findings are: arctiin, a major lignin found in burdock (*Arctium lappa* L.) significantly decreases the protein levels of PPARγ, C/EBPα and mRNA levels of PPARγ, C/EBPα, SREBP-1c, FAS, FABP4 and lipoprotein lipase (LPL) in 3T3-L1 cells (Min *et al.*, 2014). KMU-3, a novel derivative of gallic acid, down-regulates gene expression of PPARγ, C/EBPα, FAS and signal transducer and activator of transcription-3 (STAT-3) in 3T3-L1 adipocytes (Park *et al.*, 2014).

Ivy gourd, also called scarlet (fruited) gourd, little gourd, baby watermelon or gentleman's toes (http://en.wikipedia.org/wiki/Coccinia_grandis), is a tropical plant native to Africa and Asia. This plant belongs to Cucurbitaceae Family as cucumber, pumpkin and melons. Ivy gourd (*Coccinia grandis* L. Voigt or *C. cordifolia* L. Cogn. or *C. indica* Wight & Arn. or *Cephalandra indica* Naud.) is a perennial plant with climbing stem and springy coiled-like tendrils. The leaves have five lobes and vary in shape from the heart to pentagon form. The flowers are white and star-shaped. The roots are tuberous. The fruits are oval with thick and sticky skin which turns from green to bright red when they are ripe (Wasantwisut and Viriyapanich, 2003) (Figure

4). In Asia, ivy gourd has been used as a home remedy to cure several ailments, especially diabetes treatment which is mostly applied in India and neighboring countries (Satyavati *et al*., 1987). Every part of this plant contains bioactive components and its pharmacological properties have been extensively reviewed (Deokate and Khadabadi, 2012). In Thailand, however, ivy gourd leaves and shoot tips are commonly consumed as both fresh vegetables and the main ingredient in many cooking recipes. Ivy gourd has been classified as a medicinal herb in Thai traditional medicine with some properties similar to those documented in India (Wasantwisut and Viriyapanich, 2003). In addition to anti-diabetic activity, the root part has been claimed to reduce obesity (Bunyapraphatsara and Chokchaijaroenporn, 1999). Evidence of weight loss in overweight patients after the plant extract administration has been reported in India (Kuriyan *et al.*, 2008), and recently, anti-obesity effects of ivy gourd fruit have been demonstrated in high-fat diet induced obese mice (Ahmed and Manoj, 2012). Since no previous attempts have been made to study the anti-obesity property of ivy gourd in detail, the effects of ivy gourd on lipid accumulation in 3T3-L1 cells were investigated and the underlying molecular mechanisms were characterized in this study.

Figure 4 Ivy gourd (*Coccinia grandis* L. Voigt) leaves, flower and fruits.

2. Materials

2.1 Instruments

Instrument	Model	Manufacturer
Autoclave	HA-300M	Hirayama
Automatic x-ray film-developing machine	SRX-101A Medical Film Processor	Konica Minolta
CO_2 incubator	Nu-2500E	NuAire
Gel doc system	BioDoc-It™ M-20	Bioimagine System
Micro centrifuge	MIKRO 200R	Hettich Zentrifugen
Nanodrop spectrophotometer	Nanodrop 1000	Thermo Scientific
Refrigerated centrifuge	MSE Harrier 18/80	SANYO
Electroblotting apparatus	Mini-PROTEAN® Tetra System	Bio Rad
Electronic dry cabinet	KD-70	Weifo®
Electrophoresis unit	Mini Trans-Blot® Cell	Bio Rad
Freeze drier	DW6-85	Heto Drywinner
Hot air oven	FED 115	Binder
Laminar flow hood	LA2-4AI	Esco
RT-PCR	MyCycler™	Bio Rad
Real-time RT-PCR	Step One Plus™	Applied Biosystems
Rotary evaporator	BUCHI 713	BUCHI
Shaker	3D Mini Shaker	Biosan
Vortex -2 Genie®	G-560E	Scientific Industries
Water bath	WB 14	Memmert

2.2 Chemicals

Chemical	Manufacturer
Acrylamide	Merck
Agarose	Vivantis
Ammonium persulfate	Merck
Anti-actin antibody	Sigma
Anti-C/EBPα antibody	Cell Signaling Technology
Anti-C/EBPβ antibody	Cell Signaling Technology
Anti-PPARγ antibody	Millipore
Anti-FAB4 antibody	Cell Signaling Technology
Anti-adiponectin antibody	Cell Signaling Technology
Anti-GLUT4 antibody	Millipore
Anti-rabbit antibody	Cell Signaling Technology
Bromophenol blue	Sigma-Aldrich
Chloroform, AR grade	Brightchem. sdn bhd
Dexamethasone	Sigma-Aldrich
1-(4, 5-Dimethylthiazol-2-yl)-3, 5-diphenylformazan (MTT)	Sigma-Aldrich
Dimethyl sulfoxide	Riedel-de Haën®
Dithiothreitol	Sigma-Aldrich
DNA marker (1 kb plus DNA ladder)	Biolab
Dulbecco's modified Eagle's medium	Invitrogen
ECL assay kit	Thermo Scientific
Ethyl acetate, AR grade	Lab-Scan
Ethanol, AR grade	Lab-Scan
Ethylenediamine tetra acetic acid (EDTA)	Merck
Fetal bovine serum	Invitrogen/Life Technologies
Glycerol reagent kit	Sigma-Aldrich
Glycine	Vivantis
Hexane, AR grade	Lab-Scan

Chemical	Manufacturer
Human insulin	Sigma-Aldrich
Isobutylmethylxanthine	Sigma-Aldrich
Isopropanol, AR grade	Lab-Scan
β-mercaptoethanol	Fluca
Methanol, AR grade	Lab-Scan
M-MLV reverse transcriptase	Bio Rad
Novel Juice	GeneDireX®
Oil red O	Sigma-Aldrich
Penicillin (10,000 units/ml)-Streptomycin (10,000 μg/ml)	Invitrogen/Life Technologies
Silica gel 60, 0.04-0.06 cm	Scharlau Chemie S.A.
SDS	Calbiochem®
TEMED	Fluka
Tris (hydroxymethyl) amino methane	Fisher Chemical
TRIZOL™ reagent	Invitrogen
Trypsin-EDTA	Invitrogen/Life Technologies
Tween-20	Ajax Finechem
Ultrapure-DEPC-treated water	Invitrogen

3. Methods

3.1 Collection of plant materials

Ivy gourd (*Coccinia grandis* L. Voigt) samples used in this study were collected from various areas around Songkhla Province, Thailand. Once obtained, they were cleaned by thoroughly washing in tap water and left to air-dry overnight before use.

3.2 Preparation of extracts

The roots, stems and leaves of ivy gourd were separated, cut into small pieces and left at 40°C in a hot air oven until completely dried. The dried materials were ground into powder and macerated with 10 volumes of ethanol at ambient temperature. The resulting extracts were then collected and passed through a

Whatman No.4-filter paper. The filtrate obtained was evaporated under vacuum at 40°C using a rotary evaporator, freeze-dried and stored in a dry cabinet (30% humidity at room temperature).

3.3 Fractionation of the root extract

The ethanol extract of ivy gourd root was separated into five different fractions (A-E) by sequential solvent partitioning procedure as shown in the scheme (Figure 5).

3.4 Isolation and identification of active constituent in the root extract

In order to identify the active components of IGH, the IGH sample was applied to a silica gel column (Silica gel 60, 0.04-0.06 mm, 70-230 mesh) (Scharlau Chemie, Spain), and serially eluted with hexane-ethyl acetate (95: 5, v/v), hexane-ethyl acetate (50: 50, v/v) and 100% ethyl acetate. The collected fractions were then pooled into two equal portions. Each portion was concentrated to almost dryness by evaporation under vacuum at 40°C, and then applied on a Silica gel G60 F_{254} pre-coated thin-layer chromatography (TLC) plate (Merck, Germany). A mixture of hexane-ethyl acetate at a ratio of 95: 5 (v/v) acted as mobile phase for the first portion whereas that of 50: 50 (v/v) was used for the other. After elution, the TLC spots were visualized under UV light (254 and 365 nm) and phytochemical detection was performed using different types of spray reagents as described (Farnsworth *et al.* 1966).

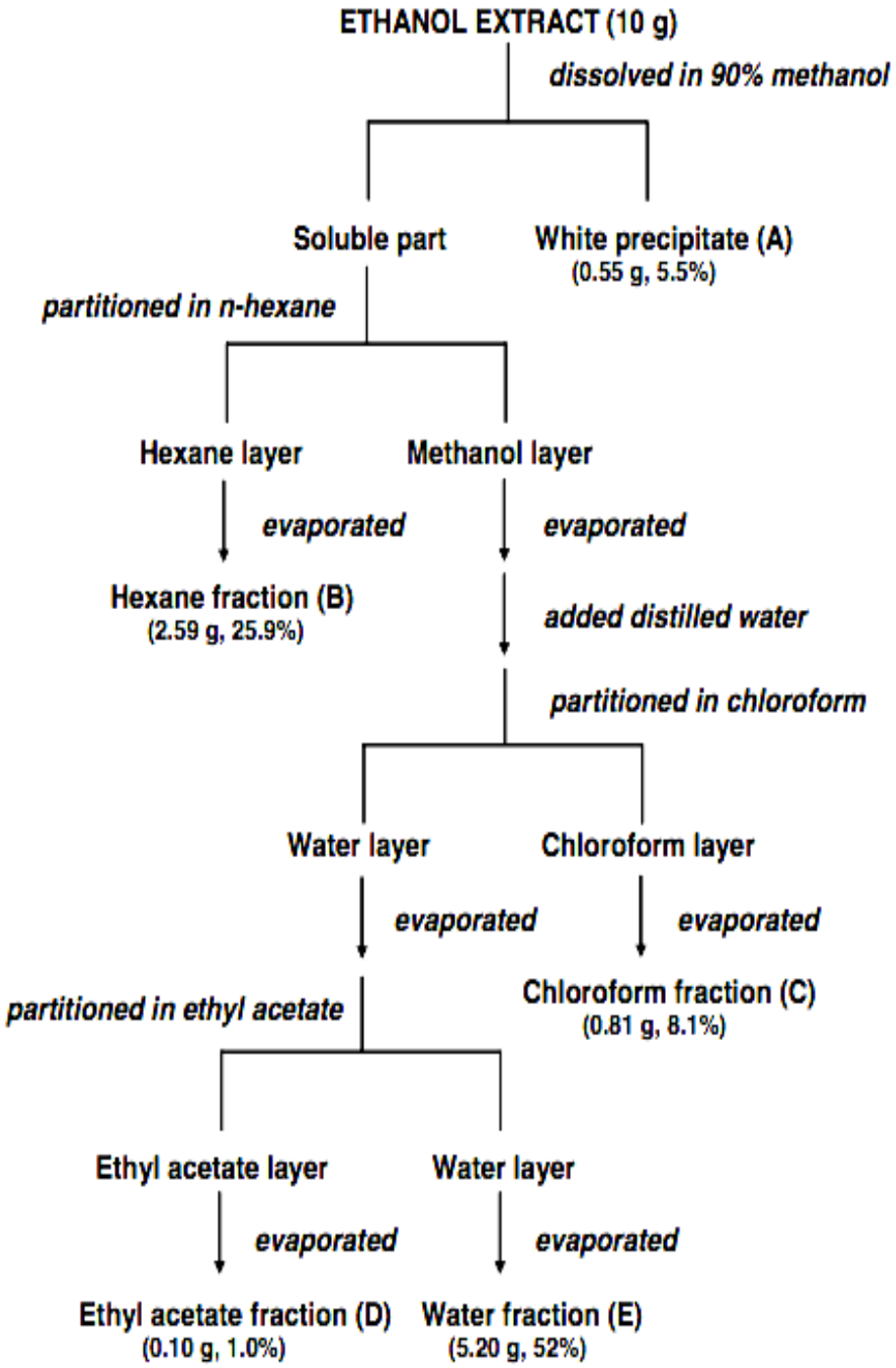

Figure 5 Fractionation scheme of the ivy gourd root extract.

3.5 Cell culture and differentiation induction

Mouse 3T3-L1 pre-adipocytes (Cat. No. CL-173, Lot No. 58358076) were purchased from American Type Culture Collection (ATCC), USA. They were routinely subcultured by growing about 10^6 cells in 10 ml of basal medium (BM) [Dulbecco's modified Eagle's medium containing 10% (v/v) fetal bovine serum and 1% (v/v) penicillin-streptomycin] on a 10 cm-diameter dish (Corning®, USA) at 37°C in a humidified atmosphere of 5% CO_2. Upon reaching 70%-80% confluence, the adherent cells were washed twice with phosphate buffered saline, pH 7.4 (PBS), followed by addition of 0.025% (v/v) trypsin-EDTA in PBS and further incubation at 37°C for 5 min. The detached cells were harvested by precipitation at 1,400 rpm in a refrigerated centrifuge for 4 min. In the experiments, the 6th passage cells were seeded into each well of 48-well plate (Nunc™, Denmark) at a density of 12,000 cells in 400 µl of BM and then grown to full confluence. To induce differentiation, 2 days post-confluence 3T3-L1 preadipocytes (designated Day 0) were treated with differentiation medium (DIM) which was BM supplemented with 1 µM dexamethasone, 0.5 mM isobutylmethylxanthine and 10 µg/ml insulin. Two days after induction (Day 2), the medium was changed to BM containing 10 µg/ml insulin only for 2 days. The differentiated cells were maintained in BM which was replaced every 2 days until harvested on Day 8.

3.6 Cell viability assay

The 6th passage 3T3-L1 cells were seeded into each well of 96-well plate (Nunc™, Denmark) at a density of 6,000 cells in 100 µl of BM and cultured to full confluence at 37°C in a humidified atmosphere of 5% CO_2. The medium was then replaced with DIM in the presence or absence of each sample. After 48 h incubation, DIM was changed to BM containing 0.25 mg/ml 1-(4, 5-dimethylthiazol-2-yl)-3, 5-diphenylformazan (MTT) for 2 h. The medium was removed, and 50 µl of dimethyl sulfoxide (DMSO) was added to dissolve the MTT-formazan complex formed (Mosmann, 1983). The optical density was measured at 570 nm. The percentage of cell viability was calculated using the equation given below.

$$\%\text{cell viability} = \frac{\text{OD sample–OD non-differentiated control}}{\text{OD untreated control–OD non-differentiated control}} \times 100$$

3.7 Oil red O staining for anti-adipogenesis assay

The 3T3-L1 adipocytes harvested on Day 8 were washed with PBS twice and fixed with 10% (v/v) formalin in PBS for 1 h at room temperature. After rinsing with 60% (v/v) isopropanol in distilled water, the cells were stained with oil red O solution [a mixture of 0.5% (w/v) oil red O dye in isopropanol and water at a ratio of 3: 2] for 10 min. The excess dye was then removed by washing with distilled water for three times. The stained intracellular lipid droplets were extracted with 150 µl of DMSO, and the absorbance was measured at 540 nm. The relative lipid contents were calculated using the equation given below.

$$\%\text{relative lipid contents} = \frac{\text{OD sample}-\text{OD non-differentiated control}}{\text{OD untreated control}-\text{OD non-differentiated control}} \times 100$$

3.8 Relative quantification of selected adipogenic proteins

The 3T3-L1 cells were cultured as previously described on a 6-cm diameter dish (Corning®, USA). At each time-point of investigation, the culture medium was discarded, and the cells were washed twice with PBS and collected using a cell scraper. Total cell lysate was prepared by mixing the scraped cells with 100 µl of lysis buffer consisting of 0.5 M Tris-HCl, pH 6.8 with 20% (v/v) glycerol, 20% (v/v) β-mercaptoethanol, 80 mM dithiothreitol (DTT), 8% (w/v) sodium dodecyl sulfate (SDS) and 0.04% (w/v) bromophenol blue for 5 min. The resulting mixture was boiled for 10 min before applying to SDS-polyacrylamide gel electrophoresis (SDS-PAGE).

The SDS-PAGE was performed according to the system developed by Laemmli (Laemmli, 1970). A discontinuous polyacrylamide gel consisting of 3.7% stacking gel and 10% separating gel was used in this experiment. Electrophoresis was carried out at a constant current of 30 mA using 25 mM Tris-HCl containing 0.192 M glycine and 0.1% SDS, pH 8.3 as electrode buffer. At the end of electrophoresis, the separating gel was placed in 20% (v/v) methanol containing 25 mM Tris and 0.19 M glycine before performing wet electroblotting. The protein bands were then transferred onto a polyvinylidene difluoride (PVDF) membrane (Amersham Hybond™-P, GE Healthcare, USA) under a condition of 100 V, for 2 h at

4°C.

The electroblotted PVDF membrane was incubated in 5% (v/v) non-fat milk in 20 mM Tris-HCl, pH 7.6 containing 0.8% (w/v) NaCl and 0.01% (v/v) Tween 20 (TBS-T) for 1 h at room temperature, to prevent non-specific binding. It was washed three times for 5 min each with TBS-T, incubated overnight at room temperature with primary antibody diluted in TBS-T (1: 500 to 1: 1,000 depending on the protein type) and then washed three times for 5 min each with TBS-T. The membrane was further incubated for 1 h at room temperature with diluted anti-rabbit IgG horse radish-peroxidase conjugated secondary antibody in TBS-T (1: 2,000). It was washed three times for 5 min each with TBS-T and subsequently incubated with ECL reagent for 30 min at room temperature. After the reagent was drained off, the membrane was wrapped with a thin plastic film and then exposed to a clear blue x-ray film (CL-XPosure™ film, Thermo Scientific, USA) for 30 min. The film was processed in an automatic x-ray film-developing machine, and dark bands on the post-developed film were then scanned and compared for density.

3.9 Quantification of relative expression of selected adipogenic genes

3.9.1 Total RNA extraction

The 3T3-L1 cells were cultured as previously described on a 6-cm diameter dish (Corning®, USA). At each time-point of investigation, the medium was discarded, and the cells were washed with PBS and lysed by adding 0.5 ml of TRIZOL™ reagent directly to the culture dish, followed by passing the resulting cell lysate several times through a Pasteur pipette. The cell lysate was then transferred to a test tube and left for 5-10 min at room temperature to allow the complete dissociation of nucleoprotein complexes. After 0.2 ml of chloroform was added, the tube was shaken vigorously for 15 sec, left at room temperature for 2-3 min and centrifuged at 12,000 rpm for 15 min at 4°C. After centrifugation, the mixture was separated into 3 phases: phenol-chloroform phase, interphase, and upper aqueous phase containing exclusively RNA.

The upper phase was collected, mixed with 2 volumes of isopropanol and subsequently incubated at -20°C for 30 min. After centrifugation at 12,000 rpm for 30 min at 4°C, RNA was precipitated as a gel-like pellet. The supernatant was

then discarded and the RNA pellet was thoroughly mixed with ethanol. The resulting suspension was centrifuged at 7,500 rpm for 10 min at 4°C. The pellet was collected, left for 2-3 h at room temperature and dissolved in RNase-free water (DEPC-treated water). The obtained solution was measured for RNA purity from the A260/A280 ratio and stored at -80°C until use.

3.9.2 First-strand cDNA synthesis for quantitative reverse transcription polymerase chain reaction (RT-PCR)

A 20 µl-reaction mixture composed of 1 µl of oligo dT (500 µg/ml), 10 µl of the total RNA (0.1 µg/µl), 1 µl of 20 mM dNTP Mix, 1 µl of 0.1 µM DTT, 4.8 µl of M-MLV reverse transcriptase reagent buffer, 0.5 µl of M-MLV reverse transcriptase (200 U/µl) and 1.7 µl of DEPC-treated water, was put in a nuclease-free microcentrifuge tube (Biotix, USA). After mixing gently, the mixture was incubated at 42°C for 60 min. The reaction was stopped by heating at 70°C for 15 min.

3.9.3 RT-PCR amplification of the selected adipogenic genes

Primer sequence of C/EBPα, C/EBPβ, PPARγ, GLUT4, FAB4, adiponectin and GAPDH genes used in this experiment are listed in Table 1.

A 20 µl-reaction mixture composed of 2 µl of 200 mM Tris-HCl, pH 8.4 containing 0.5 M KCl and 20 mM $MgSO_4$, 0.5 µl of 40 mM dNTP Mix, 1 µl of 10 µM amplification sense primer, 1 µl of 10 µM amplification antisense primer, 0.2 µl of Taq DNA polymerase (5 U/µl), 2 µl of the first-strand cDNA and 13.3 µl of distilled water, The mixture (20 µl) was put in a PCR reaction tube (Biotix, USA). After mixing gently, the mixture was subjected to RT-PCR amplification. Thirty-five cycles of PCR were performed for all studied genes by using conditions shown in Table 1.

The PCR products obtained were mixed with 4 µl of Novel Juice and subjected to 2% agarose gel electrophoresis. The DNA bands were visualized under UV illumination and relatively quantified in an automated gel doc system by employing GAPDH gene as the internal control.

Table 1 Primer sequences and conditions for RT-PCR

Gene	Forward sequence Reverse sequence	PCR conditions
Adiponectin	5'-TGTTGCAAGCTCTCCTGTTCCTCT-3'	62 °C, 35 cycles
(552 bp)	5'-AGAGAACGGCCTTGTCCTTCTTGA-3'	
C/EBPα	5'-TGGACAAGAACAGCAACGAG-3'	58 °C, 35 cycles
(225 bp)	5'-CCTTGACCAAGGAGCTCTCA-3'	
C/EBPβ	5'-GGGGTTGTTGATGTTTTTGG-3'	58 °C, 35 cycles
(149 bp)	5'-CGAAACGGAAAAGGTTCTCA-3'	
FABP4	5'-ACCTGGAAGCTTGTCTCCAGTGAA-3'	56 °C, 35 cycles
(291 bp)	5'-TGTGGTCGACTTTCCATCCCACTT-3'	
GAPDH	5'-AACTTTGGCATTGTGGAAGGGCTC-3'	58 °C, 35 cycles
(382 bp)	5'-TGGAAGAGTGGGAGTTGCTGTTGA-3'	
GLUT4	5'-GACGGACACTCCATCTGTTG-3'	58 °C, 35 cycles
(400 bp)	5'-GCAGCTGAGATCTGGTCAAAC-3'	
PPARγ	5'-GATTCTCCTGTTGACCCAGAG-3'	58 °C, 35 cycles
(408 bp)	5'-ACAGACTCGGCACTCAATGG-3'	

3.9.4 Quantitative real-time RT-PCR (qRT-PCR) assay

Total RNA was extracted from the differentiated 3T3-L1 cultured cells by using the RNeasy mini kit (Qiagen, Germany). First strand cDNA was synthesized with 2 μg of total RNA as a template using the High Capacity RNA-to-cDNA kit (Applied Biosystems, USA). The qRT-PCR was performed with the following conditions: one cycle of 95°C for 20 sec, 40 cycles of 95°C for 3 sec and 60°C for 30 sec. A melting curve analysis was performed starting at 95°C for 15 sec, 60°C for 60 sec and increasing by 0.3°C every 15 sec to determine primer specificity. Primer sequences of all studied genes are listed in Table 2. The β-actin gene was used as the internal control.

3.10 Statistical analysis

All data are presented as a mean value ± S.D. In each experiment, the inter-group differences were evaluated by one-way ANOVA, followed by Dunnett's test. Probability values of $p < 0.05$ were considered to be significant.

Table 2 Primer sequences for Real-Time PCR

Gene (product)	**Forward sequence Reverse sequence**	**Accession No.**
Actin	5'-CAGAAGGAGATTACTGCTCTGGCT-3'	NM_007393
(93 bp)	5'-GGAGCCACCGATCCACACA-3'	
ACC	5'-GGACCACTGCATGGAATGTTAA-3'	AY451393
(73 bp)	5'-TGAGTGACTGCCGAAACATCTC-3'	
Adiponectin	5'-GTTCCCAATGTACCCATTCGC-3'	NM_009605
(88 bp)	5'-TGTTGCAGTAGAACTTGCCAG-3'	
Adipor1	5'-TCTTCGGGATGTTCTTCCTGG-3'	NM_028320
(104 bp)	5'-TTTGGAAAAAGTCCGAGAGACC-3'	
C/EBPα	5'-TGGACAAGAACAGCAACGAGTAC-3'	AM_007678
(257 bp)	5'-GCAGTTGCCCATGGCCTTGAC-3'	
FABP4	5'-AGCATCATAACCCTAGATGG-3'	NM_024406.2
(115 bp)	5'-CATAACACATTCCACCACCAGC-3'	
FAS	5'- TGCTCCCAGCTGCAGGC-3'	AF_127033
(91 bp)	5'-GCCCGGTAGCTCTGGGTGTA-3'	
GLUT4	5'-CTGCAAAGCGTAGGTACCAA-3'	BC014282
(87 bp)	5'-CCTCCCGCCCTTAGTTG-3'	
IRS-1	5'-CCAGAGTCAAGCCTCACACA-3'	NM_010570.4
(179 bp)	5'-GAAGACTGCTGCTGCTGTTG-3'	
LPL	5'-AGGGCTCTGCCTGAGTTGTA-3'	NM_008509
(199 bp)	5'-AGAAATCTCGAAGGCCTGGT-3'	
MEST	5'-GTTTTTCACCTACAAAGGCCTACG-3'	NM_008590
(52 bp)	5'-CACACCGACAGAATCTTGGTAGAA-3'	

Table 2 Primer sequences for Real-Time PCR (continued)

Gene (product)	**Forward sequence Reverse sequence**	**Accession No.**
PDK4	5'-GAGAAGAGCCCAGAAGACCA-3'	NM_013743
(134 bp)	5'-TCCACTGTGCAGGTGTCTTT-3'	
SIRT1	5'-GACGACGAGGGCGAGGAG-3'	NM_019812
(79 bp)	5'-ACAGGAGGTTGTCTCGGTAGC-3'	
PPARγ	5'-AGGCCGAGAAGGAGAAGCTGTTG-3'	NM_011146
(276 BP)	5'-TGGCCACCTCTTTGCTGTGCTC-3'	
PPARγ1	5'-AAGATTTGAAAGAAGCGGTGAAC-3'	NM_001127330
(116 bp)	5'-CAATGGCCATGAGGGAGTTAG-3'	

ACC, acetyl-CoA carboxylase; Actin, cytoplasmic β-actin; Adipor1, adiponectin receptor-1; C/EBPα, CCAAT/enhancer binding protein transcription factor-α; FABP4, fatty acid binding protein-4; FAS, fatty acid synthase; GLUT4, glucose transporter-4; IRS1, insulin receptor substrate-1; LPL, lipoprotein lipase; MEST, mesoderm specific transcript; PDK4, pyruvate dehydrogenase kinase-4; PPARγ, peroxisome proliferator activated receptor-γ; PPARγ1, peroxisome proliferator activated receptor-γ1; SIRT1, sirtuin (silent mating type information regulation 2 homolog)-1; bp, base pairs.

4. Results and Discussion

4.1 Effects of the ethanol extracts from various parts of ivy gourd on intracellular lipid accumulation in 3T3-L1 adipocytes

The ethanol extracts from the root, stem, and leaf parts of ivy gourd were examined for their anti-adipogenic effects, the root extract apparently lowered the lipid levels in 3T3-L1 adipocytes. The amounts of accumulated lipid in the 3T3-L1 adipocytes following the root extract treatment, were measured in terms of the absorbance of the oil red O dye extracted from stained cells, were significantly and dose-dependently decreased (Figure 6). A smaller number of fat droplets within the mature adipocytes in the presence of the root extract were compared to the untreated cells as revealed by microscopy (Figure 7). Such an inhibitory effect, did not result from cell damage since toxicity of the root extract was not observed with any of the concentrations tested (Figure 6).

The anti-adipogenic effect of the root extract was time sensitive. When 3T3-L1 pre-adipocytes were treated with the extract during differentiation-induction period (Day 0-Day 2) and throughout the course of differentiation (Day 0-Day 6), their intracellular lipid levels decreased equally, regardless of different treatment time-lengths (Figure 8). Administration of the root extract after that period (Day 6-Day 8), however, had no effect on lipid accumulation in the fully differentiated cells (Figure 8). These results indicated that the extract was effective only if introduced early in the adipocyte differentiation program. It acted strictly on differentiating 3T3-L1 preadipocytes within the first 2 days of induction with insulin, IBMX (3-isobutyl-1-methylxanthine), and DEX (dexamethasone). A series of adipogenesis-promoting molecules are known to be activated in response to signaling by hormonal inducers (Rosen and MacDougald, 2006). The active compound(s) in the extract might inhibit any of them in some way, thereby blocking adipocyte differentiation at this early stage. Decreasing levels of such critical molecule(s) within the cells during the progress of differentiation were also implicated because the root extract when applied to mature adipocytes (Day 6-Day 8) did not cause any inhibition (Figure 8).

Extracts of stems and leaves had no effect on the differentiation of the 3T3-L1 adipocytes. The marked decrease of the lipid contents seen at 400 μg/ml of leaf

extract and at 500 μg/ml of treatments with stem extract were really a consequence of their ability to damage the cells based on the MTT assay results (Figure 6). In addition, the three ivy gourd extracts were examined for their adipolytic activities, none of them exerted lipid degrading effect on fully differentiated 3T3-L1 adipocytes, as determined from the amounts of glycerol released into the culture medium (Figure 9). From the above findings, the experiment was decided to focus on investigating the mechanisms by which the ivy gourd root inhibited the adipocyte differentiation process, and to identify the active elements.

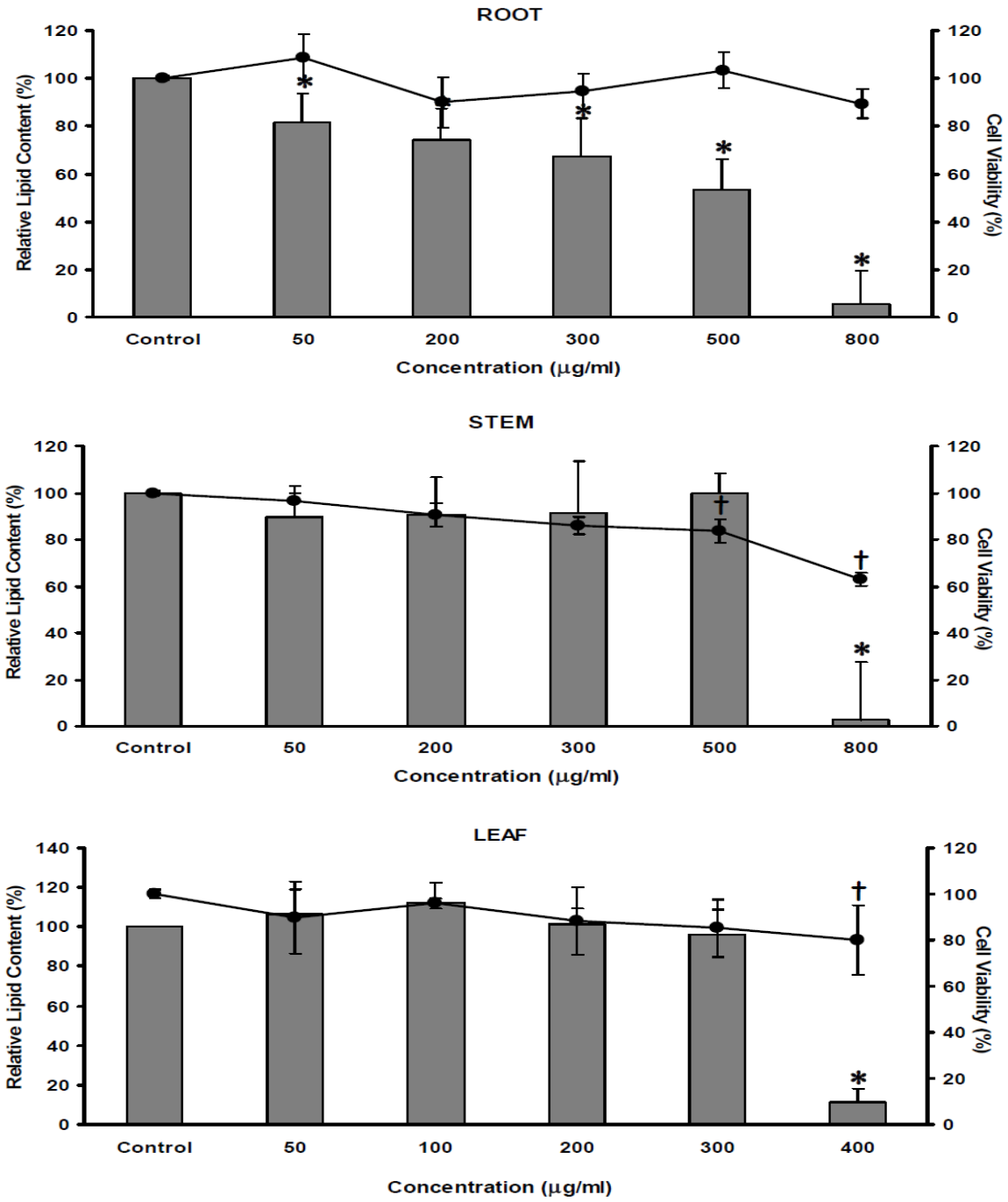

Figure 6 Effects of the ethanol extracts from different parts of ivy gourd on intracellular lipid accumulation and cell viability in 3T3-L1 cells. Results are mean ± S.D. of six-replicate measurements. Bar graphs represent the relative intracellular lipid contents; * p <0.05 *vs* Control. Percentages of cell viability are shown as a line graph; † p <0.05 *vs* Control. The standard TNFα at 10 ng/ml gave 5.00 ± 1.74% of relative lipid content in these experiments.

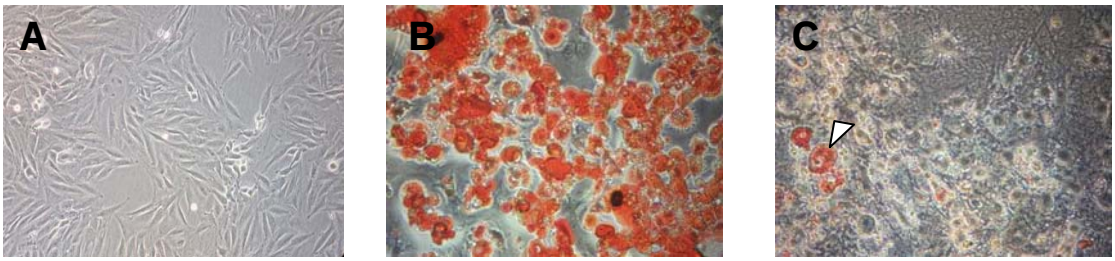

Figure 7 Effect of the root extract on intracellular lipid accumulation. (A) unstained 3T3-L1 pre-adipocytes (40x), (B) untreated adipocytes stained with oil red O (200x), (C) adipocytes treated with 800 μg/ml the root extract (200x); Arrow indicates oil red O stained intracellular fat droplets.

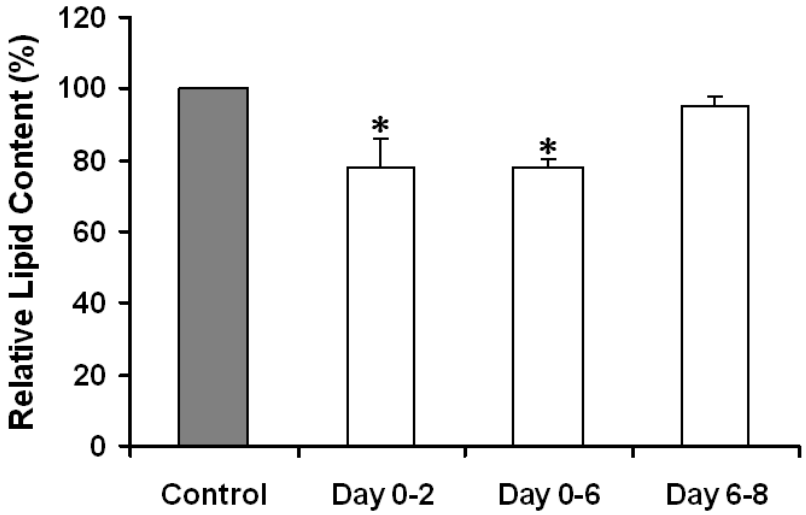

Figure 8 Effect of the root extract at different treatment times on lipid accumulation in 3T3-L1 cells. The values are shown mean ± SD of five-replicate measurements for extract at 100 μg/ml, * p <0.05 compare with control group.

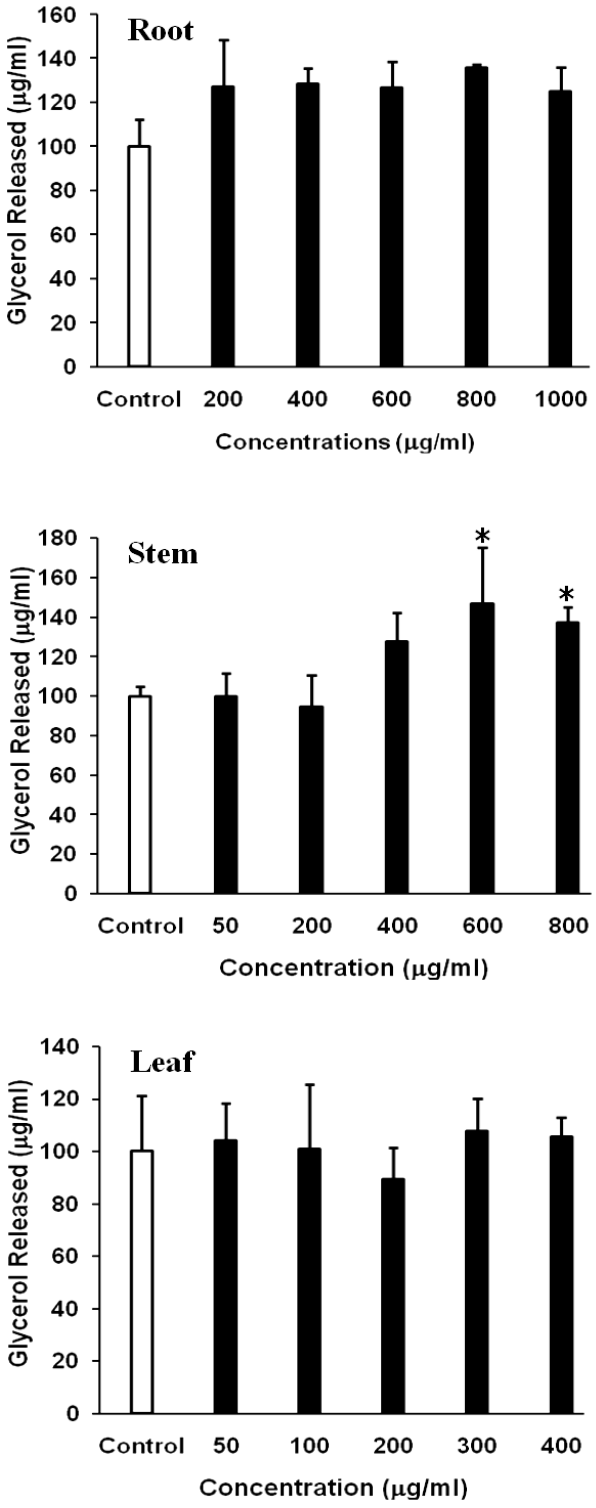

Figure 9 Effects of roots, stems and leaves on lipolysis in 3T3-L1 cells. Results are mean ± SD of three-replicate measurements. Bar graphs represent glycerol released in the culture medium. * p <0.05 compare with control.

4.2 Effects of hexane fraction of the root extract (IGH) on adipogenesis in 3T3-L1 cells

In an attempt to separate the active components from the root extract by sequential solvent partitioning, five different fractions were obtained (Figure 5). When each fraction was examined for its ability to inhibit adipogenesis, only the hexane-soluble fraction was active. The 3T3-L1 adipocyte differentiation was suppressed by hexane-soluble fraction during the early stages in a dose-dependent manner without negatively affecting the cell survival (Figure 10), whereas the other fractions were either harmful to the cells or ineffective (Figure 11). These results indicated that the main constituents in ivy gourd root with a suppressive effect on adipocyte differentiation were likely to be non-polar compounds. The presence of alkaloids (Qudrat-i-Khuda *et al*., 1965), fatty acids (Siddiqui, *et al*., 1973), carotenoids (Barua and Goswami, 1979), triterpenoids (Vaishnav *et al*., 2001), cardenolides (Orech *et al*., 2005), a long-chain polyprenol (Singh *et al*., 2007), as well as flavonoids, polyphenols, and saponins (Ajay *et al*., 2009) have been reported in this plant. Although these components have not been shown to inhibit adipogenesis, polyprenol may be one of the potential candidates since it improves dyslipidemia *in vivo* (Singh *et al*., 2007).

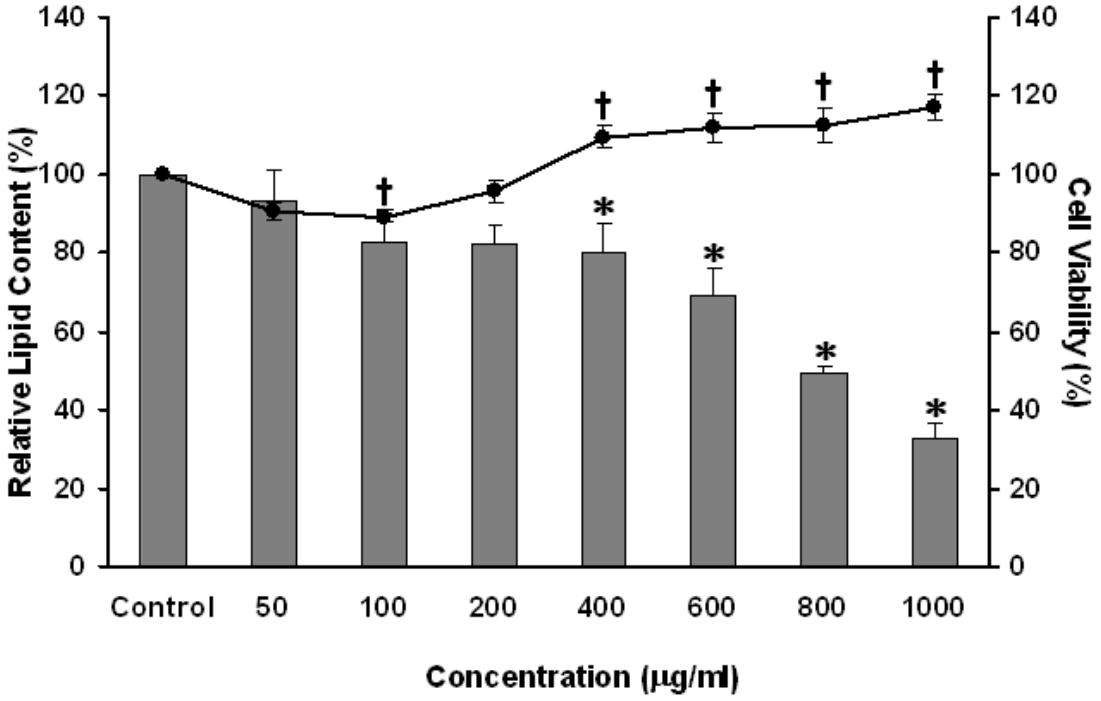

Figure 10 Effects of hexane fraction from the root extract on intracellular lipid accumulation and cell viability in 3T3-L1 cells. Results are a mean value ± S.D. of six-replicate measurements. Bar graphs represent the relative intracellular lipid contents. Asterisks indicate a significant difference at $p < 0.05$ from the untreated control (0 μg/ml). Percentages of cell viability are shown as a line graph. Crosses indicate a significant difference at $p < 0.05$ from the untreated control (0 μg/ml).

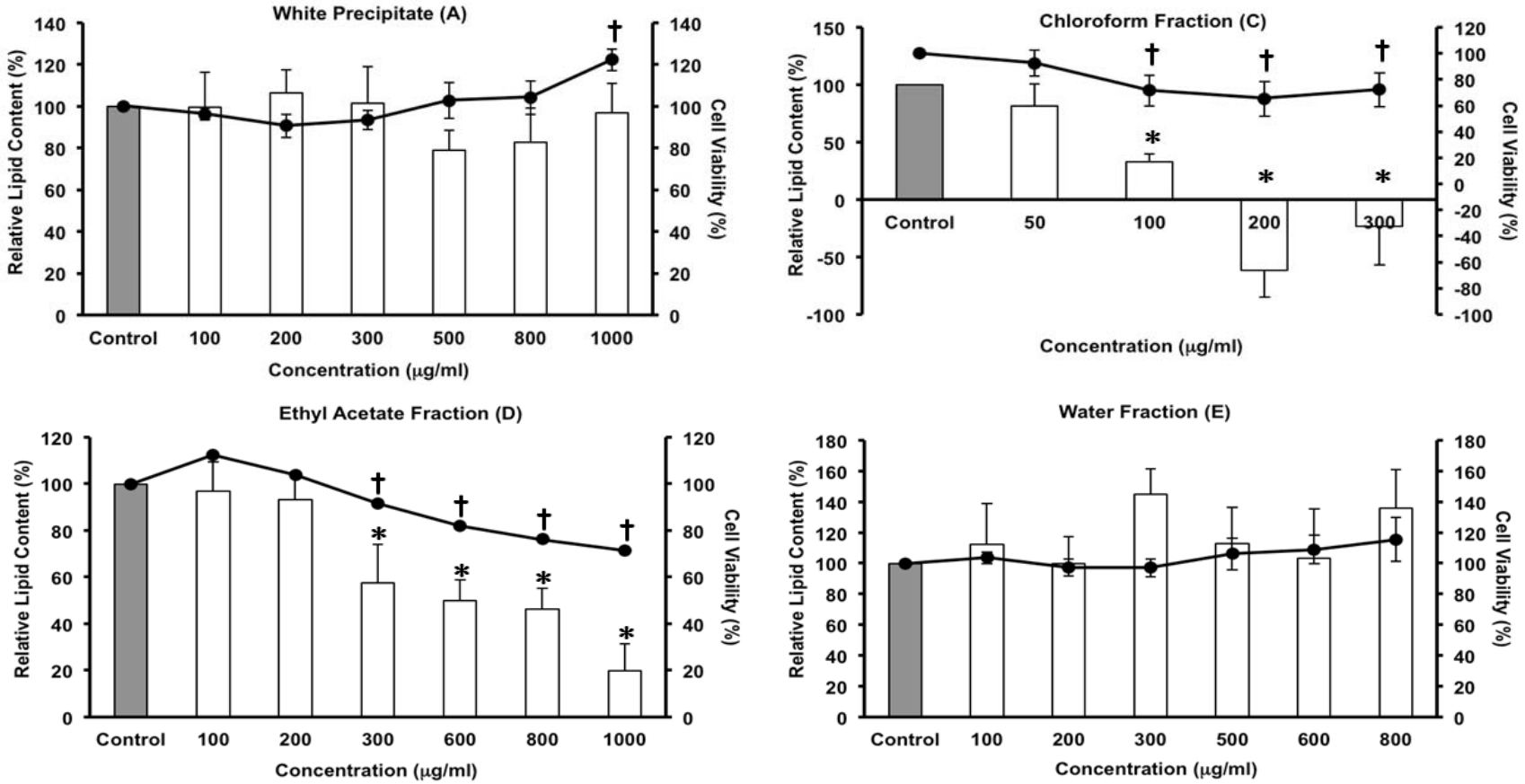

Figure 11 Effects of four different fractions from the root extract on intracellular lipid accumulation and cell viability. Results are a mean value ± S.D. of six-replicate measurements. Bar graphs represent the relative intracellular lipid contents. Asterisks indicate a significant difference at p <0.05 from the untreated control (0 μg/ml). Percentages of cell viability are shown as a line graph. Crosses indicate a significant difference at p <0.05 from the untreated control (0 μg/ml).

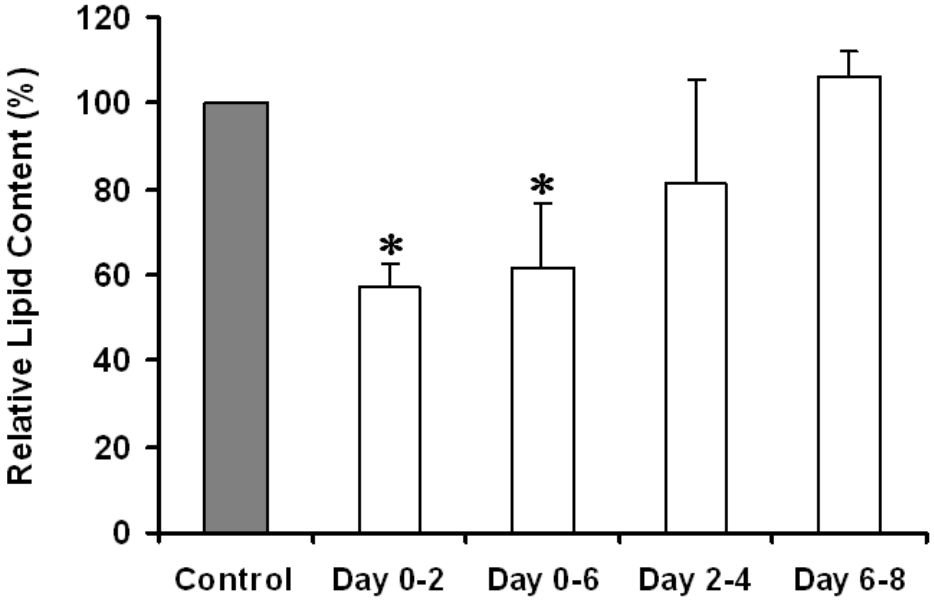

Figure 12 Suppressive effect of IGH on lipid accumulation by various treating times. The relative amounts of accumulated lipid are shown as a mean value ± S.D. of five replicate measurements for each period that the fraction (1,000 μg/ml) was present in the culture medium. Asterisks indicate a significant difference at $p < 0.05$ from the untreated control (0 μg/ml) at Day 8.

4.3 Effect of ivy gourd root extract on adipogenic gene and protein expression

Adipogenesis or the process of fat cell formation in 3T3-L1 cells is known to be sequentially regulated by a network of transcription factors and adipogenesis-related genes (Rosen and MacDougald, 2006). The effects of the root extract on the gene expression of key adipogenesis activators throughout the course of differentiation was examined, both mRNA and protein levels for PPARγ (peroxisome proliferator activated receptor-γ) and C/EBPα (CCAAT/enhancer binding protein-α) was significantly decreased but not C/EBPβ (CCAAT/enhancer binding protein-β) during the early stages as compared with those of the untreated cells (Figure 13, 14). In general, C/EBPβ induces PPARγ and C/EBPα gene expression (Ntambi and Kim, 2000). From the above findings, the PPARγ transcription was not inhibited by ivy gourd root extract through down regulation of the expression of C/EBPβ. As a result of PPARγ inhibition, adiponectin and GLUT4 (glucose transporter-4) expressions were decreased (Figure 13, 14). PPARγ is called the master regulator of adipogenesis. It is important for the promotion and maintenance of the adipocyte phenotype. Typically, PPARγ transcripts and protein levels in the 3T3-L1 cells are elevated within 2 days of the induction period (Day 0-Day 2) and reach their peaks by Day 3-Day 4 (Lee *et al.*, 2009). C/EBPα is also known as a major transcription factor of adipogenesis, which functions mainly during the terminal stages of differentiation (Ntambi and Kim, 2000). PPARγ and C/EBPα coordinately regulate adipocyte-specific gene expression. Their increased levels enhance the mRNA expression of downstream target genes such as FABP4, SCD1, leptin, adiponectin, and GLUT4, leading to the synthesis of several proteins required for intracellular lipid synthesis and storage (Otto and Lane, 2005). GLUT4 and adiponectin are adipogenic markers. They are largely produced in response to the insulin signaling pathway to facilitate cellular uptake of glucose which is ultimately converted into stored lipid. GLUT4 is a transmembrane protein which is necessary for glucose transport into adipocytes (Watson *et al.*, 2004). Adiponectin is not only an extensive marker for differentiated adipocytes but also exerts autocrine effects in these cells. This adipokine promotes adipogenesis by stimulating glucose influx through increased GLUT4 gene expression and increased GLUT4 recruitment to the plasma membrane (Fu *et al.*, 2005). The

suppressed expression of these adipogenesis-promoting genes at both transcriptional and translational levels appears to support our previous findings that the cells treated with the root extract had less intracellular lipid accumulation and fat droplet formation than the untreated controls (Figure 7).

In order to address more in detail the molecular mechanisms underlying the suppression of 3T3-L1 cell differentiation by ivy gourd root, the effects of the hexane fraction on expression of a panel of genes related to lipogenesis during the early stages of adipocyte differentiation was examined. The results are shown in Figure 15. All of the lipogenic genes were determined in this study became over-expressed by the hormonal induction of adipogenesis. In accordance with the root extract treatment (Figure 13), the hexane fraction potently suppressed the up-regulation of PPARγ gene expression in the differentiated cells harvested at Day 2. C/EBPα expression was also decreased after the same treatment. Along with the suppression of PPARγ and C/EBPα, mRNA levels of various adipogenic genes were reduced in the treated cells. FABP4 (fatty acid binding protein-4) is a carrier of fatty acids that plays a supporting role in differentiation of the adipocytes (Shan *et al.*, 2013). The down-regulated expression of this gene by the hexane fraction indicated a decline in the ability of the cells to process and metabolize fatty acids, thereby attenuating the intracellular lipid synthesis. Previously, the root extract did not produce any effect on FABP4 expression in the adipocytes (Figure 13). These conflicting results need to be clarified. It is probable that the extract may contain some chemical components which could counteract such negative effects on the FABP4 gene.

Adipor1 (adiponectin receptor-1) serves as a cell surface receptor for adiponectin. The glucose uptake was mediated by this protein through its binding with adiponectin, thereby promoting lipid synthesis in adipocytes (Rasmussen *et al.*, 2006). A large increase in expression of the Adipor1 gene was observed after the hexane fraction treatment (Figure 15), although the mRNA level of adiponectin then substantially decreased. It may be postulated that a decrease in adiponectin output would accelerate the expression of Adipor1 gene in these cells to enhance their adiponectin binding capacities. In the present study, however, the alteration only at the transcriptional level was found (Figure 15). The expression of the protein needs to be examined in order to confirm this hypothesis. The reason for the activated gene

expression of ACC1 (acetyl-CoA carboxylase-1) in the treated cells is unknown (Figure 15). The most important function of ACC1 is to provide the malonyl-CoA substrate for biosynthesis of fatty acids (Tong, 2005). This lipogenic gene is transcriptionally controlled by SREBP-1c (sterol regulator element-binding protein-1c) which is also a regulator of the PPARγ gene (Shimano, 2001). The significant down-regulation of MEST (mesoderm specific transcript), an adipocyte size marker gene (Takahashi *et al.*, 2005), means that the treated cells became smaller due to their reduced lipid content. In addition, the decreased mRNA level of LPL (lipoprotein lipase) which hydrolyzes triglycerides in lipoprotein particles to provide free fatty acids for intracellular triglycerides synthesis, would further cause reduction in the lipid synthesis and storage of these cells. From these changes in the expression of the lipogenic genes, this result was concluded that the intracellular lipid synthesis in 3T3-L1 adipocytes was suppressed by the hexane fraction by negatively modulating both up-stream and down-stream adipogenic genes in the early differentiation pathway.

Some inhibitory effects of the hexane fraction on the glucose metabolism-regulating genes that participate with lipogenesis in the adipocytes were observed. A down regulation of GLUT4 expression in the treated cells was evident. This observation and those that arose from the extract treatment (Figure 13) thus indicated that if the ivy gourd root had an anti-diabetic property, it would exert a hypoglycemic effect independently of the glucose transporter-GLUT4 system. PDK4 (pyruvate dehydrogenase kinase-4) transcripts, on the other hand, were significantly increased upon the treatment, and would cause inhibition of the pyruvate dehydrogenase complex (Harris *et al.*, 2002). Consequently, conversion of acetyl-CoA from pyruvate was attenuated, and limited its availability for use as a precursor of fatty acids. Surprisingly, SITR1 (sirtuin-1) and IRS1 (insulin receptor substrate-1) which promote cellular glucose metabolism by improving insulin sensitivity (Sun *et al.*, 2007; Gual *et al.*, 2005) were not affected by the treatment. The activation of PDK4 expression in combination with the down-regulation of FAS (fatty acid synthase), the key enzyme of fatty acid synthesis pathway, then added negative effects on lipogenesis in the treated differentiating cells.

Therefore, the overall anti-adipogenic effect of ivy gourd root in the 3T3-L1 cells seems to be primarily due to down-regulation of the expression of the PPARγ

gene early in the differentiation pathway. Our proposed mechanisms as illustrated in Figure 16 include the active element(s) that remain to be identified would initiate the inhibitory effects on the adipocyte differentiation by targeting the PPARγ and also the C/EBPα expression directly, but not through C/EBPβ or SREBP-1c. All the findings from this study imply that the ivy gourd root if applied in vivo would prevent or attenuate rather than reduce obesity by mobilizing stored fat from the adipose tissue.

4.4 Chemical Constituents of IGH

Among phytochemicals of IGH that were separated regarding to their chromatographic behaviors, those reacted with Libermann-Burchard reagent yielding blue-color spots on TLC were found most abundant. Such display of blue coloration signifies the presence of steroids (Oleszek, *et al.*, 2008). From the above results, the major constituents of IGH should be steroids and they were responsible for those observed effects both *in vitro* and *in vivo* was presumed. To date, two distinct steroids have been identified in ivy gourd. The aerial, fruit, and root parts contain β-sitosterol whereas stigmast-7-en-3-one is present in the plant roots only (Niazi *et al.*, 2013). The hypolipidemic and anti-adipogenic activities of β-sitosterol derived from various plants have been demonstrated (Jain *et al.*, 2010; Lu *et al.*, 2010; Yang *et al.*, 2011; Iyer and Patil, 2014; Luyen *et al.*, 2014), but none of stigmast-7-en-3-one has yet been reported. Hence, those observed anti-obesity effects of IGH may be due to the β-sitosterol content.

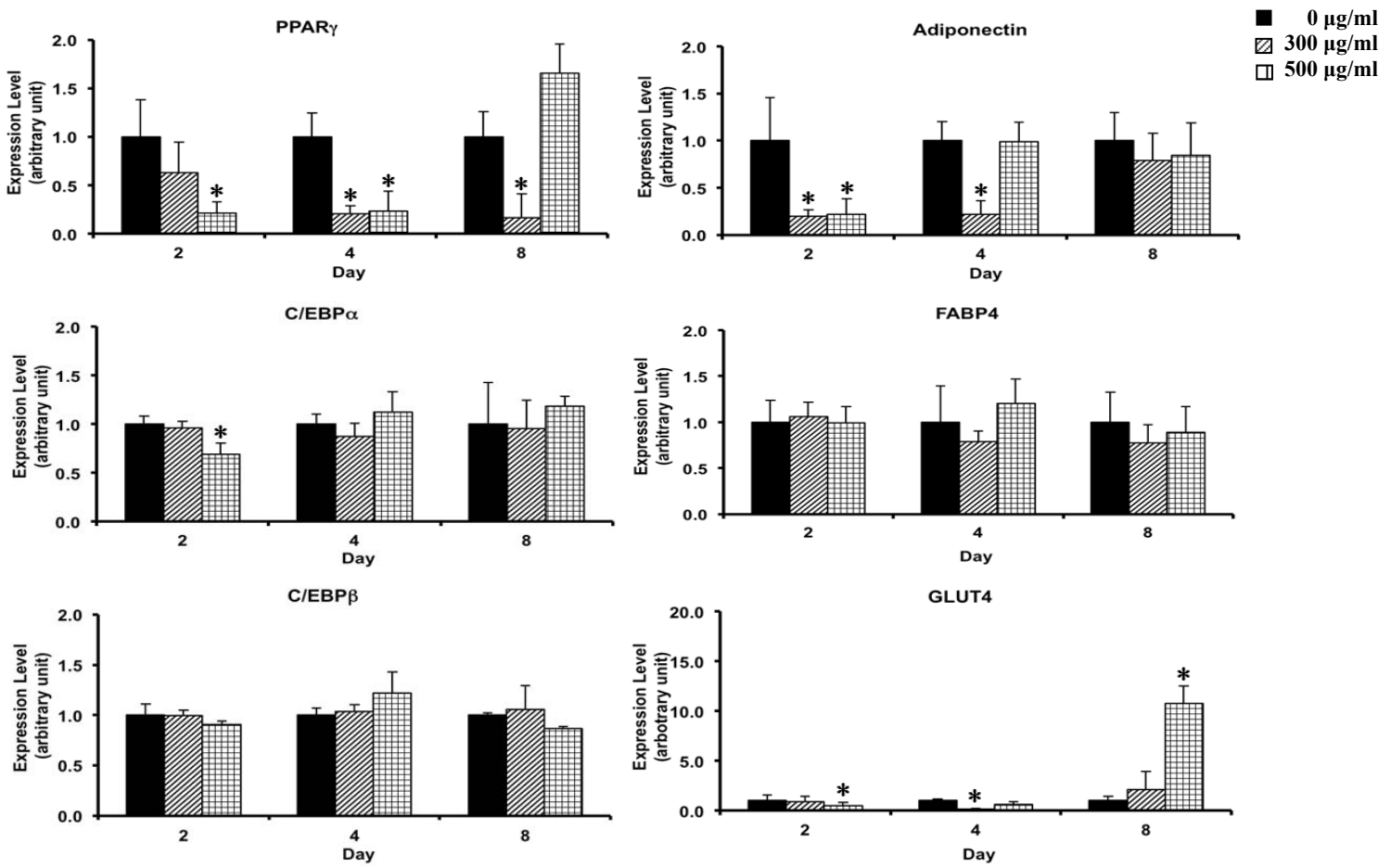

Figure 13 Effects of the root extract on mRNA expressions of adipogenesis-related genes in 3T3-L1 cells. The relative expression levels of interested genes were estimated in terms of mRNA/GAPDH ratio. Values are shown as mean ± SD of three-replicate experiments for each treatment. Asterisks indicate significant difference at $p < 0.05$ from the untreated control (0 μg/ml) at the same time point.

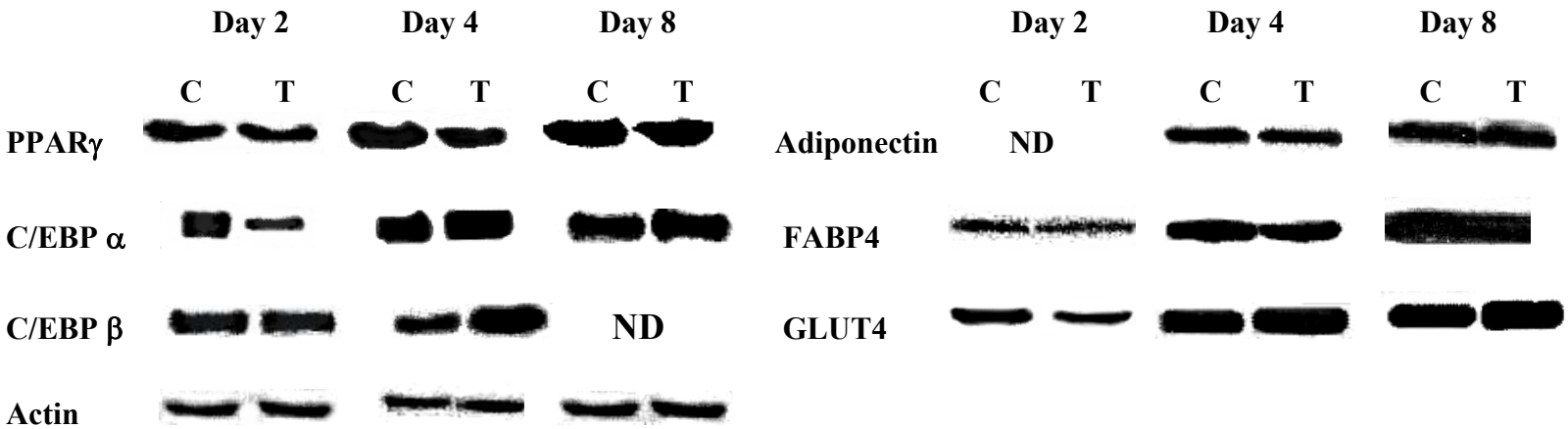

Figure 14 Effects of the root extract on protein expressions of adipogenesis-related genes in 3T3-L1 cells. The relative expression levels of interested genes were estimated in terms of mRNA/GAPDH ratio. Values are shown as mean ± SD of three-replicate experiments for each treatment. Asterisks indicate significant difference at $p < 0.05$ from the untreated control (0 μg/ml) at the same time point.

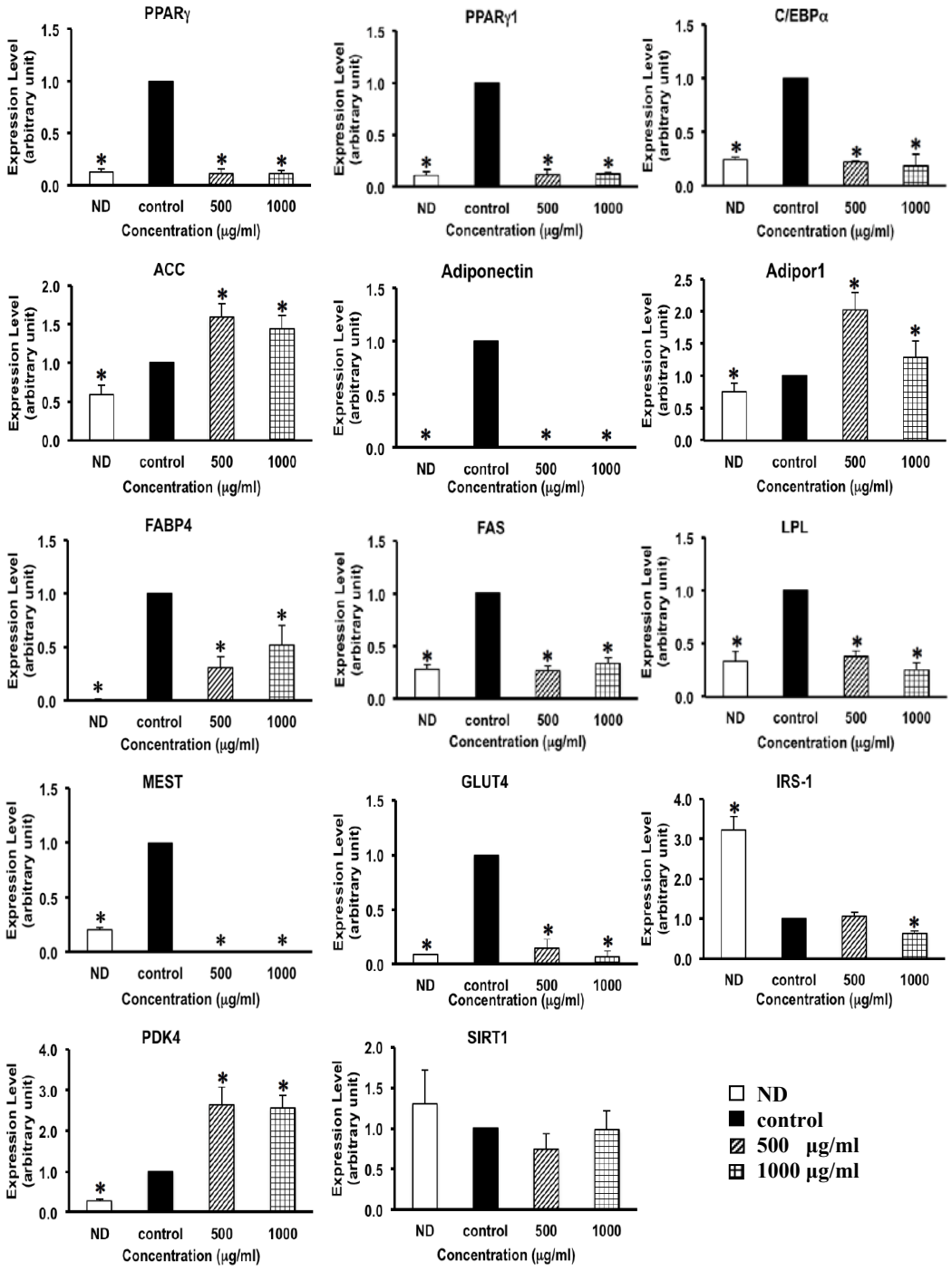

Figure 15 Effects of hexane fraction expression of adipogenesis-related genes in 3T3-L1 cells. The cells were harvested on day 2. The relative expression levels of interested genes were estimated in terms of mRNA/actin ratio. Values are shown as mean ± SD of three-replicate experiments for each treatment. Asterisks indicate significant difference at $p < 0.05$ from the untreated control (0 μg/ml). (ND = non-differentiated 3T3-L1 cells).

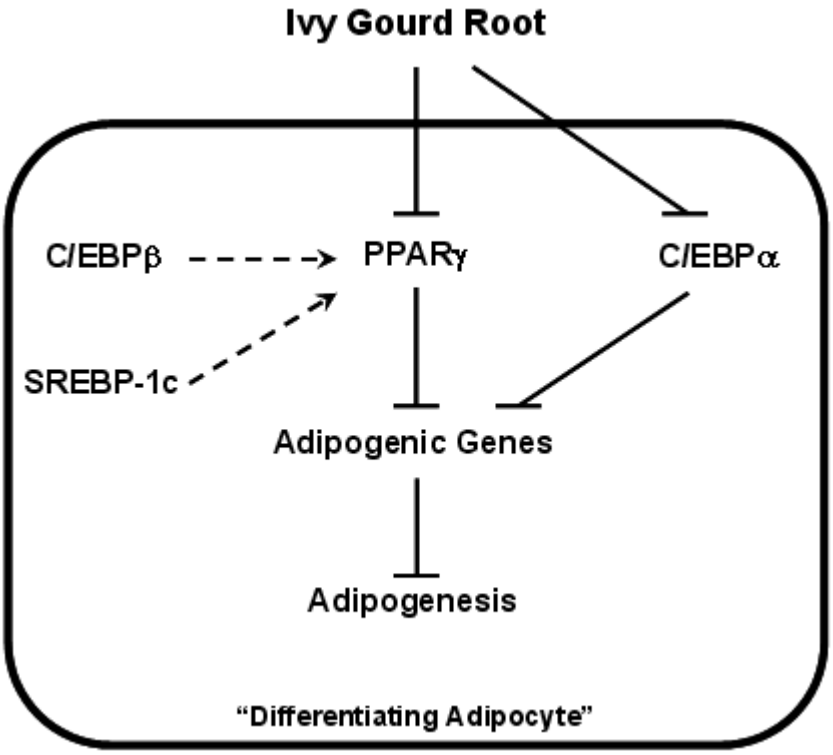

Figure 16 Proposed molecular mechanisms for the inhibition of adipogenesis by ivy gourd root. The active constituent(s) in ivy gourd root when introduced to 3T3-L1 cells during the induction of differentiation blocks PPARγ and C/EBPα expressions directly leading to a negative regulation of various adipogenic genes, and eventually inhibits adipogenesis in these cells.

CHAPTER 3

Effects of Hexane Fraction of Ivy Gourd Root in Mice Fed a High-Fat Diet

1. Introduction

In previous study, the hexane fraction of ivy gourd root extract (IGH) showed anti-obesity activity by potently suppressing adipocyte differentiation in 3T3-L1 cells, primarily through down-regulating PPARγ gene expression (Bunkrongcheap, *et al.*, 2014). To address in more detail and to ascertain the ability of ivy gourd root to counteract obesity, the effects of IGH were then examined *in vivo*.

Among animal models used in obesity study, high-fat diet induced model is frequently used because it is less expensive and easier to handle in comparison to gene-knockout models. Even though the contribution of diets with a high-fat content to human adiposity is disputed, it is clear that the exposure of animals to high-fat diet often results in the development of obesity (Willett and Leibel, 2002). Some strains of rodent prone to develop diet-induce obesity rapidly. Among them is the C57BL/6 mouse which is the most well-known inbred mouse strain derived from the original C57BL strain.

Figure 17 The C57BL/6 mouse (http://jaxmice.jax.org/strain/000664.html)

The C57BL/6 mice, also called "C57 black 6" or simply "Black 6" (Figure 17) has the advantage of strain stability and easy breeding. It has been widely used as a genetic background for congenic and mutant mice. A number of substrains have

been derived with some genetic variations (Mekada *et al*., 2008). Above all, C57BL/6J of the Jackson Laboratory and C57BL/6N of the National Institutes of Health (NIH) are the core substrains. Commercially available C57BL/6J mice are used in a wide variety of research areas including cardiovascular biology, developmental biology, diabetes and obesity, genetics, immunology, neurobiology, and sensorineural research. This animal will develop severe obesity, hyperglycemia and hyperinsulinemia when weaned onto high-fat diets, but will remain lean and euglycemic if the fat content of the diet is limited (Surwit *et al.*, 1995). The mouse of C57BL/6J strain was chosen in this study because it is particularly susceptible to the effects of dietary fat. When subjected to high dietary fat, these animals exhibit the obese phenotypes resemble to those found in humans (Lin *et al.*, 2000).

2. Materials

2.1 Instruments

Instruments	Model	Manufacturer
Analytical balance 2 digits	SE 2020	Ohaus
Analytical balance 4 digits	BP110S	Sartorius
Autoclave	HA-300M	Hirayama
Homogenizer	395 MultiPro	Dremel
Microplate reader	Infinite M200PRO	Tecan
Nanodrop spectrometer	Nanodrop 1000	Thermo Scientific
Real-time RT-PCR	Step One Plus™	Applied Biosystems
Rotary evaporator	BUCHI 713	Buchi
Spectrophotometer	UV mini-1240	Shimadzu
Ultra centrifuge	CP85P	Hitachi

2.2 Materials and Chemicals

Chemical	Manufacturer
Adiponectin (Mouse) total, HMW elisa	ALPCO™
AST and ALT assay kit	Wako
β-Hydroxybutyrate	Abcam
Ethanol, AR grade	Lab-Scan
Glucose assay kit	Wako
Insilin Elisa kit	Morinaga
Non esterified free fatty acid	Wako
QIAzol Lysis Reagent	Qiagen
RNeasy mini kit	Qiagen
Triglyceride assay kit	Wako
Total cholesterol assay kit	Wako

3 Methods

3.1 Sample preparation

The fresh roots of ivy gourd (*Coccinia grandis* L. Voigt) were collected from Phang-nga Province, Thailand, in September 2013. They were washed, dried at 40°C in a hot-air oven, and then ground with a blending machine. The ground material was extracted with ethanol. The resulting extract was prepared for IGH by sequential solvent partitioning procedure as described previously (Bunkrongcheap *et al.*, 2014). The sample obtained was kept dehumidified and away from light at room temperature. Its anti-adipogenic activity was confirmed in 3T3-L1 cells prior to be used in the *in vivo* study.

3.2 Animal experiment

Four-week-old male C57BL/6J mice weighting between 17.50 ± 0.5 g, were purchased from Japan SLC, Inc (Shizuoka, Japan). The mice were maintained on commercial chow for 7 days for acclimatization. After that period, the mice were randomly divided into 2 groups, kept in plastic cages (one mouse per cage) in a room controlling temperature 24 ± 2 °C, light-controlled room (12-h light/dark cycle) and were allowed free access to water. The day before the treatment animals, the mice were fasting and were measured for their body weight (BW) in the morning of the experiment. In the experiment, the 6 mice were fed control high fat diet (HFD) prepared based on AIN-76 purified diet formula (The American Institute of Nutrition, USA) for 4 weeks and orally administrated with water as vehicle, and another 6 mice were fed the same diet supplemented with 2% IGH (w/w) and free access to water for 4 weeks. Each mouse was weighed weekly and its daily food intake was measured at the same time of the day. On the midnight of the end of experiment, the animals were all fast 12 h and their body weight were measured on next day at 11.00 am.

3.2.1 Tissue and blood samples collection

On the end of experiment, the mice were sacrificed after 12 h of fasting. Mice were injected with pentobarbital sodium 100 µl/10 g BW and they run around a plastic cage until they became loopy and clumsy and finally their sleep. Blood sample were collected by centrifuged at 2,000 xg 10 min 2 times to obtain

serum sample. Liver tissue and white adipose tissue (WAT) were washed with PBS, weights of the liver tissue and WAT were measured immediately after collection. The tissue sample were collected in plastic zip lock bags, and stored at -80^{o}C. All experimental protocols were ethically approved under the rules and regulations of the Animal Welfare Center at University of the Ryukyus, Okinawa, Japan.

3.2.2 Lipid concentrations and biochemical parameters in the serum and liver

(1) Serum total cholesterol (TC) and triglyceride (TG) assay

The TC or TG assay kit (Wako) 750 µl was added into 1.5 ml of microcentrifuge tube and the diluted standards or samples 5 µl were added into the same tube and vortex. The tubes were incubated at 37°C 5 min. Supernatant 150 µl were transferred into 96-well microtiter plate and measured at 600 nm and reference 700 nm. The TC or TG concentrations were calculated by using standard curve.

(2) Serum glucose assay

The glucose assay kit (Wako) 750 µl was added into 1.5 ml of microcentrifuge tube and the diluted cholesterol standards or samples 5 µl were added into the same tube and vortex. The tubes were incubated at 37°C 5 min. The 150 µl of supernatant were transferred into 96-well microtiter plate and measured at 505 nm and reference 600 nm. The total cholesterol concentrations were calculated by using standard curve.

(3) Serum insulin assay

5 µl sample or working mouse insulin standard (Morinaga) per well was mixed with 95 µl of sample diluent and incubated the microplate for 2 h at 4°C. The wells were washed five times with washing buffer. Each well was added 100 µl anti-insulin enzyme conjugate per well and incubated for 30 min at room temperature. Each well was washed seven times with washing buffer and 100 µl enzyme substrate solution was added to each well. The plate was incubated at room temperature while avoiding exposure to light for 40 min. The reaction was stopped by adding 100 µl stop solution and the absorbances at 450 and 630 nm (reference)

were measured within 30 min. The insulin concentrations were calculated by using standard curve.

(4) Serum alanine aminotransferase (ALT) or aspartate amino-transferase (AST) assay

The mixer of color reagent and enzyme solution (1:1) 200 µl was added into microcentrifuge tube. The tube was incubated 37°C more than 5 min after that standard (4 µl; 100 karmen unit and 8 µl; 200 karmen unit) or sample (4 µl) was added into the tube and incubated 37°C for 20 min. The reaction was stopped by using 400 µl stop solution and measured at 555 nm. The ALT or AST concentrations were calculated by comparing with standard (Wako) and enzyme activity in IU/l unit was calculated by

Karmen unit x 0.482 = IU/l

(5) Total and high molecular weight adiponectin immunoassays

For total adionectin (ALPCO™ elisa kit), 10 µl serum was added to 100 µl of the protease buffer and 700 µl sample pretreatment buffer and stirred thoroughly. For high molecular weight adiponectin, 10 µl serum was added to 100 µl prepared protease solution and incubated for 20 min at 37°C before adding 700 µl sample pretreatment buffer and stirring thoroughly. All total and high molecular weight adiponectin were diluted with dilution buffer (1:81). The working standard and diluted samples 50 µl were added into each test well and incubated the plate for 60 min at room temperature. The solution in well was removed and washed with 350 µl of washing buffer 3 times. Biotin conjucated-PoAb (50 µl) was added to each well and the plate was incubated for 60 min at room temperature before washing three-times again. 50 µl enzyme-labeled streptavidin was added to each washed well before incubating for 30 min at room temperature and washing 3 times. The working substrate solution (50 µl) was added to each wash well and incubated for 10 min. Then, 50 µl stop reagent was added to each test well. The absorbance of each well was measured at a wavelength of 492 nm, and a reference wavelength of 600 nm.

(6) β-Hydroxybutyrate assay

The β-Hydroxybutyrate was determined by using β-hydroxybutyrate assay kit (Abcam). Serum sample 5 µl and 45 µl β-hydroxybutyrate buffer were added to 384 flat black plates (Corning). Then, 50 µl reaction mixer assay kit was added to each well containing β-hydroxybutyrate standard or samples. The plate was incubated at room temperature for 30 min and protected from light. The absorbance of each well was measured at a wavelength of 450 nm compared to β-hydroxybutyrate standard curve.

(7) Non esterified free fatty acid (NEFA) assay

Free fatty acid in serum was determined using NEFA assay kit (Wako). The sample or standard 10 µl was mixed with reagent A (200 µl) and incubated at 37°C 10 min. The solution was added with reagent B (400 µl) and incubated at 37°C 10 min. The absorbance at 550 nm wavelength and the free fatty acid concentration was determined by referring to the standard curve.

3.2.3 Lipid extraction from liver

Liver tissues (0.1 g) were homogenized in 1 ml methanol and transferred into a new glass tube. The homogenizer was washed 2 times with 1 ml methanol and the solution was transferred to the same glass tube. 6 ml chloroform were added to the tube and mixed with vortex. The homogenates were incubated at 40°C for 60 min. The glass tubes were stand for 15 min at room temperature and subsequently centrifuged at 1,500 rpm for 10 min. The supernatant (8 ml) was transferred into new glass tube and 1.6 ml distilled water was added and mixed vigorously. The mixtures were centrifuged at 1,500 rpm for 10 min. The lower phase was evaporated and weight. 10% Triton X-100 in 2-propanol (2 ml) was added to the tube and used for determining TG and TC.

3.2.4 Lipid extraction from feces

Feces from the last 3 days of the feeding were completely dried and ground into powder. The TC and TG contents in the fecal samples were extracted by the method of Folch *et al*. (1957). 5 ml of chloroform: methanol (ratio

2:1) was added into 72 mg of feces powder in a glass tube. The tubes were incubated at 40°C for 60 min, followed by on incubation at room temperature for 15 minutes. After centrifuged at 1,500 rpm for 10 min, 4 ml supernatant was transferred into new glass tube and 800 µl distilled water was added and mixed vigorously. The mixtures were centrifuged again at 1,500 rpm for 10 min. The lower phase was collected, evaporated and weighed. 10% Triton X-100 in 250 µl 2-propanol was added into the tube. TG and TC levels were subsequently determined as described previously.

3.2.5 RNA extraction from liver

50 mg liver was ground and homogenized in 1 ml QIAzol Lysis Reagent (Qiagen). The homogenates were incubated at room temperature for 5 min and 200 µl chloroform were added and shaken vigorously for 15 sec. The samples were incubated at room temperature for 2-3 min and centrifuged at 12,000 xg for 15 min at 4°C. The upper phase was transferred to a new tube and 400 µl 70% ethanol was added and vortexed. Sample was transferred to RNeasy column in 2 ml tube, centrifuged for 15 sec at 12,000 xg and flow-through was discarded. 700 µl RW1 buffer was added to RNeasy column, centrifuged for 15 sec at 12,000 xg and flow-through was discarded. 500 µl RPE buffer was added to RNeasy column and centrifuged for 15 sec at 12,000 xg. The flow-through was discarded and washed with 500 µl RPE buffer. RNeasy column was placed in 2 ml tube and centrifuged at 12,000 xg for 1 min. RNeasy column were placed with a new 1.5 ml tube. 50 µl RNase-free water was added to the column and centrifuged for 1 min at 12,000 xg. 30 µl RNase-free water was added into the same column and centrifuged for 1 min at 12,000 xg. RNA was measured at A260/280 with Nanodrop spectrophotometer.

3.2.6 Hepatic enzyme activities

To compare the hepatic enzyme activities in experiment, livers were homogenized with a buffer, containing 10 mM Tris-HCl (pH 7.4), 1 mM EDTA and 0.25 M sucrose. Liver homogenate was then subjected to centrifugation at 700xg for 10 min at 4°C. The supernatant was collected and centrifuged at 10,000 xg for 10 min at 4°C. The pellet was obtained as the mitochondrial fraction whereas the cytosolic fraction was prepared from the resulting supernatant by ultracentrifugation

at 125,000 xg for 60 min at 4°C (Shirouchi *et al*., 2007). The protein concentration of each fraction was determined by the method of Lowry *et al*. (1951). The cytosolic activities of fatty acid synthase (FAS), glucose-6-phosphate dehydrogenase (G6PDH) and malic enzyme (ME), and the mitochondrial activity of carnitine palmitoyltransferase (CPT) were measured according to the methods described previously (Kelley *et al*., 1986; Kelley and Kletzien, 1984; Ochoa, 1995; Markwell *et al*., 1973).

3.2.7 RNA Analysis

Total RNA was extracted from livers and epididymal white adipose tissue (WAT) (50 mg each) using RNeasy mini kit (Qiagen, Germany). First strand cDNA was synthesized with 2 μg of total RNA as a template using a High Capacity RNA-to-cDNA kit (Applied Biosystems, USA). The quantitative reverse transcriptase-PCR (qRT-PCR) was performed on Step One Plus™ Real-Time PCR System (Applied Biosystems, USA) with the following conditions: one cycle of 95°C for 20 sec, 40 cycles of 95°C for 3 sec and 60°C for 30 sec. A melting curve analysis was performed starting at 95°C for 15 sec, 60°C for 60 sec and increasing by 0.3°C every 15 sec to determine primer specificity. Specific primers are listed in Table 3. The mRNA levels of all genes of interest were normalized using 18S rRNA and β-actin as the internal controls for liver and WAT tissues, respectively.

3.3 Statistical analysis

The data are expressed as mean ± S.E. Differences between the means of individual groups were assessed with Student's t-test. Probability values of $p < 0.05$ were considered to be significant.

Table 3 Primer sequences used for Quantitative Real-Time PCR

Gene	Forward sequence Reverse sequence
ABCG5	5’-TCTCCGCGTCCAGAACAAC-3’
	5’-CATTGAGCATGCCGGTGTAT-3’
ABCG8	5’-CCCTCCGATTGCTTCTTTCAG-3’
	5’-CTGAGAAATGCCCCCAGATAAA-3’
Actin	5’-CAGAAGGAGATTACTGCTCTGGCT-3’
	5’-GGAGCCACCGATCCACACA-3’
ACC	5’-GGACCACTGCATGGAATGTTAA-3’
	5’-TGAGTGACTGCCGAAACATCTC-3’
ACL	5’-GCCAGCGGGAGCACATC-3’
	5’-CTTTGCAGGTGCCACTTCATC-3’
AOX	5’-TCAACAGCCCAACTGTGACTTCCATCA-3’
	5’-TCAGGTAGCCATTATCCATCTCTTCA-3’
C/EBPα	5’-TGGACAAGAACAGCAACGAGTAC-3’
	5’-GCAGTTGCCCATGGCCTTGAC-3’
CYP7A1	5’-CTGTGTTCACTTTCTGAAGCCATG-3’
	5’-CCCAGGCATTGCTCTTTGAT-3’
CYP8B1	5’-TTCGACTTCAAGCTGGTCGA-3’
	5’-CAAAGCCCCAGCGCCT-3’
FAS	5’- TGCTCCCAGCTGCAGGC -3’
	5’-GCCCGGTAGCTCTGGGTGTA-3’
GPAT	5’-CAACACCATCCCCGACATC-3’
	5’-GTGACCTTCGATTATGCGATCA-3’

ABCG5, ATP-binding cassette transporter G5; ABCG8, ATP-binding cassette transporter G8; ACC, acetyl-CoA carboxylase; ACL, ATP Citrate Lyase; AOX, acyl-CoA oxidase; C/EBPα, CCAAT/enhancer binding proteins transcription factor-α. CPT1α, carnitine palmitoytransferase 1 alpha; CYP7A1, cholesterol 7α-hydroxylase CYP8B1, Cytochrome P450, Family 8, Subfamily B, Polypeptide 1; FAS. Fatty acid synthase; GPAT, Glycerol Phosphate Acyltransferase.

Table 3 Primer sequences used for quantitative Real-Time PCR (continued)

Gene	Forward sequence Reverse sequence
HMGR	5’-AAGGTGGTGAGAGAGGTGTTAAAG-3’
	5’-AATACAGTTTGAACTCCCCACATT-3’
HSL	5’-GGTGACACTCGCAGAAGACAATA-3’
	5’-GCCGCCGTGCTGTCTCT-3’
LDLR	5’-ATCTCTCACCTCCCAGTACTCCTA-3’
	5’-AGAAGATGGACAGGAACCTCATAC-3’
MEST	5’-GTTTTTCACCTACAAAGGCCTACG-3’
	5’-CACACCGACAGAATCTTGGTAGAA-3’
PPARγ	5’-AGGCCGAGAAGGAGAAGCTGTTG-3’
	5’-TGGCCACCTCTTTGCTGTGCTC-3’
PPARγ1	5’-AAGATTTGAAAGAAGCGGTGAAC-3’
	5’-CAATGGCCATGAGGGAGTTAG-3’
SCD1	5’-CCGGAGACCCCTTAGATCGA-3’
	5’-TAGCCTGTAAAAGATTTCTGCAAACC-3’
SREBP-1c	5’-GGAGCCATGGATTGCACATT-3’
	5’-GCTTCCAGAGAGGAGGCCAG-3’
18s rRNA	5’-CGGACAGGATTGACAGATTG-3’
	5’-CAAATCGCTCCACCAACTAA-3’

HMGR, HMG-CoA reductase; HSL, hormone sensitive lipase; LDLR, Low-density lipoprotein recepter; MEST, mesoderm specific transcript; PPARγ, peroxisome proliferator activated receptor gamma; PPARγ1, peroxisome proliferator activated receptor gamma1 SCD1, steroyl-CoA desaturase 1; SREBP-1c, Sterol regulatory element-binding protein-1c.

4. Results and Discussion

4.1 Effects of IGH on Growth, Liver and Fat Tissues of Diet-Induced Obese C57BL/6 Mice

Throughout the course of this study, consumption of IGH did not cause mortality or toxic symptoms in the mice. The behavior or physical appearances among them were not observed in this study. In all cases, their feces were dry and dark indicating no abnormalities in their digestive tracts. Feeding IGH to the mice did not produce any significant effects on their food intake, growth, liver and fat tissues as shown in Table 4. Among the four different fat tissues, perirenal WAT and epididymal WAT seemed to have decreased weights in response to IGH treatment, but their values were not statistically different from those of the controls (Table 4). A crucial role of PPARγ in the development of HFD-induced obesity in mice has been demonstrated (Jones, *et al.*, 2005). Also, previous study found that IGH potently suppresses adipogenesis in differentiating 3T3-L1 cells by down-regulating PPARγ gene expression (Bunkrongcheap *et al.*, 2014). In this study, the results of adiposity-related mRNA analyses revealed a strong inhibition of gene encoding PPARγ1 in epididymal WAT of the mice given IGH (Figure 18), whereas the total PPARγ gene and its associated genes in the same tissue were not obviously affected (Figure 18). In murine WAT, PPARγ appears as PPARγ1 and PPARγ2 isoforms arisen from two distinct mRNA species, both of which are transcribed from a single PPARγ gene as a result of alternative promoter use and different splicing (Zhu *et al.*, 1995). The PPARγ1 gene has a promoter region containing C+G nucleotide-rich sequence which is lacking in that of PPARγ2 gene and this difference has been suggested to give rise to the functional diversity between both PPARγ variants (Zhu *et al.*, 1995). Although PPARγ1 is expressed at a higher degree than PPARγ2 in adipose tissue, it is less significant in promoting adipogenesis (Werman *et al.*, 1997). Because of this, non-significantly decreased epididymal fat mass found in the treatment group was necessarily a consequence of IGH-dependent inhibition of PPARγ1 gene expression. In contrast to WAT, brown adipose tissue (BAT) proportionately increases as the body mass drops (Saely *et al.*, 2012). Despite no difference in weight gain between

the treatment and control groups, a slight increase in BAT among the treated animals was noted in this study (Table 4). Based on the above findings, it is likely that an ability of IGH to prevent deposition of WAT in the HFD-induced obese mice remains to be confirmed. In this context, the IGH would work in the circumstances occurring in a body in ways analogous from those *in vitro* was presumed. Since the single dose was tested in this study, it is possible that such ineffectiveness may be attributable to a low dosage and/or low bioavailability of the potential active components in IGH. Further investigations are therefore needed to find an explanation for the observed discrepancies between our *in vitro* and *in vivo* results.

Table 4 Effect of IGH on growth parameters, liver, and adipose tissue weights.

Parameter	**Control Group**	**Treatment Group**
Food intake (g/day)	2.81 ± 0.05	2.83 ± 0.03
Initial body weight (g)	17.48 ± 0.19	17.46 ± 0.19
Final body weight (g)	26.02 ± 0.77	25.92 ± 0.22
Liver weight (g/100g BW)	3.12 ± 0.11	3.44 ± 0.11
Subcutaneous fat (g/100g BW)	1.30 ± 0.16	1.32 ± 0.11
Omental WAT (g/100g BW)	1.76 ± 0.09	1.88 ± 0.09
Perirenal WAT (g/100g BW)	1.31 ± 0.18	1.17 ± 0.13
Epididymal WAT (g/100g BW)	2.87 ± 0.32	2.77 ± 0.17
BAT (g/100g BW)	0.32 ± 0.06	0.41 ± 0.04

Values are mean ± S.E. of six mice. BW = body weight

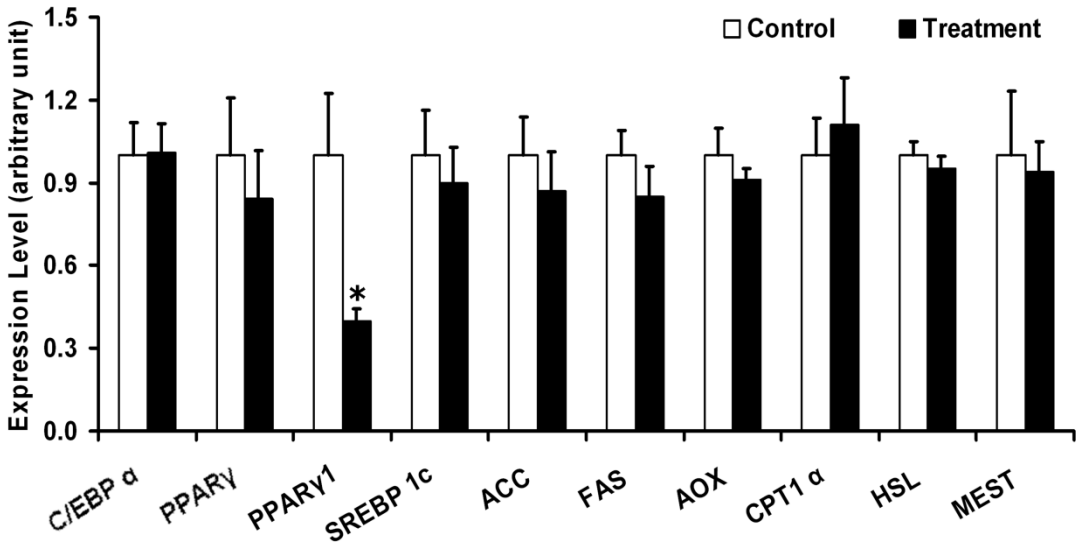

Figure 18 Effects of IGH administration on mRNA level of lipid metabolism-related genes in epididymal adipose tissue. C57BL/6J mice were fed HFD (Control) or HFD with 2% IGH (Treatment) for 4 weeks. Values are mean ± S.E. of six mice. Asterisk shows significant difference between the control group and the treatment group by Student's t-test at * p <0.05.

4.2 Effects of IGH on Serum and Liver Lipid Profiles

The most important finding of this study was that serum and liver TG levels in the HFD-fed mice receiving IGH were pronouncedly decreased (Table 7). As circulating TG molecules in the fasting state are mainly produced from liver (Mason, 1998), the decreased serum TG concentrations found among the treated animals might reflect a reduction in their hepatic TG synthesis was speculated. A decrease in lipid synthesis in the liver became evident the activity of major lipogenesis-related enzymes including FAS, G6PDH, and ME was markedly decreased upon IGH administration was found (Figure 19). In mammalian fatty acid synthesis, FAS catalyzes condensation of acetyl CoA and malonyl CoA into palmitate in the presence of NADPH which is supplied by pentose phosphate pathway and cytosolic conversion of malate to pyruvate (Wakil *et al.*, 1983). The inhibition of FAS activity therefore would cause less consumption of NADPH leading to its lower requirement, followed by a decrease in both NADPH-providing pathways as indicated by the reduced G6PDH and ME activities in our experiments (Figure 19). However, the measurement of hepatic-FAS mRNA level in the treatment group revealed that FAS gene expression was not modulated. As compared to the untreated control value, it was not statistically different ($p > 0.05$) (Figure 20). These observations suggest that the inhibitory effect on hepatic FAS activity seen in Figure 19 might be arisen from interaction between IGH-derived FAS inhibitor which is yet to be identified and the enzyme protein rather than signaling of IGH constituent(s) or G6PDH and ME inhibition. In this study, however, the inhibition of the above two enzymes was detected only at the protein level and thus their transcriptional levels need to be examined in order to confirm our hypothesis. The activity of CPT1, the rate-limiting enzyme in long-chain fatty acid (LCFA) oxidation, was also significantly decreased but to a lesser extent (Figure 19). Malonyl CoA acts as a physiological inhibitor of CPT1. This study found that the hepatic mRNA expression of acetyl carboxylase (ACC), the key enzyme responsible for malonyl CoA formation, was not altered (Figure 20). So, the inhibition of FAS mediated by IGH treatment would cause an accumulation of malonyl CoA and a subsequent decrease in CPT1 activity (Bonnefont *et al.*, 2004). This inhibition probably occur at the protein level because the mRNA expression of CPT1α, which is the primary isoform in liver (Bonnefont *et*

al., 2004), was not down-regulated (Figure 20). The inhibition of CPT1, caused a depress on of fatty acid oxidation and fatty acid oxidation became depressed resulting in a subsequent reduction of the end product, acetyl CoA. In general, acetyl CoA generated by mitochondrial fatty acid oxidation enters TCA cycle to produce ATP but in the liver some is converted to ketone bodies (Rosiers *et al.*, 1991). Such decrease in its level could attenuate the ketogenesis process as reflected by a lowering of serum β-hydroxybutyrate level (Table 5). Serum NEFA concentration in the treatment was also reduced (Table 5), which means that there was less mobilization of fat from its depots. As a result, fatty acid flux to the liver became fallen leading to a decrease in the LCFA substrate for β-oxidation pathway. Although serum NEFA is not a major source of hepatic TG synthesis, its reduction might probably affect fatty acid esterification process in the liver tissue. These changes explained the decrease in serum and hepatic TG observed in the treated mice (Table 5).

Along with decreased hepatic lipid accumulation, the serum levels of AST and ALT tended to be lower than the controls (Table 5). Feeding of HFD to C57BL/6 mice is known to induce hepatic steatosis or fatty liver (DeAngelis *et al.*, 2005). Due to liver cell damage, the activities of serum marker enzymes such as AST and ALT are elevated in this pathological abnormality (Amacher, 1998). Decreased activity of both serum aminotransferases in the treatment group (Table 5) thus gave an indication that IGH alleviated HFD-induced fatty liver in the mice at least through its hepatic TG lowering effect, and also IGH showed no adverse effects on their liver functions.

In this study, we also detected a hypoglycemic tendency in the treated mice (Table 5), though there was no difference in serum insulin levels between the treatment and control groups (Table 5). These findings indicate that IGH would probably increase insulin sensitivity among insulin-sensitive tissues, leading to stimulation in glucose utilization and/or depression of its synthesis. In this regard, the results from various studies have shown that ivy gourd possesses insulin-mimetic properties. For example, the leaf preparations depress glycogen synthesis and gluconeogenesis, as well as enhance glucose oxidation in diabetic subjects (Kumar *et al.*, 1993; Shibib *et al.*, 1993; Kamble *et al.*, 1998; Venkateswaran and Pari, 2002). The water extract from ivy gourd stem stimulates glucose uptake in myotubes by increasing glucose transporter-1 (GLUT1) protein through a direct activation of

GLUT1 promoter at the transcriptional initiation site (Graidist and Purintrapiban, 2009).

Both TG and TC were increased significantly in feces of the treated animals, accompanying with their decreased amounts in the liver (Table 5). The difference of fecal TC levels, however, was considerably greater than that of TG. When we examined the mRNA expression of key hepatic enzymes involved in cholesterol metabolism, none of them was modulated (Figure 20). These results imply that a decrease in fatty acid metabolism giving rise to lower acetyl CoA substrate abundance for TC synthesis, might not be involved in the reduced TC level in the liver. It is likely therefore that feeding of IGH could enhance liver disposal of TC, primarily by stimulating its excretion with bile.

These results demonstrated that some ingredients, presumably steroids, present in IGH had an ability to improve HFD-diet induced obesity by lowering serum and liver lipid levels through a mode of action as illustrated (Figure 21). However, more work is warranted in order to ascertain its efficiency and to identify the anti-obesity agent in this plant root.

The finding that IGH could exert its inhibitory effect on FAS activity *in vivo* is interesting. This key enzyme of fatty acid biosynthesis has received much attention as an emerging therapeutic target not only for obesity and related diseases but also for cancers (Tian, *et al.*, 2011).

Table 5 Effect of IGH on serum levels of liver marker enzymes and lipid metabolism-related parameters, and liver and feces lipid profiles.

Parameter	Control Group	Treatment Group
Serum		
AST (IU/l)	71.83 ± 15.28	49.94 ± 8.68
ALT (IU/l)	6.31 ± 2.07	5.13 ± 1.01
TC (mg/dl)	103.96 ± 13.69	96.54 ± 12.04
TG (mg/dl)	60.67 ± 2.36	46.50 ± 4.02*
Glucose (mg/dl)	212.51 ± 21.49	152.71 ± 27.12
NEFA (mEq/l)	1.63 ± 0.03	1.39 ± 0.10*
β-hydroxybutyrate (nmol/μl)	0.46 ± 0.06	0.37 ± 0.06
Total adiponectin (μg/ml)	9.47 ± 0.18	9.28 ± 0.13
HMW adiponectin (μg/ml)	3.70 ± 0.46	2.60 ± 0.30
Insulin (ng/dl)	1.02 ± 0.48	1.07 ± 0.30
Liver		
TC (mg/g liver)	7.69 ± 0.22	6.03 ± 0.35*
TG (mg/g liver)	93.77 ± 10.01	45.24 ± 4.66*
Feces		
TC (mg/g dried feces)	0.09 ± 0.00	0.22 ± 0.00*
TG (mg/g dried feces)	0.015 ± 0.00	0.02 ± 0.00*

Values are mean ± S.E. of six mice. Asterisk shows significant difference from the control group by Student's t-test at $p < 0.05$.

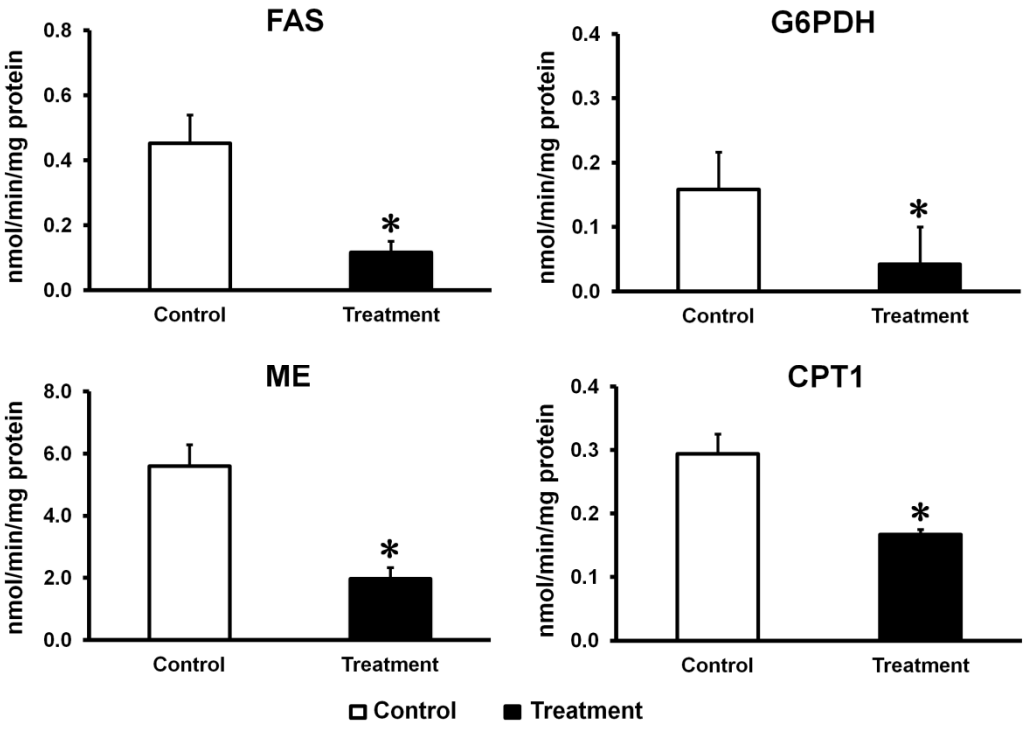

Figure 19 Effects of IGH administration on the activity of lipid metabolism-related enzymes in liver tissue. C57BL/6J mice were fed HFD (Control) or HFD with 2% IGH (Treatment) for 4 weeks. Values are mean ± S.E. of six mice. Asterisk shows significant difference between the control group and the treatment group by Student's t-test at * p <0.01.

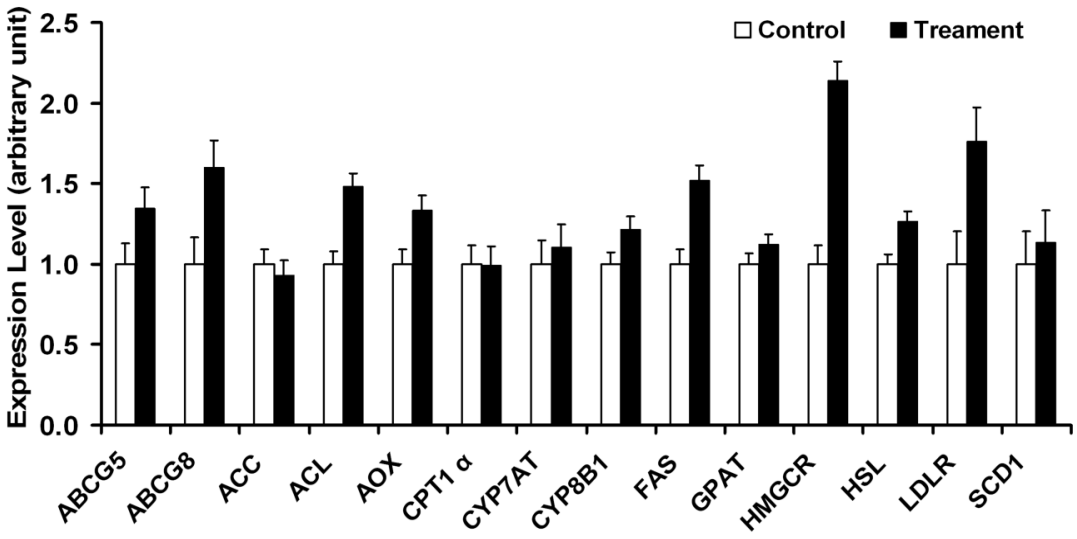

Figure 20 Effects of IGH administration on mRNA level of lipid metabolism-related genes in liver tissue. C57BL/6J mice were fed HFD (Control) or HFD with 2% IGH (Treatment) for 4 weeks. Values are mean ± S.E. of six mice.

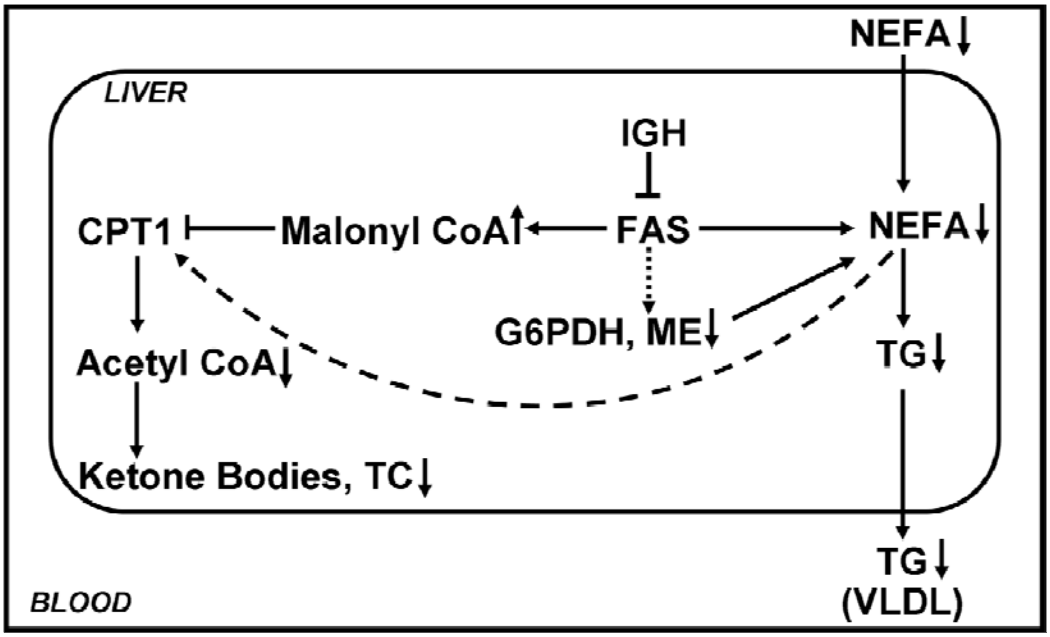

Figure 21 Proposed mode of action of IGH on lipid metabolism in mice fed HFD. The active constituent(s) in IGH when introduced to C57BL/6J mice fed HFD alters lipid metabolism by directly inhibiting FAS activity in their livers contributing to a decrease in both hepatic and serum TG levels. FAS inhibition by IGH also results in a reduction of G6PDH and ME activities in response to lower requirement of NADPH, and also a decrease in CPT1 activity due to accumulation of malonyl CoA. A decrease in acetyl CoA concentration in the liver from impaired fatty acid degradation caused by inhibited CPT1 activity combined with low serum NEFA has been expected. This would reduce both ketogenesis and *de novo* TC biosynthesis. In addition, hepatic TC level is also declined as a result of increased disposal of this lipid *via* bile excretion.

CHAPTER 4

CONCLUSIONS

The following conclusions may be drawn from this study:

1. The ethanol extract of ivy gourd root exerted anti-adipogenic effect *in vitro* by attenuating intracellular lipid accumulation in 3T3-L1 adipocytes primarily *via* suppression of PPARγ gene expression.
2. Anti-adipogenic component(s) of the ivy gourd root extract was found soluble in hexane indicating their non-polar chemical nature.
3. The hexane fraction of the extract (IGH) inhibited 3T3-L1 adipocyte differentiation by potently down-regulating gene expression of C/EBPα, PPARγ, FABP4, FAS, LPL and GLUT4.
4. The IGH exhibited anti-obesity related effects *in vivo* by significantly reducing serum TG and free fatty acid levels as well as hepatic TG and TC levels, and stimulating TG and TC excretion in feces of C57BL/6J mice fed high-fat diet.
5. The hepatic activity of lipogenesis-related enzymes including FAS, G6PDH and ME were also decreased in the IGH consumed mice, suggesting that the lowered serum lipid levels found among these animals could be a result of inhibition of lipid synthesis in their livers by IGH treatment.

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APPENDICES

APPENDIX A

Sodium dodecyl sulfate - polyacrylamide gel electrophoresis (SDS-PAGE)

Samples were electrophoresed in a slap gel, composed of 3.7% stacking gel and 10% separating gel. The gel was performed according to the method of Laemmli (1970). Compositions of the gel are as below.

	Ingredient	
	3.7%Stacking gel	**10%Separating gel**
30%acrylamide (37.5:1)	0.616 mL	3.34 mL
1.5 M Tris-HCl, pH 8.8	-	2.50 mL
0.5 M Tris-HCi, pH 6.8	1.25 mL	-
10% SDS	50 μL	100 μL
10% APS	50 μL	100 μL
TEMED	5 μL	5 μL
Distilled water	3.029 mL	4.0 mL

APPENDIX B

Composition of experimental diets

Ingredient	Group	
	Control (g)	Treatment (g)
AIN-76 M-mix	3.5	3.5
AIN-76 V-mix	1.0	1.0
β-Corn starch	15.0	15.0
Casein	20.0	20.0
Cellulose	5.0	5.0
Choline bitartrate	0.2	0.2
Corn oil	15.0	15.0
DL-methionine	0.3	0.3
Sucrose	40.0	38.0
IGH	**-**	**2.0**
Total	**100**	**100**

Malic enzyme (ME) activity assay

The reaction mixture composed of 500 µl of 32 mM triethanolamine buffer pH 7.4, 40 µl of 1.2 mM malic buffer pH 7.4, 40 µl of 4 mM $MnCl_2$ solution, 40 µl of 1.15 mM $NADP^+$ solution and 340 µl distilled water, was used to set zero at 340 nm. Then, 40 µl of cytosolic fraction was added and the change of absorbance was recorded for 2 min at 27 °C. The enzyme activity was calculated using the equation given below.

Activity (nmol/min/mg protein) = [(OD at 70 sec–OD at 10 sec) ÷ 6,220*] × 10^9× [reaction volume (1 ml) ÷ 1,000] × [1 ÷ reaction time (1 min)] × [1 ÷ protein (mg)]

*6,220 = molar absorbance coefficient of NADPH

Glucose-6-phosphate dehydrogenase (G6PD) activity assay

The reaction mixture composed of 800 µl of 160 mM Tris buffer pH 7.6 containing 30 mM $MgCl_2$ and 120 µl of distilled water, was used to set auto zero at 340 nm. Then, 20 µl of 3.3 mM glucose-6- phosphate disodium salt, 20 µl of 0.575 mM $NADP^+$ solution, 20 µl of 6-phosphogluconate dehydrogenase (0.5 Unit), 20 µl of cytosolic fraction, were added and the change of absorbance was recorded for 2 min at 30 °C. The enzyme activity was calculated using the equation given below.

Activity (nmol/min/mg protein) = [(OD at 70 sec–OD at 10 sec) ÷ 6,220] × 10^9× [reaction volume (1 ml) ÷ 1,000] × [1 ÷ reaction time (1 min)] × [1 ÷ protein (mg)] ÷ 2

Fatty acid synthase (FAS) activity assay

The reaction mixture composed of 500 µl of 100 mM potassium phosphate buffer pH 7.0 containing 0.188 mM EDTA and 390 µl of distilled water, was used to set auto zero at 340 nm. Then, 20 µl of 0.05 mM acetyl CoA, 20 µl of 0.3 mM NADPH and 50 µl of cytosolic fraction, were added and the change of absorbance at 340 nm was recorded for 2 min at 30 °C. After 2 min, 0.2 mM malonyl CoA (20 µl) was added and the change of absorbance at 340 nm was recorded for 3 min at 30 °C. The enzyme activity was calculated using the equation given below.

Activity (nmol/min/mg protein) = [{(OD at 130 sec–OD at 190 sec) – (OD at 50 sec–OD at 110 sec)} ÷ 6,220] × 10^9× [reaction volume (1 ml) ÷ 1,000] × [1 ÷ reaction time (1 min)] × [1 ÷ protein (mg)]

Carnitine palmitoyltransferase (CPT) activity assay

The reaction mixture composed of 500 µl of 58 mM Tris buffer pH 8.0 containing 1.25 mM EDTA, 0.1% Triton-100, 0.25 mM 5,5'-dithiobis (2-nitrobenzoic acid) and 440 µl of distilled water, was used to set auto zero at 412 nm. Then, 20 µl of 0.0375 mM palmitoyl CoA lithium salt, 20 µl of mitochondrial fraction, were added and the change of absorbance was recorded for 5 min at 27 °C. After 5 min, 20 µl of L-carnitine solution was added and the change of absorbance was recorded for 3 min at 27 °C. The enzyme activity was calculated using the equation given below.

Activity (nmol/min/mg protein) = [{(OD at 370 sec–OD at 310 sec) – (OD at 290 sec–OD at 230 sec)} ÷ 13,600*] × 10^9× [reaction volume (1 ml) ÷ 1,000] × [1 ÷ reaction time (1 min)] × [1 ÷ protein (mg)]

*13,600 = molar absorbance coefficient of CoA

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Ivy gourd (*Coccinia grandis* L. Voigt) root suppresses adipocyte differentiation in 3T3-L1 cells

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Abstract

Background: Ivy gourd (*Coccinia grandis* L. Voigt) is a tropical plant widely distributed throughout Asia, Africa, and the Pacific Islands. The anti-obesity property of this plant has been claimed but still remains to be scientifically proven. We therefore investigated the effects of ivy gourd leaf, stem, and root on adipocyte differentiation by employing cell culture model.

Methods: Dried roots, stems, and leaves of ivy gourd were separately extracted with ethanol. Each extract was then applied to 3T3-L1 pre-adipocytes upon induction with a mixture of insulin, 3-isobutyl-1-methylxanthine, and dexamethasone, for anti-adipogenesis assay. The active extract was further fractionated by a sequential solvent partitioning method, and the resulting fractions were examined for their abilities to inhibit adipogenesis in 3T3-L1 cells. Differences in the expression of adipogenesis-related genes between the treated and untreated cells were determined from their mRNA and protein levels.

Results: Of the three ivy gourd extracts, the root extract exhibited an anti-adipogenic effect. It significantly reduced intracellular fat accumulation during the early stages of adipocyte differentiation. Together with the suppression of differentiation, expression of the genes encoding PPARγ, C/EBPα, adiponectin, and GLUT4 were down-regulated. Hexane-soluble fraction of the root extract also inhibited adipocyte differentiation and decreased the mRNA levels of various adipogenic genes in the differentiating cells.

Conclusions: This is the first study to demonstrate that ivy gourd root may prevent obesity based mainly on the ability of its active constituent(s) to suppress adipocyte differentiation *in vitro*. Such an inhibitory effect is mediated by at least down-regulating the expression of PPARγ-the key transcription factor of adipogenesis in pre-adipocytes during their early differentiation processes.

Keywords: Anti-adipogenesis, Adipocyte, Ivy gourd, Obesity, 3T3-L1 cells

Background

Obesity, an abnormal excessive increase of adipose tissue, is an important risk factor that contributes to the development of atherosclerosis, fatty liver, hyperlipidemia, diabetes mellitus, hypertension, inflammation, and various types of cancer [1]. It is characterized at the cellular level by an increase in the number and/or size of adipocytes, round lipid-filled cells, that differentiate from their fibroblast-like precursor cells present in adipose tissue. Therefore, reducing the differentiation into adipocytes or anti-adipogenesis and/or increasing the intracellular lipid breakdown or adipolysis are possible anti-obesity mechanisms. Despite the fact that anti-obesity medication is an effective therapeutic approach, most prescribed drugs have adverse side effects. These limitations have consequently motivated investigations into the search for ingredients from natural sources that can regulate adipocyte function especially those that can suppress adipogenesis [2-8].

Ivy gourd (*Coccinia grandis* L. Voigt or *C. cordifolia* L. Cogn. or *C. indica* Wight & Arn. or *Cephalandra indica* Naud.) is a perennial plant in the family Cucurbitaceae, abundantly present in tropical countries likes India,

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Indonesia, Malaysia, the Philippines, and Thailand. It is a climbing vine with tuberous roots, and fruits throughout the year. The South-East Asians have long made use of this plant in their local cookery and traditional medicine. The leaves and roots have been well accepted in India as a medicine to treat diabetes mellitus. Their hypoglycemic effects have been demonstrated in both diabetic and normal subjects [9]. The molecular mechanisms responsible for the blood glucose lowering activity of this plant, however, remain unestablished. Ivy gourd has been classified as one of the medicinal herbs in the traditional practices of the ancient Thai medicine with some properties similar to those documented in India [10]. In addition to its anti-diabetic property, the root part has been claimed to have an ability to reduce weight. Evidence of weight loss in overweight patients after administration of the plant

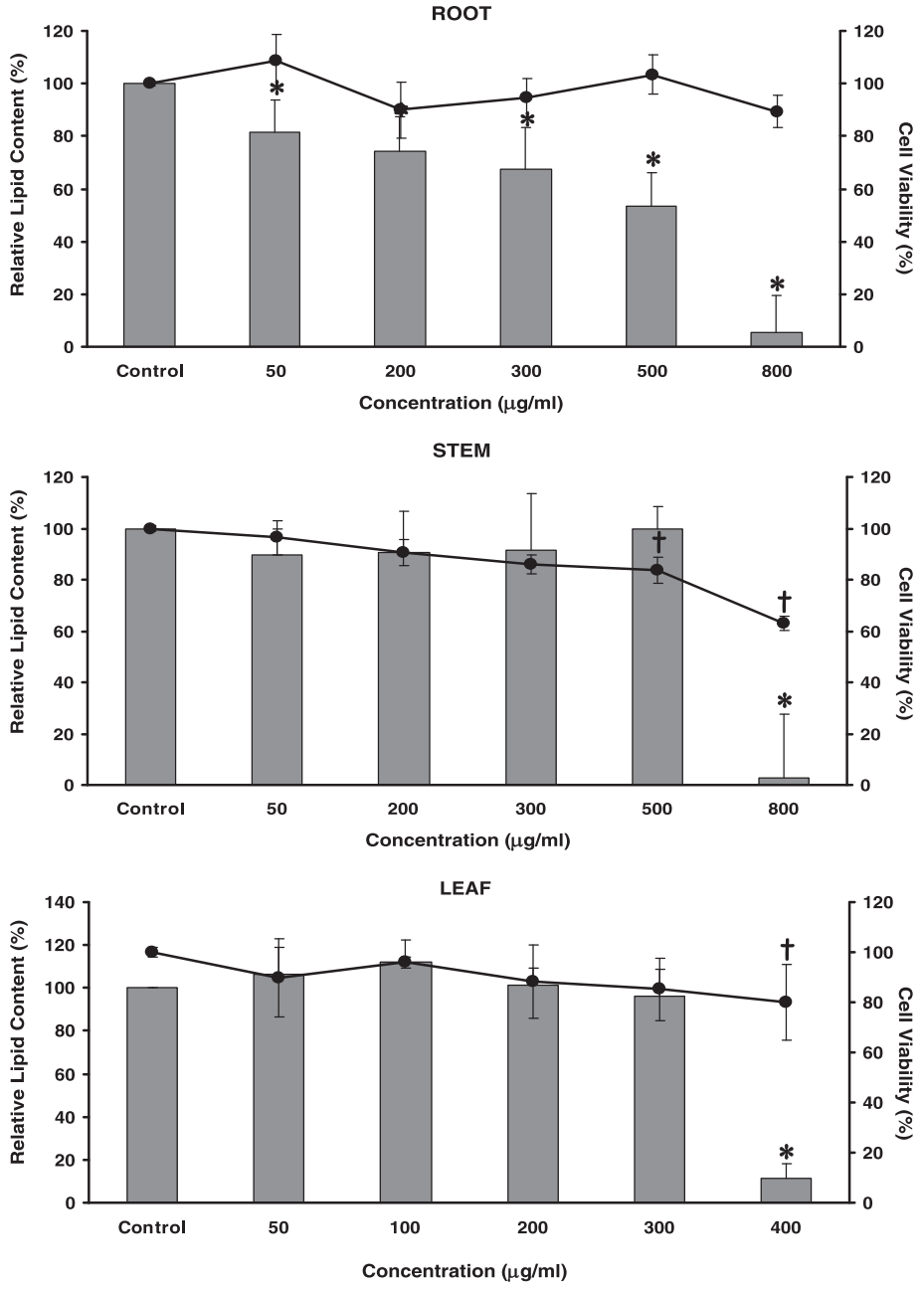

Figure 1 Effects of an ethanol extract from different parts of ivy gourd on intracellular lipid accumulation and cell viability. Results are given as a mean value ± S.D. of six-replicate measurements. Bar graphs represent the relative intracellular lipid contents. Asterisks indicate a significant difference at $p < 0.05$ from the untreated control (0 μg/mL). Percentages of cell viability are shown as a line graph. Crosses indicate a significant difference at $p < 0.05$ from the untreated control (0 μg/mL). The standard TNFα at 10 ng/mL gave 5.00 ± 1.74% of relative lipid content in these experiments.

extract has been reported in India [9]. Such claims, however, are primarily based on local wisdom and no previous attempts have been made to study the anti-obesity property of ivy gourd in detail. Singh and co-workers [11] have demonstrated that ivy gourd contains an anti-hyperlipidemic element but any profound effects of this plant on adipocyte or adipose tissue functions have not yet been examined. In this study, we have assessed the anti-adipogenic activity of different ivy gourd parts on 3T3-L1 cells. These fibroblastic pre-adipocytes can undergo differentiation in culture and exhibit similar morphology and biochemical properties to *in vivo* adipocytes. They were derived from a cloned subline of Swiss 3T3 mouse embryo fibroblasts and have been widely used to study the adipogenic process *in vitro* [12].

Results and discussion

The root extract suppresses intracellular lipid accumulation in induced 3T3-L1 adipocytes during the early stage of adipogenesis

When we examined the ethanol extracts from the root, stem, and leaf parts of ivy gourd for their anti-adipogenic effects, the root extract apparently lowered the lipid levels in 3T3-L1 adipocytes. The amounts of accumulated lipid in the 3T3-L1 adipocytes following the root extract treatment, measured in terms of the absorbance of the oil red O dye extracted from stained cells, were significantly and dose-dependently decreased (Figure 1). There was a smaller number of fat droplets within the mature adipocytes in the presence of the root extract compared to the untreated cells as revealed by microscopy (Additional file 1: Figure S1). Such an inhibitory effect, did not result from cell damage since toxicity of the root extract was not observed with any of the concentrations tested (Figure 1).

The anti-adipogenic effect of the root extract was time sensitive. When we treated 3T3-L1 pre-adipocytes with the extract during differentiation-induction period (Day 0-Day 2) and throughout the course of differentiation (Day 0-Day 6), their intracellular lipid levels decreased equally, regardless of different treatment time-lengths (Figure 2). Administration of the root extract after that period (Day 6-Day 8), however, had no effect on lipid accumulation in the fully differentiated cells (Figure 2). These results indicated that the extract was effective only if introduced early in the adipocyte differentiation program. It acted strictly on differentiating 3T3-L1 pre-adipocytes within the first 2 days of induction with insulin, IBMX (3-isobutyl-1-methylxanthine), and DEX (dexamethasone). A series of adipogenesis-promoting molecules are known to be activated in response to signaling by hormonal inducers [13]. The active compound(s) in the extract might inhibit any of them in some way, thereby blocking adipocyte differentiation at this early stage. Decreasing levels of such critical molecule(s) within the cells during the progress of differentiation were also implicated because the root extract when applied to mature adipocytes (Day 6-Day 8) did not cause any inhibition (Figure 2).

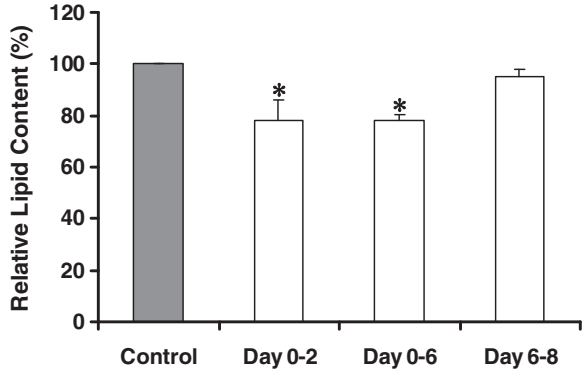

Figure 2 Effects of the root extract at different treatment times on the accumulation of intracellular lipid. The relative amounts of accumulated lipid are shown as a mean value ± S.D. of five-replicate measurements for each period that the extract (100 μg/mL) was present in the culture medium. Asterisks indicate a significant difference at $p < 0.05$ from the untreated control (0 μg/mL) at Day 8.

Extracts of stems and leaves had no effect on the differentiation of the 3T3-L1 adipocytes. The marked decrease of the lipid contents seen at 400 μg/mL of leaf extract and at 800 μg/mL of treatments with stem extract were really a consequence of their ability to damage the cells based on the MTT assay results (Figure 1). In addition, when we examined the three ivy gourd extracts for their adipolytic activities, none of them exerted lipid-degrading effect on fully differentiated 3T3-L1 adipocytes, as determined from the amounts of glycerol released into the culture medium (data not shown). From the above findings, we then decided to focus on investigating the mechanisms by which the ivy gourd root inhibited the adipocyte differentiation process, and to identify the active elements.

Hexane fraction of the root extract inhibits adipogenesis in 3T3-L1 cells

In an attempt to separate the active components from the root extract by sequential solvent partitioning, five different fractions were obtained (Additional file 2: Figure S3). When we examined each fraction for its ability to inhibit adipogenesis, only the hexane-soluble fraction was active. It could suppress 3T3-L1 adipocyte differentiation during the early stages in a dose-dependent manner without negatively affecting the cell survival (Figure 3), whereas the other fractions were either harmful to the cells or ineffective (Additional file 3: Figure S4). These results indicated that the main constituents in ivy gourd root with a suppressive effect on adipocyte differentiation were likely to be non-polar compounds. The presence of alkaloids

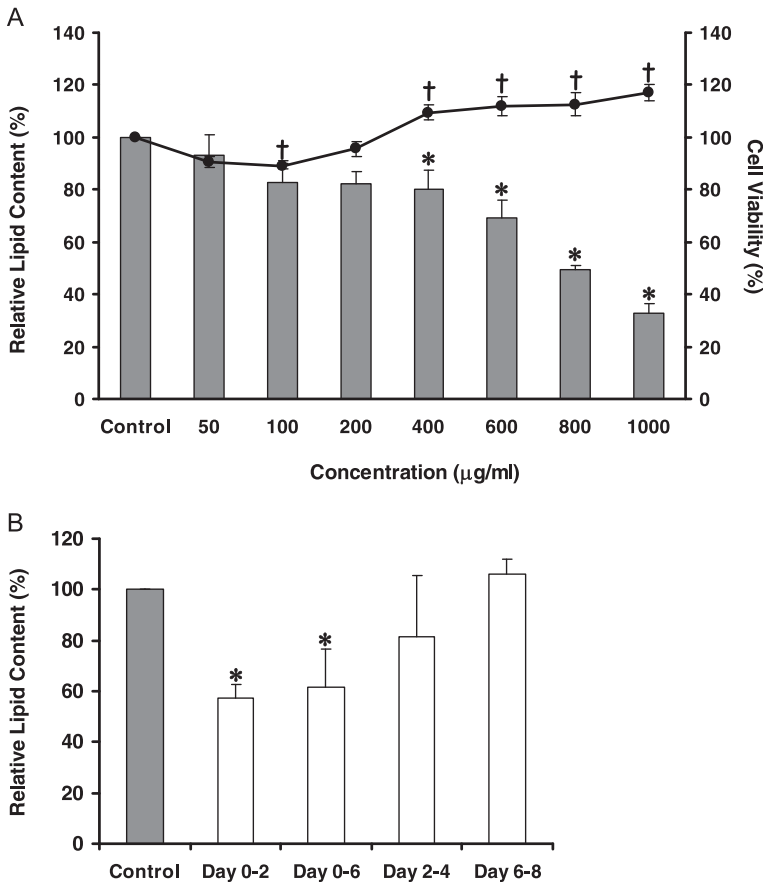

Figure 3 Effects of hexane fraction from the root extract on the intracellular lipid accumulation and cell viability. (A) Results are a mean value ± S.D. of six-replicate measurements. Bar graphs represent the relative intracellular lipid contents. Asterisks indicate a significant difference at p <0.05 from the untreated control (0 μg/mL). Percentages of cell viability are shown as a line graph. Crosses indicate a significant difference at p <0.05 from the untreated control (0 μg/mL). The standard TNFα at 10 ng/mL gave 9.17 ± 3.98% of relative lipid content in these experiments. **(B)** The relative amounts of accumulated lipid are shown as a mean value ± S.D. of five-replicate measurements for each period that the fraction (1,000 μg/mL) was present in the culture medium. Asterisks indicate a significant difference at p <0.05 from the untreated control (0 μg/mL) at Day 8.

[14], fatty acids [15], carotenoids [16], triterpenoids [17], cardenolides [18], a long-chain polyprenol [11], as well as flavonoids, polyphenols, and saponins [19] have been reported in this plant. Although these components have not been shown to inhibit adipogenesis, polyprenol may be one of the potential candidates since it improves dyslipidemia *in vivo* [11]. A current study is underway to identify the active compounds in the hexane fraction.

PPARγ is the main target for the anti-adipogenic effect of ivy gourd root

Adipogenesis or the process of fat cell formation in 3T3-L1 cells is known to be sequentially regulated by a network of transcription factors and adipogenesis-related genes [13]. When we examined the effects of the root extract on the gene expression of key adipogenesis activators throughout the course of differentiation, there was a significant decrease in both mRNA and protein levels for PPARγ (peroxisome proliferator-activated receptor-γ) and C/EBPα (CCAAT/enhancer binding protein-α) but not C/EBPβ (CCAAT/enhancer binding protein-β) during the early stages as compared with those of the untreated cells (Additional file 4: Figure S2). In general, C/EBPβ induces PPARγ and C/EBPα gene expression [20]. From the above findings, we then assumed that the ivy gourd root extract did not inhibit PPARγ transcription through down-regulation of the expression of C/EBPβ. As a result of PPARγ inhibition, adiponectin and GLUT4 (glucose transporter-4) expressions were decreased (Additional file 4: Figure S2). PPARγ is called the master regulator of adipogenesis. It is important for the promotion and maintenance of the adipocyte phenotype. Typically, PPARγ transcripts and protein levels in the 3T3-L1 cells are elevated within 2 days of the induction period (Day 0-Day 2) and reach their peaks by Day 3-Day 4 [21]. C/EBPα is also known as a major transcription factor of adipogenesis, which functions mainly during the terminal stages of differentiation [20]. PPARγ and C/EBPα coordinately

regulate adipocyte-specific gene expression. Their increased levels enhance the mRNA expression of downstream target genes such as FABP4 (aP2), SCD1, leptin, adiponectin (AdipoQ), and GLUT4, leading to the synthesis of several proteins required for intracellular lipid synthesis and storage [22]. GLUT4 and adiponectin are adipogenic markers. They are largely produced in response to the insulin signaling pathway to facilitate cellular uptake of glucose which is ultimately converted into stored lipid. GLUT4 is a transmembrane protein which is necessary for glucose transport into adipocytes [23]. Adiponectin is not only an extensive marker for differentiated adipocytes but also exerts autocrine effects in these cells. This adipokine promotes adipogenesis by stimulating glucose influx through increased GLUT4 gene expression and increased GLUT4 recruitment to the plasma membrane [24]. The suppressed expression of these adipogenesis-promoting genes at both transcriptional and translational levels appears to support our previous findings that the cells treated with the root extract had less intracellular lipid accumulation and fat droplet formation than the untreated controls (Additional file 1: Figure S1).

In order to address more in detail the molecular mechanisms underlying the suppression of 3T3-L1 cell differentiation by ivy gourd root, we then examined the effects of the hexane fraction on expression of a panel of genes related to lipogenesis during the early stages of adipocyte differentiation. The results are shown in Figure 4. All of the lipogenic genes determined in this study became over-expressed by the hormonal induction of adipogenesis. In accordance with the root extract treatment (Additional file 4: Figure S2), the hexane fraction potently suppressed the up-regulation of PPARγ gene expression in the differentiated cells harvested at Day 2. C/EBPα expression was also decreased after the same treatment. Along with the suppression of PPARγ and C/EBPα, mRNA levels of various adipogenic genes were reduced in the treated cells. FABP4 (fatty acid binding protein-4) is a carrier of fatty acids that plays a supporting role in differentiation of the adipocytes [25]. The down-regulated expression of this gene by the hexane fraction indicated a decline in the ability of the cells to process and metabolize fatty acids, thereby attenuating the intracellular lipid synthesis. Previously, the root extract did not produce any effect on FABP4 expression in the adipocytes (Additional file 4: Figure S2). These conflicting results need to be clarified. It is probable that the extract may contain some chemical components which could counteract such negative effects on the FABP4 gene.

Adipor1 (adiponectin receptor-1) serves as a cell surface receptor for adiponectin. This protein mediates glucose uptake through its binding with adiponectin, thereby promoting lipid synthesis in adipocytes [26]. A large increase in expression of the Adipor1 gene was observed after the hexane fraction treatment, although the mRNA level of adiponectin then substantially decreased. It may be postulated that a decrease in adiponectin output would accelerate the expression of Adipor1 gene in these cells to enhance their adiponectin binding capacities. In the present study, however, we found the alteration only at the transcriptional level. The expression of the protein needs to be examined in order to confirm this hypothesis. The reason for the activated gene expression of ACC1 (acetyl-CoA carboxylase-1) in the treated cells is unknown. The most important function of ACC1 is to provide the malonyl-CoA substrate for biosynthesis of fatty acids [27]. This lipogenic gene is transcriptionally controlled by SREBP1c (sterol regulator element-binding protein-1c) which is also a regulator of the PPARγ gene [28]. The significant down-regulation of MEST (mesoderm specific transcript), an adipocyte size marker gene [29], means that the treated cells became smaller due to their reduced lipid content. In addition, the decreased mRNA level of LPL (lipoprotein lipase) which hydrolyzes triglycerides in lipoprotein particles to provide free fatty acids for intracellular triglycerides synthesis, would further cause reduction in the lipid synthesis and storage of these cells. From these changes in the expression of the lipogenic genes, we then concluded that the hexane fraction suppressed intracellular lipid synthesis in 3T3-L1 adipocytes by negatively modulating both up-stream and down-stream adipogenic genes in the early differentiation pathway.

We also observed some inhibitory effects of the hexane fraction on the glucose metabolism-regulating genes that participate with lipogenesis in the adipocytes. A down-regulation of GLUT4 expression in the treated cells was evident. This observation and those that arose from the extract treatment (Additional file 4: Figure S2) thus indicated that if the ivy gourd root had an anti-diabetic property, it would exert a hypoglycemic effect independently of the glucose transporter-GLUT4 system. PDK4 (pyruvate dehydrogenase kinase-4) transcripts, on the other hand, were significantly increased upon the treatment, and would cause inhibition of the pyruvate dehydrogenase complex [30]. Consequently, conversion of acetyl-CoA from pyruvate was attenuated, and limited its availability for use as a precursor of fatty acids. Surprisingly, SITR1 (sirtuin-1) and IRS1 (insulin receptor substrate-1) which promote cellular glucose metabolism by improving insulin sensitivity [31,32] were not affected by the treatment. The activation of PDK4 expression in combination with the down-regulation of FAS (fatty acid synthase), the key enzyme of fatty acid synthesis pathway, then added negative effects on lipogenesis in the treated differentiating cells.

Therefore, the overall anti-adipogenic effect of ivy gourd root in the 3T3-L1 cells seems to be primarily due to

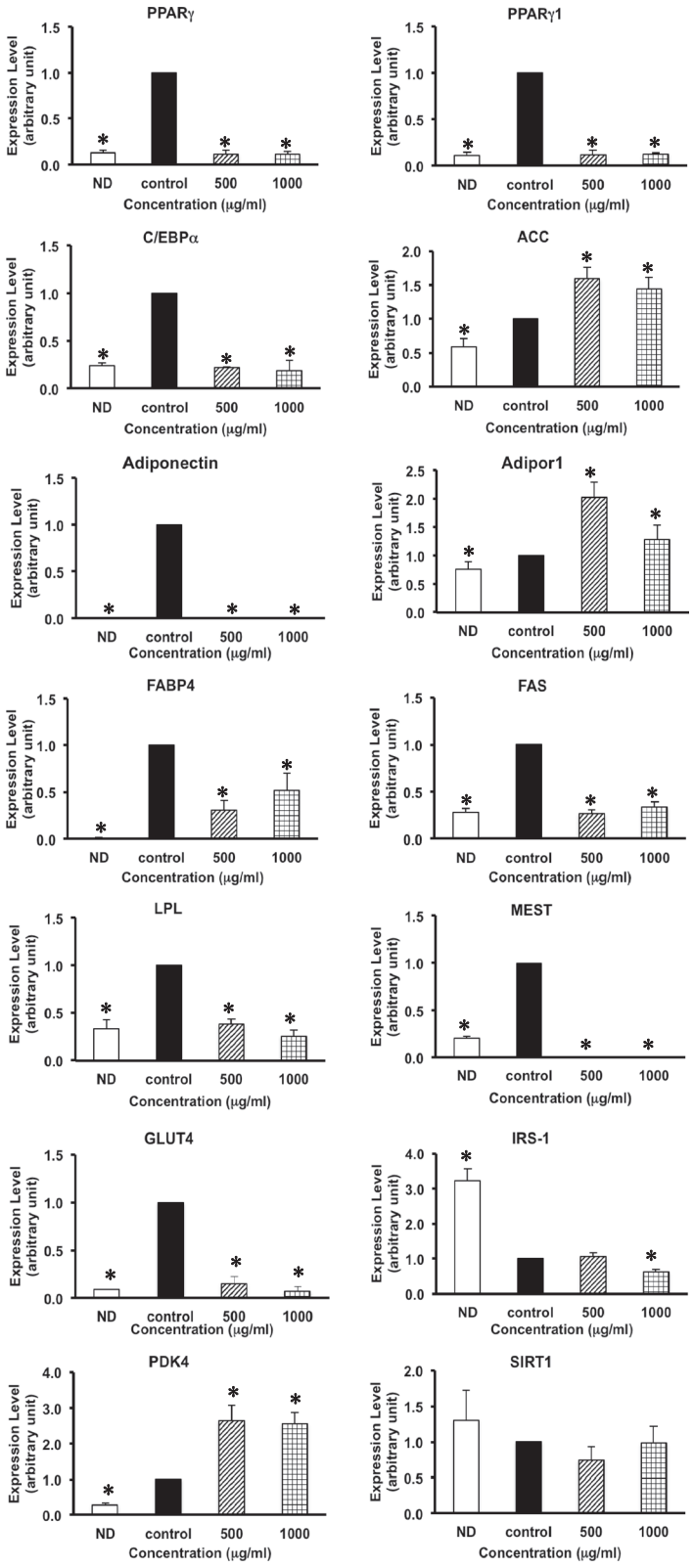

Figure 4 (See legend on next page.)

(See figure on previous page.)
Figure 4 Effects of hexane fraction on the expression of lipid metabolism-related genes in 3T3-L1 cells. The cells were cultured in the presence of an ethanol vehicle only (■); the hexane fraction at 500 μg/mL (▨); the hexane fraction at 1,000 μg/mL (▦) during the differentiation induction period (Day 0-Day 2). All cells were harvested at Day 2 to determine the mRNA levels of the interested genes. Their relative expression levels were estimated in terms of their fold-change compared to the β-actin mRNA. Values are shown as a mean value ± S.D. of three-replicateexperimentsfor each treatment. Asterisks indicate a significant difference at $p < 0.05$ from the untreated control (0 μg/mL). (ND = non-differentiated 3T3-L1 cells).

down-regulation of the expression of the PPARγ gene early in the differentiation pathway. Our proposed mechanisms as illustrated in Figure 5 include the active element(s) that remain to be identified would initiate the inhibitory effects on the adipocyte differentiation by targeting the PPARγ and also the C/EBPα expression directly, but not through C/EBPβ or SREBP-1c. All the findings from this study imply that the ivy gourd root if applied *in vivo* would prevent or attenuate rather than reduce obesity by mobilizing stored fat from the adipose tissue.

Conclusion

Our results have shown for the first time, that ivy gourd root possessed an anti-obesity property. It acted directly on pre-adipocytes by inhibiting their differentiation through down-regulation of at least the key adipogenic transcription factor-PPARγ. The presence of possible anti-adipogenic agent in this plant might be relevant to its use to improve metabolic diseases induced by obesity, in addition to having a blood sugar lowering effect. We are now attempting to identify its active component(s). Further study is also necessary to evaluate the anti-obesity effect of ivy gourd root in experimental animals.

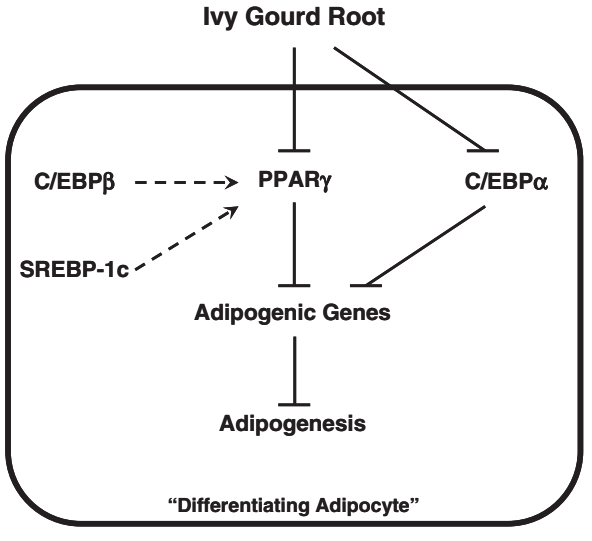

Figure 5 Proposed molecular mechanisms for the inhibition of adipogenesis by ivy gourd root. The active constituent(s) in ivy gourd root when introduced to 3T3-L1 cells during the induction of differentiation blocks PPARγ and C/EBPα expressions directly leading to a negative regulation of various adipogenic genes, and eventually inhibits adipogenesis in these cells.

Materials and methods

Plant materials

Ivy gourd (*Coccinia grandis* L. Voigt) samples were collected in and around Songkhla Province, Thailand. The voucher specimen has been deposited at the Herbarium of the Faculty of Science, Prince of Songkla University.

Preparation of the extracts

The roots, stems, and leaves were separated, cut into small pieces, and dried at 40°C in a hot-air oven. The dried materials were then ground and macerated with 10 volumes of ethanol. The resulting extracts were then collected, filtered and evaporated to dryness under reduced pressure. The percentage yields of the ethanol extracts from roots, stems, and leaves were 5.74, 8.64, and 12.74 of the initial dry weight, respectively.

Cell culture and anti-adipogenesis assay

The 3T3-L1 pre-adipocytes were obtained from the American Type Culture Collection (ATCC) and cultured in a humidified atmosphere of 95% air and 5% CO_2 at 37°C in Dulbecco's modified Eagle's medium (DMEM) (GIBCO, USA) containing 10% fetal bovine serum (FBS) (GIBCO, Canada) and penicillin (100 U/mL)-streptomycin (100 μg/mL) (PS) (GIBCO, Canada). Two days after reaching confluence, they were treated with differentiation medium containing 1 μM dexamethasone (DEX; Sigma-Aldrich, USA), 10 μg/mL of insulin (Sigma-Aldrich; USA) and 0.5 mM 3-isobutyl-1-methylxanthine (IBMX; Sigma-Aldrich, USA) in DMEM (designated Day 0). After 2 days for induction of adipogenesis (Day 2), the medium was changed to DMEM containing 10% FBS, PS and 10 μg/mL of insulin for 2 days (Day 4). They were then cultivated in post-differentiation medium (DMEM containing 10% FBS and PS only), which was replaced every 2 days until Day 8. To examine the effects of ivy gourd on adipogenesis, the 3T3-L1 pre-adipocytes were treated with various concentrations of each ivy gourd sample at the time of the induction of differentiation (Day 0-Day 2), a vehicle of dimethyl sulfoxide (DMSO) acted as a negative control and the tumor necrosis factor-α (TNFα) (Sigma-Aldrich, USA) as a positive control [33]. The extent of differentiation was assessed using oil red O staining done on Day 8 and through visual observations under the microscope from Day 1 to Day 8.

Oil red O staining of the 3T3-L1 adipocytes

The 3T3-L1 adipocytes were washed twice with phosphate buffered saline at pH 7.4 then fixed with 10% formaldehyde for 1 h at room temperature. After the cells were washed with 60% isopropanol, they were stained with oil red O (6 parts of 0.5% oil red O dye in 100% isopropanol to 4 parts of water) for 10 min. After washing with water, three times, the dye stain fixed in the cells was extracted with DMSO and the absorbance (OD) measured at 540 nm. The relative lipid contents were calculated from (OD sample–OD non-differentiated control ÷ OD untreated control–OD non-differentiated control) × 100.

Cytotoxicity assay

The viability of the cells was measured based on the reduction of yellow MTT [1-(4, 5-dimethylthiazol-2-yl)-3, 5-diphenylformazan] by mitochondrial succinate dehydrogenase to the purple formazan which can only occur in metabolically active cells [34]. In this assay, the 3T3-L1 cells were differentiated in differentiation medium containing each sample for 2 days. They were then cultured in post-differentiation medium supplemented with 0.25 mg/mL of MTT (Sigma-Aldrich, USA) for 2 h at 37°C. The culture medium was removed, and DMSO was added to dissolve the MTT-formazan complex formed. The optical density (OD) was measured at 570 nm. The percentage of cell viability was calculated from (OD sample–OD non-differentiated control ÷ OD untreated control–OD non-differentiated control) × 100.

RNA analysis

Semi-quantitative RT-PCR assay

Total RNA was extracted from the cultured cells using Trizol® reagent (Invitrogen, USA) based on the procedure described elsewhere [35]. First-strand cDNAs were synthesized with M-MLV reverse transcriptase (Bio-Rad, USA) from 2 μg RNA, and were amplified by RT-PCR (MyCycler™ Thermal Cycler System, Bio-Rad, USA) using specific primers and thermal cycling conditions as listed in Additional file 5: Table S1. The expression level of each gene transcript was normalized to the glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

Quantitative real-time RT-PCR assay

Total RNA was extracted using the RNeasy mini kit (Qiagen, Germany) according to manufacturer's instructions. First strand cDNA was generated from 2 μg RNA by the High Capacity RNA-to-cDNA kit (Applied Biosystems, USA). The quantitative real-time RT-PCR (Step One Plus™ Real Time PCR System, Applied Biosystems, USA) was performed at 60°C for 40 cycles for all genes. Their primer sequences are listed in Table 1. The mRNA level of each gene was normalized using β-actin as the internal control.

Western blot analysis

Briefly, total cell lysate was prepared using a lysis buffer consisting of 0.5 M Tris–HCl, pH 6.8 with 20% glycerol, 20% β-mercaptoethanol, 80 mM dithiothreitol (DTT), and 8% sodium dodecyl sulfate (SDS). Proteins in the lysate were separated by SDS-polyacrylamide gel electrophoresis and transferred onto a polyvinylidene difluoride (PVDF) membrane (Amersham Hybond™-P, GE Healthcare, UK) using an electroblotting apparatus (Mini-PROTEAN®

Table 1 Primer sequences for Real-Time PCR

Gene (product)	Forward sequence Reverse sequence	Accession No.
Actin	5′-CAGAAGGAGATTACTGCTCTGGCT-3′	NM_007393
(93 bp)	5′-GGAGCCACCGATCCACACA-3′	
ACC	5′-GGACCACTGCATGGAATGTTAA-3′	AY451393
(73 bp)	5′-TGAGTGACTGCCGAAACATCTC-3′	
Adiponectin	5′-GTTCCCAATGTACCCATTCGC-3′	NM_009605
(88 bp)	5′-TGTTGCAGTAGAACTTGCCAG-3′	
Adipor1	5′-TCTTCGGGATGTTCTTCCTGG-3′	NM_028320
(104 bp)	5′-TTTGGAAAAAGTCCGAGAGACC-3′	
C/EBPα	5′-TGGACAAGAACAGCAACGAGTAC-3′	AM_007678
(257 bp)	5′-GCAGTTGCCCATGGCCTTGAC-3′	
FABP4	5′-AGCATCATAACCCTAGATGG-3′	NM_024406.2
(115 bp)	5′-CATAACACATTCCACCACCAGC-3′	
FAS	5′- TGCTCCCAGCTGCAGGC -3′	AF_127033
(91 bp)	5′-GCCCGGTAGCTCTGGGTGTA-3′	
GLUT4	5′-CTGCAAAGCGTAGGTACCAA-3′	BC014282
(87 bp)	5′-CCTCCCGCCCTTAGTTG-3′	
IRS1	5′-CCAGAGTCAAGCCTCACACA-3′	NM_010570.4
(179 bp)	5′-GAAGACTGCTGCTGCTGTTG-3′	
LPL	5′-AGGGCTCTGCCTGAGTTGTA-3′	NM_008509
(199 bp)	5′-AGAAATCTCGAAGGCCTGGT-3′	
MEST	5′-GTTTTTCACCTACAAAGGCCTACG-3′	NM_008590
(52 bp)	5′-CACACCGACAGAATCTTGGTAGAA-3′	
PDK4	5′-GAGAAGAGCCCAGAAGACCA-3′	NM_013743
(134 bp)	5′-TCCACTGTGCAGGTGTCTTT-3′	
PPARγ	5′-AGGCCGAGAAGGAGAAGCTGTTG-3′	NM_011146
(276 BP)	5′-TGGCCACCTCTTTGCTGTGCTC-3′	
PPARγ1	5′-AAGATTTGAAAGAAGCGGTGAAC-3′	NM_001127330
(116 bp)	5′-CAATGGCCATGAGGGAGTTAG-3′	
SIRT1	5′-GACGACGAGGGCGAGGAG-3′	NM_019812
(79 bp)	5′-ACAGGAGGTTGTCTCGGTAGC-3′	

ACC, acetyl-CoA carboxylase; Actin, cytoplasmic β-actin; Adipor1, adiponectin receptor-1; C/EBPα, CCAAT/enhancer binding protein transcription factor-α; FABP4, fatty acid binding protein-4; FAS, fatty acid synthase; GLUT4, glucose transporter-4; IRS1, insulin receptor substrate-1; LPL, lipoprotein lipase; MEST, mesoderm specific transcript; PDK4, pyruvate dehydrogenase kinase-4; PPARγ, peroxisome proliferator activated receptor-γ; PPARγ1, peroxisome proliferator activated receptor-γ1; SIRT1, sirtuin (silent mating type information regulation 2 homolog)-1; bp, base pairs.

Tetra System, Bio-Rad, USA). The membrane was soaked in 20 mM Tris–HCl, pH 7.6 containing 0.8% NaCl, 0.1% Tween 20, and 5% non-fat dry milk for 1 h at room temperature before further incubated with a primary antibody and a horseradish peroxidase-conjugated secondary antibody for 2 h and 1 h, respectively. After incubation, the membrane was immersed in the chemiluminescent substrate using an ECL assay kit (Super Signal® West Pico, Thermo Scientific, USA). Imaging of blots was then performed on a clear blue x-ray film (CL-XPosure Film, Thermo Scientific, USA) using an automatic x-ray film developing machine (SRX-101A Medical Film Processor, Konica Minolta, Japan) for film processing.

Statistical analysis

The data are presented as a mean value ± S.D. In each experiment, the inter-group differences were evaluated by one-way ANOVA followed by the Duncan *post hoc* test. Probability values of $p < 0.05$ were considered to be significant.

Additional files

Additional file 1: Figure S1. Effects of the root extract on intracellular lipid accumulation.

Additional file 2: Figure S3. Fractionation scheme of the ivy gourd root extract.

Additional file 3: Figure S4. Effects of four different fractions from the root extract on intracellular lipid accumulation and cell viability.

Additional file 4: Figure S2. Effects of the root extract on mRNA and protein expressions of adipogenesis-related genes in 3T3-L1 cells.

Additional file 5: Table S1. Primer sequences and conditions for RT-PCR.

Abbreviations

ACC: Acetyl-CoA carboxylase; Adipor1: Adiponectin receptor-1; C/EBPα: CCAAT/enhancer binding protein-α; C/EBPβ: CCAAT/enhancer binding protein-β; DEX: Dexamethasone; DIM: Differentiation medium, DMEM, Dulbecco's modified Eagle's medium, DMSO, Dimethyl sulfoxide; DTT: Dithiothrietel; ECL: Enhanced chemiluminescence; FABP4: Fatty acid binding protein-4; FAS: Fatty acid synthase; FBS: Fetal bovine serum; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; GLUT4: Glucose transporter-4; IBMX: 3-Isobutyl-1-methylxanthine; IRS1: Insulin receptor substrate-1; LPL: Lipoprotein lipase; MEST: Mesoderm specific transcript; MTT: 1-(4, 5-dimethylthiazol-2-yl)-3, 5-diphenylformazan; PDK4: Pyruvate dehydrogenase kinase-4; PPARγ: Peroxisome proliferator-activated receptor-γ; PVDF: Polyvinylidene difluoride; RT-PCR: Reverse transcription-polymerase chain reaction; SCD1: Stearoyl-CoA desaturase-1; SDS: Sodium dodecyl sulfate; SITR1: Sirtuin (silent mating type information regulation 2 homolog)-1; SREBP1c: Sterol regulatory element-binding protein-1c; TNFα: Tumor necrosis factor-α.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

RB performed all the experiments and compiled the data. NHT wrote the manuscript and was responsible for the study concept, designing and coordinating the research, and analyzing the results. KN was responsible for development of the methods. CW was responsible for the experimental designs. MF and HO contributed to designing the study and acquisition of data. All of the authors have read and approved the final form of the manuscript.

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Lipid-Lowering Effects of Hexane Fraction of Ivy Gourd (*Coccinia grandis* L. Voigt) Root in Mice Fed a High-Fat Diet

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Running title
Lipid-Lowering Effects of Ivy Gourd Root in Mice

Abstract

Ivy gourd is an edible plant widely grown in the tropics. Its root has long been touted to possess anti-obesity property. In our previous study, the ethanolic extract of ivy gourd root exhibited anti-obesity action by potently inhibiting 3T3-L1 preadipocyte differentiation. Bioactivity-guided fractionation of the same extract also revealed that its active principles are in the hexane fraction. Here, we investigate the anti-obesity effects of the hexane fraction of ivy gourd root extract (IGH) in high-fat diet (HFD) induced obese mice and provide evidence of its underlying molecular mechanisms. C57BL/6J mice were fed HFD in the presence or absence of 2% (w/w) dietary concentration of IGH for 4 weeks. Biochemical determinants of obesity were then measured in these animals. Consumption of IGH caused a decrease of serum triglycerides (TG) and non-esterified fatty acid concentrations as well as hepatic TG and total cholesterol (TC) levels. An increase in fecal excretion of TG and TC along with a decrease in activity of hepatic lipogenesis-related enzymes including fatty acid synthase, glucose-6-phosphate dehydrogenase and malic enzyme in the liver was also detected upon the intake of IGH. These results thus suggest that IGH may have potential as an anti-hyperlipidemic agent for obesity prevention and/or management.

Keywords: Anti-obesity, ivy gourd root, high-fat fed mice, anti-hyperlipidemia, plasma lipids

Introduction

Obesity is a condition of abnormal or excessive fat accumulation that may impair health, affecting about 13 % of the world's adult population [1]. As obesity is a major risk factor for non-communicable diseases like type II diabetes, cardiovascular diseases, musculoskeletal disorders and cancers [2], its rising prevalence worldwide becomes one of the biggest public health concerns nowadays. There are many factors that can cause obesity. A regular intake of high-fat foods is one of them. It may contribute to obesity by elevating triglycerides (TG) and total cholesterol (TC) levels in both blood and tissues. Various therapeutic approaches including medications, such as phentermine for appetite suppression and oristat for gastrointestinal lipase inhibition, have been employed to treat and prevent obesity. Since most of currently available drugs used to reduce obesity have unfavorable side-effects [3], plant-based substances that give beneficial effects on obesity have received increasing attention [4, 5].

Ivy gourd (*Coccinia grandis* L. Voigt) called in Thai as "Tum-Leung", is a perennial vine belonging to the cucumber family (Cucurbitaceae). This tropical plant has been recognized for its high nutritional

value [6, 7]. In Asia, ivy gourd is both consumed as a vegetable and used as a household remedy to cure several ailments, including diabetes treatment which is widely practiced in Indian subcontinent countries [8]. Over the past decade, a number of chemical constituents and biological activities of ivy gourd have been identified. Every part of this plant contains active compounds that might be beneficial for health [9]. In addition to their hypoglycemic property, leaf and root parts of ivy gourd have anti-hyperlipidemic potential as evident from different *in vivo* studies [10-13]. Recently, we have reported that the ethanolic extract of ivy gourd root, as well as its hexane fraction (IGH), exhibit anti-obesity activity by dose-dependently inhibiting fat accumulation in 3T3-L1 pre-adipocytes, primarily through down-regulating peroxisome proliferator activated receptor-γ (PPARγ) gene expression [14]. In order to explore in more detail the ability of ivy gourd root to counteract obesity, we studied the effects of IGH on obesity-related parameters in high-fat diet induced obese C57BL/6J mice. We chose the C57BL/6J mouse strain because these mice exhibit obese phenotypes similar to humans when subjected to high dietary fat [15]. In addition to animal experiments, we performed phytochemical determination of IGH to identify associated bioactive constituents.

Materials and methods

Sample preparation

Fresh roots of ivy gourd (*C. grandis*) were collected in September 2013 from Phang-nga Province, Thailand. The plant sample was identified taxonomically by Associate Professor Dr. Kittichate Sridith of Department of Biology, Faculty of Science, Prince of Songkla University (PSU) and preserved with a voucher specimen number N. Towattana 1 (PSU) in the herbarium of PSU. The collected roots were washed, dried at 40 °C in a hot-air oven, and then ground with a blending machine. The ground material was extracted with ethanol by employing the maceration method. The resulting extract was further fractionated by solvent-solvent partitioning to produce IGH as previously described [14]. Briefly, the extract solution was filtered and evaporated to dryness under reduced pressure. The dried extract was dissolved in 90 % aqueous methanol and partitioned with *n*-hexane. Once the two liquid phases were completely separated, the upper layer (hexane fraction) was collected and evaporated to give IGH. The preparation of IGH was accomplished in 24.2 % yield. The sample obtained was kept dehumidified and away from light at room temperature. Its anti-adipogenic activity was confirmed in 3T3-L1 cells prior to *in vivo* studies.

Animal experiments

Four-week-old male C57BL/6J mice were purchased from Japan SLC, Inc. (Shizuoka, Japan). They were housed individually in similar-sized transparent plastic cages under a controlled environment (24 °C, 45-65 % humidity, and 12/12 h light/dark-cycle). The animals were maintained on standard pellet diet for 7 days to allow acclimatization to laboratory conditions. After that period, 12 mice were randomly divided into a control group (n = 6) and a treatment group (n = 6). The control mice were fed HFD prepared based on AIN-76 purified diet formula (The American Institute of Nutrition, USA) for 4 weeks, whereas the treatment group were fed the same diet supplemented with 2 % IGH (w/w) for 4 weeks. The composition of experimental diets is shown in **Table 1**. All mice were pair-fed on the diets, and water was provided *ad libitum*. The animals were observed daily for their clinical signs and behavioral changes. Each mouse was weighed weekly and its daily food intake was measured at the same time of the day. During the last 3 days of the experimental period, feces were collected and lyophilized for TC and TG analyses. At the end of the study, each animal was fasted overnight, and then sacrificed by cardiac puncture under pentobarbital anesthesia. The blood sample was collected for subsequent serum preparation. A gross necropsy was performed during which any macroscopic abnormalities were noted. Liver and fat tissues were immediately excised, thoroughly washed in an ice-cold physiological buffered saline (10 % PBS), weighed, and stored at -80 °C until further use. The experimental procedures were in accordance with the ethical guidelines for animal experiments of the University of the Ryukyus and approved by the University of the Ryukyus Animal Experiment Committee (Permit Number: 5662).

Biochemical assays

Serum levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), glucose, non-esterified fatty acid or free fatty acid (NEFA), TC and TG were measured by enzymatic kits (Wako,

Japan). Serum insulin and adiponectin levels were measured by ELISA kits (insulin, Morinaga, Japan; total and high molecular weight (HMW) adiponectins, American Laboratory Products, USA). Serum β-hydroxybutyrate was measured by an assay kit (Abcam, UK). Total lipids were extracted from livers and feces based on the method of Folch *et al.* [16]. The TC and TG levels in livers and feces were measured by enzymatic kits (Wako, Japan).

Determination of the activity of hepatic lipogenic enzymes

Liver homogenates were prepared in 10 mM Tris-HCl, pH 7.4 containing 1 mM EDTA and 0.25 M sucrose, and then separated into cytosolic and mitochondrial fractions by a two-step centrifugation (10,000 × g for 10 min at 4 °C followed by 125,000 × g for 60 min at 4 °C) [17]. The protein concentration of each fraction was determined based on the Lowry method using the DC™ protein assay kit (Bio-Rad, USA). The activity of cytosolic enzymes, fatty acid synthase (FAS), glucose-6-phosphate dehydrogenase (G6PDH) and malic enzyme (ME) was determined as described previously [18-20]. The measurement of mitochondrial carnitine palmitoyltransferase (CPT) was performed according to the method of Markwell *et al.* [21].

RNA analysis

Total RNA was extracted from epididymal white adipose tissue (WAT) (50 mg) by using the RNeasy mini kit (Qiagen, Germany). cDNA was synthesized with 2 μg of total RNA as a template using the High Capacity RNA-to-cDNA kit (Applied Biosystems, USA). Quantitative reverse transcriptase-PCR (qRT-PCR) was performed on Step One Plus™ Real-Time PCR System (Applied Biosystems, USA) with the following conditions: one cycle of 95 °C for 20 sec, 40 cycles of 95 °C for 3 sec and 60 °C for 30 sec. A melting curve analysis was performed starting at 95 °C for 15 sec, 60 °C for 60 sec and increasing by 0.3 °C every 15 sec to determine primer specificity. Specific primers are listed in **Table 2**. The mRNA levels of all genes of interest were normalized using β-actin as the internal control.

Phytochemical determination

The IGH sample (3 g) was applied to a silica gel column (Silica gel 60, 0.04 - 0.06 mm, 70 - 230 mesh) (Scharlau Chemie, Spain), and serially eluted with hexane-ethyl acetate (95: 5, v/v), hexane-ethyl acetate (50: 50, v/v) and 100 % ethyl acetate. The collected fractions were then subjected to thin layer chromatography (TLC) using a Silica gel G60 F_{254} pre-coated TLC plate (Merck, Germany) and hexane-ethyl acetate (95: 5, v/v) as mobile phase. The TLC spots were visualized under UV light (254 and 365 nm), and phytochemical screening was performed using different spray reagents as described by Farnsworth *et al.* [22]. The fractions sharing similar TLC spot patterns were combined, concentrated by evaporation under vacuum at 40 °C. To isolate steroids from IGH, the resulting material was repeatedly fractionated by column chromatography over silica gel, eluting with hexane-ethyl acetate (40: 60, v/v), hexane-ethyl acetate (55: 45, v/v) and hexane-acetone-methanol (72: 26.6: 1.4, v/v/v), respectively. The collected fractions were monitored by TLC. Only those yielding a single TLC spot which turned blue after reacting with Liebermann-Burchard reagent, were combined to give IGH-derived steroid sample (1.5 mg) for gas chromatography-mass spectrometry (GC-MS) analysis (GC-MSQP 2010, Shimadzu, Japan).

Statistical analysis

Analyses from each experiment were carried out at least in triplicate. The data are expressed as a mean value ± S.E. (standard error). Statistical analyses were performed using Student's t-test program of MEPHAS statistical software (Osaka University, Japan). Differences were judged to be significant at $p < 0.05$.

Results and discussion

Effects of IGH on growth, liver and fat tissues of diet-induced obese C57BL/6 mice

Throughout the course of this study, consumption of IGH at a dietary concentration of 2 % (w/w) (equivalent to an average dose of 1,610 mg/kg body weight/day) did not cause mortality or any unusual behavioral or phenotypic changes among the experimental mice. Feeding IGH to these animals did not produce any significant effects on their food intake, growth, and liver and fat tissues as shown in **Table 3**.

In the epididymal WAT of the mice given IGH, however, there was a decrease in their PPARγ1 mRNA levels (**Figure 1**). Typically, PPARγ1 is expressed in a higher degree than PPARγ2 in WAT, but is less significant in promoting adipogenesis [23]. Therefore, the down-regulation of PPARγ1 in epididymal WAT of the treated mice (**Figure 1**) was not sufficient to cause a significant reduction in that tissue mass (**Table 3**). In contrast to WAT, brown adipose tissue (BAT) proportionately increases as the body mass drops [24]. Despite no difference in weight gain between the treatment and control groups, a slight increase in BAT among the treated animals was noted in this study (**Table 3**). Based on the above findings, it is likely that an ability of IGH to prevent deposition of WAT in the HFD-induced obese mice remains unclear and needs to be confirmed by more extensive studies on a larger group of animals and higher doses of sample. In this context, we presume that IGH would work in the circumstances occurring in a body in ways analogous from those *in vitro*.

Effects of IGH on serum and liver lipid profile

The most important finding of this study was that serum and liver TG levels in the HFD-fed mice receiving IGH were significantly lower than those in the control group (**Table 4**). As circulating TG molecules in the fasting state mainly reside in very-low density lipoprotein (VLDL) particles, and the rate of VLDL synthesis and secretion is known to be regulated by the availability of hepatic TG synthesized from fatty acid esterification [25], we then speculated that the decreased serum TG concentrations found among the treated animals reflect a reduction in their hepatic TG synthesis. During fasting, serum NEFA is released from adipose tissue by the action of hormone-sensitive lipase (HSL) on stored TG [26]. It is the major source of fatty acid substrate for VLDL-TG production [25]. In response to IGH administration, serum NEFA became decreased (Table 4). As a result, fatty acid flux to the liver dropped, leading to the lower rate of VLDL-TG secretion. Thus, these changes could explain the decrease in both serum and hepatic TG observed in the IGH treated mice (**Table 4**).

Fatty acid *de novo* synthesis was also decreased in the treated animal livers as evident from the lower activity of enzymes involved in the process including FAS, G6PDH and ME (**Figure 2**). In mammalian fatty acid synthesis, FAS catalyzes condensation of acetyl CoA and malonyl CoA into palmitate in the presence of NADPH, which is supplied by pentose phosphate pathway and cytosolic conversion of malate to pyruvate. The inhibition of FAS activity therefore would cause the liver cells to require less NADPH leading to a decrease in both NADPH-providing pathways, as indicated by the diminished activity of G6PDH and ME (**Figure 2**). In this study, however, the inhibition of the above enzymes was detected only at the protein level and thus their transcriptional abundance needs to be examined in order to confirm our hypothesis.

In association with the decrease in FAS, G6PDH and ME activities, we found that the activity of CPT, the rate-limiting enzyme in β-oxidation of long-chain fatty acid (LCFA), was also suppressed but to a lesser extent than those of the three lipogenic enzymes (**Figure 2**). Due to the inhibition of CPT, LCFA degradation would become reduced, resulting in a decreased amount of the end product, acetyl CoA. In general, acetyl CoA generated by mitochondrial fatty acid oxidation enters the citric acid cycle to produce ATP but in the liver some is converted to ketone bodies. In the IGH treated group, however, the hepatic ketogenesis process was not attenuated as indicated by an unaltered level of circulating β-hydroxybutyrate (**Table 4**).

Feeding of HFD to C57BL/6 mice is known to induce hepatic steatosis or fatty liver [27], and the activities of serum marker enzymes such as ALT and AST are usually elevated in this pathological abnormality due to liver cell damage [28]. The non-significant differences of both serum aminotransferases between the control and treatment groups (**Table 4**) thus gave an implication that although IGH did not help alleviate HFD-induced fatty liver in the mice through its hepatic TG lowering effect, it had no adverse effects on liver functions.

In this study, we also detected a hypoglycemic tendency in the treated mice (**Table 4**), though there was no difference in serum insulin levels between the treatment and control groups (**Table 4**). These findings suggest that IGH likely increases insulin sensitivity among insulin-sensitive tissues, leading to a stimulation of glucose utilization and/or a depression of glucose synthesis. In this regard, insulin-mimetic properties of IGH on glucose metabolism have been previously demonstrated [29-32]. In addition to its hypoglycemic effect, elevation of insulin is known to lower VLDL secretion by suppressing mobilization of fat from its depots *via* HSL inhibition, followed by a subsequent decrease in NEFA available for

VLDL-TG production [25]. It is therefore possible that the lower serum NEFA and TG levels seen in our treated mice would arise from the insulin-like actions of IGH on lipoprotein metabolism.

Both TG and TC increased significantly in feces of the treated animals, while decreasing in the liver (**Table 4**). The difference of fecal TC levels between the control and treatment groups, however, was considerably greater than that of fecal TG. When we examined mRNA expression of the key hepatic enzymes involved in cholesterol metabolism such as hydroxy-3-methylglutaryl-CoA reductase, cholesterol 7α-hydroxylase, sterol 12α-hydroxylase, and also low-density lipoprotein (LDL) receptor, none of them was modulated by dietary IGH (data not shown). From these results, an enhanced biliary excretion of cholesterol was thought to be accountable for the reduction of hepatic TC level in the treatment group. An increase in fecal TG found in the treated group (**Table 4**) also suggests that IGH might exert some kind of inhibitory effect on dietary lipid absorption either by inhibiting pancreatic lipase or blocking micelle formation which is needed to be further studied.

Chemical constituents of IGH

Among phytochemicals of IGH separated based on their chromatographic behaviors on TLC, those yielding blue-color spots in Liebermann-Burchard test were most abundant (data not shown). Such display of blue coloration signifies the presence of steroids [33]. From the above findings, we then presumed that the major constituents of IGH are steroids and that they were likely responsible for the anti-obesity effects. This was evident when the fractions obtained from column chromatography of IGH were subjected to anti-adipogenesis assay. Only those giving blue spots on TLC plate showed the activity (data not shown). To date, two distinct steroids have been identified in ivy gourd. β-sitosterol is found in the aerial, fruit, and root parts, whereas stigmast-7-en-3-one is present in the plant roots only [34]. The hypolipidemic and anti-adipogenic activities of β-sitosterol isolated from various plants have previously been documented [35-39], while none of stigmast-7-en-3-one has yet been reported. The steroids contained in IGH, however, were not β-sitosterol since their migration distances on TLC plate were different (data not shown). When we subjected the IGH-derived steroid sample to GC-MS analysis, mass spectrum revealed the occurrence of sterol with molecular MS of 412 or 428 (**Figure 3**), but the results obtained were not sufficient to identify the structure of this compound. Further phytochemical investigation thus needs to be performed in order to determine the exact steroid content of IGH.

Conclusions

We demonstrated that ingredients present in IGH have an ability to improve HFD-diet induced obesity by lowering serum and liver lipid levels through a mode of action as illustrated (**Figure 4**). However, more work is warranted in order to ascertain its efficiency and to identify the anti-obesity agent in this tropical plant root.

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Table 1 Composition of experimental diets.

Ingredient (% w/w)	**Control Group**	**Treatment Group**
Casein	20	20
Cellulose	5	5
Corn oil	15	15
Corn starch	15	15
Sucrose	40	38
AIN-76 M-Mix[a]	3.5	3.5
AIN-76 V-Mix[a]	1	1
DL-Methionine	0.3	0.3
Choline bitartrate	0.2	0.2
IGH	0	2
Total	**100**	**100**

[a]Product of Oriental Yeast Co. Ltd., Tokyo, Japan

Table 2 Primer sequences used for Quantitative RT-PCR.

Gene	**Forward sequence** **Reverse sequence**
ACC	5'-GGACCACTGCATGGAATGTTAA-3'
(acetyl-CoA carboxylase)	5'-TGAGTGACTGCCGAAACATCTC-3'
Actin	5'-CAGAAGGAGATTACTGCTCTGGCT-3'
(β-actin)	5'-GGAGCCACCGATCCACACA-3'
AOX	5'-TCAACAGCCCAACTGTGACTTCCATCA-3'
(acyl-CoA oxidase)	5'-TCAGGTAGCCATTATCCATCTCTTCA-3'
C/EBPα	5'-TGGACAAGAACAGCAACGAGTAC-3'
(CCAAT/enhancer-binding proteins-α)	5'-GCAGTTGCCCATGGCCTTGAC-3'
CPT1α	5'-AAAGATCAATCGGACCCTAGACA-3'
(carnitine palmitoytransferase-1α)	5'-CAGCGAGTAGCGCATAGTCA-3'
FAS	5'- TGCTCCCAGCTGCAGGC -3'
(fatty acid synthase)	5'-GCCCGGTAGCTCTGGGTGTA-3'
HSL	5'-GGTGACACTCGCAGAAGACAATA-3'
(hormone sensitive lipase)	5'-GCCGCCGTGCTGTCTCT-3'
MEST	5'-GTTTTTCACCTACAAAGGCCTACG-3'
(mesoderm specific transcript)	5'-CACACCGACAGAATCTTGGTAGAA-3'
PPARγ	5'-AGGCCGAGAAGGAGAAGCTGTTG-3'
(peroxisome proliferator activated receptor-γ)	5'-TGGCCACCTCTTTGCTGTGCTC-3'
PPARγ1	5'-AAGATTTGAAAGAAGCGGTGAAC-3'
(peroxisome proliferator activated receptor-γ1)	5'-CAATGGCCATGAGGGAGTTAG-3'
SREBP-1c	5'-GGAGCCATGGATTGCACATT-3'
(sterol regulatory element-binding protein-1c)	5'-GCTTCCAGAGAGGAGGCCAG-3'

Table 3 Effect of IGH on growth parameters, liver and adipose tissue weights.

Parameter	Control Group	Treatment Group
Food intake (g/day)	2.81 ± 0.05	2.83 ± 0.03
Initial body weight (g)	17.5 ± 0.2	17.5 ± 0.2
Final body weight (g)	26.0 ± 0.8	25.9 ± 0.2
Liver weight (g/100g BW)	3.12 ± 0.11	3.44 ± 0.11
Adipose tissue weight (g/100g BW)		
Subcutaneous WAT	1.30 ± 0.16	1.32 ± 0.11
Omental WAT	1.76 ± 0.09	1.88 ± 0.09
Perirenal WAT	1.31 ± 0.18	1.17 ± 0.13
Epididymal WAT	2.87 ± 0.32	2.77 ± 0.17
BAT	0.317 ± 0.057	0.410 ± 0.043

Values are mean ± S.E. of six mice. BW = body weight

Table 4 Effect of IGH on serum levels of liver marker enzymes and lipid metabolism-related parameters, and liver and feces lipid profiles.

Parameter	Control Group	Treatment Group
Serum		
ALT (IU/L)	6.31 ± 2.07	5.13 ± 1.01
AST (IU/L)	71.8 ± 15.3	49.9 ± 8.7
TC (mg/dL)	104 ± 14	96.5 ± 12.0
TG (mg/dL)	60.7 ± 2.4	46.5 ± 4.0*
Glucose (mg/dL)	213 ± 22	153 ± 27
NEFA (mEq/L)	1.63 ± 0.03	1.39 ± 0.10*
β-hydroxybutyrate (pmol/μL)	464 ± 57	368 ± 65
Total adiponectin (μg/mL)	9.47 ± 0.18	9.28 ± 0.13
HMW adiponectin (μg/mL)	3.70 ± 0.46	2.60 ± 0.30
Insulin (ng/mL)	1.09 ± 0.42	1.15 ± 0.31
Liver		
TC (mg/g liver)	7.69 ± 0.22	6.03 ± 0.35**
TG (mg/g liver)	93.8 ± 10.0	45.2 ± 4.7***
Feces		
TC (μg/g dried feces)	87.0 ± 8.5	218 ± 7**
TG (μg/g dried feces)	14.6 ± 0.8	20.5 ± 1.7**

Values are mean ± S.E. of six mice. Asterisk shows significant difference from the control group by Student's t-test (*p <0.05, **p < 0.01, ***p < 0.001).

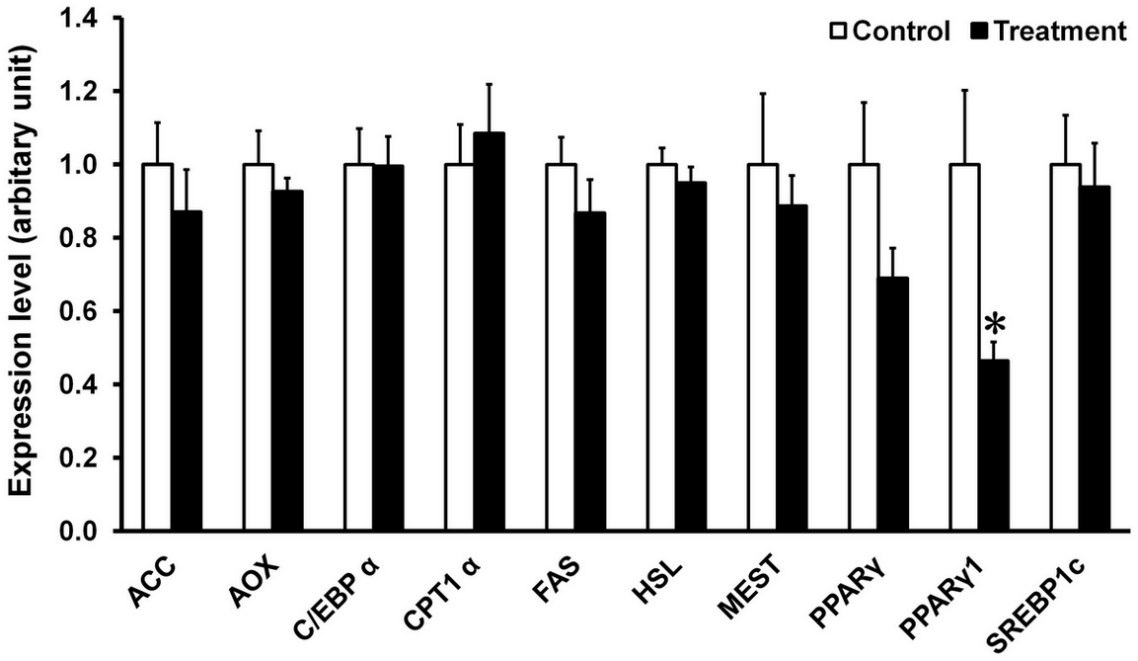

Figure 1 Effects of IGH administration on mRNA level of lipid metabolism-related genes in epididymal adipose tissue. C57BL/6J mice were fed HFD (control) or HFD with 2 % IGH (treatment) for 4 weeks. Values are mean ± S.E. of six mice. Asterisk shows significant difference between the control group and the treatment group by Student's t-test at $*p < 0.05$.

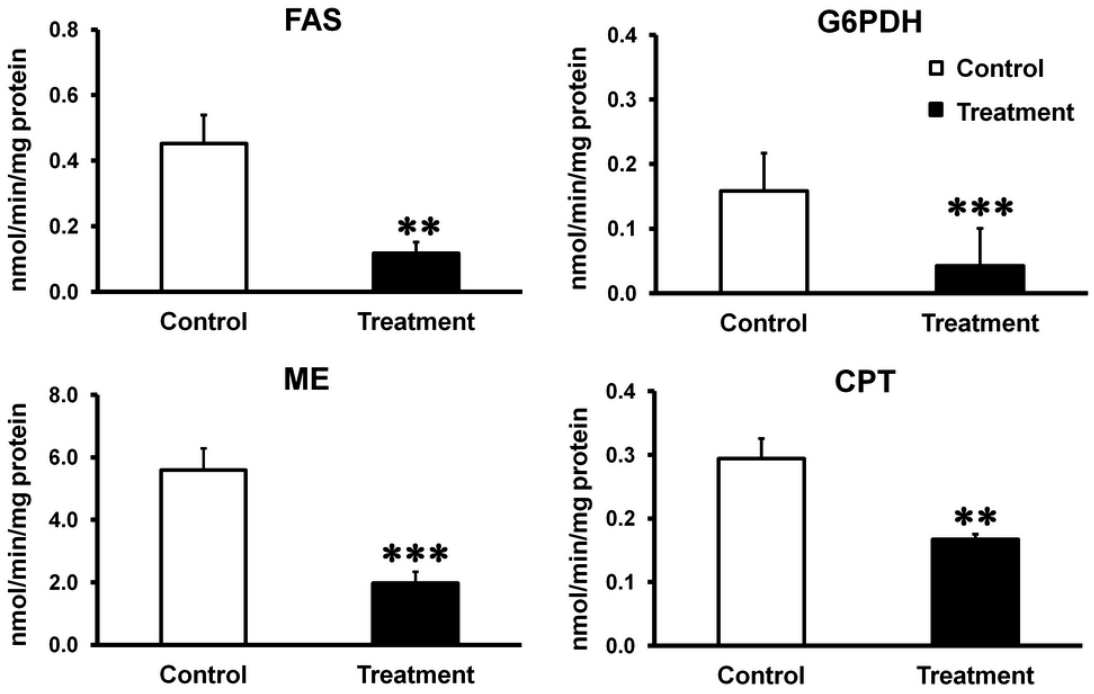

Figure 2 Effects of IGH administration on the activity of lipid metabolism-related enzymes in liver tissue. C57BL/6J mice were fed HFD (control) or HFD with 2 % IGH (treatment) for 4 weeks. Values are mean ± S.E. of six mice. Asterisk shows significant difference between the control group and the treatment group by Student's t-test at $^{**}p < 0.01$, $^{***}p < 0.001$.

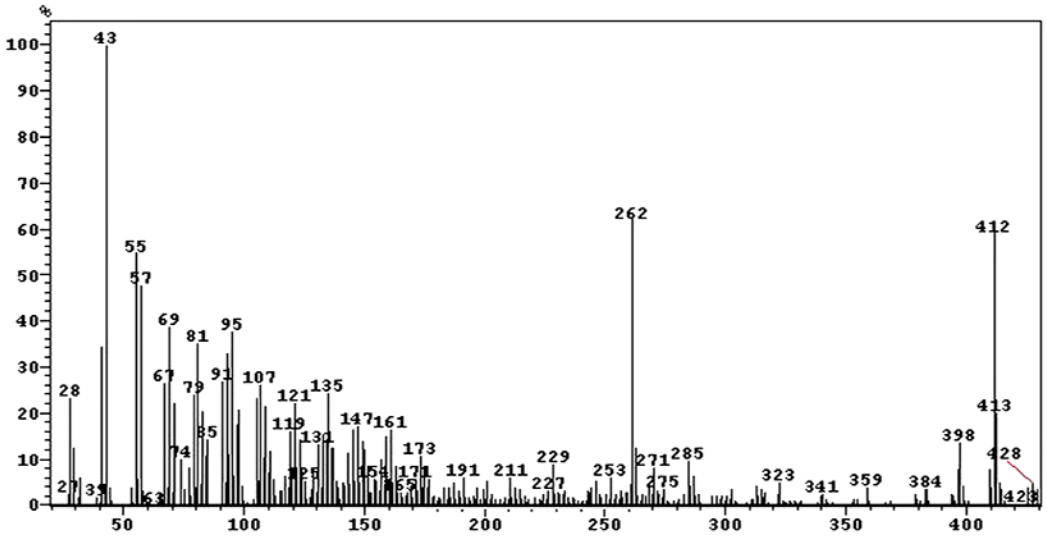

Figure 3 Mass spectrum of the IGH-derived steroid sample. The occurrence of sterol with molecular MS of 412 or 428 was detected. Its fragment peak also appears at 262. The Y axis = % relative abundance whereas X axis = mass-to-charge ratio (m/z).

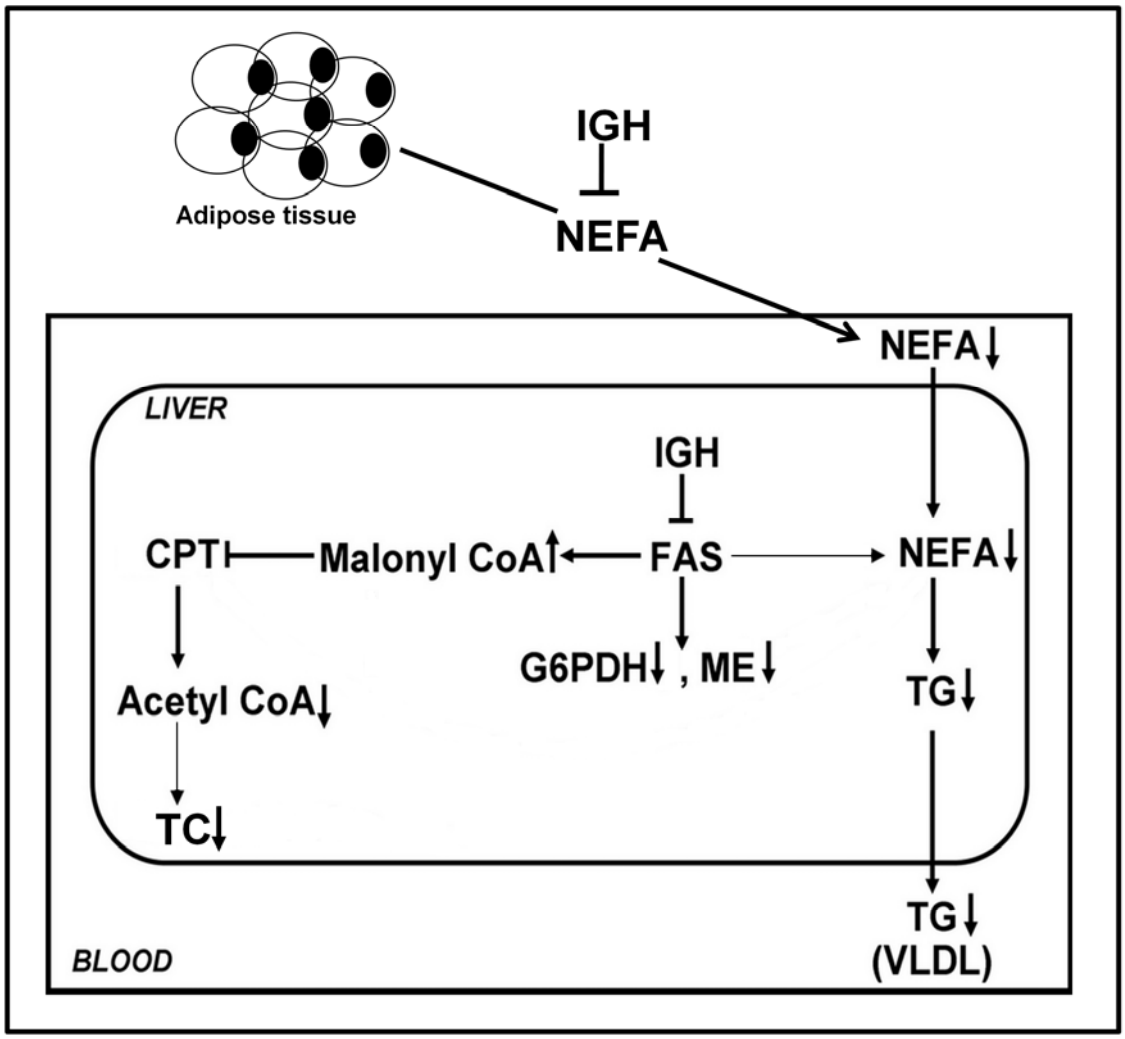

Figure 4 Proposed mode of action of IGH on lipid metabolism in C57BL/6J mouse fed HFD. The active constituents in IGH decrease serum NEFA level by attenuating degradation of fat in its depots. As a result, fatty acid supply for hepatic TG-VLDL production becomes limited and serum TG level drops. IGH also exerts inhibitory action on hepatic FAS which synthesizes fatty acid from condensation of acetyl CoA with malonyl CoA. FAS inhibition would contribute to a decrease in newly synthesized fatty acids for TG assembly. G6PDH and ME activities also decrease in response to a lower requirement of NADPH, whereas CPT (CPT1) is inhibited upon the accumulation of its physiological inhibitor, malonyl CoA [40]. A decrease in acetyl CoA concentration in the liver from impaired fatty acid degradation caused by inhibited CPT activity has been expected. This would probably also affect *de novo* cholesterol biosynthesis pathway. In addition, hepatic TC level declines mainly as a result of the increased disposal of cholesterol *via* bile excretion.

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List of Publications and Proceedings

Publications

Bunkrongcheap, R., Hutadilok-Towatana, N., Noipha, K., Wattanapiromsakul, C., Inafuku, M. and Oku, H. 2014. Ivy gourd (*Coccinia grandis* L. Voigt) root suppresses adipocyte differentiation in 3T3-L1 cells. Lipid Health and Disease. 13:88.

Bunkrongcheap, R., Inafuku, M., Oku, H., Hutadilok-Towatana, N., Wattanapiromsakul, C., and. Sermwittayawong, D. 2016. Lipid-Lowering Effects of Hexane Fraction of Ivy Gourd (*Coccinia grandis* L. Voigt) Root in Mice Fed a High-Fat Diet. Walailak Journal of Science and Technology. 13, xxx-xxx. (accepted)

Proceedings (Abstract)

1. Bunkrongcheap, R., Hutadilok-Towatana, N., Noipha, K., 2011. Ivy gourd (*Coccinia grandis* L. Voigt) extract inhibits fat accumulation in 3T3-L1 cells. The 3rd BMB International Conference, 6-8 April 2011, The Empress Convention Centre, Chiang Mai, Thailand.

2. Bunkrongcheap, R., Hutadilok-Towatana, N., Wattanapiromsakul, C., Noipha, K. and Oku, H., 2013. Ivy gourd (*Coccinia grandis* L. Voigt) inhibits adipocyte differentiation in 3T3-L1 cells. Joint meeting Japan society of nutrition and food science Kyushu & Okinawa branch, 18-19 October 2013, Faculty of Agriculture, Kyushu University, Fukuoka, Japan.
3. Bunkrongcheap, R., Inafuku, M., Oku H., Hutadilok-Towatana, N. and Wattanapiromsakul C., 2014. Lipid-Lowering Effects of Ivy Gourd (*Coccinia grandis* L. Voigt) Root in Mice Fed a High-Fat Diet. The 2014 Tokyo International Conference on Engineering and Applied Sciences, 17-19 December 2014, Toshi Center Hotel, Tokyo, Japan.