WALAILAK JOURNAL
http://wjst.wu.ac.th Health Sciences

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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Received: 14 September 2015, Revised: 27 November 2015, Accepted: 16 December 2015

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 suggest that IGH may have the 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 the 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 "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 the 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 2 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 a 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 an 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 the 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 2-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 a 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 an 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 a Step One Plus™ Real-Time PCR System (Applied Biosystems, USA) with the following conditions: one cycle of 95 °C for 20 s, 40 cycles of 95 °C for 3 s and 60 °C for 30 s. A melting curve analysis was performed starting at 95 °C for 15 s, 60 °C for 60 s and increasing by 0.3 °C every 15 s 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 $\pm$ 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 $p < 0.05$.

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'

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*.

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 6 mice. BW = body weight

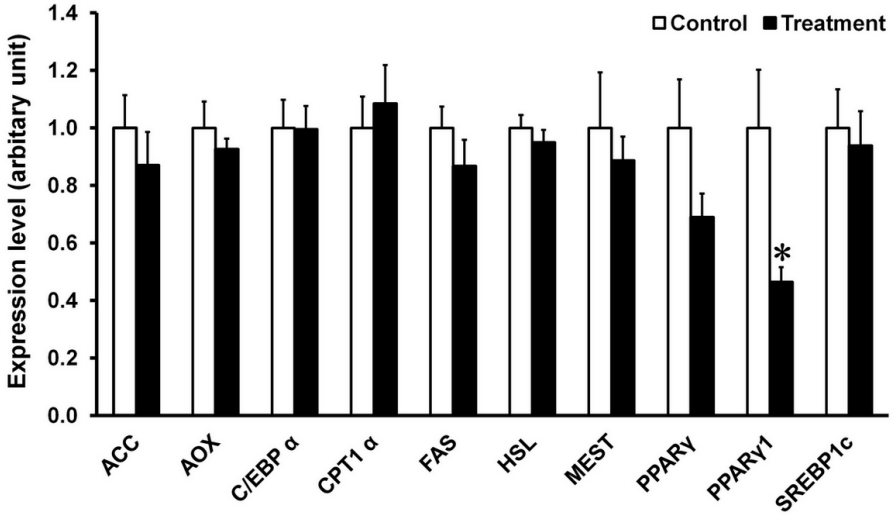

Figure 1 Effects of IGH administration on mRNA level of lipid metabolism-related genes in epididymal adipose tissue. C57BL/6J mice were fed HFD (control) or HFD with 2 % IGH (treatment) for 4 weeks. Values are mean ± S.E. of 6 mice. Asterisk shows significant difference between the control group and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

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 a 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 a 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 3 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 ivy gourd 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.

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 6 mice. Asterisk shows significant difference from the control group by Student's t-test (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

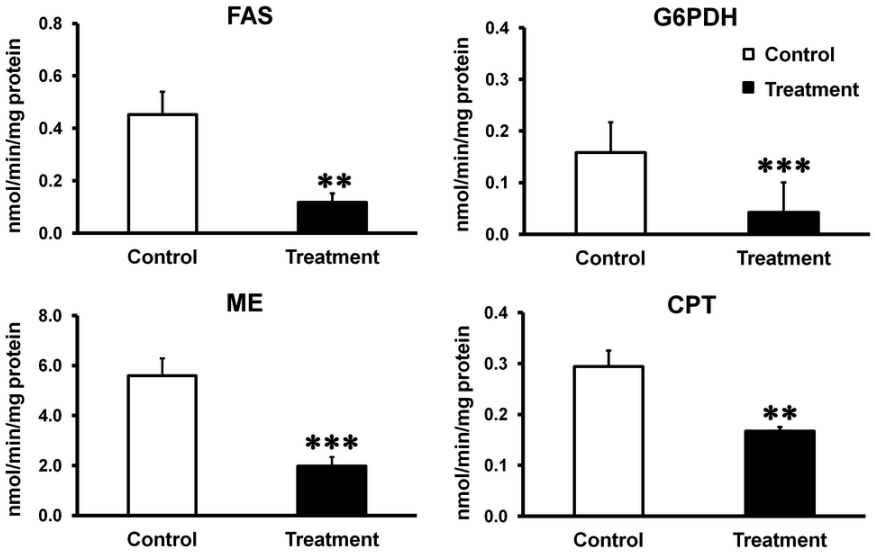

Figure 2 Effects of IGH administration on the activity of lipid metabolism-related enzymes in liver tissue. C57BL/6J mice were fed HFD (control) or HFD with 2 % IGH (treatment) for 4 weeks. Values are mean ± S.E. of 6 mice. Asterisk shows significant difference between the control group and the treatment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

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, 2 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.

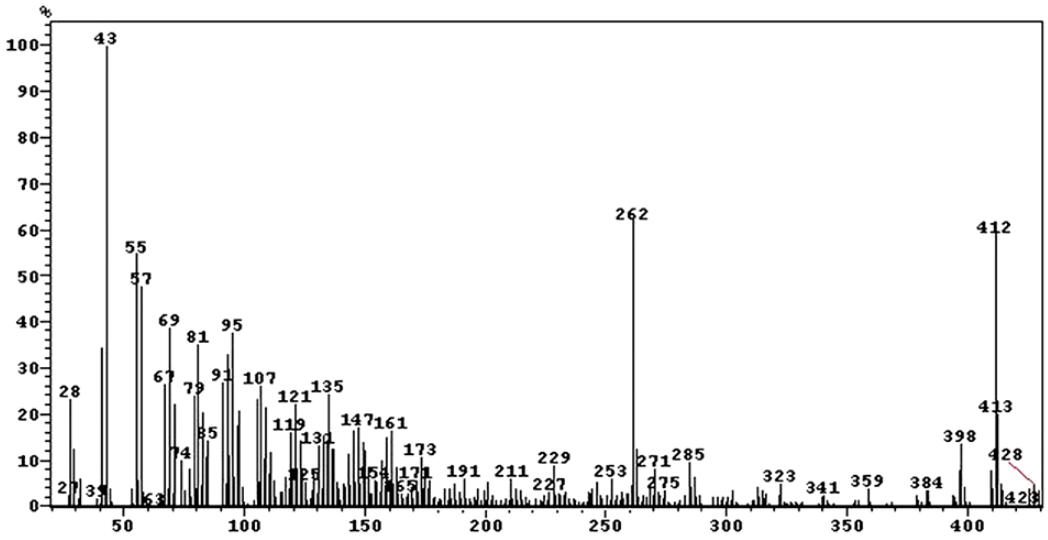

Figure 3 Mass spectrum of the IGH-derived steroid sample. The occurrence of sterol with molecular MS of 412 or 428 was detected. Its fragment peak also appears at 262. The Y axis = % relative abundance whereas X axis = mass-to-charge ratio (m/z).

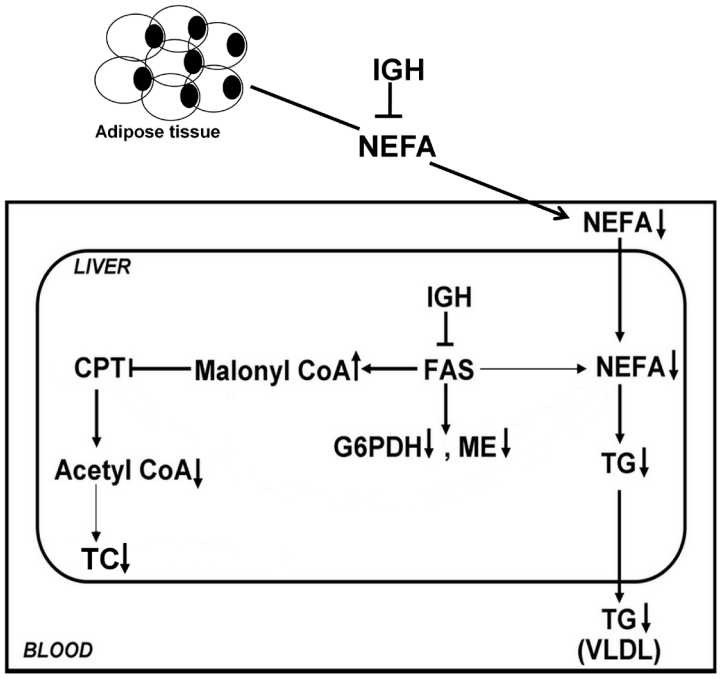

Figure 4 Proposed mode of action of IGH on lipid metabolism in C57BL/6J mouse fed HFD. The active constituents in IGH decrease serum NEFA level by attenuating degradation of fat in its depots. As a result, fatty acid supply for hepatic TG-VLDL production becomes limited and serum TG level drops. IGH 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.

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.

Acknowledgements

This work was supported by Thailand's Commission on Higher Education through the Strategic Scholarships Fellowships Frontier Research Network (Specific for Southern Region) Program, and the Higher Education Research Promotion and National Research University Project of Thailand, Prince of Songkla University. The authors thank Mr. Supphawut Benjakul for collecting ivy gourd roots and Ms. Khanitta Panjapheree for preparing the plant samples. We would also like to express our gratitude to Dr. Alexandra Surcel at the Johns Hopkins School of Medicine, USA, for her comments and suggestions that greatly improved the manuscript.

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