

***Ludwigia octovalvis* extract improves glycemic control and memory performance in diabetic mice**

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**Abstract**

*Ethnopharmacological relevance:* *Ludwigia octovalvis* (Jacq.) P.H. Raven (Onagraceae) extracts have historically been consumed as a healthful drink for treating various conditions, including edema, nephritis, hypotension and diabetes.

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*Aim of the study:* We have previously shown that *Ludwigia octovalvis* extract (LOE) can significantly extend lifespan and improve age-related memory deficits in *Drosophila melanogaster* through activating AMP-activated protein kinase (AMPK). Since AMPK has become a critical target for treating diabetes, we herein investigate the anti-hyperglycemic potential of LOE.

*Materials and methods:* Differentiated C2C12 muscle cells, HepG2 hepatocellular cells, streptozotocin (STZ)-induced diabetic mice and high fat diet (HFD)-induced diabetic mice were used to investigate the anti-hyperglycemic potential of LOE. The open field test and novel object recognition test were used to evaluate spontaneous motor activity and memory performance of HFD-induced diabetic mice.

*Results:* In differentiated C2C12 muscle cells and HepG2 hepatocellular cells, treatments with LOE and its active component ( $\beta$ -sitosterol) induced significant AMPK phosphorylation. LOE also enhanced uptake of a fluorescent glucose derivative (2-NBDG) and inhibited glucose production in these cells. The beneficial effects of LOE were completely abolished when an AMPK inhibitor, dorsomorphin, was added to the culture system, suggesting that LOE requires AMPK activation for its action in vitro. In streptozotocin (STZ)-induced diabetic mice, we found that both LOE and  $\beta$ -sitosterol induced an anti-hyperglycemic effect comparable to that of metformin, a drug that is commonly prescribed to treat diabetes. Moreover, LOE also improved glycemic control and memory performance of mice fed a HFD.

*Conclusions:* These results indicate that LOE is a potent anti-diabetic intervention that may have potential for future clinical applications.

Keywords: glucose; insulin;  $\beta$ -sitosterol; AMPK

## **1. Introduction**

An aging population is rapidly becoming a pressing issue for contemporary human society. Age-related diseases, such as metabolic syndrome, neurodegenerative diseases and cancer, pose enormous healthcare challenges and socioeconomic burdens. Dietary restriction (DR) is known to extend lifespan across species and to delay the onset of a variety of age-related diseases. However, it is impractical for humans

to adhere to a restricted diet for prolonged periods. Therefore, medications that mimic the effect of DR are attractive as preventive or therapeutic avenues for treating age-related morbidities (Lopez-Lluch and Navas, 2016).

*Ludwigia octovalvis* (Jacq.) P.H. Raven (Onagraceae) is a flowering plant widely distributed in tropical areas around the world, and extracts from *L. octovalvis* (LOE) have traditionally been consumed as a healthful drink for treating various conditions, including edema, nephritis, hypotension and diabetes (Kadum Yakob et al., 2015; Ramirez et al., 2012). Our previous study showed that LOE and one of its active ingredients,  $\beta$ -sitosterol, significantly extended the lifespan of fruit flies (*Drosophila melanogaster*) through activating AMPK (Lin et al., 2014). The effect is minimized in flies fed on a low-calorie diet, suggesting that LOE acts in a manner similar to that of DR. Unlike DR, however, flies treated with LOE do not exhibit reduced fecundity or other negative tradeoffs. LOE also attenuates age-related cognitive decline, both in flies and in senescence-accelerated-prone 8 mice (Lin et al., 2014). In order to further explore the translational potential of LOE, we began the present study by demonstrating the effect of LOE and  $\beta$ -sitosterol on AMPK activation and glucose handling in mammalian cellular models. AMPK is a major cellular energy sensor and metabolic regulator, and is considered to be a therapeutic target for treatment of diabetes mellitus (Coughlan et al., 2014). Indeed, metformin, one of the most frequently used drugs for diabetes, is an AMPK activator (Hardie et al., 2012; Zhou et al., 2001). We therefore examined the effect of LOE on both streptozotocin (STZ)-induced and high-fat diet (HFD)-induced diabetic mice, representing type 1 and type 2 diabetic models, respectively. Our findings suggest that LOE is a promising candidate for the development of anti-diabetic medications with neuro-protective potential.

## **2. Materials and methods**

### *2.1 LOE preparation and high performance liquid chromatography (HPLC) analysis*

Air-dried whole plants of *L. octovalvis* were obtained from the Hsiehyu Biotech Company Ltd., and a voucher specimen (No. 034) was deposited at the herbarium of Hsiehyu Biotech Company Ltd. LOE was

prepared as previously described (Lin et al., 2014). Briefly, 100 g of *L. octovalvis* powder were soaked in 400 ml of 70% ethanol overnight at 4 °C, and then extracted twice at 55 °C for 30 min. The supernatant was centrifuged (8000 × g for 5 min) and filtered (TOYO No. 1) to remove debris. LOE was obtained following a decompression process. The chemical composition of LOE was analyzed by gas chromatography-mass spectrometry and was reported in the previously study (Lin et al., 2014). The concentration of  $\beta$ -sitosterol ( $1.19 \pm 0.05$  mg/g) was monitored using a HPLC instrument (SYKAM) equipped with a sample injector and a UV detector (205 nm). A C18 (Alltech Alltima™) column (4.6 × 150 mm; 5  $\mu$ m particle size) was used for  $\beta$ -sitosterol analysis and the mobile phase for the HPLC system consisted of 2.5% methanol in HPLC grade water with a constant flow rate set at 1.2 ml/min. Representative HPLC chromatography traces for a  $\beta$ -sitosterol standard solution (Sigma-Aldrich) and a LOE sample are shown in Supplemental Fig. 1.

## 2.2 Cell cultures

Mouse muscle cells (C2C12) and hepatocellular cells (HepG2) were obtained from the Cell Bank of the Taiwan National Health Research Institute. C2C12 cells were grown as myoblasts in culture medium containing Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin G, and 100  $\mu$ g/ml streptomycin. C2C12 myoblasts were then cultured in DMEM medium containing 2% horse serum to promote differentiation and fusion into myotubes, as described previously (Chou et al., 2013). HepG2 cells were grown at 37 °C in DMEM medium (Gibco), supplemented with 10% FBS (Biological Industries). Different concentrations of LOE,  $\beta$ -sitosterol, metformin (Sigma-Aldrich) and/or dorsomorphin (Sigma-Aldrich) were added to culture medium for 12 h. Cells were also incubated in 0.5x medium (1:1 dilution of the 1x medium in phosphate buffered saline, PBS) for 24 h to mimic a DR condition in vitro, as described previously (Lin et al., 2014). A lactate dehydrogenase assay (BioVision) and trypan-blue (Gibco) exclusion were used to monitor the survival of cells.

### *2.3 Western blot analysis*

Cells were lysed in radioimmunoprecipitation assay buffer (Thermo), and proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes (Invitrogen), using standard procedures (Huang et al., 2015). The antibodies used were rabbit anti-phospho-AMPK (1:1000, Cell Signaling), rabbit anti-AMPK (1:500, Abcam) and mouse anti- $\alpha$ -tubulin (1:500, GeneTex). Protein signals were visualized with horseradish peroxidase-conjugated secondary antibodies and ECL reagent (Thermo). The intensity of each target protein band was quantified using Image J software.

### *2.4 Immunostaining and glucose uptake assay*

Differentiated C2C12 cells grown on cell culture chamber slides coated with Matrigel Basement Membrane Matrix (BD Matrigel™) were treated with different concentrations of LOE,  $\beta$ -sitosterol, metformin, or grown under a DR condition (0.5x medium) for 24 h. Immunocytochemistry was performed as described previously (Wang et al., 2007). Cells were stained with primary polyclonal rabbit anti-Glut4 antibody (Abcam) overnight, and with secondary TRITC-conjugated goat anti-rabbit IgG (Abcam) for 1 h. Nuclei were labeled with 4',6-diamidino-2-phenylindole (DAPI, The Jackson Laboratory), and images were captured using a Leica TCS SP5 confocal microscopy system (Leica) in the Imaging Core Laboratory of the Medical College, National Taiwan University. For the glucose uptake assay, drug-treated cells were exposed to 100  $\mu$ g/ml 2-(N-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)Amino)-2-Deoxyglucose (2-NBDG) dissolved in glucose-free, phenol red-free DMEM, for 20 min. Cells were then washed in PBS, and the fluorescence of 2-NBDG was measured with a microplate reader at excitation and emission wavelengths of 485 nm and 535 nm, respectively.

### *2.5 Glucose production assay*

HepG2 cells were treated with different concentrations of LOE,  $\beta$ -sitosterol, metformin, or were grown under a DR condition (0.5x medium) for 24 h. Afterwards, cells were exposed to the treatments described above in glucose-free, phenol red-free DMEM (Gibco), supplemented with 20 mM sodium lactate (Sigma-Aldrich) and 2 mM sodium pyruvate (Sigma-Aldrich), for 24 h. The cells were lysed in 0.5% Triton-X in PBS, and glucose concentration was measured by a glucose assay kit (Sigma-Aldrich).

### *2.6 Animals and STZ-induced hyperglycemia*

All experimental protocols for animals followed the local animal ethics regulations and were approved by the National Taiwan University College of Medicine and College of Public Health Institutional Animal Care and Use Committee. Six to eight week-old male C57BL/6 mice were obtained from the National Taiwan University College of Medicine Laboratory Animal Center, and maintained in an animal room with controlled temperature (22–24 °C) and humidity (50–55%), under a 12 h light/dark cycle. All mice were fed ad libitum with powdered AIN-93 G diet (MP Biomedicals) or HFD, supplemented with various concentrations of LOE,  $\beta$ -sitosterol or metformin (Table 1). Food intake, water consumption and change in body weight of animals were monitored regularly. For STZ-induced hyperglycemia, mice were injected intraperitoneally with freshly prepared STZ (60 mg/kg in 0.01 M citrate buffer) daily for 5 days. Glucose levels were measured from tail blood samples, using a blood glucose meter (ACCU-CHEK Active, Roche).

### *2.7 Glucose tolerance test (GTT)*

GTTs were performed after 8 weeks of LOE,  $\beta$ -sitosterol or metformin treatment. Mice were fasted for 6 h and blood glucose concentration was measured at 0, 30, 60, 90, 120, and 180 min following intraperitoneal injection of glucose (2 g/kg). Area under the curve for the GTT was calculated using the trapezoidal rule.

### *2.8 Behavioral tests*

The open field test was performed in an open box (40 cm × 40 cm × 40 cm). Each mouse was placed in the center of the open field and was allowed to freely explore the apparatus for 30 min. Moving direction and travel distance for each mouse were recorded by automated observation (Singa technology). Immediately after the open field test, mice were exposed two similar objects, and the time spent exploring each object was recorded during the subsequent 5-min period (defined as the training session for the novel object recognition test). The mice were then returned to their home cage. Twenty-four hours later, the same animals were re-tested for 5 min in the box with a familiar and a novel object (defined as the memory test session). The object discrimination index was calculated by subtracting the time spent on exploring a familiar object from the time spent on exploring a novel object, and dividing by total time spent exploring both objects. A higher score for the discrimination index indicates better memory performance in object recognition.

### **3. Results**

#### *3.1 LOE activates AMPK and enhances glucose uptake in C2C12 muscle cells*

To investigate the glycemic regulatory effect of LOE, we began by evaluating the function of LOE on muscle cells in vitro. The viability of differentiated C2C12 muscle cells was unaffected by treatment for 24 h with different concentrations of LOE,  $\beta$ -sitosterol, metformin or by growth under a DR condition (0.5x medium), compared to controls (Fig. 1A). Higher doses of LOE and  $\beta$ -sitosterol significantly enhanced AMPK phosphorylation, mimicking levels induced by treatments under a DR condition (0.5x medium) or metformin (Figs. 1B and 1C). These results are consistent with our previous study showing that LOE and  $\beta$ -sitosterol induce AMPK phosphorylation in *Drosophila* S2 cells (Lin et al., 2014). Importantly, this effect was ameliorated by co-administration of dorsomorphin, an AMPK inhibitor (Figs. 1D and 1E). AMPK activation in muscle cells induces translocation of glucose transporter type 4 (Glut4) to the plasma membrane to facilitate glucose uptake (Hardie et al., 2012; Richter and Hargreaves, 2013). Indeed, in contrast with vehicle-treated cells, we detected strong Glut4 immunoreactivity in the plasma

membrane of C2C12 cells following 24 h of LOE treatment (Fig. 1F). Moreover, LOE,  $\beta$ -sitosterol and metformin all dramatically increased the uptake of the fluorescent glucose derivative, 2-NBDG (Fig. 1G). Glut4 translocation and 2-NBDG uptake were blocked when dorsomorphin was also present in the culture medium (Figs. 1F and 1G). These results strongly suggest that LOE may regulate glucose uptake in muscle cells through AMPK activation.

### *3.2 LOE activates AMPK and attenuates glucose production in HepG2 cells*

In HepG2 cells, we also demonstrated that the viability of cells was not jeopardized by DR, LOE,  $\beta$ -sitosterol, or metformin treatment, respectively (Fig. 2A). AMPK phosphorylation was induced by higher doses of LOE and  $\beta$ -sitosterol, analogous to our findings in C2C12 cells, and this effect was abolished by co-administration of dorsomorphin (Figs. 2B-2E). As for the higher dose of metformin, LOE exhibited an inhibitory effect on glucose production in HepG2 cells, an effect that was also prevented by co-administration of dorsomorphin (Fig. 2F). However,  $\beta$ -sitosterol inhibiting glucose production in these cells (Fig. 2F).

### *3.3 LOE improves glycemic control in STZ-induced diabetic mice*

To test the effect of LOE on systemic glucose homeostasis, the STZ-induced diabetic mouse model was used. These mice exhibit polyphagia, polydipsia, poor body weight gain, fasting hyperglycemia, hypoinsulinemia, and impaired glucose tolerance (Figs. 3A-3G), mimicking human type 1 diabetes. Treatment with either LOE or  $\beta$ -sitosterol led to partial improvement in polyphagia, polydipsia, hyperglycemia and glucose tolerance, similar to improvements observed in metformin-treated STZ mice (Figs. 3B-3D and 3F-3G). Unlike some anti-diabetic drugs that act as insulin secretagogues, our treatments did not result in increased plasma insulin levels (Fig. 3E).

### *3.4 LOE improves glycemic control and memory performance in HFD-induced diabetic mice*

We next tested the effects of LOE in the HFD-induced diabetic mouse model, which exhibits exaggerated body weight gain, fasting hyperglycemia, and impaired glucose tolerance (Figs. 4A and 4D-4F),



simulating human type 2 diabetes. LOE treatment corrected glucose intolerance and fasting hyperglycemia, but did not significantly affect food intake, water consumption or body weight in these mice (Figs. 4A-4C).

Neurologic function is frequently impaired in patients with type 2 diabetes, thus we assessed motor and memory performance in these mice. Open field tests showed no significant difference in spontaneous motor activities among controls and HFD mice with or without LOE treatment (Figs. 5A and 5B). Control mice showed normal memory retention 2 h after training on the novel object recognition test, with a significant decline 24 h after training (Figs. 5C and 5D). However, HFD mice already exhibited diminished memory performance 2 h after training compared to controls, and this deficit was rescued by prior LOE treatment (Figs. 5C and 5D). Furthermore, the memory decay 24 h after training that was observed in control and HFD mice was abolished by LOE treatment (Figs. 5C and 5D).

#### **4. Discussion**

Our study provides *in vitro* and *in vivo* evidence for an anti-diabetic action of LOE. Liver and muscle are the major metabolic tissues involved in glucose homeostasis, and are responsible for glucose production and utilization, respectively. Diabetes mellitus is characterized by increased hepatic glucose production, as well as by impaired Glut4 glucose receptor translocation to the plasma membrane and decreased glucose uptake in muscle cells (Musi and Goodyear, 2006; Zierath et al., 1996). Both are counteracted by LOE in a cell-autonomous manner in our *in vitro* cellular models. In HepG2 cells, LOE activates AMPK, which is known to suppress expression of rate-limiting enzymes of hepatic gluconeogenesis, i.e., glucose-6-phosphatase and phosphoenolpyruvate carboxykinase (Foretz et al., 2005). This likely contributes to the reduced glucose production observed in our study. Indeed, inhibition of AMPK blocked the effect of LOE on glucose production. Glucose uptake into skeletal muscle can be either AMPK- or insulin-dependent. The former usually occurs in a starved state or during muscle contraction, while the latter occurs in the fed state (Hardie et al., 2012; Musi and Goodyear, 2006). From our standpoint, the effect of LOE on muscle cells appears to mimic the effect of DR or exercise, both of which are beneficial to multiple

aspects of physiology beyond glycemic control. Therefore, additional medical applications of LOE are worth future exploration.

In line with our previous report on the effect of LOE on *Drosophila* S2 cells (Lin et al., 2014), the present study indicates that evolutionarily conserved AMPK mediates, at least in part, the actions of LOE in various cell types. In addition, we have demonstrated that  $\beta$ -sitosterol, one of the active ingredients of LOE, contributes to AMPK activation in these cells. It is possible that AMPK in different tissues works coordinately in response to intrinsic (e.g., FGF21) or extrinsic (e.g., LOE) factors to regulate systemic glucose metabolism (Salminen et al., 2017). Our cellular and animal studies fit well with this picture, since LOE-treated diabetic mice exhibit improvements in both baseline glycemia and response to glucose loading. In addition to  $\beta$ -sitosterol, LOE is rich in polyphenols that may also contribute to glycemic control, and will be worthy of future investigation (Bozzetto et al., 2015; Lin et al., 2014; Tresserra-Rimbau et al., 2016).

Although AMPK activation was thought to be responsible for the metformin-induced inhibition of hepatic glucose production (Zhou et al., 2001), it has been shown that metformin is able to inhibit glucose production in AMPK-deficient hepatocytes, and there is evidence that metformin can act to suppress hepatic gluconeogenesis by inhibiting mitochondrial glycerophosphate dehydrogenase (Madiraju et al., 2014). Likewise, although we have demonstrated that LOE induces AMPK phosphorylation in cellular models and attenuates hyperglycemia in diabetic mice, we cannot exclude the possibility that they exert the anti-hyperglycemic effect through pathways independent of AMPK. AMPK activation can be achieved through diverse mechanisms (Hardie et al., 2012; Hawley et al., 2010), and the precise steps in LOE activation of AMPK are not known. Many phytochemicals such as resveratrol activate AMPK through inhibition of mitochondrial ATP synthesis, presumably as a strategy for plants to deter pathogens or herbivores (Hawley et al., 2010). Whether this is the case for LOE remains to be determined.

Obesity is an important risk factor for diabetes mellitus, and control of body weight ameliorates insulin resistance (Musi and Goodyear, 2006). It is plausible that LOE alters the whole-body energy balance

through AMPK activation, thereby inducing a change in body weight (Lopez, 2017). However, the body weight trajectories of STZ-induced and HFD-induced diabetic mice showed no significant differences between LOE-treated and control groups during the experimental period, suggesting that the effects of LOE on glucose disposal are largely independent of body weight regulation.

Patients with diabetes mellitus are at increased risk for a variety of neurologic complications, ranging from peripheral neuropathy to cognitive impairment (Gaspar et al., 2016; Mayeda et al., 2015; Stoeckel et al., 2016). Therefore, it would be a great benefit for anti-diabetic treatment to also alleviate accompanying neurologic problems (Hasanvand et al., 2016). Relevant studies have been limited, and have given conflicting results. For instance, metformin was found to be detrimental to the spatial memory of old male mice in one study (Thangthaeng et al., 2017), whereas it prevented HFD-induced decline in spatial memory in another study (Allard et al., 2016). In addition, metformin use was reported to be associated with an increased risk of cognitive impairment in diabetic patients (Moore et al., 2013). Although the issue remains controversial, LOE is promising in this regard, since in the mouse model, it rescued the HFD-induced memory decline seen 2 h after training, and enhanced memory performance 24 h after training. Further studies are needed to confirm these findings.

## **5. Conclusions**

Our data indicate that LOE modulates AMPK signaling in multiple tissues and improves systemic glucose homeostasis. Furthermore, it exhibits memory-enhancing effects in diabetic mice. Overall, these findings suggest that LOE is a promising nutraceutical for diabetic care.

### **Author's contributions**

Conceived and designed the experiments: WSL, SZF, JHY, PYW. Performed the experiments and analyzed the data: JHL, JHY, HWW, PYW. Funding acquisition: WSL, SZF, PYW. Wrote the paper: WSL, PYW.

### **Conflict of interest**

None declared.

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### **Abbreviations**

LOE, *Ludwigia octovalvis* extract; AMPK, AMP-activated protein kinase; STZ, streptozotocin; HFD, high fat diet; DR, dietary restriction; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; Glut4, glucose transporter type 4; 2-NBDG, 2-(N-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)Amino)-2-Deoxyglucose; GTT, Glucose tolerance test; HPLC, high performance liquid chromatography

### **Appendix A. Supplementary material**

Supplementary data associated with this article can be found in the online version at [doi:10.1016/j.jep.2017.06.044](https://doi.org/10.1016/j.jep.2017.06.044).

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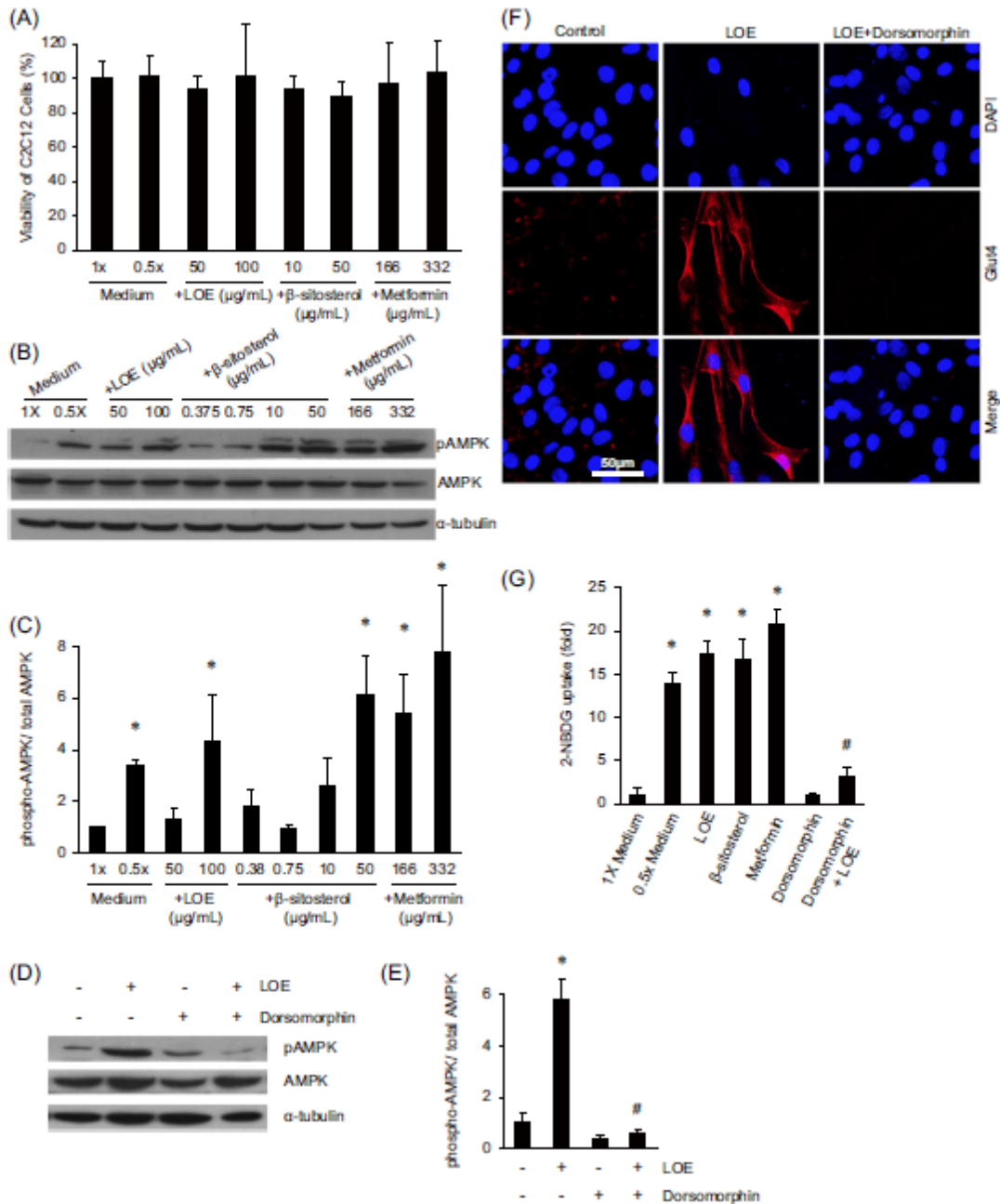
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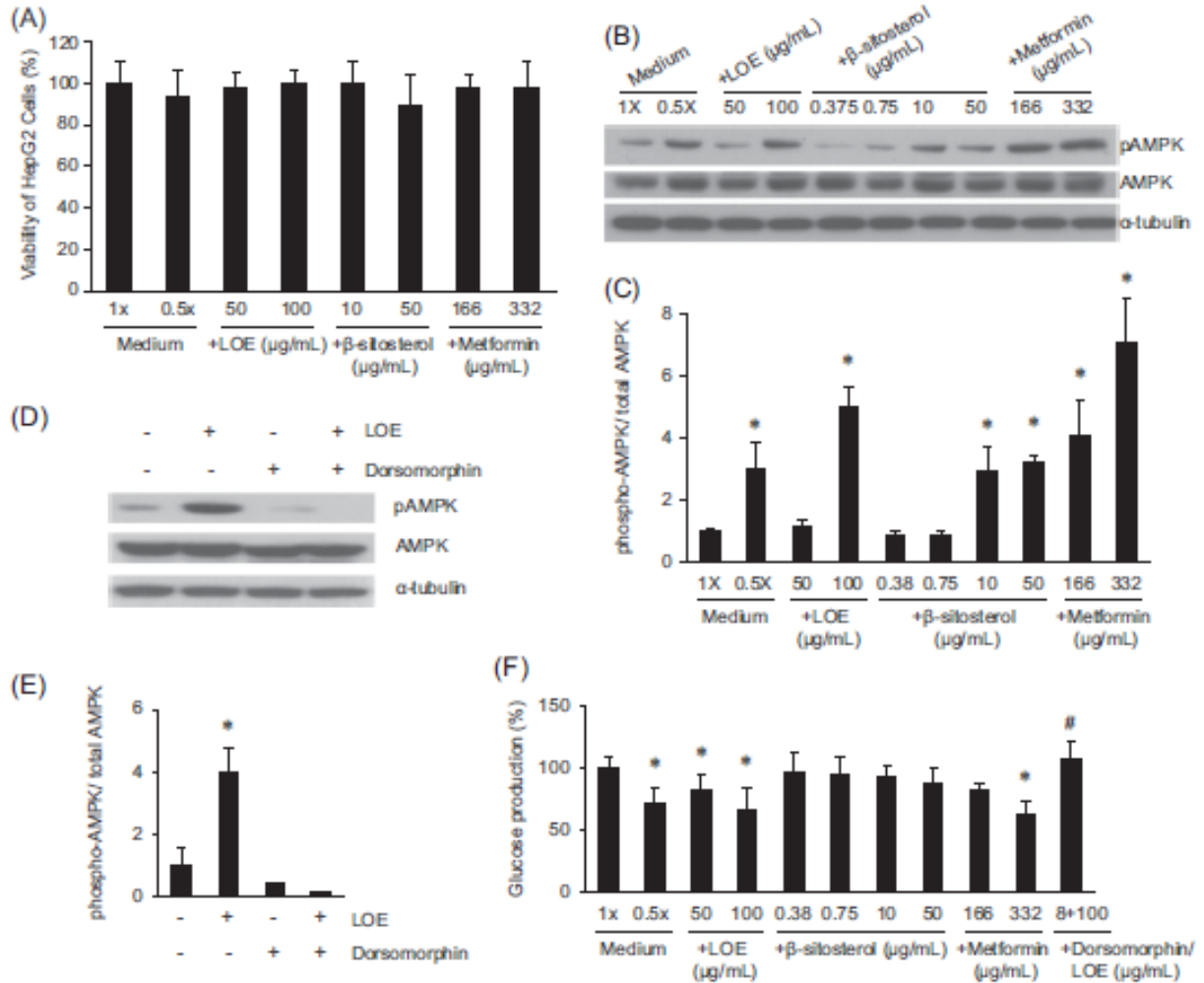
Table 1. Dietary composition of mice receiving different drug treatments.

Composition (g/ kg)	Group					
	Control	LOE	$\beta$ -sitosterol	Metformin	HFD	HFD+ LOE
Casein lactic	200	200	200	200	200	200
Soy oil	70	70	69.96	70	–	–
Lard	–	–	–	–	300	300
Solka floc-40	50	50	50	50	50	50
Dextrin	132	132	132	132	132	132
Granular sugar	100	100	100	100	100	100
AIN-93 mineral mix	35	35	35	35	35	35
AIN-93 vitamin mix	10	10	10	10	10	10
L-Cystine	3	3	3	3	3	3
Choline bitartrate	2.5	2.5	2.5	2.5	2.5	2.5
LOE	–	0.1	–	–	–	0.1
$\beta$ -sitosterol	–	–	0.04	–	–	–
Metformin	–	–	–	0.3	–	–

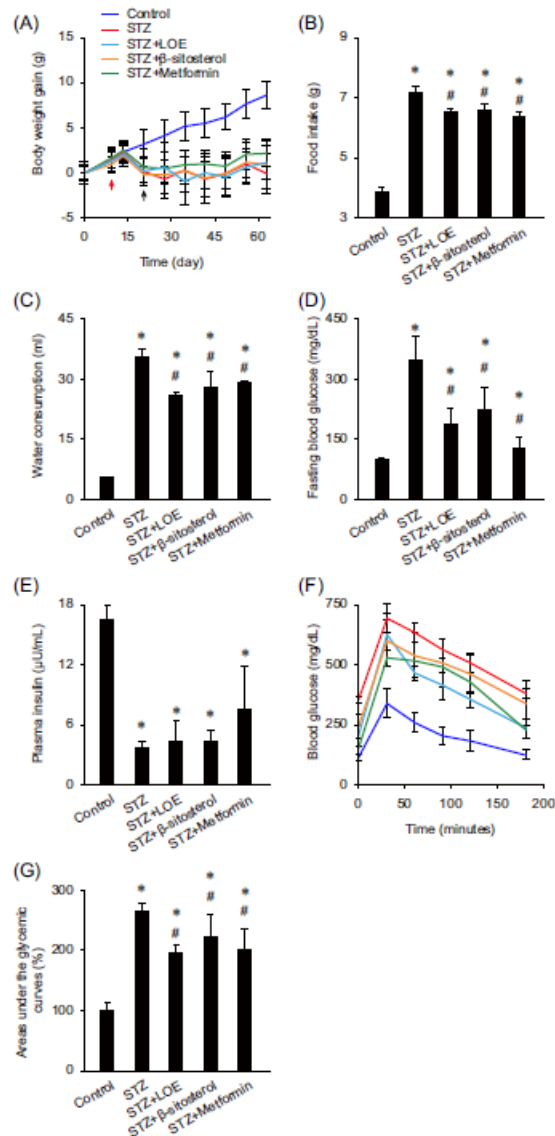
**Figure 1.** LOE and  $\beta$ -sitosterol induce AMPK phosphorylation and enhance glucose uptake in C2C12 muscle cells. (A) LOE,  $\beta$ -sitosterol and metformin treatment do not affect the viability of C2C12 muscle cells. (B and C) Representative Western blot and quantitative results show that LOE and  $\beta$ -sitosterol can induce AMPK phosphorylation. Reduced nutrients (0.5x medium) and metformin are known to activate AMPK, and are included as positive controls. (D and E) Representative Western blot and quantitative results show that LOE-induced AMPK phosphorylation is attenuated by an AMPK inhibitor (dorsomorphin). (F) LOE-induced glucose transporter type 4 (Glut4) translocation to the plasma membrane is blocked by dorsomorphin. (G) LOE-induced glucose uptake is blocked by dorsomorphin. Data are presented as mean  $\pm$  SD ( $n = 3$  for each group). \*  $p < 0.05$  compared to 1x medium or vehicle controls; #  $p < 0.05$  compared to LOE by one-way ANOVA with Fisher's LSD post hoc test.



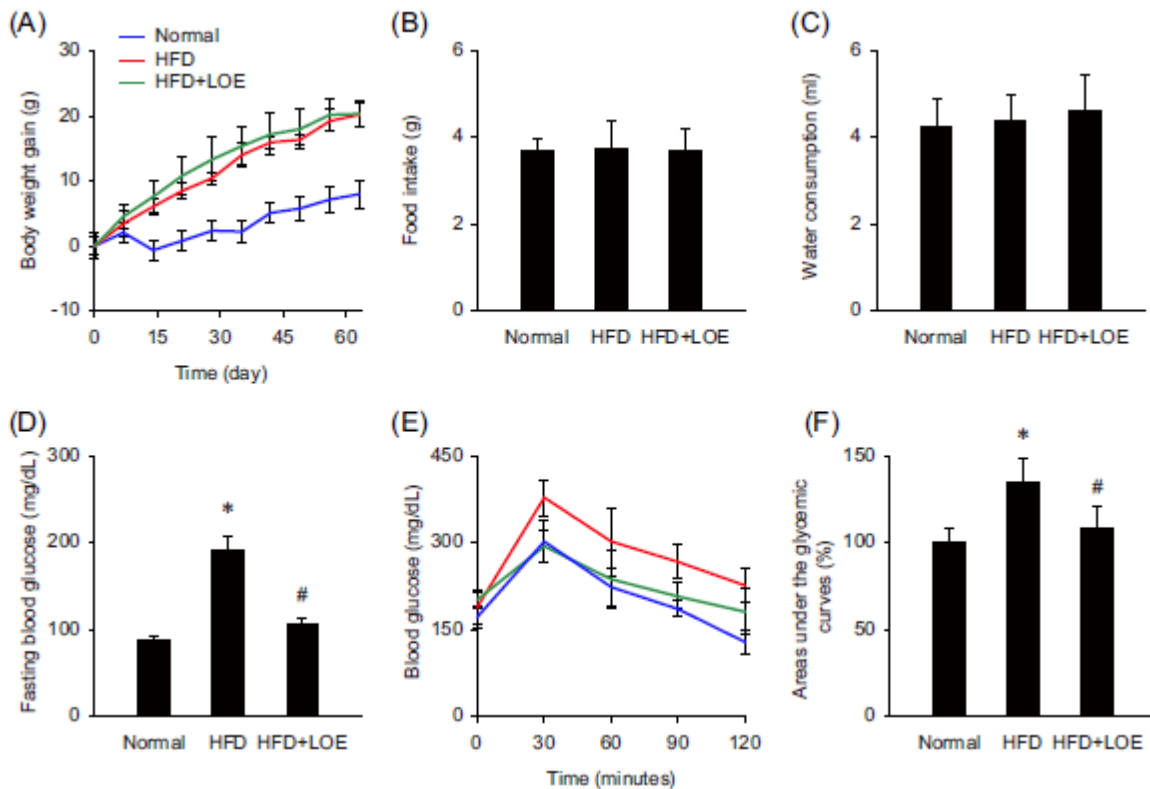
**Figure 2.** LOE and  $\beta$ -sitosterol induce AMPK phosphorylation and reduce glucose production in HepG2 cells. (A) LOE and  $\beta$ -sitosterol do not affect the viability of HepG2 cells. (B and C) Representative Western blot and quantitative results show that LOE and  $\beta$ -sitosterol can induce AMPK phosphorylation. Reduced nutrients (0.5x medium) and metformin are known to activate AMPK and are included as positive controls. (D and E) Representative Western blot and quantitative results show that LOE-induced AMPK phosphorylation can be attenuated by an AMPK inhibitor (dorsomorphin). (F) LOE-induced reduced glucose production can be blocked by dorsomorphin. Data are presented as mean  $\pm$  SD (n = 3–9 for each group). \* p < 0.05 compared to 1x medium or vehicle controls; # p < 0.05 compared to 100  $\mu$ /ml LOE by one-way ANOVA with Fisher's LSD post hoc test.



**Figure 3.** Anti-hyperglycemic activity of LOE and  $\beta$ -sitosterol on STZ-induced diabetic mice. (A) Body weight gain of normal control and STZ-induced diabetic mice with or without LOE or  $\beta$ -sitosterol added to the food. Metformin is an oral antidiabetic drug and is included as a positive control. Red arrow and black arrow indicate the time points of STZ injection and beginning of drug treatments, respectively. (B and C) LOE,  $\beta$ -sitosterol and metformin significantly reduce food intake and water consumption of STZ-induced diabetic mice. (D) LOE,  $\beta$ -sitosterol and metformin significantly reduce fasting blood glucose of STZ-induced diabetic mice after 6 weeks of drug treatments. (E) Fasting plasma insulin levels are not affected after 6 weeks of drug treatments. (F and G) Plasma glucose levels are measured during the GTT test in different groups of mice. The area under the glycaemic curves is significantly lower after 6 weeks of LOE,  $\beta$ -sitosterol and metformin treatments compared to STZ group. Data are presented as mean  $\pm$  SD (n = 8–9 for each group). \*  $p < 0.05$  compared Control; #  $p < 0.05$  compared to STZ by one-way ANOVA with Fisher's LSD post hoc test.



**Figure 4.** Anti-hyperglycemic activity of LOE on HFD-induced diabetic mice. (A) Body weight gain of normal control and HFD-induced diabetic mice with or without LOE added to the food. (B and C) LOE does not affect food intake and water consumption of HFD-induced diabetic mice. (D) LOE significantly reduces the fasting blood glucose of HFD-induced diabetic mice after 6 weeks of treatment. (E and F) Plasma glucose levels are measured during the GTT test in different groups of mice. The area under the glycemic curves is significantly lower after 6 weeks of LOE treatment compared to HFD group. Data are presented as mean  $\pm$  SD (n = 8–9 for each group). \* p < 0.05 compared Control; # p < 0.05 compared to STZ by one-way ANOVA with Fisher's LSD post hoc test.



**Figure 5.** LOE enhances recognition memory of HFD-induced diabetic mice. (A and B) Representative moving path (A) and total travel distance (B) during the 30 min open field test is shown. HFD and LOE treatment do not affect general locomotor activity of mice. (C and D) The short-term (2 h after training) and long-term (24 h after training) recognition memory of mice was assessed using the novel object recognition (NOR) test. The time mice spent on both novel and familiar objects is shown in (C), and the discrimination index is presented in (D). LOE treatment significantly enhances both short-term and long-term recognition memory of HFD-induced diabetic mice. Data are presented as mean  $\pm$  SEM (n = 5 for each group). \*p < 0.05 compared to 2 h normal control among 3 different dietary groups; # p < 0.05 compared to 24 h normal control among 3 different dietary groups; + p < 0.05 comparison between 2 and 24 h in each dietary group by two-way ANOVA with Fisher's LSD post hoc test.

