Methanol extraction fraction from *Citrus Sudachi* peel exerts lipid reducing effects in cultured cells

(スダチ果皮由来画分による細胞内脂質を減らす薬効メカニズムの研究)

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Introduction

Diabetes mellitus is characterized by elevated blood glucose levels. The estimated number of people with diabetes worldwide in 2017 is 425 million, the number is expected to increase to 642 million by 2040. Type 2 diabetes (T2D), characterized as multiple organ (e.g., muscle, liver, and adipose) insulin resistance (IR), makes up about 90% of cases of diabetes.

Recent research showed that increasing ectopic triglyceride (TG) accumulation was associated with IR and T2D. Adipose tissue is well known lipid storage, but has a limited maximal capacity to store lipids. Once beyond this limitation, excess fatty acids would spill over into the blood, lead to their ectopic storage in the tissues which normally contain only small amount of fat, such as the liver, skeletal muscle, heart, and pancreas[1]. Ectopic fat accumulation induces metabolic processes disruption and organ function impaired[2].

Plants are abundant in biologically active molecules that play critical roles in pharmacolog[3]. Most of the available therapeutic agents that widely used for treating diabetes, natural antidiabetic products which are believed to have minimal side effects have been highlighted for the treatment of diabetes[4, 5]. *Citrus sudachi* is an evergreen tree that was found mainly in Tokushima Prefecture of Japan, and it has been demonstrated that *Citrus sudachi* had the capability of inhibiting the rising trend of blood glucose and fatty acid in human subjects[6, 7],though its mechanism has not been clarified yet.

AMPK (AMP-activated protein kinase) is a fuel-sensing enzyme that plays a crucial role

in response to starvation by increasing AMP/ATP ratio. AMPK activated by phosphorylation of the threonine residue (Thr172) on its catalytic α-subunit, which initiates processes such as fatty acid oxidation that restore ATP, and inhibits ATP consumption such as triglyceride and protein synthesis, cell proliferation[8]. It is known that AMPK have effect on carbohydrate and lipid metabolism, it is already clear that the system is a major player in the development and/or treatment of obesity, diabetes, and other metabolic syndrome[9].

Peroxisome proliferator-activated receptors (PPARs) belongs to a subgroup in the family of nuclear hormone receptors and highly expressed in the most of metabolic tissues. PPARα was the first discovered PPARs and is known to contribute to oxidative processes including fatty acid metabolism[10]. PPARα exists in skeletal muscles and liver, which is closely correlated with fatty acid oxidation[11]. PPARα has been suggested as an important therapeutic target for metabolic diseases with hyperlipidemia [12].

1 Methods and Materials

1-1 Materials

We purchased AICAR, BML-275 and Gw6471 from Funakoshi Co., Ltd. (Tokyo, Japan), respectively. Other reagents were obtained from Kanto Chemical (Tokyo, Japan), Wako Pure Chemical (Osaka, Japan), or Tokyo Chemical Industry (Tokyo, Japan). The following commercially available antibodies were used: anti-AMPK (Sigma-Aldrich, St Louis, MO, USA); anti-sirt1 (Cell Signaling Technology Japan, K.K.); anti-Phospho-

AMPKα (Thr172) (Cell Signaling Technology Japan, K.K.); anti-β-actin, as a loading control, from Cell Signaling Technology Japan, K.K.; anti-GAPDH, as a loading control, from Wako Pure Chemical Industries, Ltd..

1-2 The extract process of sudachi peel

The extract process of *Citrus sudachi* peel was shown in Figure1. The sudachi peel dry powders were extracted with hexane at room temperature for 24hr, the residue was extracted with methanol again at the same condition, hexane extract part and methanol extract part were separated. The methanol extract was concentrated to a residue which was partitioned between hexane and 90% methanol. The 90% methanol-soluble fraction was chromatographed over Diaion HP 20 column, eluted with methanol and H_2O solution, and then gradient and removing the sugar dissolved in water. 5 fractions named by hydrophobicity from M-F1 to M-F5 were obtained at last.

In order to screen the function of Sudachi peel extracts, the extracts were dissolved in dimethyl sulfoxide (DMSO), made to different concentration.

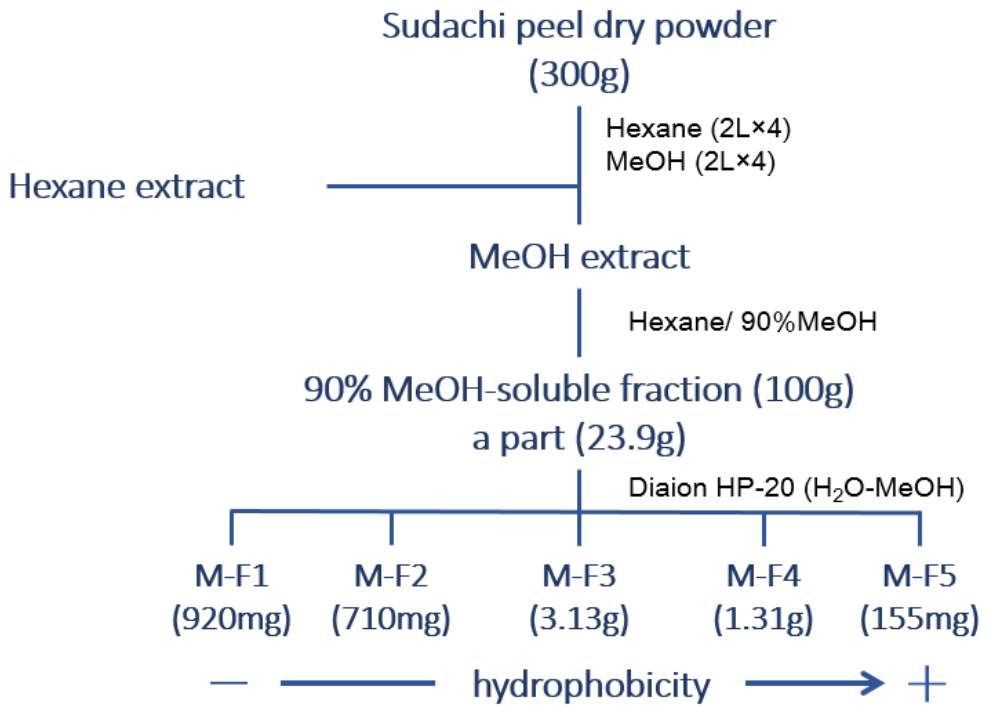


Figure 1: The extraction and separation process of *Citrus sudachi* peels

1-3 Cell culture and treatment

Cell culture and differentiation was conducted as reported previously[13]. C2C12 myoblasts, which are derived from mouse thigh muscle cell, were obtained from American Type Culture Collection. The C2C12 myoblasts were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 4.5 g/L D-(+)-Glucose (nacalai tesque), supplemented with 10% (v/v) fetal bovine serum in a humidified atmosphere with 5% CO_2, and grown in DMEM supplemented with 2% horse serum for 1 week to be induced differentiation into myotubes.

1-4 Triglyceride and non-essential fatty acid measurement

Following to the stimulation, lipids were extracted with 2-propanol at 4°C overnight, and concentrations of triglyceride (TG) and NEFA (non-essential fatty acid) were determined by Triglyceride E-test Kit and NEFA C-test Kit (Wako Pure Chemical, Osaka,

Japan) according to the manufacturer's instruction, respectively.

1-5 Oil Red O staining

Cells were fixed with 10% formalin for 1 h, rinsed with PBS for 2 times, and stained with 0.5% Oil Red O dye for 20 minutes. After washing again with PBS, cells were visually monitored by OLYMPUS microscopic observation.

1-6 Western blotting

After the stimulations, cells were washed with ice-cold PBS and lysed with lysis buffer (500 mM Tris-HCl, 10 mM NaCl, 1 mM $MgCl_2$, 250 mM sucrose, 0.5% NP-40, 0.1 mM EGTA) as previously reported[14-17]. Protein expression and phosphorylation was detected by western blot analysis. Equal amounts of proteins were separated by 7.5% SDS-PAGE, and electrotransferred to PVDF membranes (Millipore, USA), and then were blocked with PBS solution with 1% Tween20 and 5% skim milk for 1 hr at room temperature. Membranes were incubated with appropriately diluted primary antibodies overnight at 4°C. Then incubated with the relevant secondary antibodies for 2 hr at room temperature. The immune complexes were visualized with the enhanced chemiluminescence (ECL) detection system according to the manufacturer's instructions, in conjunction with ImageQuant LAS 4000 luminescent image analyzer (GE Healthcare, USA). Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH) or β-actin was served as an internal control protein.

1-7 Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted from cells and was reverse-transcribed using the Superscript II^{TM} First Strand Kit (Invitrogen). We evaluated the mRNA levels of peroxisome

proliferator-activated receptor alpha (PPARα) , sterol regulatory element-binding protein 1 (SREBP1) , peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC1α) , carnitine palmitoyltransferase 1b (CPT1b) , acyl-coenzyme A oxidase (Aco) , medium chain acyl-coenzyme A dehydrogenase (MCAD) , mitochondrial transcription factor A (mtTFA) , uncoupling protein 2 (UCP2) , and housekeeping gene GAPDH, primer sequences are shown in Table1. Real-time PCR was performed on an ABI PRISM 7500 PCR System (Applied Biosystems, USA) using THUNDERBIRD SYBR qPCR Mix (TOYOBO, Japan). PCRs were carried out in a total of 20 µL as follows: one cycle at 95 °C for 1min, followed by 40 cycles of 95 °C for 15 sec, 65 °C for 30 sec and 72 °C for 40 sec. The gene expression from each sample was analyzed in duplicates and normalized against GAPDH. The results are expressed as relative gene expression using the ΔC_t method.

GENE	FORWARD PRIMER	REVERSE PRIMER
PPARA	AGGCTGTAAGGGCTTCTTTCG	GGCATTTGTTCCGGTTCTTC
SREBP1	GGCTATTCCGTGAACATCTCCTA	ATCCAAGGGCATCTGAGAACTC
PGC1A	TTCCACCAAGAGCAAGTAT	CGCTGTCCCATGAGGTATT
CPT1B	CGGAAGCACACCAGGCAGTA	GCAGCTTCAGGGTTTGTCGGAATA
ACO	GCCGTCGAGAAATCGAGAACT	TCTTAACAGCCACCTCGTAACG
MCAD	GAGCCTGGGAACTCGGCTTGA	GCCAAGGCCACCGCAACTTT
MTTFA	GAAGGGAATGGAAAGGTAGA	AACAGGACATGGAAAGCAGAT
UCP2	CTACAAGACCATTGCACGAGAGG	AGCTGCTCATAGGTGACAAACAT
GAPDH	AACACTGAGCATCTCCCTCA	GTGGGTGCAGCGAACTTTAT

Table1: **Sequences of the primers used in real-time PCR**

1-8 Evaluation of viability

The cell viability was evaluated by MTT ((3-(4,5-di-methylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay as previously described [18, 19].

1-9 Statistical Analysis

All the data are shown as the means ± standard deviation (SD). Student's t-test was performed to determine the difference between two groups, and one-way ANOVA and post-hoc Tukey-Kramer test were performed to evaluate multiple groups. $P<0.05$ was considered statistically significant.

2 Result

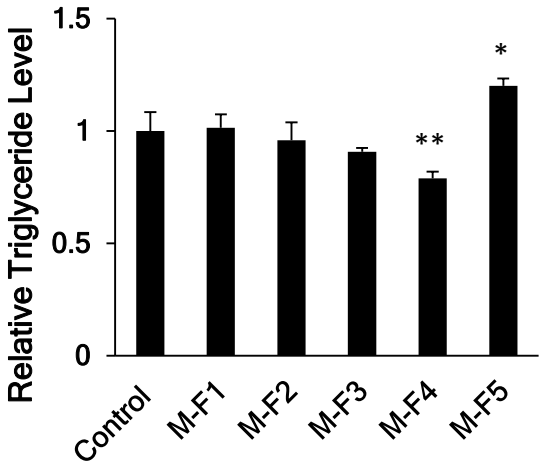
2-1 The Crude sudachi peel and M-F4 reduce intracellular triglyceride level of C2C12 cell

Our previous study demonstrated that the peel dry powders of *Citrus sudachi* had the capability of inhibiting the rising trend of blood glucose and fatty acid in human. This result probably caused by increasing TG consumption in blood.

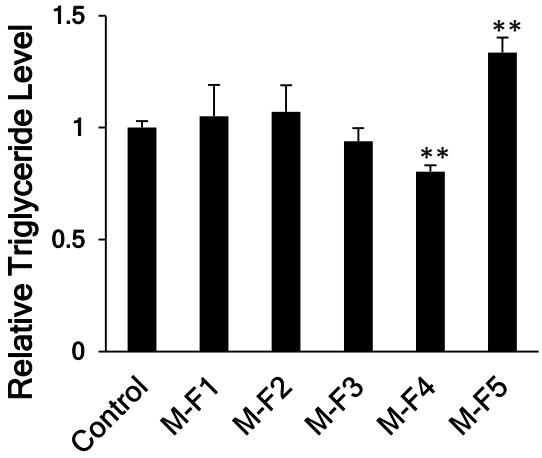
We supposed that if the sudachi peel extract could reduce intracellular TG level in C2C12 cells.

We try to find out which fraction of this crude extract cause this result. C2C12 myoblast were stimulated by 5 kinds of sudachi peel methanol extracts (M-F1~M-F5) (100 μg/mL) for 24 hours, only M-F4 obviously down-regulated TG level (Fig 2A). The similar results were also observed from C2C12 myotubes treated with sudachi peel methanol extracts (Fig 2B). Consistent with TG level, the neutral lipids, cholesterol esters, and triglycerides, staining in C2C12 myotubes which treatment with M-F4 (Fig 2C-b) was lower than the control group (Fig 2C-a). We purposed to investigate the mechanism of this effect.

(A)



(B)



(C)

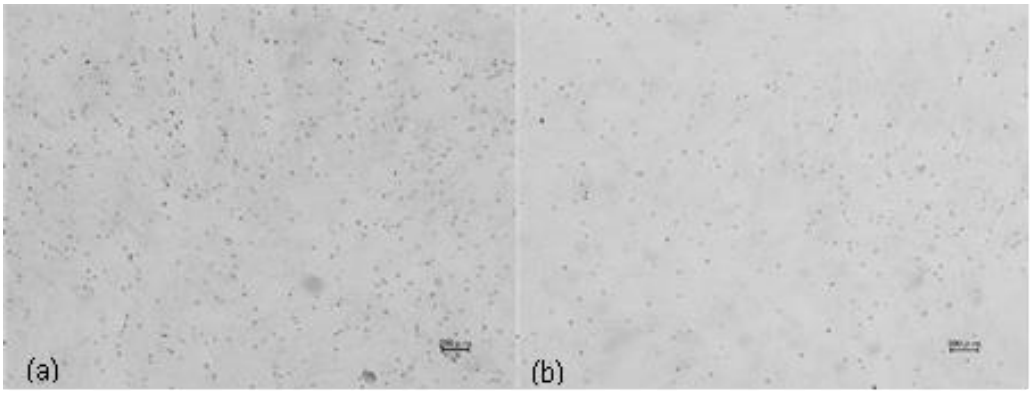


Figure 2 : **Crude sudachi peel extraction and M-F4 reduced intracellular triglyceride level in C2C12.**

Crude sudachi peel extract reduced intracellular triglyceride level in C2C12 myoblasts (A) and myotubes (B) were stimulated by 5 kinds of extract (100 μg/mL) for 24 hr. The Oil Red O positive lipid storage in C2C12 myotubes which treatment with M-F4 (C-b) was lower than the control group (C-a). Data are expressed as the mean±SD. **:p < 0.05*, ***:p < 0.01* compared to vehicle control .

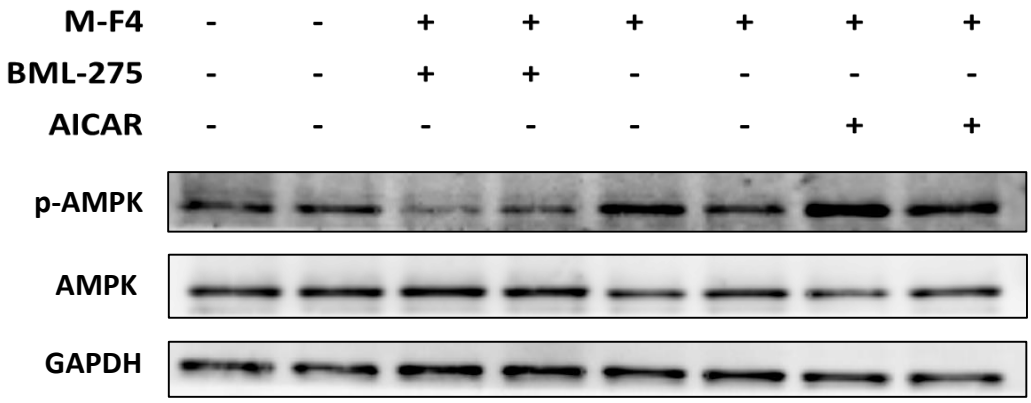
2-2 M-F4 increases AMPK phosphorylation but did not alter Sirt1 expression.

AMPK is a fuel-sensing enzyme that plays a crucial role in TG consumption for energy supply. To elucidate whether M-F4 exert a TG-lowering effect via AMPK, C2C12 myotubes were treated with 100 μg/mL M-F4 for 24 hours and the protein levels were examined firstly. As expected, M-F4 increased the phosphorylation of AMPK in C2C12 myotubes obviously in Western blotting (Fig 3A). BML-275 (compound C) is a potent, selective, and reversible ATP-competitive inhibitor of AMPK[20, 21]. 5-aminoimidazole-4-carboxamide 1-β-D-ribofuranoside (AICAR) used as a positive control of AMPK activation to treatment cells[22]. In absence or presence of the stimulation by 15μM BML-275 and 1mM AICAR, as shown in Fig. 3A the BML-275 prevent the phosphorylation level of AMPK increasing induced by M-F4, and AICAR improve this trend and had a combination impact. The p-AMPK/AMPK radio showed in Fig 3B also determined this opinion. In TG measurement, the TG reducing effect caused by M-F4 also decreasing with BML-275, and increasing by AICAR (Fig. 3C). Those data were consistent with previous western blotting results. In summary, the M-F4 caused TG-lowering effect mediated by AMPK signaling pathway.

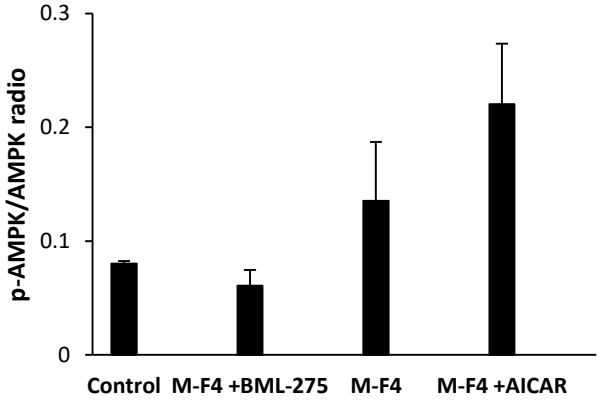
On the contrary, as shown in Figure 3D, the protein expression level of Sirt1 did not significantly increased by M-F4. Sirtinol is a non-specific Sirt1 inhibitors. Same as the treatment by M-F4, sirtinol (25 μM) induced a statistically insignificant trend toward reduced triglyceride (Fig 3E), which means that Sirt1 had no substantive role in

reducing TG. Those datum were consistent with previous western blotting results. In summary, the M-F4 caused TG-lowering effect mediated by AMPK signaling pathway but not involved Sirt1.

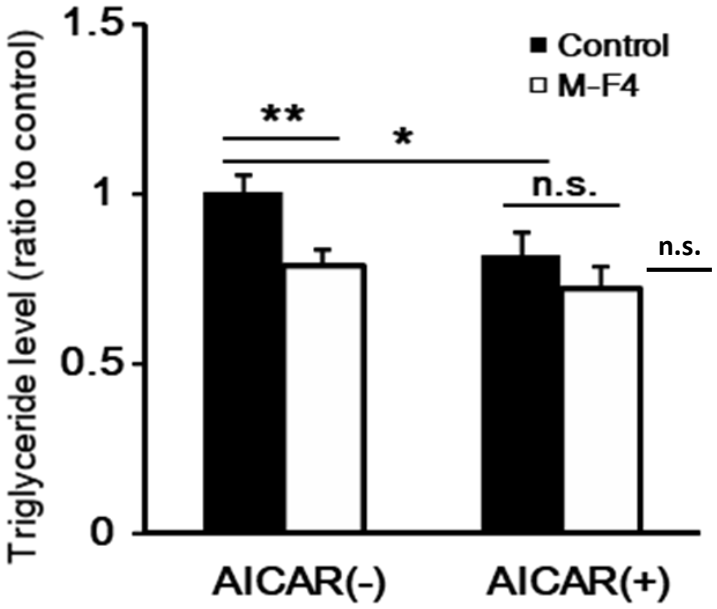
(A)



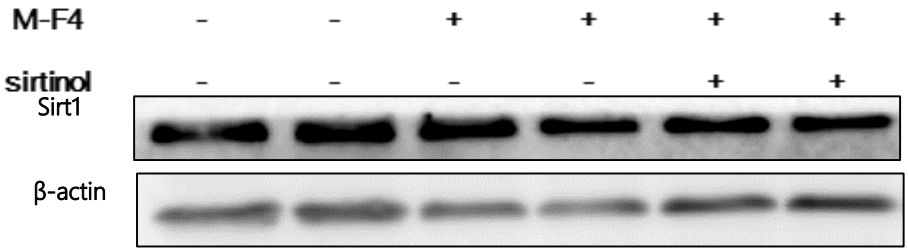
(B)



(C)


Triglyceride level (ratio to control)
1.5
1
0.5
0
Control
M-F4
**
*
n.s.
n.s.
AICAR(-)
AICAR(+)

(D)


M-F4
sirtinol
Sirt1
β-actin

(E)

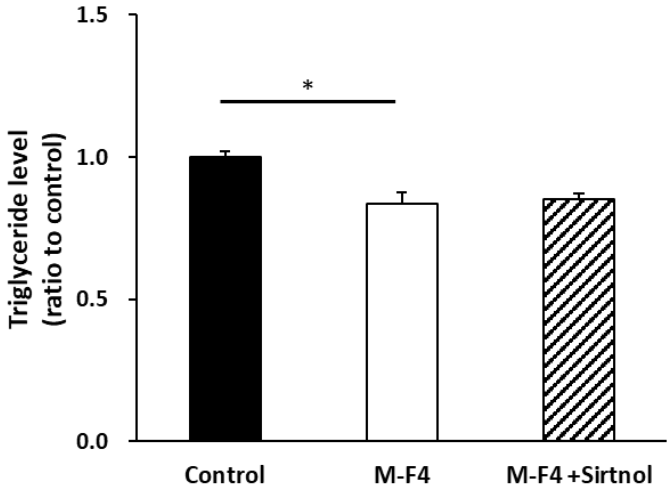

Triglyceride level (ratio to control)
1.5
1.0
0.5
0.0
*
Control
M-F4
M-F4 +Sirtnol

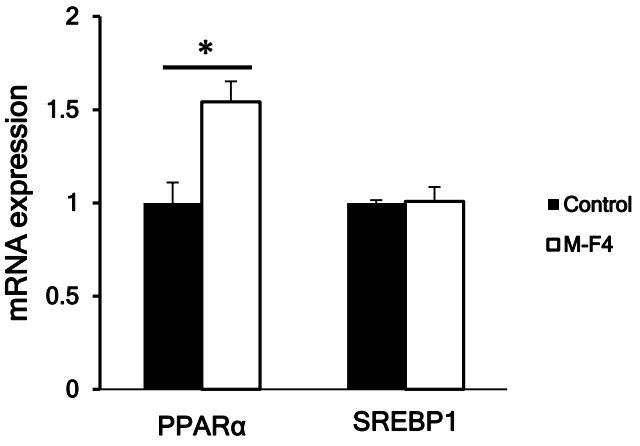
Figure 3: **M-F4 increases AMPK phosphorylation but did not alter Sirt1 expression.**

The protein expression levels (A) and the TG levels (C) in C2C12 myotube which were treated with 100 μg/mL M-F4, and per-stimulated with or without the 15μM BML-275 and 1mM AICAR. Quantified phosphorylation levels of AMPK(B). The protein expression levels (D) and the TG levels (E) in C2C12 myotube which treated with or without 25 μM sirtinol. **; $p < 0.05$, **; $p < 0.01$.* n.s.; not significantly different.

2-3 PPARα was involved in the AMPK signaling pathway which caused the TG-lowering effect by M-F4.

As a master regulator of cellular energy homeostasis, AMPK has a lot of relative substrates. PPARα and SREBP1 are importance targets in lipid metabolism related to AMPK. Overexpression of PPARα in the skeletal muscle increases the expression of genes for fatty acid uptake and oxidation[23]. SREBP-1 is a transcription factor of lipid metabolism involved in the synthesis of fatty acids and cholesterol[24]. Considering whether those two targets were involved in this triglyceride reducing effect, we used the real-time PCR to measure the mRNA level in myotubes pre-treatment with 100 μg/mL M-F4 for 24 hr. In the result, PPARα was increased significantly by M-F4 stimulated. PPARα expression was increased by M-F4, while M-F4 did not change expression levels of SREBP1 (Fig 4A). This triglyceride reducing effect probably mediated by augment of lipid oxidation through PPARα. GW6471 is PPARα specific antagonist[25]. We use Gw6471 as a PPARα inhibitor to treat cells. In the triglyceride measurement, the reducing effect caused by M-F4 was relieve when treated by 25μM Gw6471 (Fig 4B).

(A)



(B)

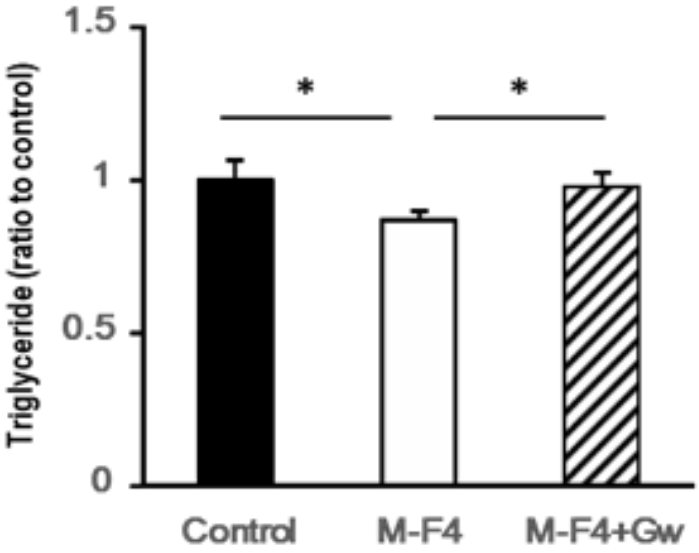


Figure 4: **Effects of M-F4 on mRNA levels in the expression of AMPK-related genes.** Relative mRNA expression of metabolism-related genes in the C2C12 myotubes were treated with M-F4 or vehicle. Gene transcription of PPARα and SREBP1 (A) were normalized for GAPDH. The TG level in C2C12 myotube stimulated by M-F4 with or without Gw6471 (B). Data are expressed as the mean ± SD; n = 3. *$p < 0.05$ and **$p < 0.01$ vs. the control.

2-4 PPARα and its downstream targets mediate the TG and NEFA reducing effect caused by M-F4.

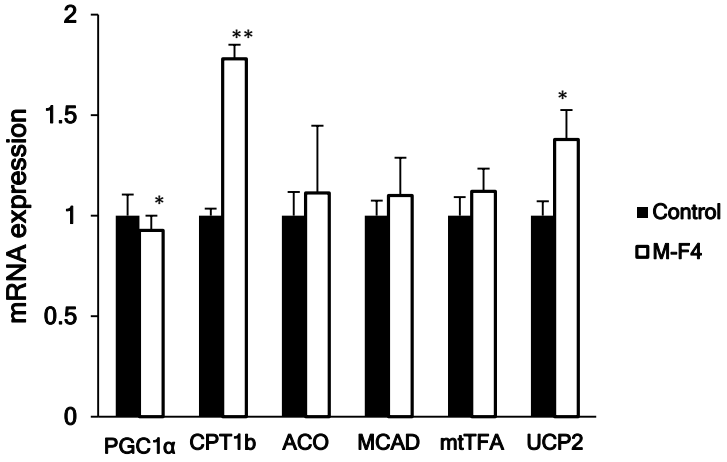
In the PPARα signal pathway, a lot of downstream genes were concerned to be

involved (Fig 5A). PPAR gamma coactivator 1-alpha (PGC-1α) is a regulator of mitochondrial biogenesis and function, but in our experiment, PGC1α were not activated by M-F4. Carnitine palmitoyl transferase1 (CPT1b) which considered to be the gene that controlled fatty acid mitochondrial β-oxidation were mightily activated in 24hr by M-F4 stimulated. Considering the genes involved in lipid oxidation, the expression of ACO, MCAD and mtTFA were slightly increased following M-F4 stimulate, but there were no significant differences. Mitochondrial uncoupling protein 2 (UCP2) gene expression was increased in myotubes of M-F4 treated with statistically significant.

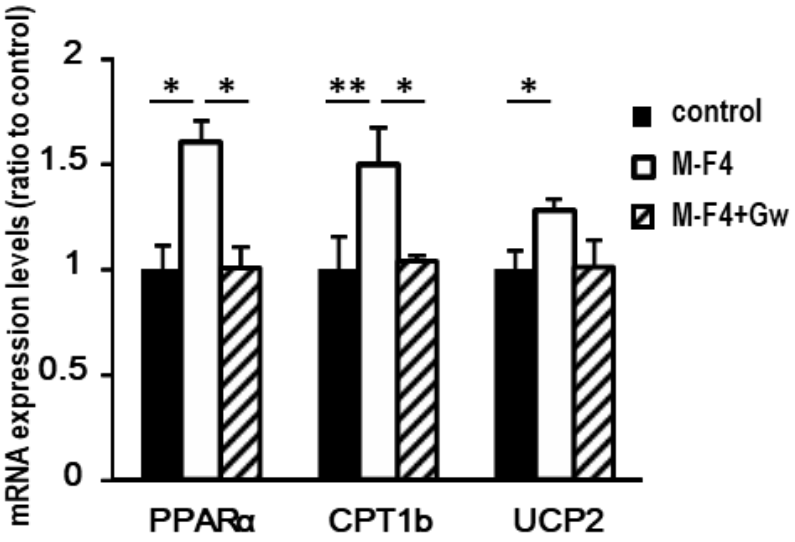
M-F4 led the increasing trends of PPARα, CPT1b and UCP2 were mitigated when treated with Gw6471 (Fig 5B). This result was consistent with our expectation that CPT1b and UCP2 were the downstream targets of PPARα to reduce triglyceride level.

CPT1b is a rate-limiting enzyme on the mitochondrial outer membrane, governing long-chain fatty acid entry into mitochondria. UCPs increase the proton conductance of the mitochondrial inner membrane, eventually promote fatty acid oxidation in the mitochondria. Therefore we conjecture the lipid reducing the effect of M-F4 not only reveal in triglyceride but also in the fatty acid. In figure 5C, we measure the non-essential fatty acid in the myotubes, the result showed that M-F4 assuredly decreased the NEFT level and decreasing tendency alleviated by Gw6471 intervening.

(A)


mRNA expression
2
1.5
1
0.5
0
**
*
*
PGC1α
CPT1b
ACO
MCAD
mtTFA
UCP2
Control
M-F4

(B)


mRNA expression levels (ratio to control)
2
1.5
1
0.5
0
* *
** *
*
PPARα
CPT1b
UCP2
control
M-F4
M-F4+Gw

(C)

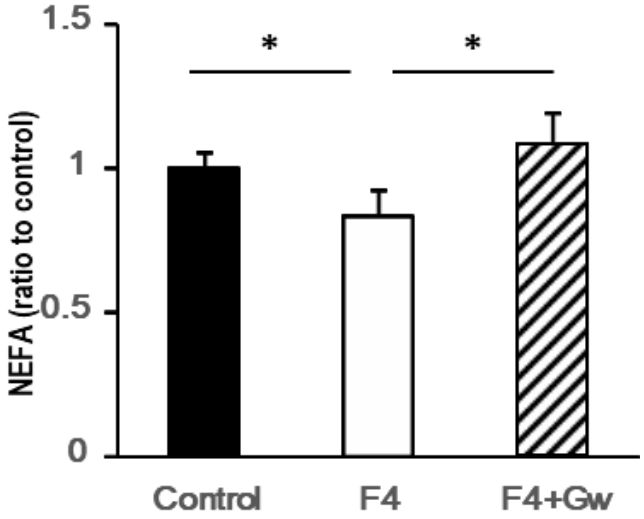


Figure 5: **PPARα and its downstream targets mediate the TG and NEFA reducing effect caused by M-F4.**
(A)Relative mRNA expression of PPARα-related genes in the C2C12 myotubes stimulated by M-F4. C2C12 myotubes were stimulated by M-F4 (100 μg/mL) and treated with or without Gw6471. The mRNA expression (B), and non-esterified fatty acid (C) in the C2C12 myotube stimulated by M-F4 with or without Gw6471 (Gw). Data are expressed as the mean ± SD. **;p < 0.05, **;p < 0.01* vs. the control.

2-5 PPARα was the downstream target of AMPK signal pathway and involved in TG and NEFA reducing effect caused by M-F4.

According to some papers, the relationship between AMPK and PPARα was a kind of complicated. In most cases, AMPK was the upstream target of PPARα, but in some special cases, PPARα was the upstream one. In the WB result, the level of p-AMPK was increased by M-F4 stimulated, and this increasing trend did not change in presence of Gw6471, but reduced in presence of BML-275(Fig 5A). On the other side, the PPARα increasing level induced by M-F4 reduced not only intervened by Gw6471 but also by BML-275. Those results shown that PPARα is the downstream target of AMPK signal pathway in this TG and NEFA reducing effect caused by M-F4.

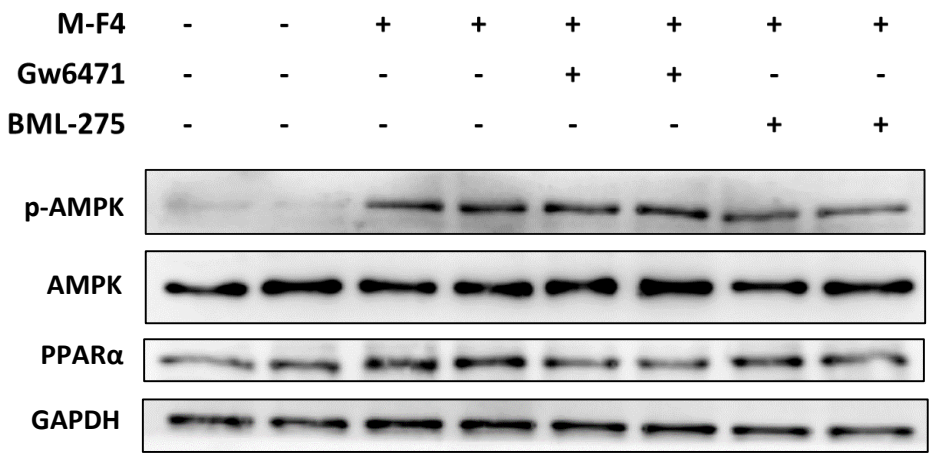


Figure 6: **PPARα was the downstream target of AMPK signal pathway.**
C2C12 myotubes were stimulated by M-F4 (100 μg/mL) and treated with or without 25μM Gw6471 and 15μM BML-275. The proteins were examined by western blotting analysis to check PPARα and phosphorylation and total levels of AMPK.

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The proposed scheme for M-F4-stimulated signaling pathway in C2C12 myotubes was shown in Figure7. In the lipid metabolism process, M-F4 is primarily phosphorylated AMPK-Thr172, but does not involve Sirt1 pathway. Then mediated by PPARα and its downstream targets CPT1b and UCP2, ultimately leading to improve lipid metabolism characterized by TG and NEFA reducing.

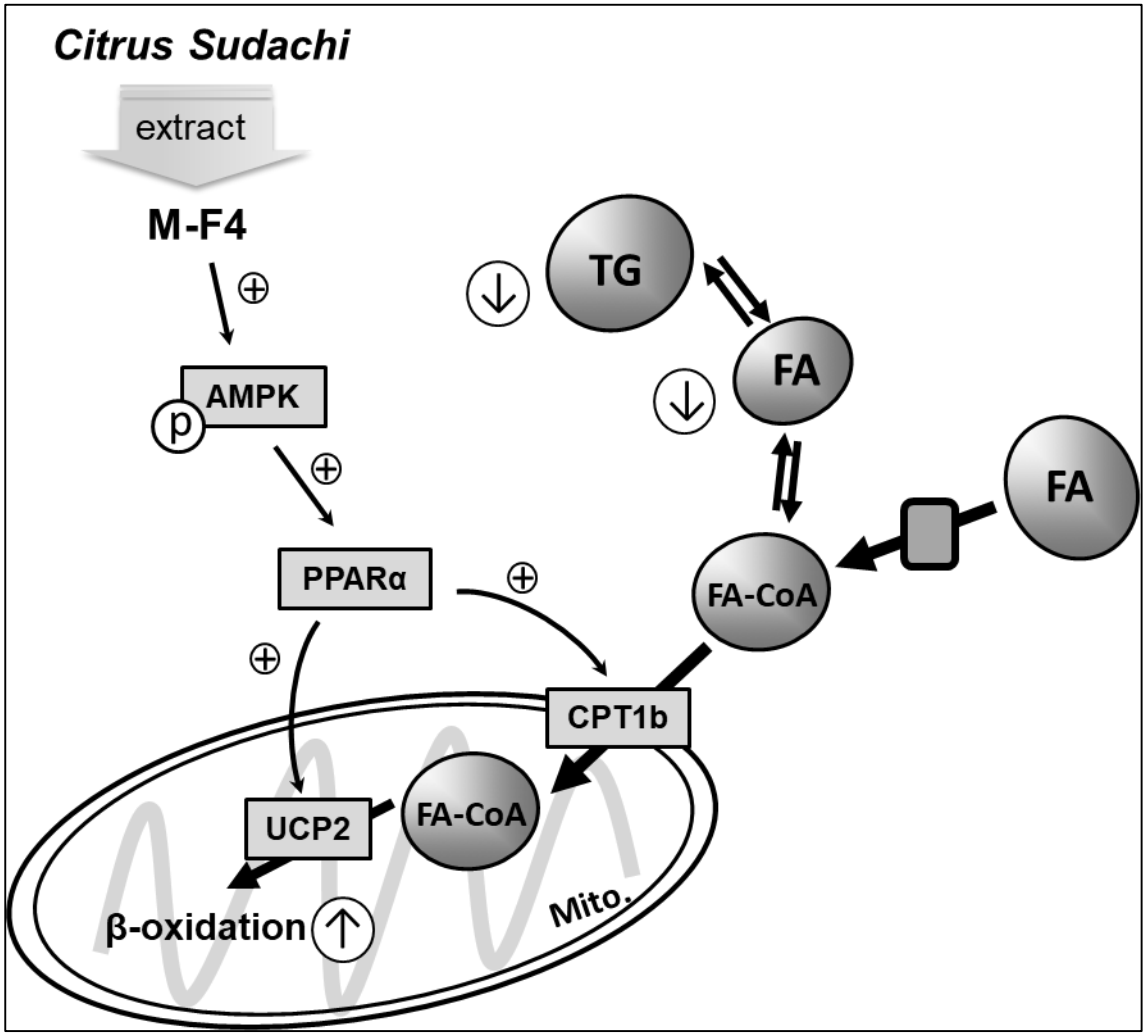


Figure 7: Proposed scheme for M-F4-stimulated signaling pathway in C2C12 myotubes. In the lipid metabolism process, M-F4 is primarily dependent on AMPK activation. Then mediated by PPARα, CPT1b and UCP2, ultimately lead to amplifying the β-oxidation. Simultaneously induce fatty acid and triglyceride level reduce in the C2C12 myotubes.

3 Discussion

The aim of this research is to find the lipid lowering fraction in *Citrus sudachi*, and investigate its mechanism. We found that the sudachi peel crude extract could reduce intracellular TG level of C2C12 cells. Moreover, M-F4, a fraction obtained from the sudachi peel extract, reduce triglyceride of C2C12 cells. In this lipid-lowering effect, the PPARα and its downstream targets were activated by AMPK, suggesting that M-F4

improved the lipid metabolism characterized by TG and NEFA reducing, via activated AMPK and mediated by PPARα and its downstream targets CPT-1b and UCP2.

Several reports have demonstrated that citrus played important roles in regulating glucose and lipid metabolic disorders[26-28]. In our previous study, the peel dry powders of *Citrus sudachi* inhibited the rising trend of blood glucose and fatty acid in human. We hypothesized that if this fatty acid rising trend inhibiting effect caused by TG consumption increasing in blood, the sudachi peel dry powders probably could reduce intracellular TG level in C2C12 cells. In the extracting process, we got five fractions of methanol sudachi peel extractions. Among those of fractions, the M-F4 had the strongest effect on triglyceride reducing effect in both myoblasts and myotubes. Because the C2C12 myotubes were differentiated cells and have mature cell function, we chose the myotubes to use in the next experiments which was more close to the skeletal muscle in vivo.

In order to find out the proper condition for the mechanism research, we tested a series of varied concentration (1μg/mL - 500 μg/mL) and stimulation time (2 hr- 48hr) (Supplement 1(A)). In the concentration-variable experiment, while the triglyceride reduces the effect of M-F4 as 500 μg/mL was most intensely, but considering about the cell viability result we got in MTT assay (Supplement 2), we chose concentration as 100 μg/mL in the stimulation process. In the stimulation time-variable experiment, the reduction effect started from 2 hr slightly and became remarkably in 24 hr(Supplement 1(B)). We chose concentration as 100 μg/mL for 24 hr stimulation in the next step of study. Since the myotubes were differentiated by grown in DMEM

supplemented with 2% horse serum for 1 week, the differentiation also in progress during our stimulation experiment periods. We intended the control group at every time point to avoid result changed.

AMPK is an important metabolism target with diverse functions in many cell types, include regulate lipid and carbohydrate metabolism in skeletal muscle[29]. We first hypothesized that sudachi peel extract reduces triglyceride effect is likely to be related to AMPK. In addition, Sirt1 always has a complicated relationship with AMPK in many metabolic regulate fields. But in our Western Blotting assay, only AMPK related proteins were activated obviously. In the following experiment, sirtinol and AICAR were used to intervene the expression of Sirt1 and AMPK, the results were consistent with previous ones. It means that AMPK should be involved in the TG reducing effect caused by M-F4, and this effect may depended on some other AMPK- related target but not Sirt1.

PPARs are ligand-activated transcription factors including PPARα, PPARγ and PPARδ (also known as PPARβ) belonging to the nuclear receptor family. The PPARs act as fatty acid sensors to control many metabolic programs that are essential for systematic energy homeostasis[10, 30]. As shown in the result, M-F4 exerted the triglyceride reducing effect by activating PPARα. This is supported by some studies, which confirmed the role of PPARα in fatty acid catabolism in peripheral tissues[23]. There also reported that naringenin treatment lowers triglyceride and cholesterol in plasma and liver by increasing the PPARα expression in rat liver[31]. PPARα null mice have decreased expression of cardiac fatty acid oxidation genes and decreased rates of fatty

acid oxidation, but maintain normal cardiac function[32, 33]. PPARα/γ may exert the synergic inhibitory effects on fatty acid synthesis by controlling the expressions of the transcription factor SREBP-1c[34], though this does not correspond with our results.

The target genes regulated by PPARα and involved in lipid metabolism and obesity include PGC1α, CPT1b, MCAD, UCP2, ACO and mtTFA[35-39]. PGC1α has been implicated in increasing the oxidation of fatty acids via increasing mitochondrial capacity and function, making this co-factor a key candidate for the treatment of lipotoxicity recently[35]. Unexpectedly, the expression level of PGC1α was declined by stimulation, which is probably because PGC1α activity is also altered via AMPK and Sirt1 pathways[6]. Some reports showed that synergistic activation of the CPT1b gene is promoted by the heterodimer of nuclear receptors PPARα-RXRα[36]. Slight increases in CPT1 expression in muscle can recover insulin sensitivity and reduced lipid accumulation[40, 41]. Our current results exactly suggest that TG reducing effect is mediated by augment of lipid oxidation through the PPARα-CPT1b pathway. UCP2 is expressed and functionally active in both central and peripheral tissues involved in glucose and lipid metabolism[42]. It was reported that obesity could be treated by promoting the activity of native UCP in muscle[43]. There also reported that UCP2 gene expression was significantly increased in skeletal muscle of mice which improved glucose and lipid metabolism[6]. Molecular mechanisms how M-F4 exerts the lipid-lowering effects we propose is summarized in Fig. 7.

We considered if M-F4 improved the lipid metabolism by reducing TG only, or it could effect on other lipid substance. Consistent with our expectation, M-F4 improved the

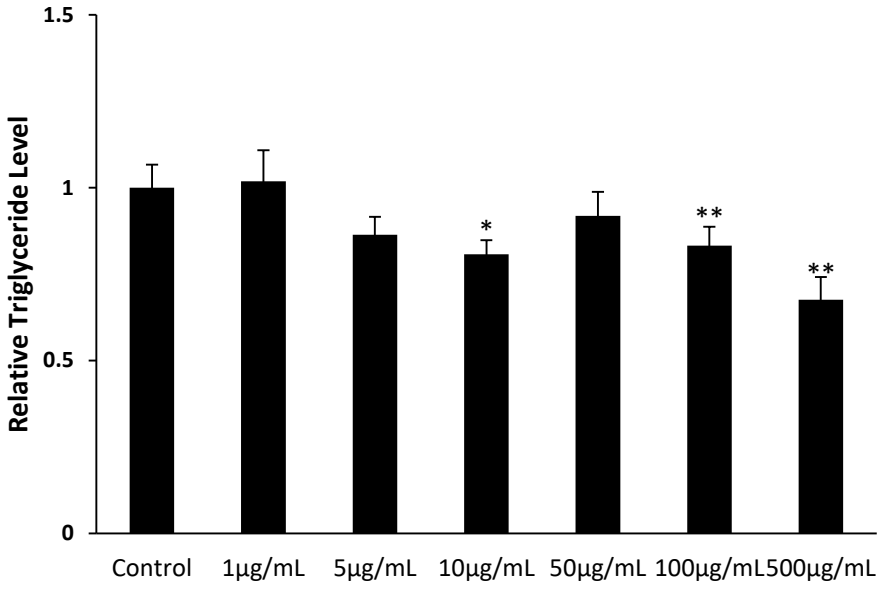
lipid metabolism characterized by both TG and NEFA reducing in C2C12 myotubes.

Conclusion

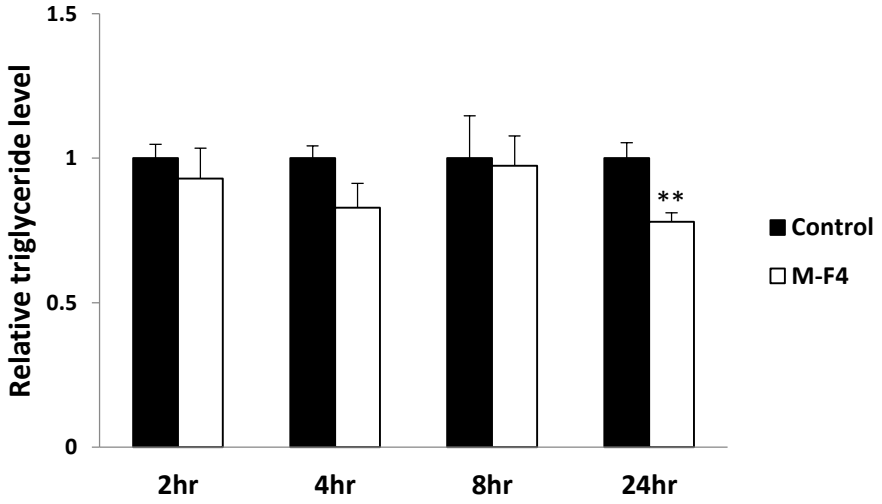
In summary, our study is the first to demonstrate that sudachi peel methanol extract reduces triglyceride and fatty acid level in the skeletal muscle cell. Additionally, our findings suggest that M-F4 improves the lipid metabolism through AMPK, PPARα and their downstream targets, CPT-1b and UCP2. Furthermore, these observations indicate that this extract may be useful for preventing obesity and diabetes-related diseases.

Supplement

(A)

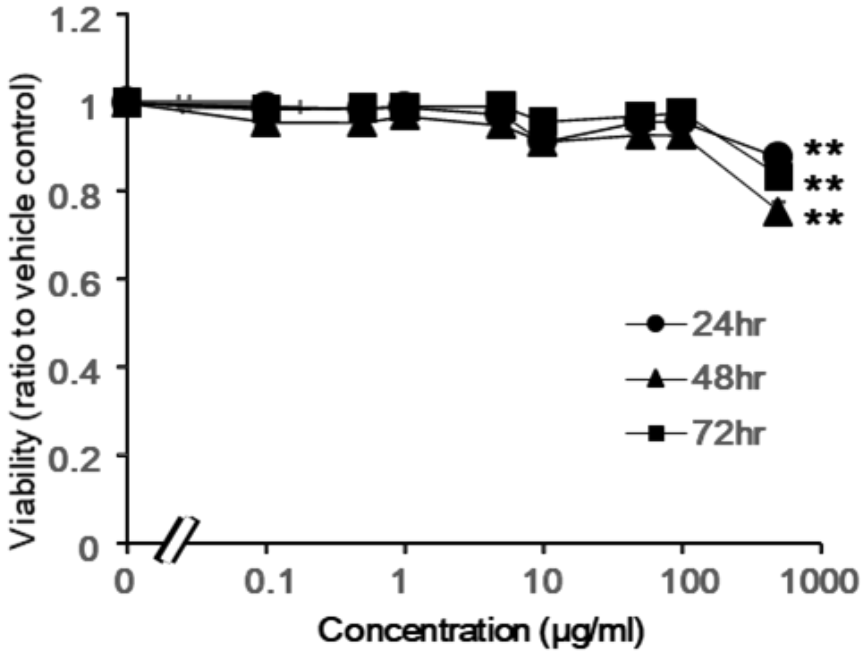


(B)



Supplement 1 :

Triglyceride content in cellular lysates is reported in the graph. C2C12 myotube were treated by M-F4 with (A) different concentration (0.1 μg/mL~500 μg/mL) and (B) different periods (2 hr-24 hr). **;p < 0.05, **;p < 0.01* vs. the control.



Supplement 2:

The effects of M-F4 on cell viability.

Viability of C2C12 cells were determined by MTT assay 24 (circle), 48 (triangle), or 72 (square) hr after exposure to indicated concentration of M-F4, respectively. ***; p<0.01* vs vehicle control.

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