

## ORIGINAL

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

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**Abstract :** Ectopic fat accumulation is associated with insulin resistance and type 2 diabetes mellitus. *Citrus sudachi* is an evergreen tree that is found mainly in Tokushima Prefecture in Japan. Previously, it was demonstrated that *Citrus sudachi* could inhibit the rising trend of blood glucose and fatty acid in human subjects. In the current study, we illustrated the function of methanol extracts from sudachi peel and investigated the mechanism of this effect. We got the five kinds of methanol extracts by using diaion HP-20, and those were named by hydrophobicity from M-F1 to M-F5. Among the 5 kinds of sudachi methanol extracts, only M-F4 significantly decreased the intracellular triglyceride of C2C12 cells. It augmented the AMPK activity and increased the transcription of PPAR $\alpha$  and its downstream targets CPT-1b and UCP2. In conclusion, M-F4 improved the lipid metabolism possibly through AMPK, PPAR $\alpha$  and their downstream targets like CPT-1b and UCP2. Furthermore, this extract may be useful for preventing obesity and diabetes related diseases. *J. Med. Invest.* 65 : 225-230, August, 2018

**Keywords :** *Citrus Sudachi*, Ectopic fat, lipid metabolism, 5'AMP-activated protein kinase

## INTRODUCTION

Diabetes mellitus is characterized by elevated blood glucose levels. The estimated number of people with diabetes worldwide in 2015 is 415 million, the number is expected to increase to 642 million by 2040(1). Type 2 diabetes (T2D) makes up about 90% of cases of diabetes(2), insulin resistance (IR) as one of the most important defect appears in various tissues (e.g., muscle, liver, and adipose)(3).

Recent research showed that increasing ectopic triglyceride (TG) accumulation in non-adipose tissues were 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. Ectopic fat accumulation induce metabolic processes disruption and organ function impaired(4).

Plants are abundant in biologically active molecules that play critical roles in pharmacology(5). Most of the available therapeutic agents widely used for treating diabetes are associated with side effects(6). However, natural antidiabetic products which are believed to have minimal side effects have been highlighted for the treatment of diabetes(7, 8). *Citrus sudachi* is an evergreen tree that was found mainly in Tokushima Prefecture in Japan(9, 10), 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, though its mechanism has not been clarified yet (11).

AMPK (AMP-activated protein kinase) is a fuel-sensing enzyme that plays a crucial role in response to starvation. AMPK is activated by phosphorylation of the threonine residue (Thr172) on its catalytic  $\alpha$ -subunit, which initiates processes such as fatty acid oxidation that restore ATP, and inhibits ATP consumption such as triglyceride and protein synthesis, and cell proliferation (12).

Peroxisome proliferator-activated receptors (PPARs) belong to a subgroup in the family of nuclear hormone receptors and highly expressed in the most of metabolic tissues. PPAR- $\alpha$  was the first discovered PPARs and is known to contribute to oxidative processes including fatty acid metabolism(13). PPAR- $\alpha$  exists in skeletal muscles and liver, which is closely correlated with fatty acid oxidation(14). PPAR- $\alpha$  has been suggested as an important therapeutic target for metabolic diseases with hyperlipidemia (15).

## METHODS AND MATERIALS

### Materials

We purchased AICAR, sirtinol and Gw6471 from Sigma-Aldrich, Cosmo bio or 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-AMPK $\alpha$  (Thr172) (Cell Signaling Technology Japan, K.K.) ; anti- $\beta$ -actin, as a loading control, from Cell Signaling Technology Japan, K.K. ; anti-GAPDH, as a loading control, from Wako Pure Chemical Industries, Ltd..

### The extract process of sudachi peel

The extract process of *Citrus sudachi* peel was shown in Figure1. Sudachi peel dry powder were extracted with hexane at room

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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 in 90% methanol by portioned between hexane and 90% methanol. The fraction was chromatographed on diaion HP 20 column, eluted with methanol and H<sub>2</sub>O solution, and then gradient and removing the sugar dissolved in water. 5 kinds of fraction named by hydrophobicity from M-F1 to M-F5 were got at last.

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

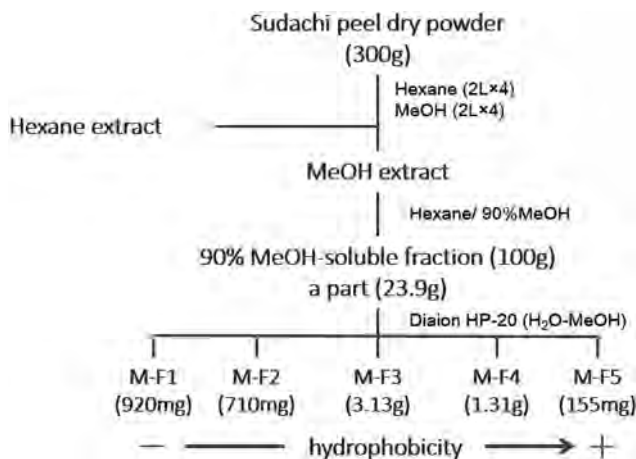


Figure 1 : The extract process of *Citrus sudachi* peel

#### Cell culture and treatment

Cell culture and differentiation was conducted as reported previously (16). 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<sub>2</sub>, and grown in DMEM supplemented with 2% horse serum for 1 week to be induced differentiation into myotubes.

#### Triglyceride and non-essential fatty acid measurement

Following to the stimulation, lipids were extracted with 2-pro-

panol 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.

#### 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.

#### 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<sub>2</sub>, 250 mM sucrose, 0.5% NP-40, 0.1 mM EGTA) as previously reported (17-20). 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.

#### Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted from cells, and was reverse-transcribed using the Superscript II™ 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 palmitoyl transferase 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 Table 1. 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 1 min, 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 analyze in duplicates and normalized against GAPDH. The results are expressed as relative

Table 1 : Sequences of the primers used in real-time PCR

GENE	FORWARD PRIMER	REVERSE PRIMER
PPARA	AGGCTGTAAGGGCTTCTTCG	GGCATTGTTCGGTTCTTC
SREBP1	GGCTATTCCGTGAACATCTCCTA	ATCCAAGGGCATCTGAGAACTC
PGC1A	TTCACCAAGAGCAAGTAT	CGCTGTCCCATGAGGTATT
CPT1B	CGGAAGCACACCAGGCAGTA	GCAGCTTCAGGGTTTGTCGGAATA
ACO	GCCGTGCAGAAATCGAGAACT	TCTTAACAGCCACCTCGTAACG
MCAD	GAGCCTGGGAACCTCGGCTTGA	GCCAAGGCCACCGCAACTTT
MTTFA	GAAGGGAATGGAAAGGTAGA	AACAGGACATGGAAAGCAGAT
UCP2	CTACAAGACCATTGCACGAGAGG	AGCTGCTCATAGGTGACAAACAT
GAPDH	AACACTGAGCATCTCCCTCA	GTGGGTGCAGCGAACTTTAT

gene expression using the  $\Delta C_t$  method.

#### Evaluation of viability

Cell viability was evaluated by MTT ((3-(4,5-di-methylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay as previously described (12, 19, 21).

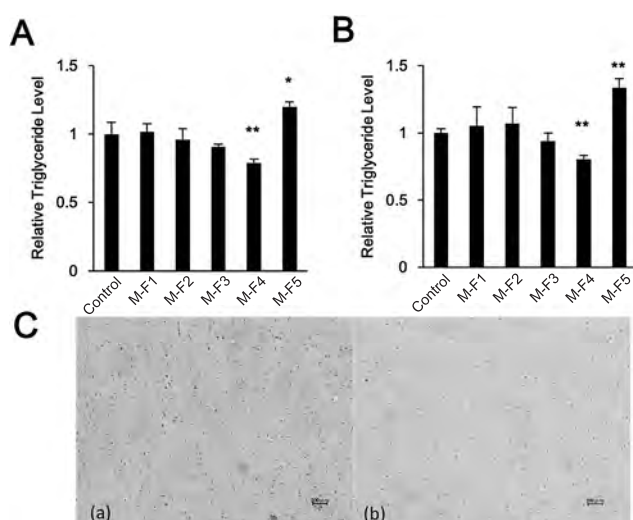
#### Statistical Analysis

All the data are shown as the means  $\pm$  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.

## RESULT

### Crude sudachi peel and M-F4 reduce intracellular triglyceride level significantly in C2C12 cell.

In another paper, we demonstrated that *Citrus sudachi* had the capability of inhibiting the rising trend of blood glucose and fatty acid in human (11). We supposed that if the crude sudachi peel could reduce intracellular Triglyceride (TG) level in C2C12 cells. We try to find out which part of this crude extraction cause this result. C2C12 myoblast were stimulated by 5 kinds of sudachi peel methanol extractions (M-F1~M-F5) (100  $\mu\text{g}/\text{mL}$ ) for 24 hours, only M-F4 obviously down-regulated TG level, but M-F5 had the opposite effect (Fig 2A). The similar results were also observed from C2C12 myotubes treated with sudachi peel methanol extractions (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.



**Figure 2 : Crude sudachi peel extraction and M-F4 reduced intracellular triglyceride level in C2C12.** Crude sudachi peel extraction reduced intracellular triglyceride level in C2C12 myoblasts (A) and myotubes (B) were stimulated by 5 kinds of extraction (100  $\mu\text{g}/\text{mL}$ ) 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  $\pm$  SD. \* :  $p < 0.05$ , \*\* :  $p < 0.01$  compared to vehicle control.

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

To elucidate whether M-F4 exert a TG-lowering effect via AMPK or Sirt1, C2C12 myotubes were treated with 100  $\mu\text{g}/\text{mL}$  M-F4 for 24hr and the protein levels were examined by western blotting. As expected, M-F4 increased the phosphorylation of AMPK in C2C12 myotubes obviously (Fig 3A). 5-aminoimidazole-4-carboxamide 1- $\beta$ -D-ribofuranoside (AICAR), a specific AMPK activator, was reported to be used as a positive control to increase fatty acid oxidation levels(22). Both AICAR and M-F4 increased phosphorylation level of AMPK, but had no further combination impact. (Fig 3D)

On the contrary, as shown in Figure 3C, 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  $\mu\text{M}$ ) 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.

### PPAR $\alpha$ and downstream targets were 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 downstream substrates. PPAR $\alpha$  and SREBP1 are two targets in lipid metabolism related to AMPK. 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 after 100  $\mu\text{g}/\text{mL}$  M-F4 treated for 24 hr. PPAR $\alpha$  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 though PPAR $\alpha$ .

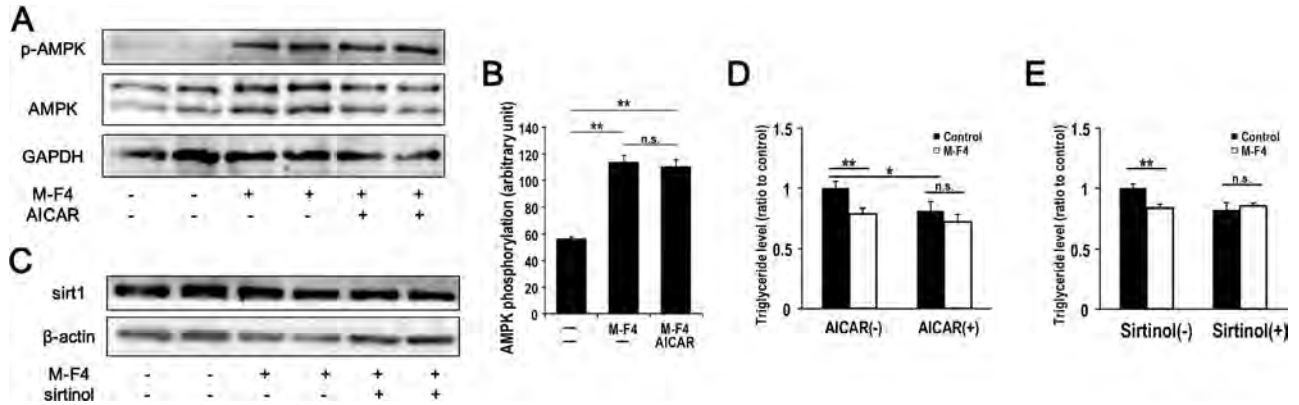
In the PPAR $\alpha$  signal pathway, a lot of downstream genes were concerned to be involved (Fig 4B). PPAR gamma coactivator 1-alpha (PGC-1 $\alpha$ ) is a regulator of mitochondrial biogenesis and function, but in our experiment PGC1 $\alpha$  were not activated by M-F4. Carnitine palmitoyl transferase1 (CPT1b) which considered to be the gene that controlled fatty acid mitochondrial  $\beta$ -oxidation were mightly 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 treatment, but there were no significant differences. Mitochondrial uncoupling protein 2 (UCP2) gene expression was increased in myotubes of M-F4 treated with statistically significant.

### PPAR $\alpha$ was the downstream target of AMPK signaling pathway and involved in TG and NEFA reducing effect caused by M-F4.

According to some papers, the relationship between AMPK and PPAR $\alpha$  was a kind of complicated. In most cases, AMPK was the upstream target of PPAR $\alpha$ , but in some special cases PPAR $\alpha$  was the upstream one. In order to confirm this relationship, we use Gw 6471 as a PPAR $\alpha$  inhibitor to treat cells. In the WB result, the level of p-AMPK were increased by M-F4, and this increasing trend did not change in the presence of Gw6471 (Fig 5A). In the triglyceride measurement, the reducing effect caused by M-F4 was relieve when treated by Gw6471 (Fig 5B).

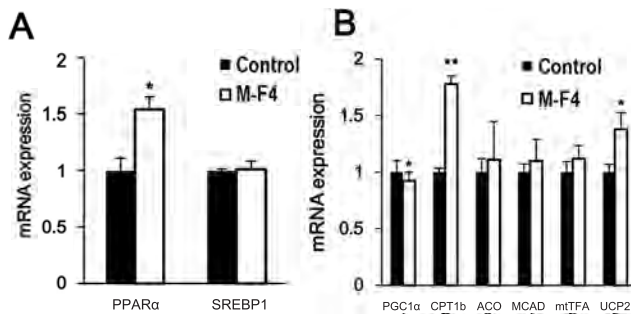
M-F4 led the increasing trends of PPAR $\alpha$ , CPT1b and UCP2 were mitigated when treated with Gw6471 (Fig 5C). This result was consistent with our expectation that CPT1b and UCP2 were the downstream target of PPAR $\alpha$  to reduce triglyceride level.

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



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

C2C12 myotube were treated with 100  $\mu$ g/mL M-F4. The phosphorylation and expression levels of AMPK (A) and Sirt1 (C). Quantified phosphorylation levels of AMPK (B). C2C12 myotubes were stimulated by M-F4 and treated with or without 1mM AICAR (D) and 25  $\mu$ M sirtinol (E) for 24 hr. \* ;  $p < 0.05$ , \*\* ;  $p < 0.01$ . n.s. ; not significantly different



**Figure 4 : Effects of M-F4 on mRNA levels in the expression of AMPK-related genes.**

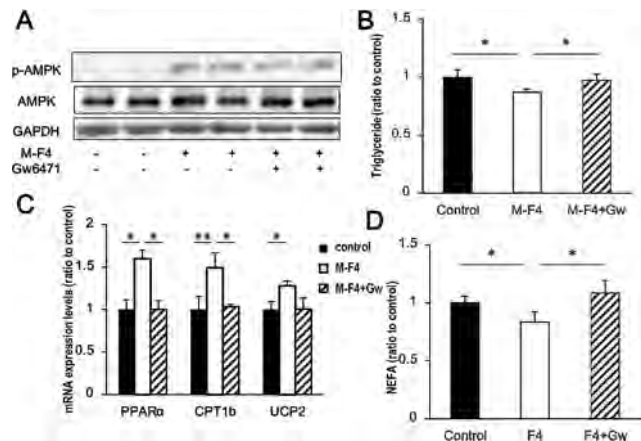
Relative mRNA expression of metabolism-related genes in the C2C12 myotubes were treated with c or vehicle. Gene transcription of PPAR $\alpha$  and SREBP1 (A) were normalized for GAPDH. C2C12 myotube treated with 100  $\mu$ g/mL M-F4, the downstream genes of PPAR $\alpha$  mRNA expression were normalized for GAPDH (B). Data are expressed as the mean  $\pm$  SD ; n = 3. \* $p < 0.05$  and \*\* $p < 0.01$  vs. the control.

mitochondrial. Therefore we conjecture the lipid reducing effect of M-F4 not only reveal in triglyceride but also in fatty acid. In figure 5D, we measure the non-essential fatty acid (NEFA) in the myotubes, the result shown that M-F4 assuredly decreased the NEFA level and decreasing tendency alleviated by Gw6471 intervening.

The proposed scheme for M-F4-stimulated signaling pathway in C2C12 myotubes was shown in Figure 7. In the lipid metabolism process, M-F4 is primarily phosphorylated AMPK-Thr172, but does not involve Sirt1 pathway. Then mediated by PPAR $\alpha$  and its downstream targets CPT1b and UCP2, ultimately leading to improve lipid metabolism characterized by TG and NEFA reducing.

## DISCUSSION

The aim of this research is to find the lipid lowering substance in *Citrus sudachi*, and investigate its mechanism. We found that the sudachi peel crude could reduce intracellular TG level of C2C12 cells. Moreover, M-F4, a kind of sudachi peel extractions, reduces triglyceride of C2C12 cells. In this lipid-lowering effect, the PPAR $\alpha$



**Figure 5 : The effects of M-F4 on plasma lipids are dependent on PPAR $\alpha$ .**

C2C12 myotubes were stimulated by M-F4 (100  $\mu$ g/mL) and treated with or without Gw6471. The proteins were examined by western blotting analysis to check phosphorylation and total levels of AMPK (A). Triglyceride (B), mRNA expression (C), and non-esterified fatty acid (D) in the C2C12 myotube stimulated by M-F4 with or without Gw6471 (Gw). Data are expressed as the mean  $\pm$  SD. \* ;  $p < 0.05$ , \*\* ;  $p < 0.01$

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 PPAR $\alpha$  and its downstream targets CPT-1b and UCP2.

Several reports have demonstrated that citrus played important roles in regulating glucose and lipid metabolic disorders. In the present research, we got five kinds of methanol sudachi peel extractions. Among the kinds of extracts, M-F4 had the strongest effect on triglyceride reducing in both myoblasts and myotubes. Because the C2C12 myotubes were differentiated cells and have mature cell function, we thought use the myotubes in the next experiments were more close to the skeletal muscle in vivo. It is interesting that the M-F5 was opposite to M-F4 in triglyceride measurement, but we have not found the answer yet.

In order to find out the proper condition for the mechanism research, we tested a series of varied concentration (1  $\mu$ g/mL - 500  $\mu$ g/mL) and duration (2 hr- 48 hr).

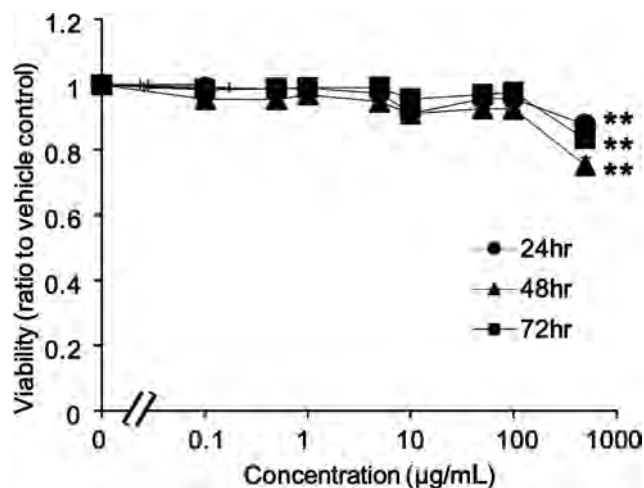


Figure 6 : The effects of M-F4 on cell viability. Viability of C2C12 cells were determined by MTT assay 24 (circle), 48 (triangle), or 72 (square) hr after exposure to indicated concentration of M-F4, respectively. \*\*:  $p < 0.01$  vs vehicle control.

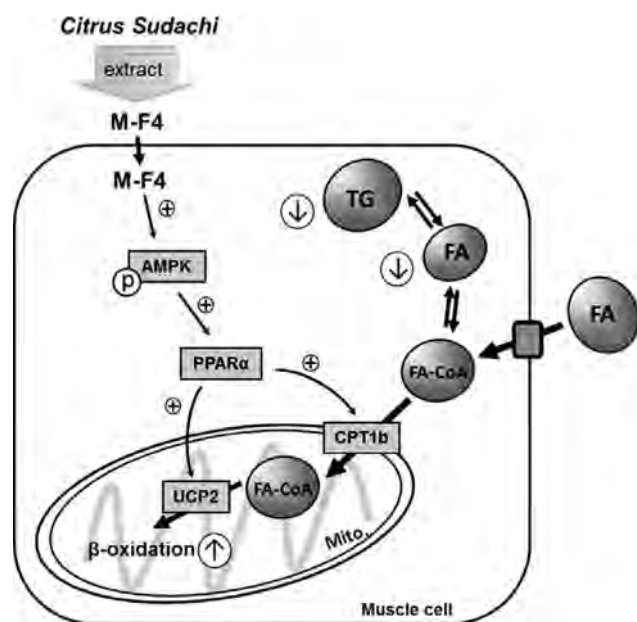


Figure 7 : **Proposed scheme for M-F4-stimulated signaling pathway in C2C12 myotubes.**

In the lipid metabolism process, M-F4 is primarily dependent on AMPK activation, which does not involve Sirt1 pathway. Then mediated by PPAR $\alpha$ , CPT1b and UCP2, ultimately lead to amplify the  $\beta$ -oxidation. Simultaneously induce to fatty acid and triglyceride level reduce in the C2C12 myotubes.

In the concentration-variable experiment, while the triglyceride reduce effect of M-F4 as 500  $\mu\text{g/mL}$  was most intensely, but considering the cell viability result we got in MTT assay, we chose concentration as 100  $\mu\text{g/mL}$  in the stimulate step (Fig. 6). 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 in every time point to avoid result changed. The reduction effect was start from 2 hr slightly, and became remark-

ably in 24 hr. Taking together with the MTT assay result, we chose concentration as 100  $\mu\text{g/mL}$  for 24 hr stimulation in the next step of study.

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. Several natural ingredients have been shown to exert antidiabetic effects, accompanied by AMPK activation. Thus, we first hypothesized that sudachi peel extraction reduce 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 act as fatty acid sensors to control many metabolic programs that are essential for systematic energy homeostasis. Fig. 4A and 5B-D demonstrates that M-F4 exerts triglyceride reducing effect by activating PPAR $\alpha$ . There also reported that naringenin treatment lowers triglyceride and cholesterol in plasma and liver with increasing PPAR $\alpha$  expression in rat liver (23). PPAR $\alpha$  null mice have decreased expression of cardiac fatty acid oxidation genes and decreased rates of fatty acid oxidation, but maintain normal cardiac function. PPAR $\alpha/\gamma$  may exert the synergic inhibitory effects on fatty acid synthesis by controlling the expressions of the transcription factor SREBP-1c, though this does not correspond with our results.

The target genes regulated by PPAR $\alpha$  and involved in lipid metabolism and obesity include PGC1 $\alpha$ , CPT1b, MCAD, UCP2, ACO and mtTFA. PGC1 $\alpha$  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. Unexpectedly, the expression level of PGC1 $\alpha$  were declined by stimulation, which is probably because PGC1 $\alpha$  activity is also altered via AMPK and Sirt1 pathways. Some reports showed that synergistic activation of CPT1b gene is promoted by heterodimer nuclear receptors PPAR $\alpha$ -RXR $\alpha$ . Increase in CPT1 expression in muscle can recover insulin sensitivity and reduce lipid accumulation. Our current results exactly suggest that TG reducing effect is mediated by augment of lipid oxidation through PPAR $\alpha$ -CPT1b pathway. UCP2 is expressed and functionally active in both central and peripheral tissues involved in glucose and lipid metabolism. It was reported that obesity could be treated by promoting the activity of native UCP. Tsutsumi also reported that UCP2 gene expression was significantly increased in skeletal muscle of mice which improved glucose and lipid metabolism (9). Molecular mechanisms how M-F4 exerts the lipid-lowering effects we propose is summarized in Fig. 7.

We considered if M-F4 improves 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 (Fig. 5D).

## CONCLUSION

In summary, our study is the first to demonstrate that sudachi peel methanol extract reduces triglyceride and fatty acid level in skeletal muscle cell. Additionally, our findings suggest that M-F4 improves lipid metabolism through AMPK, PPAR $\alpha$  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.

## CONTRIBUTIONS AND DISCLOSURES

*L.M. designed and conducted the study, and contributed to manuscript preparation. X.W. performed most of the experiments, wrote the manuscript, and contributed to the study design. The others contributed to the experiments and/or discussion.*

Disclosures ; The authors declare no conflict of interest.

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