Doctoral Dissertation

Research on Food Functionality of Insect Feces

（昆虫のフンの食品機能性の研究）

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Table of contents

Page

GENERAL INTRODUCTION

Insects are expected to be the next generation of food, but until now they have not been studied scientifically. In our research on insects as functional foods, we focused on their byproducts, feces, and studied its food functionality.

In Part I, we investigated the use of insect feces as insect tea, and examined the fat accumulation-suppressing effect of the feces of *Locusta migratoria*. We examined the anti-obesity effect of insect tea of *Locusta migratoria* (LT) using in vitro and in vivo assays. From the results of oil red o staining experiments using 3T3L1 cells, it was confirmed that LT suppresses the differentiation into adipocytes, not the process of accumulation of lipid droplets. Furthermore, it suppressed the master regulators of differentiation PPARγ and C/EBPα. In mice fed a high-fat diet, the body weight of the mice administered LT did not change when compared to the control mice, but the white fat weight decreased significantly; in particular, the visceral fat was reduced. The total cholesterol level was significantly decreased in the LT-administered mice, but the glucose, free fatty acid, and triglycerol levels remained unchanged. These results indicated that LT can suppress fat accumulation by inhibiting adipocyte differentiation.

In Part II, we attempted to investigate the the efficacy of cherry caterpillar (larvae of *Phalera flavescens*) feces tea (PT) for treating sarcopenia, particularly concerning muscle building and atrophy suppression using C2C12 cells. PT treatment (0.2 mg/mL) increased myotube widths by approximately 40% and increased the expression levels of Myod, Myog, and MYHC. Additionally, PGC1α, TFAM, SDHA, BCAT, and BCKDH were upregulated in a PT concentration-dependent manner. For PGC1α which is the transcription coactivator, the protein expression level also increased in a concentration-dependent manner. The findings demonstrate that PT stimulates PGC1α and activates

mitochondria via branched-chain amino acid metabolism and the electron transport chain in C2C12 myoblasts. Furthermore, PT suppressed LPS-induce expression of IL6 and TNFα, and reduced the protein expression levels of the ubiquitin ligases Atrogin1 and MuRF, which are major cause of muscle atrophy. These results indicated that PT may be effective for muscle building and suppression of atrophy.

In the past, insect food was looked down upon and was generally not eaten, except in a few areas. However, insects are now recognized as food all over the world, and it may help solve food shortage problems in the future. Furthermore, the use of insect food may have environmental benefits. Since ancient times, insect tea has been used as an herbal medicine in parts of China, and it is said to have various beneficial effects. In this study, we could demonstrate one of its efficacies, and showed the potential of insect food as functional food.

Chapter I

Anti-obesity effect of insect tea of locust (*Locusta migratoria*) through the suppression of *PPARγ* and *C/EBPα* in mice.

1.1 ABSTRACT

In 2013, the Food and Agriculture Organization of the United Nations proposed to actively use insects as food and feed. However, the function of insect foods has not yet been studied. In particular, insect tea has been used as a Chinese medicine since ancient times. Obesity is a main factor of lifestyle diseases, so prevention and improvement of obesity are indispensable for living a healthy life. We examined the anti-obesity effect of insect tea of *Locusta migratoria* (LT) using *in vitro* and *in vivo* assays. From the results of oil red o staining experiments using 3T3L1 cells, it was confirmed that LT suppresses the differentiation into adipocytes, not the process of accumulation of lipid droplets. Furthermore, it suppressed the master regulators of differentiation *PPARγ* and *C/EBPα*. In mice fed a high-fat diet, the body weight of the mice administered LT did not change when compared to the control mice, but the white fat weight decreased significantly; in particular, the visceral fat was reduced. The total cholesterol level was significantly decreased in the LT-administered mice, but the glucose, free fatty acid, and triglycerol levels remained unchanged. These results indicated that LT can suppress fat accumulation by inhibiting adipocyte differentiation.

1.2 INTRODUCTION

It has been speculated that the human population on Earth will reach 10 billion in 2070 and that the temperature will rise by 7.5 °C from the current temperature due to excessive global warming (Fedoroff, 2015) (C. Xu et al., 2020). Consequently, a serious food crisis is predicted to occur worldwide because there will not be enough food for the entire human population. In 2013, the Food and Agriculture Organization of the United Nations proposed the active use of insects as food and feed (van Huis et al., 2013). Furthermore, the European Union began to regulate insects as a novel food in 2015. In other words, insects, which have been used as common foods in Asia and Africa, joined the food category in Europe, where insect-eating was not a habit. One of the main reasons for the inclusion of insects as food is attributed to their high nutritional value comparable to that of animal meats, including cattle, pigs, and chickens. Insects contain high levels of protein, as well as large amounts of lipids, beneficial fatty acids, vitamins, and minerals (T. K. Kim et al., 2019) (de Castro et al., 2018) (Ghosh et al., 2017). In Southeast Asia, particularly in Thailand and Laos, the farming of lepidopteran insects has already been put into practical use, and in Japan, research on the farming of crickets and locusts has begun. It is believed that insect farming will spread and progress worldwide in the near future.

The feces of insects have been consumed as insect tea in China since ancient times. Insect tea is prepared from the feces of the moth larvae fed with naturally fermented and finely crushed leaves, which undergo a further step of fermentation by the internal enzymes of the larvae. Insect tea has not only a very good taste and flavor but also contains various ingredients, such as amino acids, minerals, fatty acids, and volatile oils.

In addition, insect tea contains many antioxidants, including polyphenols (L. Xu et al., 2013). This tea is considered to have antioxidant activity that can eliminate free radicals in the body as well as anti-inflammatory effects (Zhao et al., 2018). Additionally, several functionalities of insect tea have also been reported. For instance, Sanye insect tea has been shown to have a hypoglycemic effect, whereas Hawk insect tea has been reported to have serum total cholesterol (TC), triglyceride (TG), and low-density lipoprotein cholesterol-reducing effects (L. Xu et al., 2013), indicating the possible anti-diabetic and anti-obesity effects of insect tea. It is assumed that there will be an increase in the number of areas in which the cultivation of edible insects will increase with the spread of insect food consumption. As a consequence, it is expected that the amount of insect feces discharged by the farmed insects will increase and become a waste burden. Therefore, using feces to prepare insect tea, which has high functionality and health benefits similar to commonly used teas, such as green tea and black tea, could practically be helpful.

As obesity is the cause of lifestyle diseases, the prevention and improvement of obesity are indispensable for living a healthy life in modern society. In particular, an increase in visceral fat can cause metabolic syndromes in which dyslipidemia, hyperglycemia, and hypertension are triggered by visceral fat obesity (Reaven, 2006). Patients with metabolic syndrome have been reported to be about three times more likely to develop type 2 diabetes or die from cardiovascular disease than those who do not (Ford, 2005). Obesity is attributed to the increase in adipocyte counts and hypertrophy. Recent investigations have demonstrated that peroxisome proliferator-activated receptor (PPAR)γ, CCAAT/enhancer-binding protein (C/EBP)α and β, and sterol regulatory element-binding transcription factor 1 are transcription factors that

regulate adipocytes differentiation (de sá et al., 2017). Adipocytes differentiate via the *PPARγ* and *C/EBPα* pathway (Rosen et al., 2002). Therefore, it is important to inhibit these transcription factors to suppress the differentiation of adipocytes. Functional foods and ingredients have garnered attention as a means to maintain health. Indeed, many foods have been reported to have fat accumulation-suppressing effects (Hasumura et al., 2012) (Suk et al., 2016) (Neil et al., 2019). However, the fat accumulation-suppressing effects of insect feces tea have not yet been reported. In this study, we investigated the use of insect feces of *Locusta migratoria* as a tea and examined its lipid accumulation-suppressing effect.

1.3 MATERIALS AND METHODS

Extraction from feces of *L. migratoria*.

The feces of L. migratoria bred with wheat (Bromus catharticus) at 30 °C in the National Institute of Agrobiological Sciences (Ibaraki, Japan) were used. Feces were collected as appropriate during breeding. Feces tea of L. migratoria (1 g) was extracted using boiled water (50 mL) for 10 min. The solution of the feces of L. migratoria extracted with boiled water was centrifuged at 10,000 × g for 10 min and filtered through a 0.45-μm-pore filter membrane. The filtrate (~20 mg feces/ mL) was used as L. migratoria tea (LT). The test solutions with 0.25, 0.5 and 1.0 mg feces/mL were prepared by diluting LT with assay medium and used for in vitro assays using 3T3-L1 cells.

Cell culture and differentiation.

3T3-L1 cells were obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA) and cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% fetal calf serum (Gibco, Thermo Fisher Scientific, Waltham, MA, USA), 100 U/mL penicillin, and 100 U/mL streptomycin (Sigma-Aldrich) as the growth medium at 37 °C under 5% CO2. Penicillin and streptomycin were added to all of the culture media used thereafter. Briefly, cells were seeded at 5.0 × 104 cells per well in 24-well plates and cultured in growth medium until 100% confluent for 4 days (period 1). The medium was changed to a differentiation medium (DMEM supplemented with 10% fetal bovine serum (Gibco, Thermo Fisher Scientific), isobutylmethylxanthine, dexamethasone, and insulin

(Adipogenesis Assay Kit, Cayman Chemical, MI, USA)) to induce differentiation for 3 days (period 2). After 3 days, the medium was replaced with a maintenance medium (DMEM supplemented with 10% fetal bovine serum), and the cells were cultured for 4 days (period 3). Three tests were performed: in Test I, LT was added to the medium in both periods 2 and 3; in Test II, LT was added only in period 2; and in Test III, LT was added only in period 3. Cells not treated with LT were used as the control. The cytotoxicity of LT on 3T3-L1 cells was examined in Test I with Cell Counting Kit-8 (Dojindo Laboratories, Kumamoto, Japan).

Oil Red O staining.

Intercellular lipid accumulation was measured by Oil Red O staining. 3T3-L1 cells on a 24-well plate were incubated in a differentiation medium (500 μL) with or without LT. The final concentration of LT was 0.25, 0.5, and 1.0 mg/mL. After 3 days of incubation, the medium was replaced with a maintenance medium (500 μL) with or without LT. After 4 days of incubation, cells were washed twice with phosphate-buffered saline and fixed in a 10% formalin (Nacalai Tesque, Osaka, Japan). The fixed cells were washed twice with water, then stained by 0.3% Oil Red O for 15 min at room temperature. After discarding the solution, the stained droplets in the cells were washed three times with water. The droplets were extracted from the cells with isopropanol for 20 min at room temperature, then the absorbance of the extracts was measured at 520 nm (Ramírez-Zacarías et al., 1992). Results are shown as the relative percentage of differentiated cells in comparison to the control cells without LT.

mRNA preparation and real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR).

3T3-L1 cells on a 24-well plate were incubated in differentiation medium (500 μL) containing LT (0.25, 0.5, or 1.0 mg/mL). After the induction of differentiation, the cells were harvested every day for 3 days, and total RNA was extracted from the cells using an RNeasy Kit (Qiagen N.V., Venlo, Netherlands) according to the manufacturer's instructions. cDNA was synthesized using random primers and PrimeScript Reverse Transcriptase (Takara Bio, Shiga, Japan). An aliquot of cDNA was used as a template for qRT-PCR in a StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). The target cDNAs were amplified using Fast SYBR Green Master Mix (Applied Biosystems) together with the following gene-specific primers: *PPARγ* (MA029808; Takara Bio), *C/EBPα* (MA024950; Takara Bio), and *βACTIN* (MA050368; Takara Bio). The relative expression level of each mRNA was normalized to the expression of the housekeeping gene *βACTIN*.

Animal treatments.

Four-week-old male C57BL/6NCrSlc mice were obtained from Japan SLC, Inc. (Shizuoka, Japan). The mice were preliminarily bred for 3 days as the acclimatization period. For the entire study, including the preliminary breeding period, the mice were bred in an environment with a 12-h light-dark cycle and free access to food and water. During the preliminary breeding period, all mice were kept in cages with six mice per cage and were fed the solid diet CLEA Rodent Diet CE-2 (CLEA Japan, Tokyo, Japan) as a normal diet. After the preliminary breeding period, all mice were weighed and

assigned to one of three weight-matched groups—the first group was fed a normal diet (ND) with water (n = 4), the second group was fed a high-fat diet (HFD) with water (n = 5), and the third group was fed an HFD with LT (n = 6) for 11 weeks. As the high-fat feed, HFD-60 (Oriental Yeast, Tokyo, Japan) was used. The concentration of LT was 8 g/400 mL. After 11 weeks of dietary treatment, blood was collected from the abdominal vena cava of the group fed an ND with water (n = 4), and the groups fed an HFD with water (n = 3) or LT (n = 6). Liver and adipose tissues (epididymal fat, perirenal fat, inguinal fat, and interscapular fat) were collected from the group fed an ND with water (n = 4), the groups fed an HFD with water (n = 5) or LT (n = 6). After collection, they were weighed, frozen in liquid nitrogen, and stored at -80 °C. The experimental protocol for this study was approved by the Animal Care and Use Committee at Yamaguchi University (number 276), accredited by AAALAC International.

Measurement of blood and liver components.

The plasma was obtained by centrifuging blood at 600 × g and 4 °C for 10 min. The liver fragment was weighed in a tube, homogenized in a mixed solution of methanol: chloroform (1:2), then extracted by sonication. After centrifugation at 16,000 × g and 4 °C for 10 min, the solvent was collected into a new tube, phosphate-buffered saline was added and mixed with a vortex mixer. After centrifugation at 16,000 × g and 4 °C for 10 min, the chloroform layer was recovered. Subsequently, the solvent was volatilized at 4 °C and redissolved in isopropanol and was used as the sample. For TG measurement, free fatty acid, TC, and blood sugar levels, Lab Assay™ Triglyceride, Lab Assay™ NEFA, Lab Assay™ Cholesterol, and Lab Assay™ Glucose (Wako Pure Chemical, Osaka, Japan) were used.

Statistical analysis.

Data were analyzed using one-way analysis of variance followed by Dunnett's test or Tukey test using GraphPad Prism (GraphPad Software, San Diego, CA, USA). Statistical significance was denoted by values of $p < 0.01$ or $p < 0.05$.

1.4 RESULTS

Suppressing effect of LT on lipid accumulation in 3T3-L1 cells.

First, to examine whether LT has a lipid accumulation-suppressing effect. For this purpose, 3T3-L1 cells to which LT was added (0.25, 0.5, or 1.0 mg/mL) were subjected to Oil Red O staining (Figure 1A). LT attenuated the Oil Red O staining level in a concentration-dependent manner and suppressed more than 75% of the staining at all concentrations tested (Fig. 1B). Therefore, it was confirmed that LT had a lipid accumulation-suppressing effect. Next, to investigate the mechanism by which LT suppresses cell differentiation, LT was added to the cells in the experimental scheme shown in Fig. 2A, and Oil Red O staining was performed (Fig. 2B). The staining level of cells in Test III was the same as that in the control cells, i.e., LT did not suppress lipid accumulation when added only in period 3. In contrast, cells in Tests I and II showed almost the same staining level, and their level was less than that in the control cells (Fig. 2C). These results indicated that LT suppressed lipid accumulation during the process of 3T3-L1 differentiation into adipocytes but not during the process of lipid droplet accumulation. The viability of 3T3-L1 cells treated with LT was also measured. LT showed no toxicity during the experimental process at concentrations below 1.0 mg/mL (data not shown).

Effect of LT on the expression of PPARγ and C/EBPα in 3T3-L1 cells.

Because *PPARγ* and *C/EBPα* are the master regulators of adipocyte differentiation in 3T3-L1 cells, the expression of these genes was examined by qRT-PCR in cells during period 2 (incubation in differentiation medium; Fig. 2A). LT did not suppress the

expression of *PPARγ* on the 1st day after the induction of differentiation, but after 2 days, it significantly suppressed the expression of *PPARγ* in a concentration-dependent manner compared to the control cells not treated with LT (Fig. 3). In contrast, LT significantly suppressed the expression of *C/EBPα* from the 1st day of differentiation induction, and the suppression became stronger with each passing day (Fig. 3).

Effect of LT on the body weight, organs, and adipose tissue of mice fed a high-fat diet.

Following 11 weeks of breeding, the body weight of mice fed an HFD was higher than those of mice fed an ND, whereas there was no difference in between the group fed an HFD with water and that with LT (Fig. 4A). Additionally, the feed intake of the mice fed an HFD with water was 2.91 ± 0.15 g/day mouse and that with LT was 2.88 ± 0.11 g/day/mouse, which was almost same. Furthermore, all WAT weights were higher in mice fed an HFD than in mice fed an ND (Fig. 4B). The epididymal WAT weight was significantly lower in the group fed an HFD with LT than that with water. There was no significant difference in other adipose tissues, but it tended to decrease in the group fed an HFD with LT (Fig. 4B). When the epididymal WAT and perirenal WAT were considered to constitute the visceral WAT, and the inguinal WAT and interscapular WAT were considered to constitute the subcutaneous WAT, the visceral WAT in the LT-treated mice group was significantly decreased (Fig. 4C). Furthermore, the total WAT weight was significantly lower in the group fed an HFD with LT than that with water (Fig. 4C). These results showed that LT suppressed the accumulation of fat induced by the HFD.

Effect of LT on blood and liver components in mice fed a high-fat diet.

TG, free fatty acid, TC, and glucose levels were measured in the collected plasma. TC and glucose increased when mice were fed an HFD (Fig. 5A). In addition, TC in mice fed an HFD with LT tended to decrease with respect to that with water ($P = 0.078$). Furthermore, there were no significant changes in free fatty acids and TG content among the three groups (Fig. 5A). TC and TG levels were measured in the collected liver. TC and TG increased when mice were fed an HFD (Fig. 5B). TC was significantly higher in the group fed an HFD with LT than that with water, and TG did not decrease in the group fed an HFD with LT (Fig. 5B).

1.5 DISCUSSIONS

Although there are varying opinions on the diagnostic criteria for obesity (Reaven, 2006) (Elabbassi & Haddad, 2005), obesity has been considered a risk factor for diseases, such as diabetes, hypertension, and arteriosclerosis, since the World Health Organization announced the term "metabolic syndrome" in 1998. Recently, metabolic syndrome in children, especially in developed countries, has become recognized as a problem because poor eating habits during childhood can become a cause of diabetes in adults (Al-Hamad & Raman, 2017). Metabolic syndrome has become a serious problem in all generations, and there is an urgent need to control and eliminate obesity. In this study, we investigated the lipid accumulation-suppressing effect of LT both *in vitro* and *in vivo* as the consumption of insect tea represents a method of waste utilization from insect cultivation, which will increase with the expected spread of insect food.

The differentiation of cells into adipocytes and the accumulation of lipid droplets are considered to be the cellular mechanisms that lead to obesity. In this study, we found that LT suppressed *PPARγ* and *C/EBPα*, which are master regulators of differentiation. Various studies have reported that the mechanism of action that suppresses PPARγ differs depending on the food. It is known that ginger, coffee, and green tea are typical foods that have an anti-obesity effect. Ginger suppresses the accumulation of lipid droplets by mainly suppressing the expression of fatty acid synthase and acetyl CoA carboxylase (J. Wang et al., 2017). In other words, ginger is an example of a food that can suppress the accumulation of fat during period 3 of this experiment. However, as was seen with LT in the present study, coffee and green tea have also been reported to suppress the differentiation of progenitor cells into adipocytes (Raseetha et al., 2017)

(Lin et al., 2005) (Aoyagi et al., 2014) (Maki et al., 2017). Coffee suppresses adipocyte differentiation by suppressing *PPARγ* in 3T3-L1 cells (Aoyagi et al., 2014). It has also been reported that coffee suppresses obesity by suppressing the expression of insulin receptor substrate 1 (IRS1), an insulin receptor adapter protein located upstream of PPARγ and C/EBPα (Maki et al., 2017). The active ingredient of coffee is considered to be the ingredient of roasted coffee beans and has not yet been specified. Tea catechin, one of the main active ingredients of green tea, has been reported to suppress PPARγ and C/EBPα in 3T3-L1 cells (Suzuki et al., 2016). 5-Aminoimidazole-4-carboxamide ribonucleoside (AICAR), which is an AMP - activated protein kinase (AMPK) activator, has been shown to suppress the expression of PPARγ and C/EBPα in 3T3-L1 cells, suggesting that these transcription factors are regulated by AMPK (Habinowski & Witters, 2001). In addition, it has been reported that obesity was suppressed in a group of mice administered a green tea extract because AMPK was mainly induced in adipose tissue, and the expression of PPARγ was suppressed (Rocha et al., 2016). In this experiment, *B. catharticus* was used as feed in the breeding of locust, and the active ingredient was considered to be derived from *B. catharticus* or the locust itself. Some studies have shown that saponarin, a flavonoid present in barley belonging to the same family as *B. catharticus*, suppresses lipid accumulation (J. H. Lee et al., 2015) (Y. J. Kim et al., 2017). In a previous study, saponarin suppressed fat accumulation in mice via AMPK activation (Y. J. Kim et al., 2017). Saponarin was also reported to suppress fat accumulation in 3T3-L1 cells (J. S. Kim et al., 2020). It was highly possible that saponarin suppressed the expression of PPARγ by activating AMPK. Through these published results, it was considered that LT contained *B. catharticus*-derived

polyphenols like saponarin, which activated AMPK and suppressed PPARγ, thereby suppressing fat accumulation.

Based on the findings of the *in vitro* experiments that demonstrated the fat accumulation-suppressing effect of LT, an *in vivo* experiment was performed by administering LT to mice, and the same effect was verified in the mice. The findings revealed that visceral WAT was significantly reduced by LT treatment. Though visceral WAT and subcutaneous WAT are both white fat, their properties differ—Subcutaneous WAT has a high ability to synthesize and accumulate fat, while visceral WAT synthesizes and secretes adipocytokines that control sugar and lipid levels in the blood as well as the blood pressure (Dutheil et al., 2018). The accumulation of visceral WAT causes the abnormal secretion of these adipocytokines, leading to the development of various diseases (Kaisanlahti & Glumoff, 2019). For example, after decomposition, the accumulated fat can reenter the circulatory system, leading to increased cholesterol and TG levels in the blood, which can cause arteriosclerosis. Alternatively, the secretion of tumor necrosis factor α, which blocks the function of insulin, is increased, leading to insulin resistance and contributing to diabetes. When these symptoms occur, it leads to metabolic syndrome. Although not at a statistically significant level, LT tended to decrease the blood TC. It was considered that the reason why the blood TG tended to decrease in this experiment was that the visceral fat decreased. These results suggested that LT has the possibility to improve metabolic syndrome.

A study comparing the polyphenols of Kuding tea and insect feces tea prepared from the insects fed with the Kuding tea leaves has demonstrated that polyphenol from insect feces tea increased the activity of superoxide dismutase, glutathione peroxidase and glutathione, and reduced activity of nitric oxide and malonaldehyde in mice than

polyphenols in Kuding tea (Zhao et al., 2018). Although these polyphenols were not identified, it was predicted that the transformation of Kuding tea polyphenols in insects could have increased the antioxidant activity in the latter. Several studies have shown that plant polyphenols act as defensive agents against insects (Orozco-Cardenas et al., 1993) (Chung et al., 2013). Reportedly, polyphenols act as digestive inhibitors for insects, hence insects neutralize the action of polyphenols by secreting glycine in the body. Briefly, upon feeding, polyphenols are oxidized and converted to quinones that bind to proteins to form macromolecules, consequently making protein nonnutritive (Konno *et al*., 1997). To avoid this, insects use glycine that prevents the binding of quinones to proteins. Additionally, it has been shown that quinones have high reactivity and can transform into other polyphenols (Masuda et al., 2005). For instance, purpurin, a quinone, has been shown to have the ability to suppress lipid accumulation (Nam et al., 2019).

Moreover, insect feces are fermented by gut bacteria as they pass through the digestive system of the insect (Dillon & Dillon, 2004). It has been reported that the flavonoids, epigallocatechin and gallocatechin, are converted by gut bacteria, Adlercreutzia equolifaciens JCM 14793, Asaccharobacter celatus JCM 14811, Slackia equolifaciens JCM 16059 and Slackia isoflavoniconvertens JCM 1613712, that metabolize isoflavones (Takagaki & Nanjo, 2015). Similarly, daidzein, a soy isoflavone, is metabolized to equol, which has stronger estrogenic activity, by the lactic acid bacterium, *Lactococcus garvieae* (Mayo et al., 2019) (Shimada et al., 2012). Collectively, these studies indicate that the polyphenols in plants upon feeding are converted into other active ingredients in insects, which is passed into their feces; thereby increasing the functionalities of the latter. There are no reports on the

components of *B. catharticus* and their ability to suppress lipid accumulation. However, these facts suggest that LT might have the polyphenols derived from *B. catharticus*, which could have gained higher functionalities being modified by locust gut microbiota.

1.6 CONCLUSIONS

In the past, insect food was looked down upon and was generally not eaten, except in a few areas. However, insects are now recognized as food globally, which may help solve food shortage problems in the future. In fact, insects can be nutritious as food. Furthermore, the use of insect food may have environmental benefits. Since ancient times, insect tea has been used as an herbal medicine in parts of China, and it is said to have various beneficial effects. In this study, we demonstrate one of its efficacies and show the potential of insect food as a functional food. We hope that research on the functionality of various insects will progress in the future and that insect food will be used as a functional food that can relieve adult diseases as well as food shortages.

1.7 FIGURES

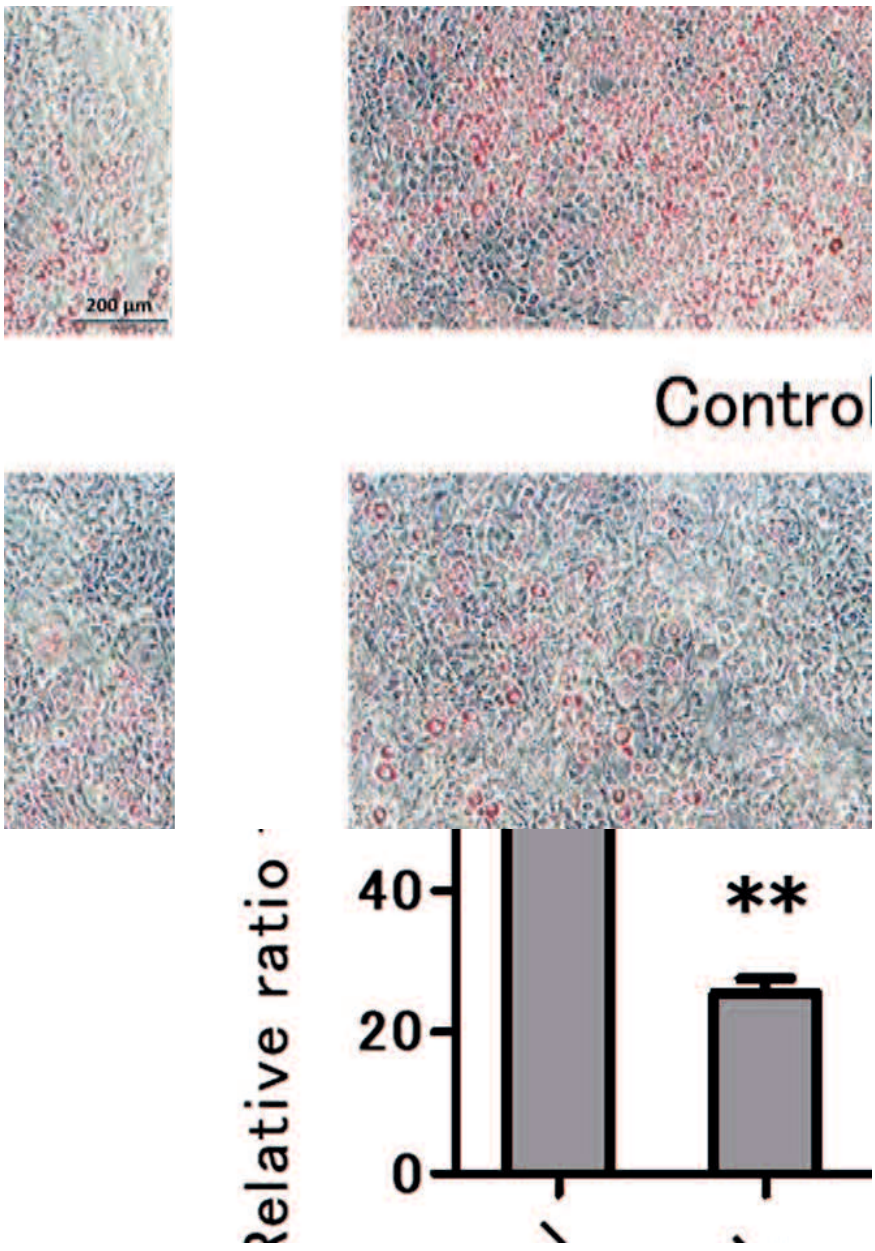

Fig. 1.1 Effect of insect tea of *Locusta migratoria* (LT) on adipocyte differentiation in 3T3-L1 cells.

(A) Representative images of Oil Red O staining. Adipogenesis was induced in the presence or absence of LT (0, 0.25, 0.5, or 1.0 mg/mL LT). (B) The droplets were extracted from the cells with isopropanol. Then, the absorbance of the extracts was measured at 520 nm. Results are shown as the relative percentage of the control values. Values are expressed as the mean ± standard error of the mean (n = 3). $^{**}p < 0.01$ when compared to the control.

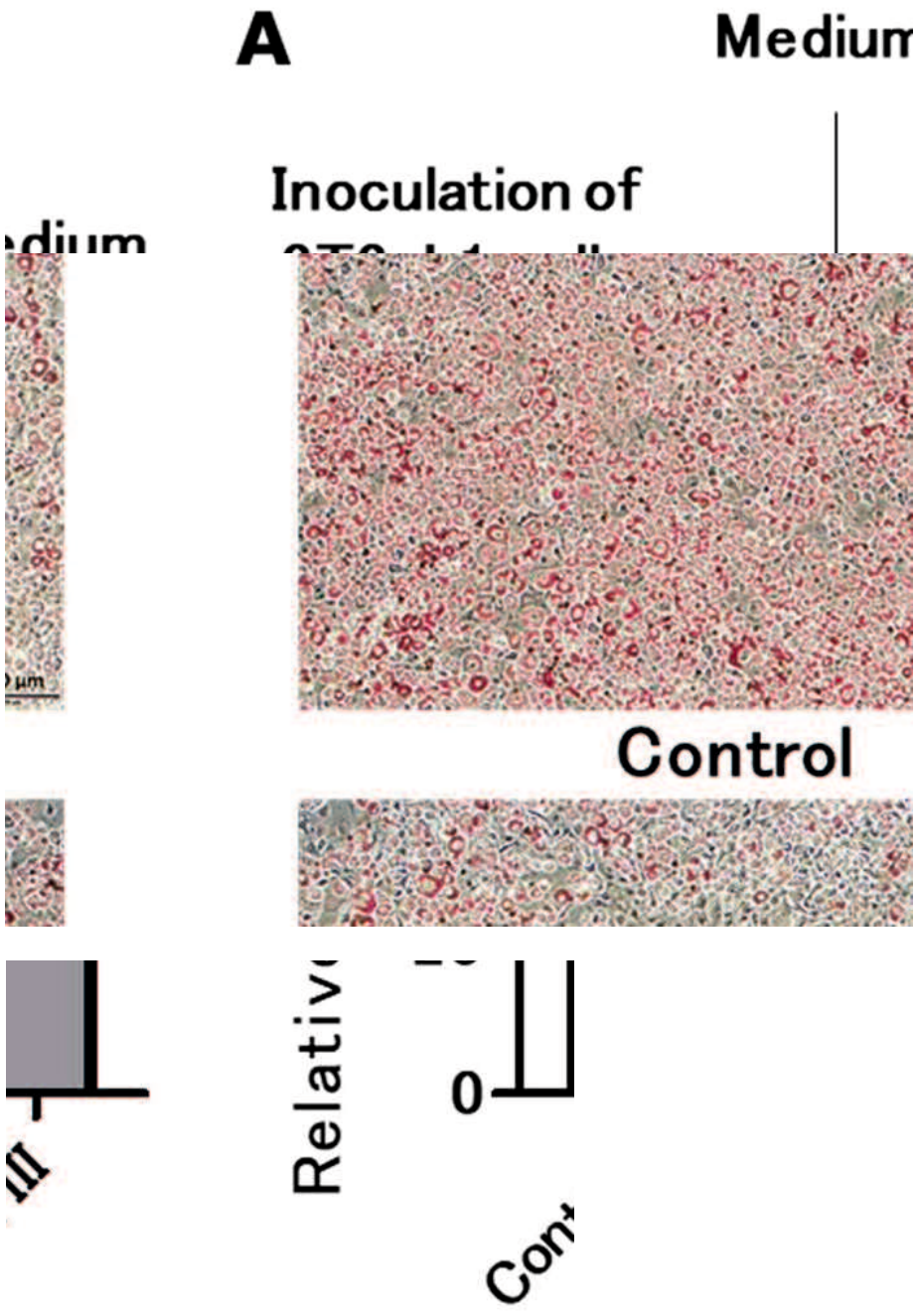
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Fig. 1.2 The inhibitory effect of insect tea of *Locusta migratoria* (LT) occurred during the first stage of adipogenesis.

(A)The scheme of the investigation. Intracellular lipid accumulation was assessed by staining with Oil Red O solution. In Test I, LT was added to the medium in both periods 2 and 3; in Test II, LT was added only in period 2; and in Test III, LT was added only in period 3. The concentration of LT added was 1.0 mg / mL. (B) Representative images of Oil Red O staining. (C) The droplets were extracted from the cells with isopropanol. Then, the absorbance of the extracts was measured at 520 nm. Results are shown as the relative percentage of the control values. Values are expressed as the mean ± standard error of the mean (n = 3). $^{**}p < 0.01$ when compared to the control.

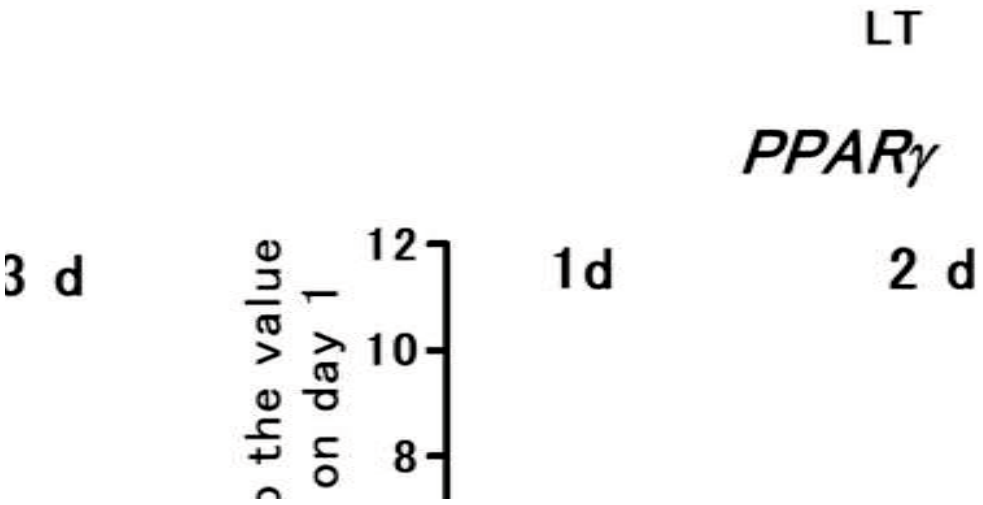

Fig. 1.3 Effect of insect tea of *Locusta migratoria* (LT) on the expression of *PPARγ* and *C/EBPα* in 3T3-L1 cells.

The expression of *PPARγ* and *C/EBPα* was examined by real-time quantitative reverse transcription-polymerase chain reaction every day during the 3 days of period 2. Values are expressed as the mean ± standard error of the mean (n = 3). $^{**}p < 0.01$ when compared to the value of the control on day 1.

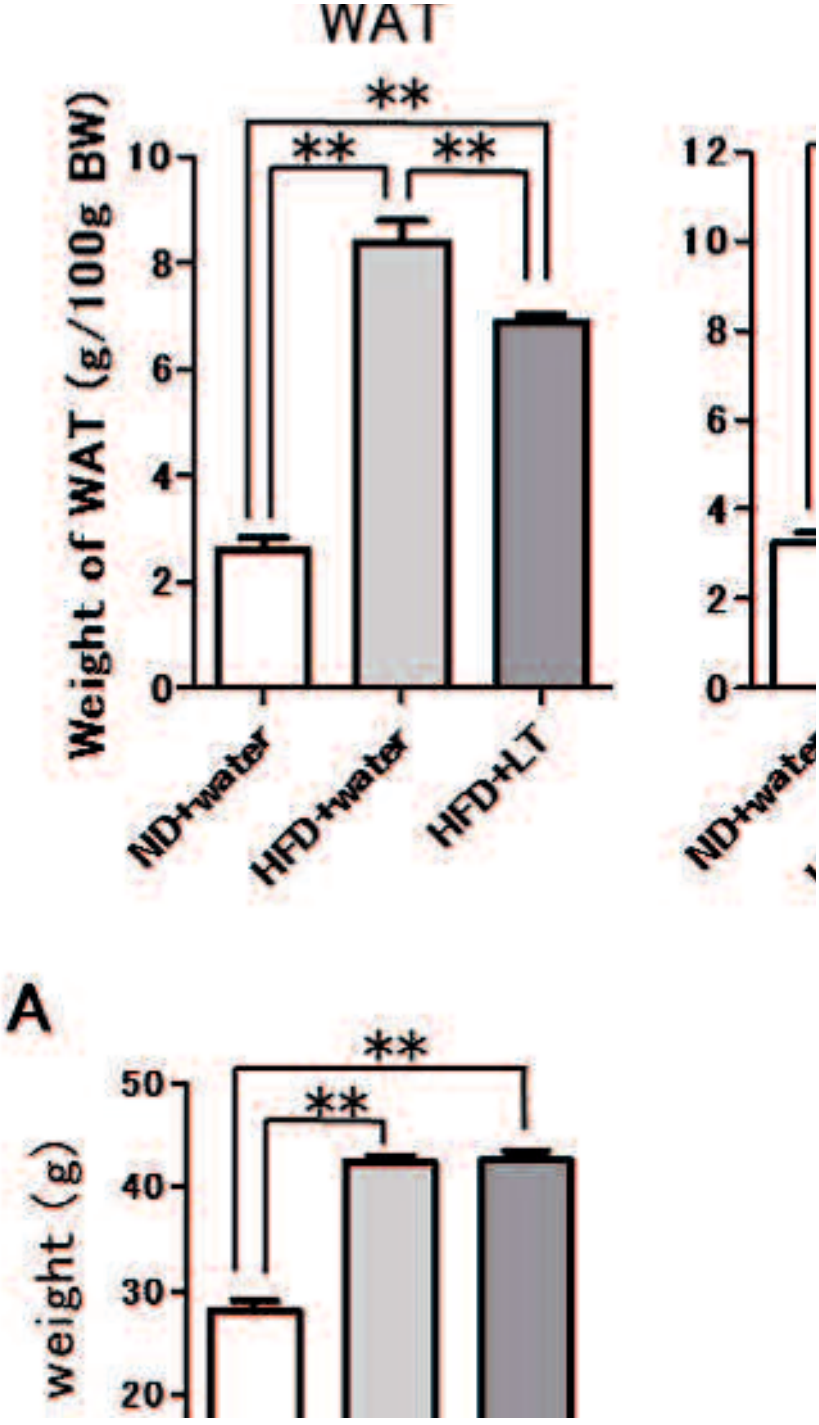

Fig. 1.4 Effect of insect tea of *Locusta migratoria* (LT) on the body weight of mice and white adipose tissue (WAT)

(A) Effect of LT on the body weight in male C57BL/6NCrSlc mice fed a normal diet with water (ND + water; n = 4), a high-fat diet with water (HFD + water; n = 5) or LT (HFD + LT; n = 6) at the end of the experimental period. (B) Effect of LT on the weights of WAT in C57BL/6NCrSlc mice at the end of the experimental period. (C) The epididymal WAT and perirenal WAT were considered to constitute the visceral WAT, and the total inguinal WAT and interscapular WAT were considered to constitute the subcutaneous WAT. The sum of all WAT masses was taken to be the total WAT mass. Values are expressed as the mean ± standard error of the mean. Data were analyzed by one-way ANOVA with Tukey test; *$p < 0.05$ and **$p < 0.01$.

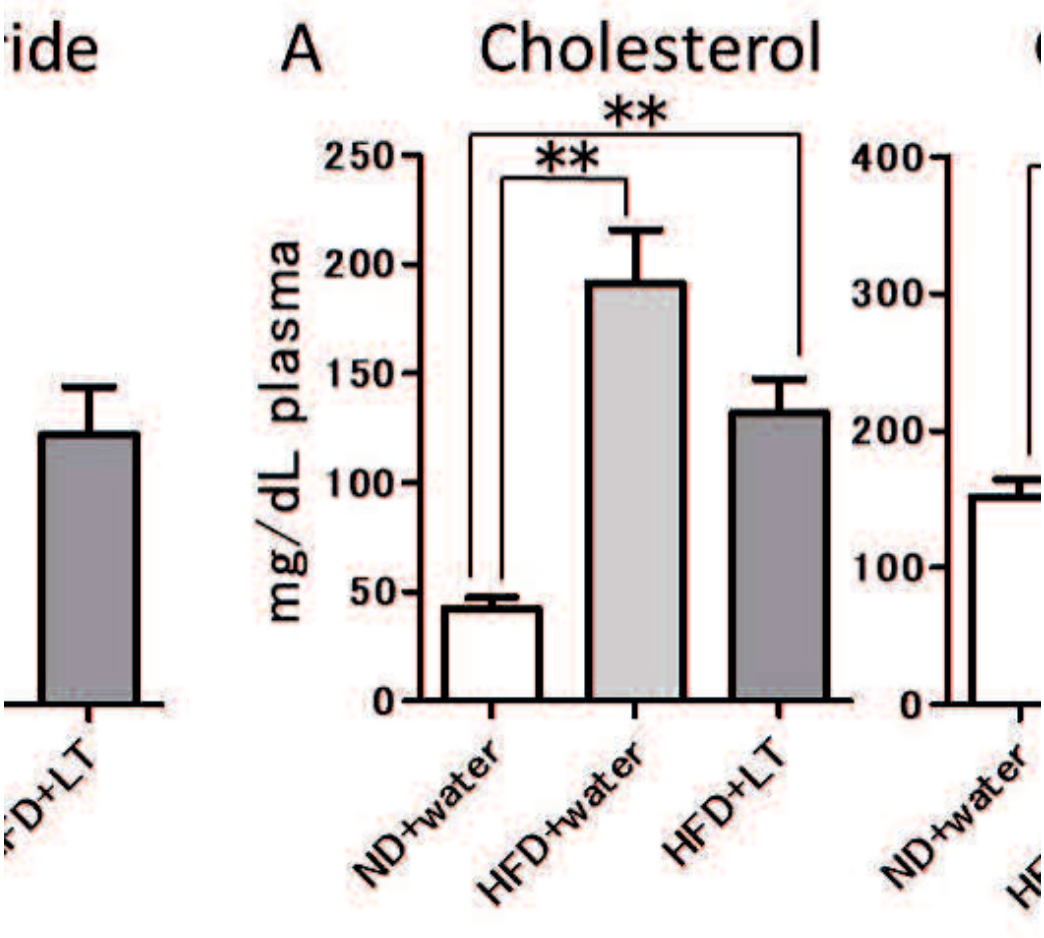

Fig. 1.5 Effect of insect tea of Locusta migratoria (LT) on plasma and liver components.

(A) Effect of LT on the cholesterol, glucose, free fatty acid, and triglyceride levels of plasma in male C57BL/6NCrSlc mice fed a normal diet with water (ND + water; n = 4), a high-fat diet with water (HFD + water; n = 3) or LT (HFD + LT; n = 6) at the end of the experimental period. (B) Effect of LT on the cholesterol and triglyceride levels of the liver in male C57BL/6NCrSlc mice fed a normal diet with water (ND + water; n = 4), a high-fat diet with water (HFD + water; n = 5) or LT (HFD + LT; n = 6) at the end of the experimental period. Values are expressed as the mean ± standard error of the

mean. Data were analyzed by one-way ANOVA with Tukey test; *$p < 0.05$ and **$p < 0.01$.

CHAPTER II

Feces tea of Cherry caterpillar (larvae of *Phalera flavescens*) promotes differentiation into myotubes, activates mitochondria, and suppresses the protein expression of ubiquitin ligase in C2C12.

2.1 ABSTRACT

Sarcopenia is a syndrome characterized by progressive and systemic loss of skeletal muscle mass and strength. In order to prevent sarcopenia and lead a healthy life, it is necessary to maintain muscle mass and prevent loss of muscle mass. Insect feces have long been consumed as tea in China, both as a medicine and as a functional food. In this study, we investigated the efficacy of cherry caterpillar feces tea (PT) for treating sarcopenia, particularly concerning muscle building and atrophy suppression using C2C12 cells. PT treatment (0.2 mg/mL) increased myotube widths by approximately 40% and increased the expression levels of *Myod*, *Myog*, and *MYHC*. Additionally, *PGC1α*, *TFAM*, *SDHA*, *BCAT*, and *BCKDH* were upregulated in a PT concentration-dependent manner. For PGC1α which is the transcription coactivator, the protein expression level also increased in a concentration-dependent manner. The findings demonstrate that PT stimulates PGC1α and activates mitochondria via branched-chain amino acid metabolism and the electron transport chain in C2C12 myoblasts. Furthermore, PT suppressed LPS-induce expression of IL6 and TNFα, and reduced the protein expression levels of the ubiquitin ligases Atrogin1 and MuRF, which are major cause of muscle atrophy. These results indicated that PT may be effective for muscle building and suppression of atrophy.

2.2 INTRODUCTION

Sarcopenia is a disorder characterized by the reduction in muscle mass due to aging and illness, resulting in muscle weakness and deterioration of physical function. Since the discovery of sarcopenia in 1989, the loss of muscle due to aging and disease has become a major problem in an aging society worldwide. One of the major causes of sarcopenia is the degradation of skeletal muscle due to inflammation. Inflammation causes mitochondrial dysfunction and activates the ubiquitin ligases, MuRF and Atrogin-1(Bodine & Baehr, 2014). As a result, muscle decomposition by the proteasome progresses and muscle mass decreases. Recent studies showed that sarcopenia induces the development of diabetes and pneumonia (Mesinovic et al., 2019)·(Okazaki et al., 2020). It has been reported that quercetin, catechin, and resveratrol, which have anti-inflammatory effects in foods, may prevent muscle atrophy by suppressing the expression of these ubiquitin ligases (Mukai et al., 2010) (Hemdan et al., 2009). It is also important to improve sarcopenia by promoting muscle building as much as preventing atrophy. Muscle, which is the skeletal muscle tissue, grows and enlarges through the action of satellite cells in muscle fibers. Stimulated satellite cells differentiate into progenitor cells known as myoblasts, which proliferate via division and then differentiate into myocytes. Subsequently, myocytes fuse to form myotubes, leading to skeletal muscle growth by fusing with existing muscle fibers(Hartman & Spudich, 2012). Differentiation into myotubes is mainly regulated by transcription factors in the myogenic regulatory factor (MRF) family, including myoblast determination protein (MYOD), myogenin (MYOG), myogenic factor 6 (MRF-4), and myogenic factor 5 (MRF-5) (Hernández-Hernández et al., 2017). Among these, MYOD

and MYF-5 determine whether satellite cells differentiate into myoblasts and play an important role in their maintenance. MYOG exerts an essential function in the early stages of differentiation into myotubes; MRF4 is expressed in the late stages of differentiation and maintains myotubes (Hughes et al., 1993). It is important to obtain protein from the diet to gain muscle mass. In recent years, consumption of foods that activate MRFs and show a muscle-building effect has attracted attention as a preventive and countermeasure for sarcopenia. Catechin, which is present in green tea, promotes the expression of *Myog* by activating TAZ, a transcription coactivator of *Myod*(A. R. Kim et al., 2017). Folic acid is abundant in spinach and chicken liver and promotes the expression of *Myod* and *Myog* by regulating the protein kinase B (AKT) signaling pathway(Hwang et al., 2015). It has also been shown that insulin growth factor (*Igf*)-*1* stimulates myoblast proliferation and differentiation. In addition, plum-derived polyphenols increase *Igf-1* levels and lead to the development of muscle hypertrophy (Alsolmei et al., 2019). C2C12 cells, which are typical myoblasts, are often used in in vitro skeletal muscle differentiation experiments. A few studies identified functional foods that promote differentiation into myotubes using C2C12 cells. Such foods have the possibility to build muscle and improve sarcopenia. Although exercise is the best approach for gaining muscle mass, the elderly population must gain muscle mass by consuming food when exercise is not viable.

In 2013, the Food and Agriculture Organization of the United Nations suggested the proactive use of insects as food and feed. The European Union also regulated insects as a novel food in 2015. Therefore, insect-based food is recognized as a food in Europe but is not consumed daily. Insects as food contain large amounts of important nutrients, such as lipids, fatty acids, and vitamins, in addition to high levels of protein, which are

the main components of these foods (T. K. Kim et al., 2019) (de Castro et al., 2018). The functional properties of edible insects have recently been investigated, such as their anti-blood pressure and antioxidant effects (Vercruysse et al., 2005) (Dutta et al., 2016). In Japan, it is customary to eat salted cherry leaves because of their pleasant scent. The raw leaves do not contain a scent; however, when they are crushed or salted, coumalic acid glycosides react with enzymes in the leaves to produce coumarin, imparting a pleasant fragrance to the crushed leaves(Takahashi et al., 2006). The larvae of *Phalera flavescens*, known as the cherry caterpillar in Japan, eat cherry leaves as their staple food; therefore, both the cherry caterpillar and its feces contain the same scent as salted cherry leaves. “Feces tea” is prepared using insect feces in China (L. Xu et al., 2013) after feeding the moth larvae naturally fermented leaves that are crushed into small pieces that are further fermented by enzymes in the insect digestive tract(L. Xu et al., 2013). Feces tea contains not only several nutrients, such as amino acids, minerals, and fatty acids, but also antioxidants, including polyphenols (L. Xu et al., 2013) (Zhao et al., 2018). Various studies have explored the functional properties, such as anti-inflammatory and anti-obesity effects, of feces tea (Zhao et al., 2018) (Zhao et al., 2017). However, the functions of feces tea in cherry caterpillar have not been examined. We investigated the possibility of improving sarcopenia in the fecal tea of cherry caterpillar in the two aspects of muscle building and suppression of atrophy for the first time using C2C12 cells, which are mouse striated muscle cells and are frequently used in skeletal muscle differentiation experiments in vitro.

2.3 MATERIALS AND METHODS

Preparation of feces tea of *P. flavescens*.

The feces of *P. flavescens* larvae bred cherry leaf (*Prunus yedoensis* Matsumura) in Yamaguchi University (Yamaguchi, Japan). Feces were collected as appropriate during breeding. Feces tea of *P. flavescens* (1 g) was extracted using boiled water (20 mL) for 10 min. The feces solution was centrifuged at 10,000 ×*g* for 10 min and filtered through a 0.2-μm pore Steradisc (KURABO, Osaka, Japan). The filtrate obtained was used as the PT. PT was diluted with the assay medium and used for *in vitro* assays involving C2C12 cells.

Cell culture and differentiation.

C2C12 cells were obtained from the European Collection of Authenticated Cell Cultures and cultured in Dulbecco's modified Eagle's medium (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (Gibco, Grand Island, NY, USA), 100 U/mL penicillin, and 100 U/mL streptomycin (Sigma-Aldrich) at 37°C under 5% CO_2. Penicillin and streptomycin were added to all culture media used thereafter. Briefly, cells were seeded at 1.5×10^4 cells per well in 24-well plates and cultured in growth medium for 3 days until reaching 100% confluency. The medium was changed to differentiation medium (Dulbecco's modified Eagle's medium supplemented with 2% fetal horse serum [Gibco]) to induce differentiation. The cytotoxicity of PT on C2C12 cells was examined using a Cell Counting Kit-8 (Dojindo Laboratories, Kumamoto, Japan).

Immunocytochemistry.

For immunostaining, the cells were fixed in 4% formaldehyde for 15 min after 3 days of culture in differentiation medium containing PT (0.05, 0.1, or 0.2 mg/mL), washed with phosphate-buffered saline (PBS), and blocked in 2% fetal bovine serum at room temperature for 1 h. The primary antibody was incubated overnight at 4°C. After several washes with PBS, the secondary antibody was incubated for 1 h at room temperature. The primary antibody was monoclonal anti-slow skeletal myosin heavy chain (ab11083; Abcam, Cambridge, UK; 1:300), and the secondary antibody was anti-IgG + IgM (H + M), mouse, goat-poly, Fluorescein isothiocyanate (Funakoshi, Tokyo, Japan). The cells were washed with PBS and counterstained with anti-fade solution containing 4',6-diamidino-2-phenylindole (SouthernBiotech, Birmingham, AL, USA). Fluorescence images were captured using a microscope equipped with CellSens Pro (Olympus, Tokyo, Japan). The width of the immunostained myotubes was measured using ImageJ software (National Institutes of Health, Bethesda, MD, USA). The average width was obtained from five randomly selected fields for each treatment, and the experiments were performed independently in triplicate.

mRNA preparation and qRT-PCR.

C2C12 cells in a 24-well plate were incubated in differentiation medium containing PT (0.05, 0.1, or 0.2 mg/mL). At 2 days after inducing differentiation, the cells were harvested, and total RNA was extracted from the cells using an RNeasy Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. cDNA was synthesized using random primers and the PrimeScript Reverse Transcriptase kit (Takara Bio, Shiga, Japan). An aliquot of cDNA was used as a template for qRT-PCR in a StepOnePlus

Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). The target cDNAs were amplified using Fast SYBR Green Master Mix (Applied Biosystems) with the following gene-specific primers: *Myod* (MA128901; Takara Bio), *Myog* (MA127738; Takara Bio), *Myhc* (MA117697; Takara Bio), *Igf-1* (MA109541; Takara Bio), *Tfam* (MA027412; Takara Bio), *ESRα* (MA128422; Takara Bio), *Acad* (MA078709; Takara Bio), *Acox* (MA061629; Takara Bio), *Cpt1* (MA031170; Takara Bio), *Bcat* (MA102865; Takara Bio), *Bckdh* (MA111227; Takara Bio), *Sdha* (MA115726; Takara Bio), and *PGC1α* (sense, 5′-CACCAAACCCACAGAAAACAG-3′; antisense, 5′-GGGTCAGAGGAAGAGATAAAGTTG-3′). The relative expression level of each mRNA was normalized to that of the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*; MA050371, Takara Bio).

Protein quantification by the Jess Simple Western System.

Protein quantification was performed with the Jess Simple Western System (Proteinsimple, San Jose, CA, USA). Proteins extracted from C2C12 cells with cOmplete™ Lysis-M (Sigma-Aldrich) and protein lysates (0.5mg/mL) were mixed with fluorescent standards, Master Mix, dithiothreitol, and Simple Western Sample Buffer (Proteinsimple) and then were loaded into Wes 25-well plates. Primary antibody against Gapdh was purchased from Cell Signaling Technology (MA, Danvers, USA), and primary antibody against Pgc1α was purchased from Proteintech (IL, Rosemont, USA). Muscle Atrophy Ubiquitin Ligase Antibody Sampler Kit (ECM biosciendes, KY, Versailles, USA) was used as primary antibody. The appropriate secondary antibodies, stacking and separation gel matrix were added according to the manufacturer's instructions. Protein bands were obtained using the software, Compus for Simple

Western (Proteinsimple). The relative expression level of each protein was normalized to that of the housekeeping protein Gapdh.

Mitochondrial staining.

Active staining of mitochondria was performed using the JC-1 MitoMP Detection Kit (Dojindo Laboratories). After 2 days of incubation in differentiation medium containing PT (0.05, 0.1, or 0.2 mg/mL), the medium was changed to 20 μmol/L JC-1, and the cells were incubated at 37°C under 5% CO_2 for 1 h. After washing the cells with PBS, the imaging buffer provided with the kit was added to the cells. Fluorescence images were captured using a microscope equipped with CellSens Pro.

Lipopolysaccharide (LPS) -induced inflammation in C2C12 cells.

C2C12 cells in a 24-well plate were incubated in differentiation medium. At 5 days after inducing differentiation, the medium was changed to 500 μL differentiation medium containing PT (0.05, 0.1, or 0.2 mg/mL) and pre-incubated for 2 hours. After adding 2 μg of LPS and incubating for 2 hours, the cells were harvested, and total RNA was extracted from the cells using an RNeasy Kit. The expression levels of Atrogin-1, Murf, TNFα, and IL6 were examined by qRT-PCR. Atrogin-1 (MA117269, Takara), MuRF (MA061093, Takara), TNFα (MA031450, Takara), and IL6 (MA152279, Takara) were used as primers. When examining the protein expression levels of Atrogin1 and MuRF, the same procedure was performed, cells were collected 24 hours after LPS addition, and their expression levels were measured by the Jess Simple Western System.

Statistical analysis.

Data were analyzed by one-way analysis of variance followed by Dunnett's test or an unpaired *t*-test with Welch's correction using GraphPad Prism (GraphPad, Inc., San Diego, CA, USA). Statistical significance was denoted by values of $p < 0.01$ or $p < 0.05$.

2.4 RESULTS

Effect of *P. flavescens* larval feces tea (PT) treatment on C2C12 cell differentiation into myotubes.

Before studying the effects of PT on muscle differentiation, its effects on cell viability were determined during differentiation. C2C12 cells were cultured in differentiation medium containing PT (0, 0.05, 0.1, and 0.2 mg/mL) for 3 days. There were no significant differences in cell viability among all concentrations (date not shown). Subsequently, the effect of PT on morphological changes in C2C12 cells related to the differentiation process was investigated by immunostaining of the myosin heavy chain (Fig. 1). PT treatment promoted the myogenic differentiation of C2C12 cells. Furthermore, the thickndths and number of immunostained myotubes were measured to confirm the effects of PT treatment on myogenic differentiation. The results showed that the width of myotubes increased in a concentration-dependent manner. Particularly, at a PT concentration of 0.2 mg/mL, the width increased by approximately 40% (Fig. 2a). There was no significant difference in the number of cells; however, a concentration-dependent increase was observed (Fig. 2b).

Effect of PT treatment on gene expression of MRFs and energy metabolism in C2C12 cells.

The expression of *Myod* and *Myog* coding for MRFs and *Myhc* was examined by real-time quantitative reverse transcription-polymerase chain reaction (qRT-PCR) two days after the initiation of differentiation of cells treated with PT (Fig. 3a). The expression levels of all three genes increased in a concentration-dependent manner

following addition of PT. In addition to MRFs, energy metabolism is involved in the gain of muscle mass(Gill et al., 2018); therefore, we investigated the expression levels of genes related to energy metabolism via qRT-PCR two days after the initiation of differentiation of cells treated with PT (Fig. 3b). Addition of PT increased the expression levels of *Pgc1α* and *Tfam*. The expression levels of *Esrα* and *Cpt1*, which are β-oxidation-related genes, were increased, whereas there was no change in the expression of *Acox* and *Acad.* Additionally, PT induced a concentration-dependent increase in the expression of *Sdha*, which is involved in the electron transport chain. Furthermore, the expression levels of *Bcat* and *Bckdh*, factors in branched-chain amino acid (BCAA) metabolism, were significantly increased. The expression level of Pgc1α, which was a transcription coactivator, was examined by Western blotting (Fig. 4). PT increased the protein expression level of Pgc1α.

Effect of PT treatment on mitochondria in C2C12 cells.

To confirm whether PT activates mitochondria, active staining of mitochondria in C2C12 cells was performed two days after the initiation of differentiation in cells treated with PT (Fig. 5). The intensity of the red color clearly increased in cells treated with PT compared to in control cells, indicating that PT activated mitochondrial biosynthesis and metabolism.

Effect of PT on LPS-induced inflammation in C2C12 cells.

LPS was added to C2C12 cells to induce inflammation, and the effect of PT on inflammation was investigated. The gene expression levels of the inflammatory cytokines IL-6 and TNFα were significantly suppressed in C2C12 cells 2 h after LPS

addition, suggesting that PT had an anti-inflammatory effect (Fig. 4A). *Atrogin1* and *MuRF* decreased slightly but not significantly (Fig. 4A). However, the expression level of these proteins was significantly suppressed 24 hours after the addition of LPS (Fig.4B).

2.5 DISCUSSION

For C2C12 cells, the expression levels of *Myod*, *Myog*, and *Igf-1* increased after PT treatment. It has been shown that *Igf-1* promotes skeletal muscle cell differentiation by upregulating *Myog* via the phosphatidylinositol 3-kinase/AKT signaling pathway (Delling et al., 2000) (Machida & Booth, 2004). Additionally, daidzein, a soybean-derived isoflavone, increases muscle mass by stimulating *Igf-1*(Zheng et al., 2018). Betaine present in various foods stimulates *Igf-1* to promote the differentiation of C2C12 cells (Senesi et al., 2013). These studies indicate that PT induces the expression of *Igf-1* to promote differentiation. In addition to MRFs, we confirmed that the gene and protein expression level of Pgc1α were upregulated in the process of C2C12 cell differentiation via PT treatment. Pgc1α is a major regulator of mitochondrial biosynthesis (Luo et al., 2016). In addition to its role as a transcription factor, Tfam also has a role as a structural protein that non-specifically binds to mtDNA and maintains its structure and stability, and is activated by Pgc1α via nuclear respiratory factor, resulting in activation of mitochondrial biosynthesis (Gureev et al., 2019) (Matsukawa et al., 2017). In fact, the gene expression level of Tfam increased in this experiment. Despite the upregulation of *Esrα* and *Cpt1*, there was no change in the expression of *Acox* and *Acad*. Because *Acox* is localized in the peroxisomes, it was predicted that addition of PT, which is thought to activate mitochondria, did not affect the expression level of *Acox*. Esrα is known to activate the expression of genes involved in energy metabolism, such as those associated with β-oxidation and the electron transport chain of mitochondria, in skeletal muscles (Ventura-Clapier et al., 2019). In addition, as Esrα targets Cpt1 (Ventura-Clapier et al., 2019), Cpt1 was predicted to be upregulated via

activation of Esrα. However, although Cpt1 is an early initiation gene for β-oxidation localized in the outer mitochondrial membrane, the expression level of *Acad* did not change. PT treatment activated only the uptake of long-chain fatty acids and did not affect β-oxidation. Furthermore, in experiments using transgenic mice overexpressing *Pgc1α* in skeletal muscles, the expression levels of *Bcat* and *Bckdh* are significantly increased compared to those of wild-type mice (Hatazawa et al., 2014). Bcat is an enzyme that transfers BCAA to branched-chain keto acids in the first step of the BCAA cycle. In the second step, the enzyme encoded as BCKDH decarboxylates branched-chain keto acid and converts it to a coenzyme A compound (Karlsson et al., 2006). In our study, *Bcat* and *Bckdh* were upregulated by activation of Pgc1α in C2C12 cells, indicating that they affected BCAA metabolism. BCAA metabolism leads to activation of the electron transport chain via the ATC cycle (Li et al., 2017). Moreover, we found that PT activated *Sdha*; therefore, PT affects the electron transport chain by activating upstream BCAA metabolism. Mitochondrial activity and muscle mass are closely related, as supported by the fact that mitochondrial activation restores the differentiation ability of C2C12 cells (Watanabe et al., 2020). Therefore, mitochondria activation is involved in promoting the differentiation of cells into myosin, which may lead to an increase in muscle mass. Collectively, these results suggest that PT treatment promotes the differentiation of C2C12 cells into myotubes via signal transduction, as shown in Fig. 5. In addition to promoting the differentiation of C2C12, PT also suppressed the protein expression levels of the ubiquitin ligases Atrogin1 and MuRF. In skeletal muscle, muscle degradation is promoted by Atrogin1 and MuRF induced by signals from inflammatory cytokines such as TNF-α and IL-6 (Grounds, 2002).

There are many reports that plant polyphenols have anti-inflammatory and antioxidant effects and suppress muscle atrophy (Mukai et al., 2010) (Hemdan et al., 2009), and that they activate PGC1α in skeletal muscle (L. Wang et al., 2016) (Ray Hamidie et al., 2015). The cherry blossoms used as feed in this study have been reported to have antioxidant and anti-inflammatory effects (Takahashi et al., 2006) (J. Lee et al., 2013). The fermented ingredients in feces of cherry caterpillars feeding on the leaves that undergo fermentation by gut bacteria (Dillon & Dillon, 2004) may have contributed to the beneficial effects of PT. There was a report that, comparing the polyphenols of Kuding tea, which used Kuding tea leaves as feed, and insect feces tea, insect feces tea had more antioxidant effect (Zhao et al., 2018). Although these polyphenols have not been identified, it is predicted that the polyphenols of Kuding tea have increased antioxidant activity as a result of transformation in insects. The flavonoids, epigallocatechin and galocatechin, are converted by gut bacteria, *Adlercreutzia equolifaciens* JCM 14793, *Asaccharobacter celatus* JCM 14811, *Slackia equolifaciens* JCM 16059 and *Slackia isoflavoniconvertens* JCM 1613712, that metabolize isoflavones (Takagaki & Nanjo, 2015). Daidzein, a soy isoflavone, is metabolized to equol, which has stronger estrogenic activity, in gut bacteria such as the lactic acid bacterium, *Lactococcus garvieae* (Mayo et al., 2019) (Shimada et al., 2012). These facts indicate that polyphenols in plants that feed insects may be converted into other active ingredients in insects and contained in insect feces. Since PT has antioxidant activity (data not shown), it is highly possible that it contains polyphenols derived from cherry leaves. Further studies are needed to isolate and identify the active ingredients in PT.

2.6 CONCLUSIONS

During aging, the rate of protein degradation required to generate muscle mass exceeds that of protein synthesis. Muscle loss due to aging and illness is associated with a risk of being bedridden and movement disorders. This weakens the muscles, potentially leading to the development of sarcopenia, which can be ameliorated by supplementation of proteins, as exercise is not a viable option for the elderly. Insect-based food consumption has gained attention because of its nutritional and various other benefits. This study investigated the efficacy of PT for treating sarcopenia using C2C12 mouse myoblasts. It was suggested that PT induces myoblast differentiation into myotubes and activates C2C12 mitochondria. Furthermore, it was suggested that PT has an anti-inflammatory effect and suppresses muscle atrophy. From the above results, PT may be an effective beverage for the treatment of sarcopenia.

2.7 FIGURES

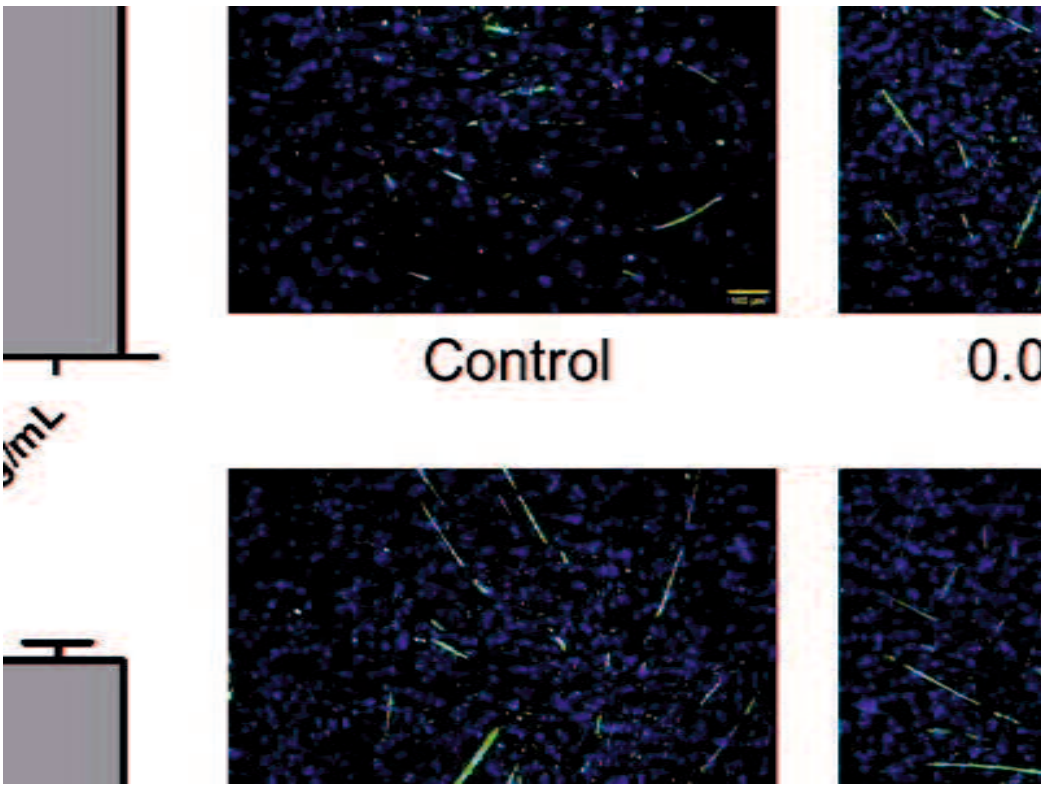

Fig. 2.1 Effects of PT treatment on C2C12 myoblast differentiation.

(A) Representation images of differentiated cells after treatment with several concentrations of PT showing nuclei stained in blue and myotubes stained in green. Pictures were taken using an Olympus fluorescence microscope. The bar represents a length of 100 μm. (B) The width of the immunostained myotubes from five random fields were measured using the ImageJ software. The numbers above the bars indicate the average width (μm). (C) The effect of PT treatment on the number of myotubes. Values are expressed as the mean ± standard error of the mean (n = 3). *$p < 0.05$. compared to the control. PT, *Phalera flavescens* larval feces tea.

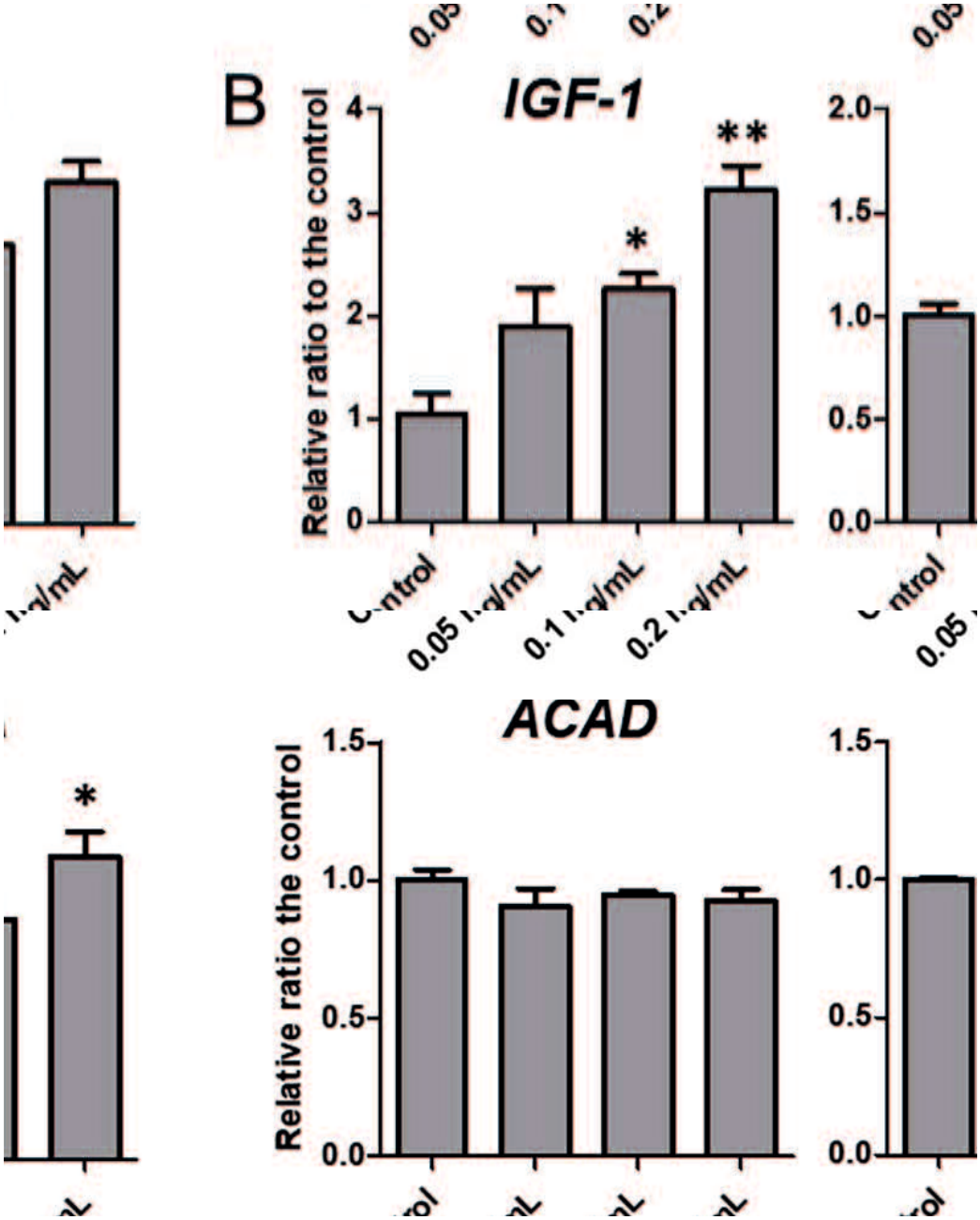
B
IGF-1
Relative ratio to the control
4
3
2
1
0
*
**
2.0
1.5
1.0
0.5
0.0
0.05
0.1
0.2
ACAD
Relative ratio the control
1.5
1.0
0.5
0.0

Fig. 2.2 Effect of PT treatment on the expression of myogenic regulatory factors family genes and energy metabolism genes, and the protein expression of PGC1α in C2C12 cells.

(A)(B)The expression of each gene was examined via real-time PCR 2 days after the initiation of differentiation of cells treated with PT. (A) The effect of PT treatment on the expression of MRF genes; (B) The effect of PT treatment on the expression of energy metabolism genes. Each mRNA level was normalized to the Gapdh level. (C) The protein on of PGC1α was examined via the Jess Simple Western System 2 days after the initiation of differentiation of cells treated with PT. Protein level was normalized to the Gapdh level. Values are expressed as the mean ± standard error of the mean (n = 3). *$p < 0.05$ and **$p < 0.01$ when compared to the control group as the mean ± standard error of the mean (n = 3). PT, *Phalera flavescens* larval feces tea; PCR, polymerase chain reaction; Myod, myoblast determination protein; Myog, myogenin; Myhc, Myosin heavy chain; Igf-1, insulin growth factor 1; Pgc1α, peroxisome proliferator-activated receptor gamma coactivator 1-alpha; Tfam, mitochondrial transcription factor A; ESRα, estrogen-related receptor alpha; Acad, acyl-CoA dehydrogenase; Acox, acyl-CoA oxidase; Cpt1, carnitine/choline acetyltransferase 1; Sdha, succinate dehydrogenase complex subunit A flavoprotein variant; Bcat, branched-chain aminotransferase; Bckdh, α-ketoacid dehydrogenase

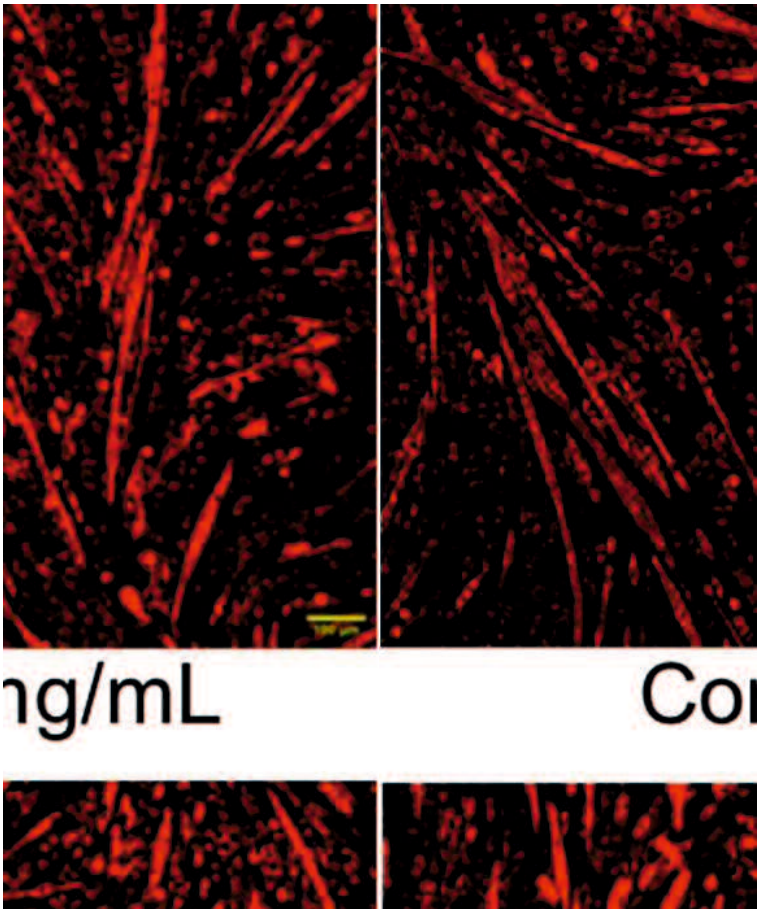

Fig. 2.3 Mitochondria active staining via the JC-1 MitoMP Detection Kit.

After two days of incubation in a differentiation medium containing PT (0.05, 0.1, or 0.2 mg/mL) and control, the medium was changed to 200 nmol/L MitoRed (Dojindo Laboratories), and the cells were incubated at 37 °C under 5% CO_2 for 1 h. Fluorescence images were captured using a microscope equipped with CellSens Pro. Mitochondria were stained red. PT, *Phalera flavescens* larval feces tea.

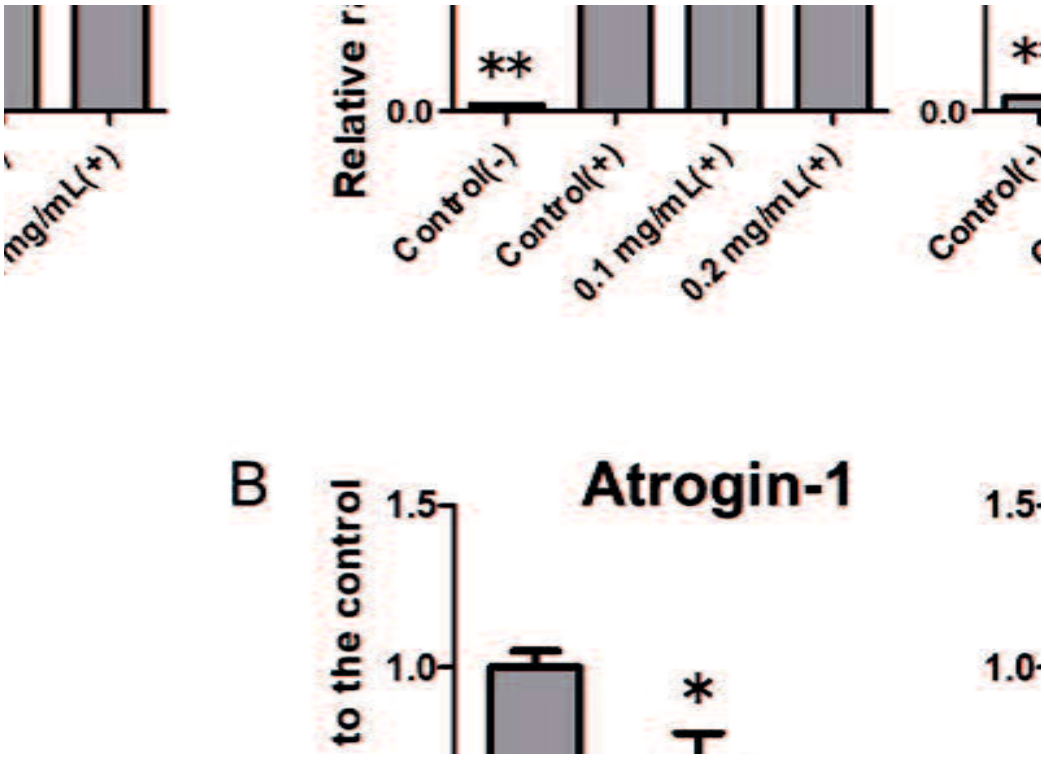

Fig. 2.4 PT-mediated effects on LPS-treated C2C12 myotubes.

The C2C12 cells were cultured in differentiation medium for 5 day. After 2 h of pretreatment with PT (0.1 and 0.2 mg/mL), the cells were stimulated with LPS. (A) Two hours after stimulated with LPS, the total RNA was extracted from the C2C12 cells, and RT-PCR for IL-6, TNFα, Atrogin-1 and MuRF were performed. Each mRNA level was normalized to the Gapdh level. (B) A day after stimulated with LPS, the protein was extracted from the C2C12 cells, and the Jess Simple Western System for Atrogin-1 and MuRF were performed. Each protein level was normalized to the Gapdh level. Values are expressed as the mean ± standard error of the mean (A; n = 3, B; n=4). *$p < 0.05$ and **$p < 0.01$ when compared to the control or control (+) group as the mean ±

standard error of the mean. PT, *Phalera flavescens* larval feces tea; PCR, polymerase chain reaction; IL-6, interleukin-6; TNFα, tumor necrosis factor alpha; MuRF, muscle specific ring finger protein; Gapdh, glyceraldehyde-3-phosphate dehydrogenase; LPS, lipopolysaccharide.

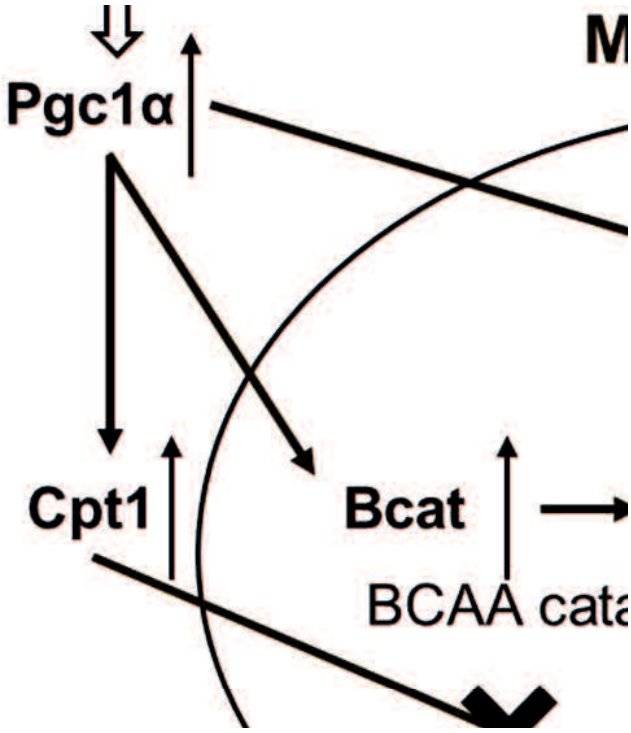

Fig. 2.5 Schematic representation of the signal transduction activated via PT treatment of C2C12 cells.

PT induces *Myod* and *Igf-1* to promote differentiation. PT stimulates *Pgc1α* and activates *Tfam*. It was found that BCAA metabolism was activated by PT based on the upregulation of *Bcat* and *Bckdh*. The electron transport chain downstream of BCAA metabolism was activated based on upregulation of *Sdha*. These results indicated that the activation of mitochondria, which led to an increase in myotubes. The upward arrows indicate upregulation. PT, *Phalera flavescens* larval feces tea; Myod, myoblast determination protein; Myog, myogenin; Myhc, Myosin heavy chain; Igf-1, insulin growth factor 1; Pgc1α, peroxisome proliferator-activated receptor gamma coactivator 1-alpha; Tfam, mitochondrial transcription factor A; Acad, acyl-CoA dehydrogenase;

Cpt1, carnitine/choline acetyltransferase 1; Sdha, succinate dehydrogenase complex subunit A flavoprotein variant; Bcat, branched-chain aminotransferase; Bckdh, α-ketoacid dehydrogenase.

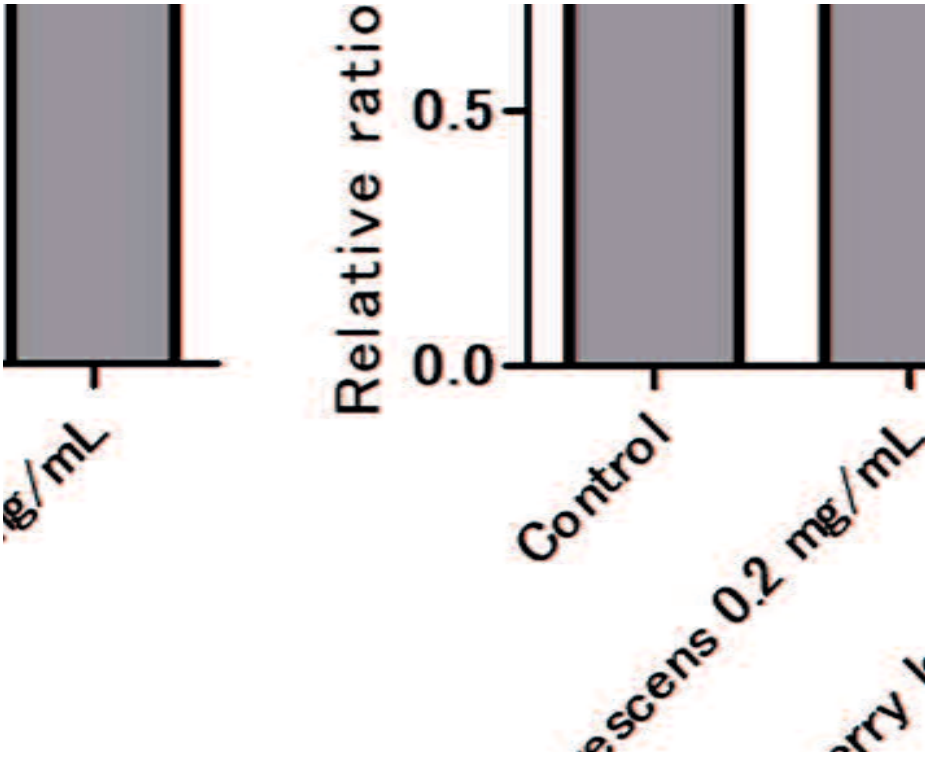

Fig. 2.6 Effect of *Phalera flavescens* and Cherry leaf on the expression of *Myog* and *MYHC* in C2C12 cells.

Both samples were extracted with boiled water in the same manner as the PT extraction method. The expression of each gene were examined with real time-PCR on the 2nd days after the initiation of differentiation treated samples.

GENERAL DISCUSSION

For this study, we clarified the functionality of the insect feces tea in *Phalera flavescens* and *Locusta migratoria*. Migratory locust is one of the most eaten insects in the world, and research on aquaculture is advanced in Japan, and it is expected that it will be on our table in the near future. Cherry caterpillar is an insect that has a scent of cherry blossoms and is evaluated to be very delicious, and has been commercialized as a stick confectionery. Like these insects, when the insect food spreads, insect farming will be carried out all over the world. In that case, insect feces become waste, but we think that they can be used as tea for reuse of resources. Insect feces tea is drunk as a Chinese medicine in Asian countries. In fact, we confirmed that migratory locust feces tea has an anti-metabolic syndrome effect and cherry caterpillar feces tea has an anti-locomotive syndrome effect. We also consider that components such as polyphenols contained in plant leaves are converted into more effective components in the process of becoming feces. Insect feces tea is a very interesting beverage because it is thought that various effects can be imparted by the food given to insects. In other words, the function of insect feces tea can be controlled more easily than other teas. In the future, we hope that not only insect food but also insect feces tea will become more widely known, and research as a functional food will proceed.

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List of Publications

The contents of this thesis have been published in the following original papers.

Chapter 1

Insect feces tea of locust *(Locusta migratoria*) suppresses lipid accumulation in 3T3-L1 cells and mice.

Yushi Takahashi, Hiromi Kuribayashi, Eisuke Tasaki, Izumi Yoshida, Masahiro Ide, Kazuhiro Fujita, Tomoji Igarashi, Shinjiro Saeki, Yoshihito Iuchi

Food Science and Technology Research, 2021, 27(5), 807-816

Chapter 2

Feces tea of Cherry caterpillar (larvae of *Phalera flavescens*) promotes differentiation into myotubes, activates mitochondria, and suppresses the protein expression of ubiquitin ligase in C2C12.

Yushi Takahashi, Izumi Yoshida, Kazuhiro Fujita, Tomoji Igarashi, Yoshihito Iuchi.

International Food Research Journal, in press