博士論文

Functional analysis of lemon myrtle extract, a novel functional food ingredient that activates skeletal muscle satellite cells

（筋衛星細胞を活性化する新規機能性食品素材レモンマートル抽出物の機能解析）

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2022 年 9 月

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２．参考論文

Lemon myrtle (*Backhousia citriodora*) extract and its active compound, casuarinin, activate skeletal muscle satellite cells in vitro and in vivo
Ayumi Yamamoto, Shinichi Honda, Mineko Ogura, Masanori Kato, Ryuichi Tanigawa, Hidemi Fujino, and Seiji Kawamoto
Nutrients, 14(5), 1078 (2022).

主論文

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General introduction

Japan has the longest life expectancy in the world. According to a survey by Japan's Ministry of Health, Labor and Welfare, the average life expectancy in 2019 is 81.41 years for men and 87.45 years for women, and it is estimated to continue to increase [1]. In contrast, healthy life expectancy, the period during which activities of daily living (ADL) are not limited, is approximately 9 years shorter for men and 12 years shorter for women than the average life expectancy [1]. In other words, long-term care is needed for approximately 10 years before the end of life. About 40% of the causes of the need for long-term care are related to the decline in motor function, including frailty, falls, fractures, and articular diseases [2].

Human skeletal muscle decreases at a rate of approximately 1–2% per year after the age of 50 [3]. This age-related skeletal muscle atrophy is termed sarcopenia and is defined as a progressive and generalized skeletal muscle disorder with the accelerated loss of muscle mass and function [4]. Sarcopenia is a major component of frailty [5] and increases the risk of falls and fractures, which results in loss of ADL and quality of life [6]. Therefore, prevention and improvement of sarcopenia is an important issue for extending healthy life expectancy.

The primary intervention for improving sarcopenia is exercise, which has been shown to be effective for older adults with sarcopenia [4]. Among exercise methods, resistance exercise is recommended for improving sarcopenia, but carries the risk of temporary blood

pressure elevation [7] and injury [8]. In contrast, nutritional interventions are simple and safe methods. For example, several studies have reported the effects of protein [9], vitamin D [10], omega-3 polyunsaturated fatty acids [11], and leucine [12] intake on older adults. However, effective nutritional interventions for sarcopenia have not yet been established [4], partly because the differences in skeletal muscle hypertrophy mechanisms between exercise and nutritional interventions are not well understood.

Skeletal muscle is composed of multinucleated cells called myofibers. Since the nuclei of myofibers (myonuclei) are post-mitotic and cannot divide to produce new myonuclei, new myonuclei are supplied by the proliferation of skeletal muscle satellite cells (SCs) [13]. SCs are localized between the basal membrane and the plasma membrane of myofibers [14]. In adult muscle, SCs normally exist in a quiescent and undifferentiated state. However, when skeletal muscle is stimulated by injury or exercise, SCs are activated and enter the cell cycle, dividing to produce myoblasts. Myoblasts further proliferate and differentiate, fuse into myofibers, and form new myonuclei [15]. In addition, some myoblasts return to a quiescent state to maintain the SC pool [16]. With aging, the SC pool diminishes, and the remaining SCs have less capacity for activation, resulting in poor myogenic potential [17]. Verdijk et al. reported that age-related skeletal muscle atrophy is accompanied by a decrease in SC numbers, but resistance exercise increases both myofiber size and SC numbers in older adults [18].

Exercise induces skeletal muscle hypertrophy through both activation of SCs and promotion of muscle protein synthesis [19]. In contrast, most nutritional interventions promote muscle protein synthesis, but little is known about their effects on SC activation [20]. Kaneka Corporation focused on the differences in skeletal muscle hypertrophy mechanisms induced by exercise and most nutritional interventions and searched for novel functional food ingredients that activate SCs. Approximately 200 plant extracts and phytochemicals were preliminarily evaluated for their effects on SC activation *in vitro*, and an extract of lemon myrtle (*Backhousia citriodora*) leaves was found to activate SCs *in vitro* (in-house data from Kaneka Corporation).

Lemon myrtle is a plant of the genus *Backhousia* in the family Myrtaceae. Various solvent extracts of lemon myrtle have been reported to have biological activities, including antimicrobial [21,22], anti-inflammatory [23–25], and antioxidant [24–27] activities, but its effect on SC activation remains to be investigated.

To develop and commercialize lemon myrtle extract as a novel functional food ingredient for sarcopenia, the objective of this thesis is to clarify the function of lemon myrtle extract in terms of SC activation. In Chapter 1, I aimed to establish a general manufacturing method of lemon myrtle extract. To this end, I evaluated the effect of solvents used for extraction from lemon myrtle leaves to maximize its SC activation capacity. I also examined whether the lemon myrtle extract specifically activates SCs (quoted from [28]). In Chapter 2,

to gain mechanistic insights into the activation of SCs by lemon myrtle extract, I attempted to identify the active compound in lemon myrtle extract and elucidate the signaling molecule associated with SC activation by lemon myrtle extract (quoted from [28]).

Chapter 1: Lemon myrtle extract activates skeletal muscle satellite cells (SCs) both *in vitro* and *in vivo*

1.1. Introduction

For a novel nutritional intervention against sarcopenia, an extract of lemon myrtle leaves was selected as a candidate to activate skeletal muscle satellite cells (SCs) in a preliminary study by Kaneka Corporation. It is known that in the preparation of plant extracts, pretreatment of plant samples (e.g., grinding and homogenization), drying methods (e.g., air-drying and freeze-drying), and extraction conditions (e.g., type of extraction solvent, extraction time and temperature) affect extraction yield and composition of plant extracts [29]. In particular, it has been reported that in various plant extracts, the type of extraction solvent affects the content of phenolic compounds (the major bioactive compounds in plant extracts) and their bioactivity [22,30,31].

In this chapter, I examined the type of extraction solvent to maximize the effect of lemon myrtle extract on SC activation. Lemon myrtle leaves have long been used as a food flavoring and herbal tea [32] and are listed as "Ingredients that are not deemed to be pharmaceuticals unless they are labelled with pharmaceutical effects" in Japan [33]. Also, only water and ethanol can be used as solvents for food extraction without restrictions in Japan [34,35]. Therefore, to produce lemon myrtle extract as a food product in Japan, I used only

water and ethanol as extraction solvents and evaluated the effect of their mixing ratio. Then, to clarify the functional properties of lemon myrtle extract, I evaluated its effect on SC activation *in vitro* and *in vivo*. In addition, to elucidate the specificity of the cell proliferative effect of lemon myrtle extract on cell types, I also evaluated its effect on myoblasts, which are produced by SCs in the process of fusion to myofibers.

1.2. Materials and methods

1.2.1. Preparation of lemon myrtle extracts

Cut and dried samples of lemon myrtle leaves were supplied by Australian Native Lemon Myrtle Farms (Airlie Beach, Queensland, Australia). The weighed samples were extracted with 5-fold volume of solvent for 2 h at 50°C. Water, aqueous ethanol (20%, 40%, 60%, or 80% ethanol concentration), or ethanol was used as the solvent. Each extract was filtered through an α-cellulose membrane, and the supernatant was collected. These procedures were repeated twice. The supernatants from the two extractions were combined, and the solvent was evaporated under reduced pressure at 50°C using a rotary evaporator. Each concentrated extract was then freeze-dried under reduced pressure, and each lyophilized powder obtained was referred to as "lemon myrtle extract" and stored at 4°C until further use.

1.2.2. Total phenolic content analysis

Total phenolic content in each extract was determined using the Folin–Ciocalteu method previously described by Do et al. [31] with some modifications. Each extract was dissolved in dimethyl sulfoxide (DMSO) to a concentration of 200 μg/mL. The calibration curve was prepared with gallic acid (0–100 μg/mL, ChromaDex, Los Angeles, CA, USA). Each extract or gallic acid solution (200 μL) was added to 300 μL of Folin–Ciocalteu phenol reagent

(4-fold diluted with distilled water; MP Biomedicals, Irvine, CA, USA) and 1.5 mL of 0.4 M sodium carbonate (Nacalai Tesque, Kyoto, Japan) and mixed. The mixture was then incubated at 30°C for 60 min. After cooling at room temperature for 15 min, the mixture was filtered through a PTFE filter (0.45 μm) and the absorbance was measured at 760 nm using a UV–VIS spectrophotometer (UV-1280, Shimadzu, Kyoto, Japan). Total phenolic content was calculated as milligram gallic acid equivalent per gram dry weight of extract.

1.2.3. Animals

Male Sprague-Dawley rats were purchased from Japan SLC (Shizuoka, Japan) at 13-week-old for an *in vivo* assay and over 6-month-old for *in vitro* assays. Animals were housed at constant humidity (55% ± 10%) and temperature (22°C ± 2°C) in a 12 h light/dark cycle and had free access to food (CE-2; Clea Japan, Tokyo, Japan) and water. Animals were acclimated to the environment for one week prior to the experiments. All experimental procedures were approved by the Animal Care and Use Committee of Kaneka Corporation (approval number: 2014-16 and 2014-21, approval date: 25 March 2014), and conducted in accordance with the guidelines for animal experiments of Kaneka Corporation.

1.2.4. SC isolation and culture

SC isolation and culture were performed according to previously described methods with slight modifications [36–38]. Briefly, after the rats were euthanized, the upper hindlimb and back muscles were excised, and adipose and connective tissues were trimmed. The muscle tissues were minced with scissors and digested for 1 h at 37°C with 1.25 mg/mL protease type XIV (Sigma-Aldrich, St. Louis, MO, USA). The cells were separated from muscle fiber fragments and tissue debris by differential centrifugation and filtration through cell strainers (100 and 40 μm). Following further centrifugation, cells were suspended in 10% HS-DMEM (Dulbecco's modified Eagle medium [DMEM; Gibco, Grand Island, NY, USA] supplemented with 10% horse serum [HS; Gibco], 1% antibiotic-antimycotic solution [AA; Gibco], and 0.5% gentamicin [Gibco]). For 5-bromo-2′-deoxyuridine (BrdU)-incorporation assay, the cells were seeded into 48-well plates coated with poly-L-lysine (Sigma-Aldrich) and fibronectin (Sigma-Aldrich) at a density of 0.5 g tissue/cm^2. The cells were cultured for 24 h prior to the assay. All cultures were maintained in a humidified atmosphere of 5% CO_2 at 37°C.

1.2.5. Myoblast culture

L6 rat myoblast and C2C12 mouse myoblast cell lines were purchased from KAC (Kyoto, Japan) and cultured in DMEM supplemented with 10% fetal bovine serum (FBS;

Gibco), 1% AA, and 0.5% gentamicin. For the BrdU-incorporation assay, the cells were seeded into 48-well plates at a density of 1×10^4 cells/well and cultured for 24 h prior to the assay.

1.2.6. *In vitro* BrdU-incorporation assay

The effect of each lemon myrtle extract on cell proliferation was evaluated using BrdU incorporation, which is an indicator of cell activation (entry into the cell cycle) and subsequent proliferation. Samples used for the assay were each lemon myrtle extract and recombinant human hepatocyte growth factor (HGF; positive control for SC activation [39], Gibco). Each sample was dissolved in water or DMSO and then resuspended in 10% HS-DMEM to the following final concentrations: lemon myrtle extract (1–10 μg/mL) and HGF (5 ng/mL). The cells were prepared as described in Sections 1.2.4 and 1.2.5. The BrdU-incorporation assay was performed according to previously described methods with some modifications [37,40]. Briefly, each culture was washed three times, and the cell culture medium was replaced with 10% HS-DMEM (as the control, Ctrl) or 10% HS-DMEM containing each sample described above and incubated for 22 h. At least three independent cultures were performed for each treatment. The cultures were pulse-labeled with 10 μM BrdU (Sigma-Aldrich) in 10% HS-DMEM for 2 h followed by fixation with cold methanol–H_2O_2 for 10 min. BrdU-positive ($BrdU^+$) cells were detected by immunocytochemistry using a monoclonal anti-BrdU antibody

(1:500, Sigma-Aldrich), a horseradish peroxidase (HRP)-conjugated anti-mouse IgG antibody (1:500, Exalpha Biologicals, Shirley, MA, USA), and 3,3′-diaminobenzidine tetrahydrochloride (Sigma-Aldrich). The number of cells was counted under a microscope and the ratio of $BrdU^+$ cells to the total number of cells was calculated. The data are shown as fold changes compared to the Ctrl.

1.2.7. *In vivo* BrdU-incorporation assay

The effect of lemon myrtle extract on SC activation in rat skeletal muscle was evaluated *in vivo* by administration of the extract and BrdU to rats. The assay was performed according to a previously described method [37]. Eighteen rats were divided into three groups (n = 6), and the rats in each group were orally administered the following samples daily for 4 d: Ctrl group received a mixture of COCONAD RK medium chain triglyceride (MCT; Kao, Tokyo, Japan) and POEM DOV-100 emulsifiers (E; Riken Vitamin, Tokyo, Japan) (1:1, v/v; MCT-E); Water Ext group received lemon myrtle water extract (500 mg/kg/day, dissolved in water); and 60% EtOH Ext group received lemon myrtle 60% ethanol extract (500 mg/kg/day, dissolved in MCT-E). Eight hours after the last administration, the animals were all intraperitoneally administered BrdU (50 mg/kg, dissolved in saline). The animals were sacrificed 16 h after BrdU administration, and SCs were isolated from the upper hindlimb muscle of each rat as

described in Section 1.2.4. Cells suspended in 10% HS-DMEM were seeded into 48-well plates (three wells per rat) and cultured for 24 h. $BrdU^+$ cells were detected by immunocytochemistry as described in Section 1.2.6, and the ratio of $BrdU^+$ cells to the total number of cells was calculated. The values of the three wells for each rat were averaged and then further averaged for each group. The data are shown as fold changes compared to the Ctrl group.

1.2.8. Statistical analysis

Data are expressed as the mean ± standard deviation (SD). Statistical differences were analyzed by one-way ANOVA with Dunnett's multiple comparison test, and statistical significance was defined as $p < 0.05$. A correlation between extraction yield and total phenolic content was analyzed using Pearson correlation. Statistical analyses were performed using the statistical software package R (version 4.0.2, https://www.R-project.org/, accessed on 3 March 2022, R Foundation for Statistical Computing, Vienna, Austria).

1.3. Results

1.3.1. The extraction solvents affect the properties of the lemon myrtle extracts

To examine the effect of the type of solvent used for extraction from lemon myrtle leaves on the properties of lemon myrtle extracts, I prepared each lemon myrtle extract using water, aqueous ethanol (ethanol concentrations of 20%, 40%, 60%, and 80%), and 100% ethanol as extraction solvents and evaluated the extraction yield and total phenolic content. The extraction yield increased with ethanol concentrations up to 40% but decreased with ethanol concentrations above 60% (Table 1). A similar trend was observed for total phenolic content calculated in terms of gallic acid equivalent (GAE) (Table 1). There was a positive correlation between extraction yield and total phenolic content (Pearson $r = 0.66$).

Next, I evaluated the effect of extraction solvents on SC activation using an *in vitro* BrdU-incorporation assay. Compared to the control, HGF treatment (positive control) significantly increased the ratio of $BrdU^+$ SCs ($p < 0.01$) (Figure 1). Treatment with lemon myrtle water and aqueous ethanol extracts (20%, 40%, and 60% ethanol concentrations) also significantly increased the ratio (water and 20% and 40% ethanol extracts: $p < 0.01$; and 60% ethanol extract: $p = 0.01$) (Figure 1). In contrast, treatment with 80% and 100% ethanol extracts did not significantly change the ratio (Figure 1). Collectively, I concluded that the mixing ratio of water and ethanol in the extraction solvent used for lemon myrtle extract must contain at

least 40% water to activate SCs.

Table 1. Extraction yield and total phenolic content of lemon myrtle extracts in different types of extraction solvents.

Extraction solvent	Extraction yield (%) [*1]	Total phenolic content (mg GAE/g) [*2]
Water	17.3	273.4 ± 5.5
20% aqueous ethanol	22.1	331.3 ± 14.7
40% aqueous ethanol	22.4	383.2 ± 12.8
60% aqueous ethanol	21.4	373.9 ± 14.4
80% aqueous ethanol	18.2	311.0 ± 12.9
100% ethanol	8.8	297.1 ± 14.6

[*1] Extraction yield was calculated as dry weight of extract per initial dry weight of lemon myrtle leaves (%).

[*2] Total phenolic content was calculated as milligram gallic acid equivalent (GAE) per gram dry weight of extract. Data represent the mean ± standard deviation (SD) (n = 3).

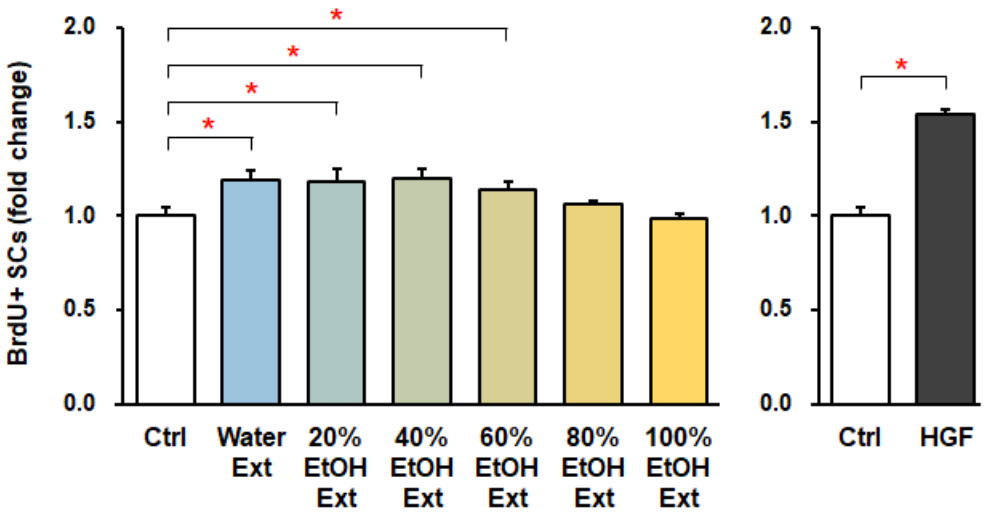

Figure 1. Effects of lemon myrtle extracts in different extraction solvents and hepatocyte growth factor (HGF) treatment on BrdU incorporation into skeletal muscle satellite cells (SCs) *in vitro*. SCs were treated with each lemon myrtle extract or HGF for 22 h and then incubated with BrdU for an additional 2 h. The concentration of each lemon myrtle extract used was 2.5 µg/mL. The HGF concentration used was 5 ng/mL. BrdU-positive ($BrdU^+$) cells were detected by immunocytochemistry and the number of cells was counted. BrdU incorporation into SCs is expressed as $BrdU^+$ SCs per total SCs and is shown as a fold change compared to the control (Ctrl). Data represent the mean ± SD of three independent cultures. * Significant differences ($p < 0.05$) relative to the Ctrl. EtOH: ethanol; Ext: extract.

1.3.2. Oral administration of lemon myrtle extracts activates SCs *in vivo*

To determine whether lemon myrtle extract also activates SCs *in vivo*, I orally administered lemon myrtle extract to rats and evaluated SC activation in rat skeletal muscle. The experimental design is illustrated in Figure 2a. Rats in the Ctrl, Water Ext, and 60% EtOH Ext groups were orally administered solvent, lemon myrtle water extract, and lemon myrtle 60% ethanol extract, respectively, followed by intraperitoneal injection of BrdU, and BrdU incorporation into SCs was evaluated. The results showed that the ratio of $BrdU^+$ SCs was significantly increased in the Water Ext and 60% EtOH Ext groups compared to the Ctrl group (Water Ext group: $p < 0.01$, and 60% EtOH Ext group: $p = 0.01$) (Figure 2b). Thus, I found that oral administration of both water and 60% ethanol extracts of lemon myrtle activated SCs in skeletal muscle. Thereafter, a water-soluble lemon myrtle water extract was selected for development of lemon myrtle extract as a variety of food products.

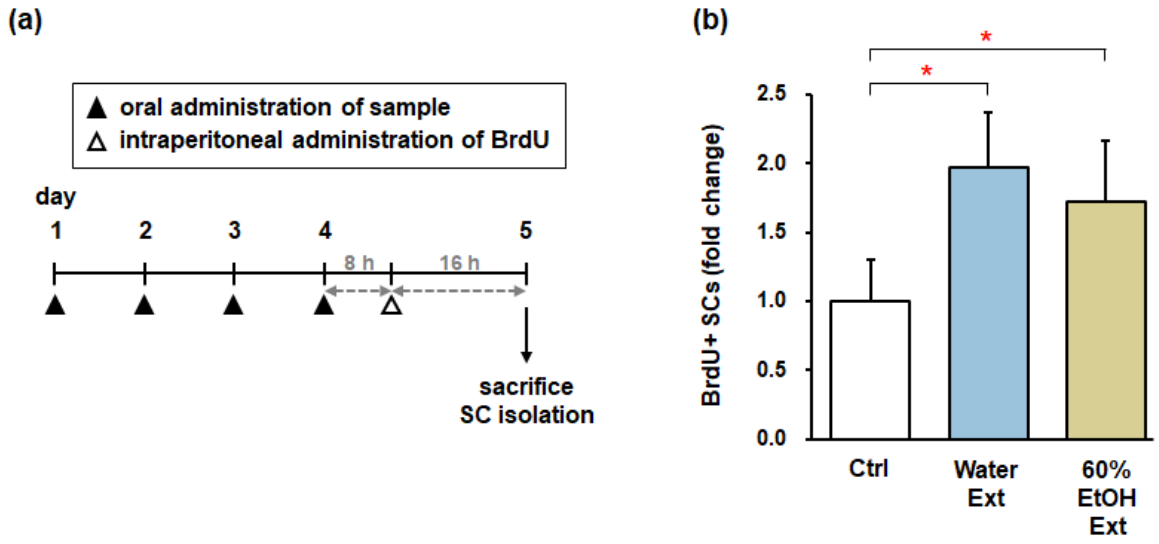

Figure 2. Lemon myrtle water and 60% ethanol extracts promote BrdU incorporation into SCs *in vivo*. (**a**) Experimental design of *in vivo* BrdU-incorporation assay. Rats in each group were orally administered samples (Ctrl group: a mixture of medium chain triglyceride and emulsifiers [MCT-E]; Water Ext group: lemon myrtle water extract [500 mg/kg in water]; or 60% EtOH Ext group: lemon myrtle 60% ethanol extract [500 mg/kg in MCT-E]) daily for 4 d. Eight hours after the last administration of each sample, all animals were intraperitoneally administered BrdU (50 mg/kg), and then 16 h later, animals were sacrificed, and SCs were isolated. (**b**) Effects of lemon myrtle water and 60% ethanol extracts administration on BrdU incorporation into SCs. BrdU incorporation into SCs is expressed as $BrdU^+$ SCs per total SCs and is shown as a fold change compared to the Ctrl. Data represent the mean ± SD of each group (three independent cultures per rat). * Significant differences ($p < 0.05$) relative to the Ctrl.

1.3.3. Lemon myrtle water extract treatment does not activate myoblasts *in vitro*

SCs are normally maintained in a quiescent state, but when skeletal muscle is stimulated, SCs are activated and divide to produce myoblasts. To investigate whether lemon myrtle water extract (LM) specifically activates SCs, I compared the effects of LM treatment on the activation of primary rat SCs and myoblast cell lines (L6 rat and C2C12 mouse myoblasts) by *in vitro* BrdU-incorporation assays. In SCs, LM treatment as well as HGF treatment (positive control) significantly increased the ratio of $BrdU^+$ cells compared to the control, with the effect plateauing at 1.0–10 μg/mL of LM (1.0 and 5.0 μg/mL: $p < 0.01$, and 10 μg/mL: $p = 0.03$) (Figure 3a). By contrast, in any myoblast cell lines, LM treatment did not significantly change the ratio of $BrdU^+$ cells at any concentration (Figure 3b). Therefore, I concluded that LM specifically promoted the proliferation of SCs.

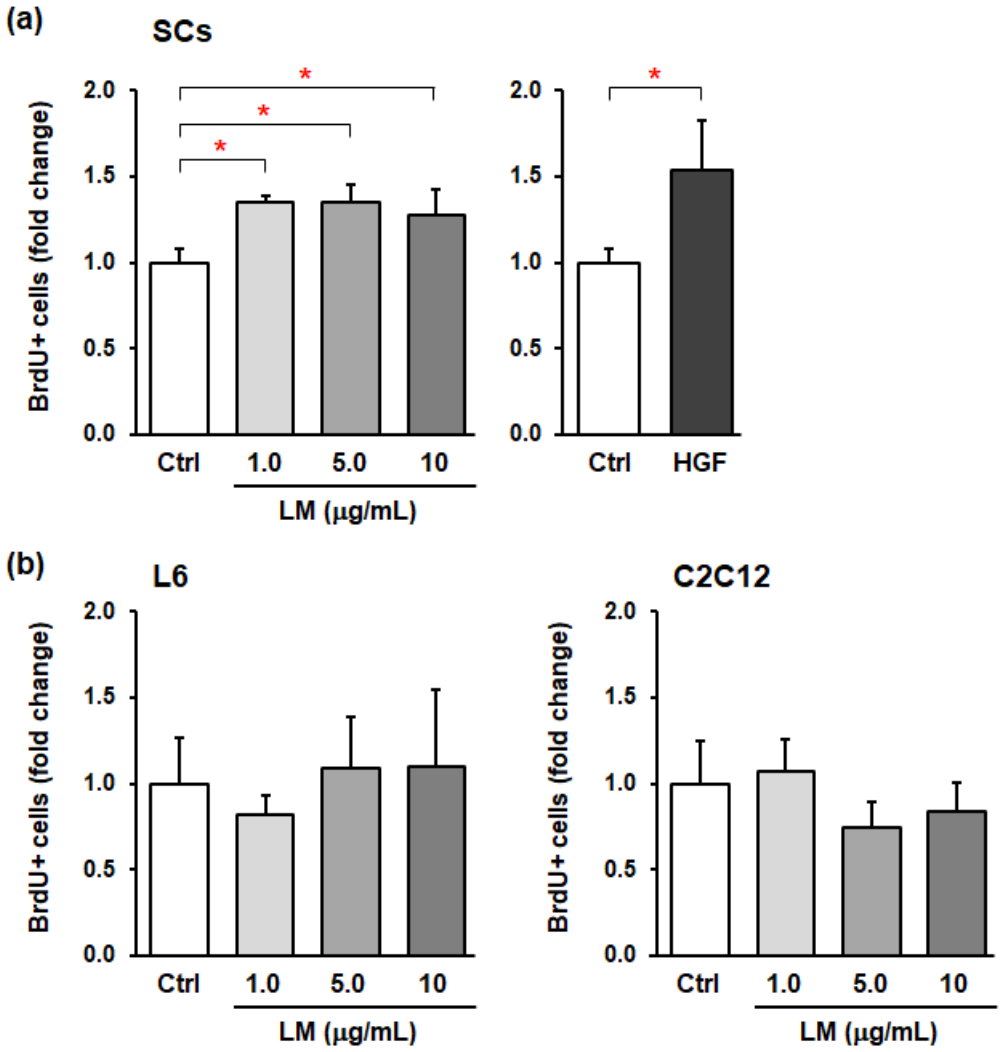

Figure 3. Lemon myrtle water extract (LM) promotes BrdU incorporation into SCs but not into myoblasts *in vitro*. (**a**) Effects of LM and HGF treatment on BrdU incorporation into SCs. (**b**) Effects of LM treatment on BrdU incorporation into L6 rat myoblast cells (left) and C2C12 mouse myoblast cells (right). SCs and myoblast cells were treated with LM or HGF for 22 h and then incubated with BrdU for an additional 2 h. The LM concentration is indicated in the figure. The HGF concentration used was 5 ng/mL. BrdU incorporation into each cell is expressed as $BrdU^+$ cells per total cells and is shown as a fold change compared to the Ctrl. Data represent the mean ± SD of three independent cultures. * Significant differences ($p < 0.05$) relative to the Ctrl. This figure was quoted and modified from the literature [28].

1.4. Discussion

In this chapter, I showed that the type of extraction solvent affected the extraction yield and total phenolic content of lemon myrtle extract. Alderees et al. also prepared water and ethanol extracts of lemon myrtle and reported the extraction yield and total phenolic content [22]. The extraction yield (16.3%) and total phenolic content (281.7 mg GAE/g) of the water extract in the study by Alderees et al. [22] were both similar to those in the present study (17.3% and 273.4 mg GAE/g, respectively, Table 1). In contrast, the extraction yield and total phenolic content of the ethanol extract in the study by Alderees et al. (17.9% and 373.2 mg GAE/g, respectively) [22] were both higher than those in this work (8.8% and 297.1 mg GAE/g, respectively, Table 1). One possible reason for the differences in the ethanol extracts may be due to the origin of the lemon myrtle. In this study, lemon myrtle was sourced from Airlie Beach, Queensland, Australia, whereas in the study by Alderees et al., it was sourced from Lismore, New South Wales, Australia. Another possibility is the difference in the extraction methods. In this study, cut samples were extracted with solvent at 50°C for 2 h, whereas in the study by Alderees et al., powder samples were extracted at 60°C for five cycles of pressurized liquid extraction. In contrast, the water extracts prepared in this study and in the study by Alderees et al. had similar extraction yield and total phenolic content, suggesting that water extraction would be suitable for production of lemon myrtle extract with stable quality, since

this procedure is less affected by the origin of the materials and extraction methods.

Next, I found that the mixing ratio of water and ethanol in the extraction solvent affected the activation of SCs by lemon myrtle extract. Lemon myrtle extract activated SCs when the extraction solvent contained more than 40% water, suggesting that the relatively hydrophilic compounds in lemon myrtle extract may be responsible for SC activation. Furthermore, I showed that administration of lemon myrtle water and 60% ethanol extracts activated SCs in rat skeletal muscle. In the study by Kim et al. on the preparation of muscadine seed extracts with antimicrobial activity, a water extract was selected because of its water solubility for addition to beverages [41]. Similarly, in lemon myrtle extracts, the water extract is soluble in water and could be processed into a variety of foods, including beverages, whereas the 60% ethanol extract is insoluble in water and may be limited in food processing. In addition, since sarcopenia reduces swallowing muscles and causes dysphagia [42,43], it is difficult for some older adults to intake dietary supplements in regular capsule form, and food processing suitable for older adults may be required. Therefore, to develop lemon myrtle extract as a variety of food products that can be taken by older adults, I decided to select the water extract, considering its water solubility as well as its SC activation properties.

Finally, I showed that lemon myrtle water extract did not activate myoblast cell lines *in vitro*. In contrast, Sakulnarmrat et al. reported that an 80% aqueous methanol extract of lemon

myrtle reduced the proliferation of colon, stomach, bladder, and liver cancer cell lines [44]. Although there are differences between the present study and the study by Sakulnarmrat et al. in terms of extraction procedures and evaluation methods, lemon myrtle extract may have a specific proliferative effect on SCs. Future *in vivo* studies are required to clarify the specificity of cell proliferation by lemon myrtle extract to confirm that supplementation with lemon myrtle extract does not induce unexpected cell proliferation *in vivo*.

Chapter 2: Casuarinin is the active compound in lemon myrtle extract involved in activation of skeletal muscle satellite cells (SCs)

2.1. Introduction

In Chapter 1, I examined the effects of the extraction solvents used in the preparation of lemon myrtle extracts on total phenolic content and skeletal muscle satellite cell (SC) activation properties, and selected the water extraction method that exhibited high SC activation capacity and could be produced with stable quality. It has been suggested that relatively hydrophilic compounds in lemon myrtle water extract (LM) may be involved in SC activation, but the active compounds involved in SC activation were unknown. It was also unclear by what mechanism LM activates SCs.

In this chapter, I attempted to elucidate the mechanism of action of SC activation by LM. First, I sought to identify the active compound responsible for SC activation by LM. Next, I performed structure–activity relationship analysis of the active compound to further elucidate its SC activation capacity. I also evaluated the effect of LM and its active compound on the expression of interleukin-6 (IL-6), a signaling molecule important for SC activation and proliferation. Finally, I examined the effect of oral administration of the active compound on SC activation *in vivo*.

2.2. Materials and methods

2.2.1. Preparation of lemon myrtle water extract (LM)

Cut and dried samples of lemon myrtle leaves were supplied by Australian Native Lemon Myrtle Farms (Airlie Beach, Queensland, Australia). The weighed samples were extracted with 5-fold volume of water for 2 h at 50°C. The extract was filtered through an α-cellulose membrane, and the supernatant was collected. These procedures were repeated twice. The supernatants from the two extractions were combined, and the solvent was evaporated under reduced pressure at 50°C using a rotary evaporator. The concentrated extract was then freeze-dried under reduced pressure, and the lyophilized powder (LM) obtained was stored at 4°C until further use.

2.2.2. Liquid chromatography–tandem mass spectrometry (LC-MS/MS) analysis

The major compounds in LM were analyzed using LC-MS/MS. A Nexera X2 UHPLC system (Shimadzu, Kyoto, Japan) equipped with a binary pump, a column oven, and a photodiode array (PDA) detector was coupled to a maXis 4G quadrupole-time-of-flight mass spectrometer equipped with an electrospray ionization (ESI) source (Bruker, Billerica, MA, USA). Chromatographic separation was performed using a YMC-Pack ODS-A column (250 × 4.6 mm, 5 μm; YMC, Kyoto, Japan). The mobile phases consisted of acetonitrile/methanol (1:1,

v/v) (A) and 0.1% formic acid in water (B) in a gradient elution analysis programmed as follows: 95–85% (B) at 0–20 min, 85–70% (B) at 20–100 min, 70% (B) at 100–120 min, and 70–95% (B) at 120–140 min. The flow rate was 0.7 mL/min. LM was dissolved in distilled water (1 mg/mL) and 10 μL was injected. The column temperature was set at 40°C. UV spectra were recorded at 260 nm. The ESI interface was operated in the negative mode, and the mass (MS) spectra were acquired in the mass range of 50–1500 m/z. Tentative identification of compounds was performed by comparing MS and MS/MS spectra with data available on MassBank (http://www.massbank.jp/, accessed on 3 March 2022) and MetFrag (https://ipb-halle.github.io/MetFrag/, accessed on 3 March 2022) online databases.

2.2.3. High-performance liquid chromatography (HPLC) analysis

Identification and quantification of peaks tentatively identified by LC-MS/MS were performed using a prominence HPLC system (Shimadzu). Among the peaks, four peaks were identified and quantified by HPLC using analytical standards (the same materials as described in Section 2.2.7), whereas one peak (casuarinin) was identified by nuclear magnetic resonance (NMR) analysis (described in Section 2.2.4) and quantified by HPLC using chemically synthesized casuarinin [45], a kind gift from Dr. Yamada and Dr. Wakamori (Kwansei Gakuin University and Tokyo University of Agriculture, respectively). The separation conditions were

the same as those described for LC-MS/MS in Section 2.2.2, except for the mobile phases, sample preparation, and sample injection volumes. The mobile phases consisted of acetonitrile/methanol (1:1, v/v) (A) and 20 mM phosphoric acid in water (B) in a gradient elution analysis programmed as follows: 95–85% (B) at 0–20 min, 85–82.2% (B) at 20–35 min, 82.2–70% (B) at 35–55 min, and 70% (B) at 55–75 min. Each sample was prepared as follows: LM was dissolved in water (2 mg/mL), and each standard was dissolved in methanol (2–200 μg/mL). Sample injection volumes were 5 μL. Peaks were detected in the range of 200–800 nm and identified by the retention time and spectrum compared with each standard. Quantification of each compound was performed using standard curves at 260 or 270 nm, and the results are shown as milligram of each compound per gram dry weight of LM.

2.2.4. Casuarinin isolation and nuclear magnetic resonance (NMR) analysis

To determine the molecular structure of casuarinin peak estimated by LC-MS/MS, I performed the peak isolation and NMR analysis. The peak was isolated from LM using three-step column chromatography. First, LM was applied to a Diaion HP-20 column (Sigma-Aldrich, St. Louis, MO, USA), and eluted with 0%, 10%, 20%, 30%, and 40% aqueous methanol to obtain 10 fractions. Based on the HPLC chromatogram of each fraction, the fractions eluted with 20% and 30% aqueous methanol were collected. Next, the fractions were applied to a

Toyopearl HW-40C column (Tosoh, Tokyo, Japan) and eluted with 50% aqueous methanol. Following fractionation, samples enriched with target peak were collected. Finally, the samples were reapplied to a Toyopearl HW-40C column using the same method as described above, and a pure target sample was isolated. The isolated sample was subsequently analyzed using an AVANCE NEO 700 NMR spectrometer (Bruker). ^{1}H NMR (700 MHz) and ^{13}C NMR (176 MHz) spectra were obtained in methanol-d_4 at 30°C using tetramethylsilane as the internal standard. The molecular structure of the sample was determined by LC-MS/MS (described in Section 2.2.2) and NMR data compared with the literature data [46].

2.2.5. Animals

Male Sprague-Dawley rats were purchased from Japan SLC (Shizuoka, Japan) at 13-week-old for an *in vivo* assay and over 6-month-old for *in vitro* assays. Animals were housed at constant humidity (55% ± 10%) and temperature (22°C ± 2°C) in a 12 h light/dark cycle and had free access to food (CE-2; Clea Japan, Tokyo, Japan) and water. Animals were acclimated to the environment for one week prior to the experiments. All experimental procedures were approved by the Animal Care and Use Committee of Kaneka Corporation (approval number: 2019-17 and 2020-6, approval date: 29 March 2019, and 31 March 2020, respectively), and conducted in accordance with the guidelines for animal experiments of Kaneka Corporation.

2.2.6. SC isolation and culture

SC isolation and culture were performed according to previously described methods with slight modifications [36–38]. Briefly, after the rats were euthanized, the upper hindlimb and back muscles were excised, and adipose and connective tissues were trimmed. The muscle tissues were minced with scissors and digested for 1 h at 37°C with 1.25 mg/mL protease type XIV (Sigma-Aldrich). The cells were separated from muscle fiber fragments and tissue debris by differential centrifugation and filtration through cell strainers (100 and 40 μm). Following further centrifugation, cells were suspended in one of the following media: (1) 10% HS-DMEM; Dulbecco's modified Eagle medium (DMEM; Gibco, Grand Island, NY, USA) supplemented with 10% horse serum (HS; Gibco), 1% antibiotic-antimycotic solution (AA; Gibco), and 0.5% gentamicin (Gibco) for 5-bromo-2′-deoxyuridine (BrdU)-incorporation assay; and (2) 20% FBS-Ham's F-10; Ham's F-10 nutrient mixture medium (Ham's F-10; Gibco) supplemented with 20% fetal bovine serum (FBS; Gibco), 1% AA, and 0.5% gentamicin for mRNA expression assay. For the BrdU-incorporation assay, the cells were seeded into 48-well plates coated with poly-L-lysine (Sigma-Aldrich) and fibronectin (Sigma-Aldrich) at a density of 0.5 g tissue/cm^2. The cells were cultured for 24 h prior to the assay. For the mRNA expression assay, the cells were seeded into 90 mm dishes coated with poly-L-lysine and fibronectin at a density of 0.1 g tissue/cm^2 and cultured for 16 h. To enrich the cell density,

the cells were washed three times, harvested using 0.25% trypsin-EDTA (Gibco), and reseeded into 24-well plates coated with poly-L-lysine and fibronectin at a density of 3.5×10^4 cells/well. The cells were cultured for a further 8 h prior to the assay. All cultures were maintained in a humidified atmosphere of 5% CO_2 at 37°C.

2.2.7. *In vitro* BrdU-incorporation assay

The effects of LM-derived compounds on cell proliferation were evaluated using BrdU incorporation, which is an indicator of cell activation (entry into the cell cycle) and subsequent proliferation. Samples used for the assay are listed as follows: casuarinin (isolated from LM, described in Section 2.2.4), gallic acid (ChromaDex, Los Angeles, CA, USA), myricitrin (Adooq Bioscience, Irvine, CA, USA), hyperin (Extrasynthese, Genay Cedex, France), quercitrin (Extrasynthese), ellagic acid (Fujifilm Wako Pure Chemical, Osaka, Japan), casuarictin (Nagara Science, Gifu, Japan), castalagin (Sigma-Aldrich), and recombinant human hepatocyte growth factor (HGF; positive control for SC activation [39], Gibco). Each sample was dissolved in water or dimethyl sulfoxide (DMSO) and then resuspended in 10% HS-DMEM to the following final concentrations: casuarinin (13–400 nM), gallic acid, myricitrin, hyperin, and quercitrin (25–100 nM each); ellagic acid, casuarictin, and castalagin (250 nM each); and HGF (5 ng/mL). The cells were prepared as described in Section 2.2.6. The BrdU-

incorporation assay was performed according to previously described methods with some modifications [37,40]. Briefly, each culture was washed three times, and the cell culture medium was replaced with 10% HS-DMEM (as the control, Ctrl) or 10% HS-DMEM containing each sample described above and incubated for 22 h. At least three independent cultures were performed for each treatment. The cultures were pulse-labeled with 10 μM BrdU (Sigma-Aldrich) in 10% HS-DMEM for 2 h followed by fixation with cold methanol–H_2O_2 for 10 min. BrdU-positive ($BrdU^+$) cells were detected by immunocytochemistry using a monoclonal anti-BrdU antibody (1:500, Sigma-Aldrich), a horseradish peroxidase (HRP)-conjugated anti-mouse IgG antibody (1:500, Exalpha Biologicals, Shirley, MA, USA), and 3,3′-diaminobenzidine tetrahydrochloride (Sigma-Aldrich). The number of cells was counted under a microscope and the ratio of $BrdU^+$ cells to the total number of cells was calculated. The data are shown as fold changes compared to the Ctrl.

2.2.8. *In vitro* mRNA expression assay

The effects of LM and its derived compounds on *IL-6* mRNA expression in SCs were evaluated *in vitro*. Each sample was dissolved in water or DMSO and then resuspended in 20% FBS-Ham's F-10 to the following final concentrations: LM (2.5 μg/mL), casuarinin (330 nM), hyperin, and quercitrin (220 nM each). The cells were prepared as described in Section 2.2.6.

The supernatant of each culture was replaced with 20% FBS-Ham's F-10 (Ctrl) or 20% FBS-Ham's F-10 containing each sample described above and incubated for 16 h. Four independent cultures were performed for each treatment. The cultures were washed twice, and total RNAs were isolated using the RNeasy Plus Micro Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. RNA samples were quantified and qualified with a NanoDrop 2000 spectrophotometer (Thermo Scientific, Waltham, MA, USA), and then reverse transcribed using SuperScript IV VILO Master Mix (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. Quantitative real-time polymerase chain reaction (PCR) was performed using a QuantStudio 3 real-time PCR system (Applied Biosystems, Foster City, CA, USA). Reaction samples were prepared using TaqMan Fast Advanced Master Mix (Applied Biosystems) and TaqMan Gene Expression Assays (Applied Biosystems) according to the manufacturer's protocol. The following two probes were used: glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) (Rn99999916_s1) and *IL-6* (Rn01410330_m1). *IL-6* gene expression levels were normalized to *GAPDH* using the ΔΔCt method. The data are shown as fold changes compared to the Ctrl.

2.2.9. *In vivo* BrdU-incorporation assay

The effect of casuarinin on SC activation in rat skeletal muscle was evaluated *in vivo*

by administration of casuarinin and BrdU to rats. The assay was performed according to a previously described method [37]. Twenty rats were divided into four groups (n = 5), and the rats in each group were orally administered samples (Ctrl group: water; LM group: LM [250 mg/kg/day, dissolved in water]; or casuarinin groups [two doses]: casuarinin [4 or 8 mg/kg/day, dissolved in water]) daily for 4 d. Eight hours after the last administration, the animals were all intraperitoneally administered BrdU (50 mg/kg, dissolved in saline). The animals were sacrificed 16 h after BrdU administration, and SCs were isolated from the upper hindlimb muscle of each rat as described in Section 2.2.6. Cells suspended in 10% HS-DMEM were seeded into 48-well plates (three wells per rat) and cultured for 24 h. $BrdU^+$ cells were detected by immunocytochemistry as described in Section 2.2.7, and the ratio of $BrdU^+$ cells to the total number of cells was calculated. The values of the three wells for each rat were averaged and then further averaged for each group. The data are shown as fold changes compared to the Ctrl group.

2.2.10. Statistical analysis

Data are expressed as the mean ± standard deviation (SD). Statistical differences were analyzed by one-way ANOVA with Dunnett's multiple comparison test, and statistical significance was defined as $p < 0.05$. A correlation between casuarinin concentration and *in*

vitro BrdU incorporation was analyzed using Pearson correlation. Statistical analyses were performed using the statistical software package R (version 4.0.2, https://www.R-project.org/, accessed on 3 March 2022, R Foundation for Statistical Computing, Vienna, Austria).

2.3. Results

2.3.1. Identification and quantification of the major compounds in LM

LC-MS/MS, HPLC, and NMR analyses were performed to identify the major compounds in LM. First, five peaks in LM were tentatively identified by LC-MS/MS analysis (Table 2). Next, four of these five compounds, for which analytical standards were available, were confirmed by HPLC analysis to be gallic acid, myricitrin, hyperin, and quercitrin. However, the remaining one peak estimated by LC-MS/MS analysis had no available analytical standard. Therefore, the peak was isolated from LM and analyzed by NMR. Based on ^{1}H and ^{13}C NMR spectra compared to literature data, this peak was identified as casuarinin (Table 3 and Figure 4). In addition, the content of these five compounds in LM was determined by HPLC analysis (Table 4); among the five compounds, the highest content in LM was quercitrin (38.8 mg/g) and the lowest was gallic acid (2.5 mg/g). A representative HPLC chromatogram of LM with the identified peaks is shown in Figure 5.

Table 2. Tentative identification of major compounds in lemon myrtle water extract (LM) detected by LC-MS/MS.

Compound	Retention time (min)	Molecular formula	$[M-H]^-$ (*m/z*)	Fragment ions (*m/z*)
Gallic acid	9.6	$C_7H_6O_5$	169.0141	125.0236
Casuarinin	26.3	$C_{41}H_{28}O_{26}$	935.0769	300.9978 169.0133
Myricitrin	64.2	$C_{21}H_{20}O_{12}$	463.0862	316.0211
Hyperin	67.8	$C_{21}H_{20}O_{12}$	463.0862	300.0261
Quercitrin	84.5	$C_{21}H_{20}O_{11}$	447.0915	300.0262

This table was quoted from the literature [28].

Table 3. ^{1}H and ^{13}C NMR spectra of casuarinin (comparison of this study and reported data [46]).

^{1}H NMR position		**δ (this study) (multiplicity, *J* [Hz])[a]**		**δ (reported) (multiplicity, *J* [Hz])[b]**		**Δδ[c]**
2,3-HHDP	H-3′	6.39	(1H, s)	6.37	(1H, s)	0.02
4,6-HHDP	H-3	6.82	(1H, s)	6.81	(1H, s)	0.01
	H-3′	6.51	(1H, s)	6.49	(1H, s)	0.02
Galloyl	H-2,6	7.08	(2H, s)	7.07	(2H, s)	0.01
Glucose	H-1	5.52	(d, *J* = 5.0)	5.50	(d, *J* = 4.9)	0.02
	H-2	4.70	(dd, *J* = 2.1, 5.0)	4.68	(dd, *J* = 2.4, 4.9)	0.02
	H-3	5.38	(t, *J* = 2.0)	5.36	(m)	0.02
	H-4	5.45	(dd, *J* = 1.9, 8.7)	5.44	(dd, *J* = 1.2, 9.8)	0.01
	H-5	5.31	(dd, *J* = 3.0, 8.8)	5.30	(dd, *J* = 2.4, 8.5)	0.01
	H-6	4.91	(dd, *J* = 3.5, 13.4)	4.90	(dd, *J* = 3.7, 13.4)	0.01
	H-6	4.06	(d, *J* = 13.1)	4.04	(d, *J* = 13.4)	0.02

Table 3. (continued).

^{13}C NMR position		δ (this study)[a]		δ (reported)[b]		Δδ[c]
2,3-HHDP	C-1	117.0		117.0		0.0
	C-1′	116.7		116.7		0.0
	C-2	120.2		120.2		0.0
	C-2′	125.2		125.2		0.0
	C-3	118.0		118.0		0.0
	C-3′	105.3		105.3		0.0
	C-4	147.0		147.0		0.0
	C-4′	146.5		146.5		0.0
	C-5	140.1		140.1		0.0
	C-5′	135.9		135.8		0.1
	C-6	145.0		145.0		0.0
	C-6′	145.0		144.9		0.1
	C-7	167.1		167.0		0.1
	C-7′	171.0		171.0		0.0
4,6-HHDP	C-1	116.7		116.7		0.0
	C-1′	116.0		116.0		0.0
	C-2	127.6		127.6		0.0
	C-2′	127.1		127.1		0.0
	C-3	109.3		109.2		0.1
	C-3′	107.7		107.7		0.0
	C-4	146.0		145.9		0.1
	C-4′	146.0		146.0		0.0
	C-5	137.9		137.9		0.0
	C-5′	137.1		137.1		0.0
	C-6	144.4		144.4		0.0
	C-6′	144.5		144.5		0.0
	C-7	169.5		169.5		0.0
	C-7′	170.4		170.4		0.0
Galloyl	C-1	120.8		120.8		0.0
	C-2,6	110.5	(2C)	110.4	(2C)	0.1
	C-3,5	146.7	(2C)	146.7		0.0
	C-4	140.4		140.3		0.1
	C-7	167.2		167.2		0.0

Table 3. (continued).

^{13}C NMR position		δ (this study)[a]	δ (reported)[b]	Δδ[c]
Glucose	C-1	67.8	67.8	0.0
	C-2	78.1	78.0	0.1
	C-3	70.7	70.7	0.0
	C-4	74.8	74.8	0.0
	C-5	71.8	71.7	0.1
	C-6	65.2	65.1	0.1

Spectra were acquired in methanol-d_4. [a] ^{1}H NMR (700 MHz) and ^{13}C NMR (176 MHz). [b] ^{1}H NMR (500 MHz) and ^{13}C NMR (125 MHz). [c] Difference between δ (this study) − δ (reported). This table was quoted from the literature [28].

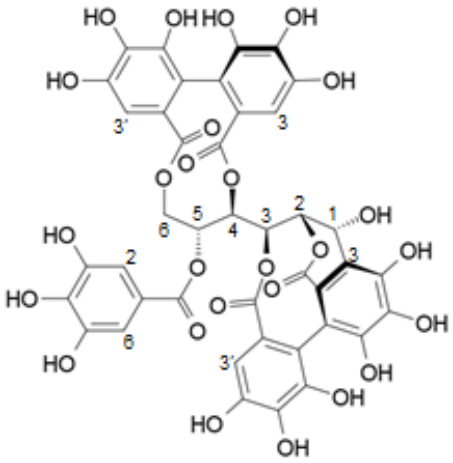

Figure 4. Chemical structure of casuarinin. The numbers correspond to ^{1}H and ^{13}C NMR positions (Table 3).

Table 4. Content of the major compounds in LM.

Compound	Content (mg/g)
Gallic acid	2.5
Casuarinin	16.1
Myricitrin	12.6
Hyperin	19.1
Quercitrin	38.8

Data are shown as milligram of each compound per gram dry weight of LM. This table was quoted from the literature [28].

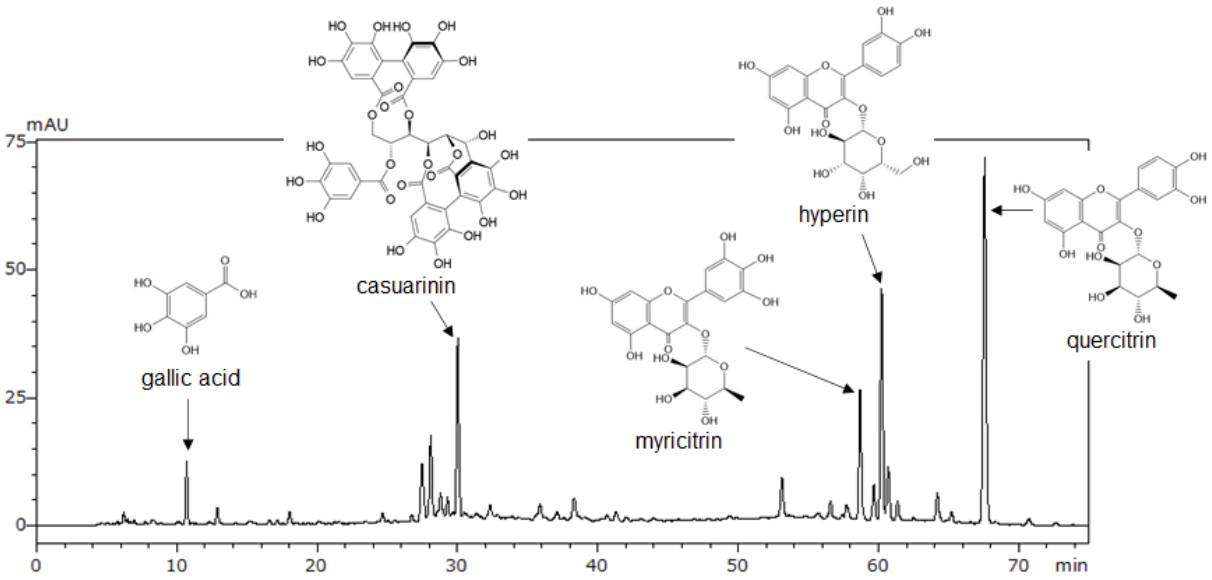

Figure 5. HPLC chromatogram of LM at 270 nm. Gallic acid, myricitrin, hyperin, and quercitrin were identified by comparison with standard compounds in HPLC analysis following LC-MS/MS. Casuarinin was identified by NMR analysis following LC-MS/MS. This figure was quoted from the literature [28].

2.3.2. Only casuarinin activates SCs among the major compounds in LM

To determine the active compounds in LM involved in SC activation, I evaluated the effect of the major compounds identified in LM on SC activation *in vitro*. I found that treatment with casuarinin significantly promoted BrdU incorporation into SCs ($p < 0.05$), whereas the other four compounds did not (Figure 6a). Casuarinin treatment significantly increased BrdU incorporation into SCs in the range of 50–400 nM (50, 100, and 200 nM: $p < 0.01$; and 400 nM: $p = 0.02$) (Figure 6b). There was little correlation between casuarinin concentration and BrdU incorporation (Pearson $r = 0.22$). The effective concentration of casuarinin (50–400 nM) corresponds to 47–375 ng/mL, calculated from its molecular weight of 936.7. From the effective concentration of LM (1.0–10 μg/mL; Chapter 1, Figure 3a) and casuarinin content in LM (16.1 mg/g, Table 4), the effective concentration of casuarinin in LM was calculated to be 16–161 ng/mL, which was equivalent to the measured effective concentration of casuarinin described above (47–375 ng/mL, Figure 6b). Taken together, these data suggest that casuarinin is one of the active compounds in LM involved in SC activation *in vitro*.

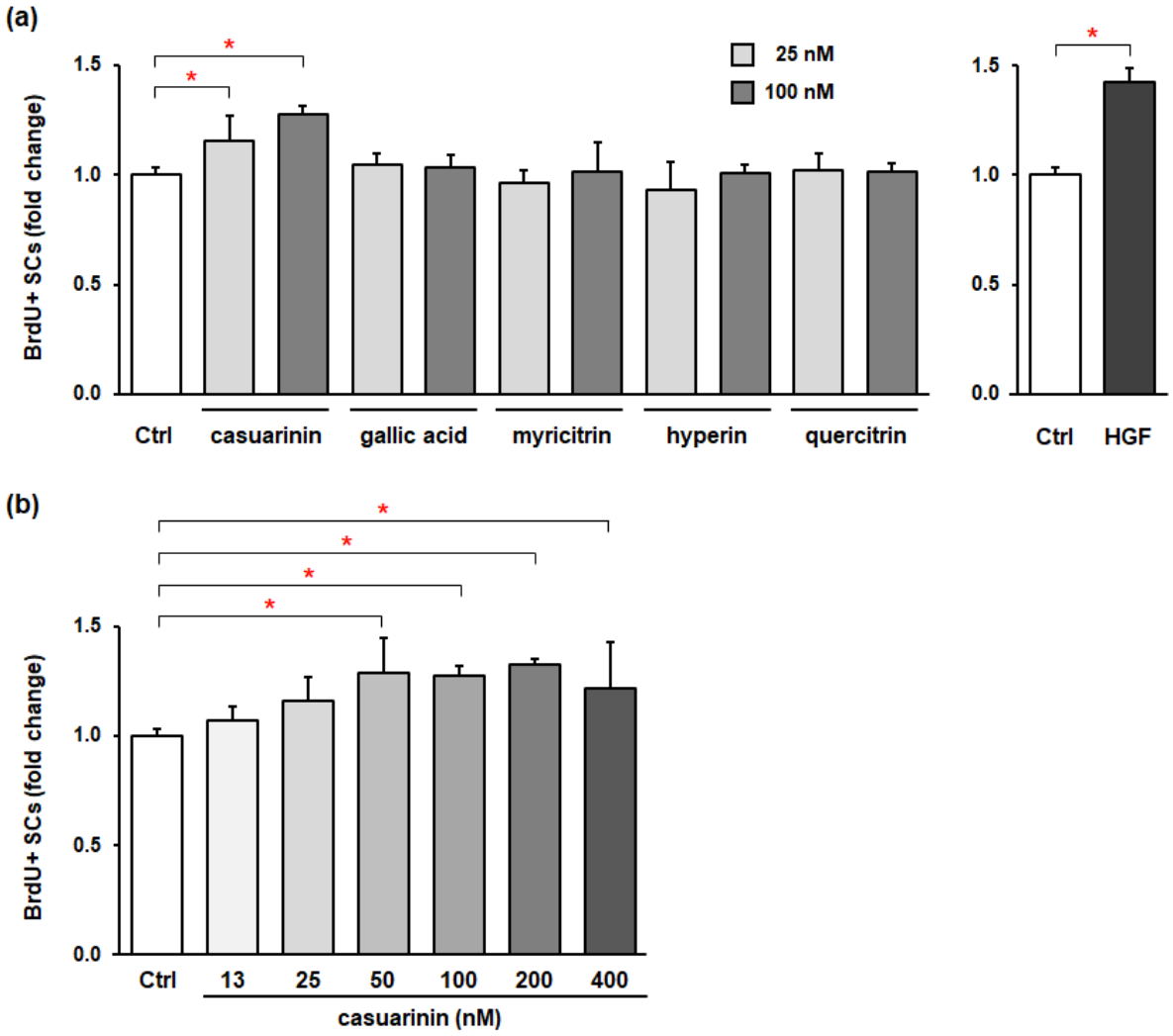

Figure 6. Casuarinin promotes BrdU incorporation into SCs *in vitro*. (**a**) Effects of the major compounds identified in LM on BrdU incorporation into SCs. (**b**) Effects of casuarinin concentration on BrdU incorporation into SCs. The concentration of each sample (except for HGF) is indicated in the figure. The HGF concentration used was 5 ng/mL. BrdU incorporation into SCs is expressed as $BrdU^+$ SCs per total SCs and is shown as a fold change compared to the Ctrl. Data represent the mean ± SD of three independent cultures, except for Ctrl and HGF (nine independent cultures). * Significant differences ($p < 0.05$) relative to the Ctrl. This figure was quoted and modified from the literature [28].

2.3.3. Structurally related compounds of casuarinin do not activate SCs

Casuarinin is one of the ellagitannins, which consists of two hexahydroxydiphenoyl (HHDP) groups and a galloyl group linked to an open-chain form of glucose core. The chemical structure of casuarinin is shown in the top left of Figure 7a. As shown in this figure, one HHDP group links to O-2 and O-3 of glucose (called the 2,3-HHDP group), the other HHDP group links to O-4 and O-6 of glucose (called the 4,6-HHDP group), and the galloyl group links to O-5 of glucose (called the 5-galloyl group). The 2,3-HHDP group is additionally linked to C-1 of glucose; therefore, casuarinin is called a *C*-glycosidic ellagitannin.

I showed that among the major compounds in LM, casuarinin activated SCs, whereas gallic acid, a partial structure of casuarinin, did not activate SCs (Figure 6a). Therefore, I focused on the molecular structure of casuarinin and evaluated whether compounds structurally related to casuarinin activate SCs *in vitro*. To this end, three compounds were selected (shown in Figure 7a): (1) ellagic acid, a derivative of casuarinin; (2) casuarictin, a structural isomer of casuarinin; and (3) castalagin, an ellagitannin that has intramolecular coupling of the 2,3-HHDP and 5-galloyl groups in casuarinin. Treatment of SCs with these compounds showed that only casuarinin significantly promoted BrdU incorporation into SCs ($p < 0.01$), whereas the other related compounds had no effect (Figure 7b). The structural similarities and differences between casuarinin and the related compounds are as follows: (1) ellagic acid is a

lactonized compound of HHDP, which is a partial structure of casuarinin; (2) casuarictin has the same 2,3-HHDP and 4,6-HHDP groups as casuarinin, but differs from casuarinin in the number of hydroxyl groups and the position of the galloyl group due to the glucose moiety in closed form; and (3) castalagin has the same 4,6-HHDP group linked to the open-chain glucose moiety as casuarinin, but differs from casuarinin in the nonahydroxytriphenoyl (NHTP) group linked to the 2,3,5-positions of glucose moiety. None of these compounds activated SCs, suggesting that SC activation is not induced by all ellagitannins and their derivative, but rather by the casuarinin molecular structure—i.e., the 2,3-HHDP, 4,6-HHDP, and 5-galloyl groups linked to the open-chain glucose moiety.

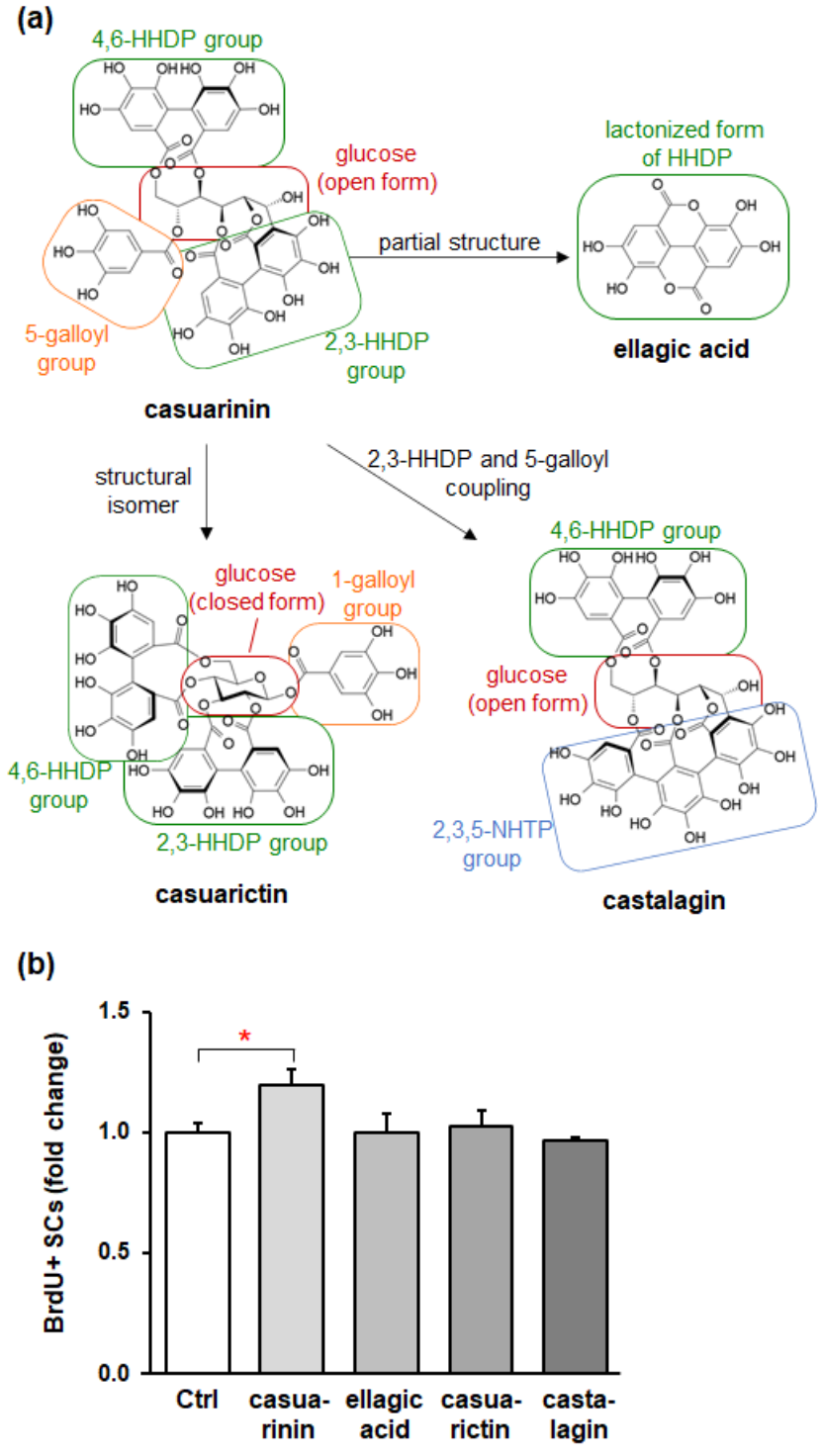

Figure 7. Structurally related compounds of casuarinin do not promote BrdU incorporation into SCs *in vitro*. (**a**) Chemical structures of the ellagitannins (casuarinin, casuarictin, and castalagin) and their derivative (ellagic acid). (**b**) Effects of the ellagitannins and their derivative (250 nM each) on BrdU incorporation into SCs. BrdU incorporation into SCs is expressed as $BrdU^+$ SCs per total SCs and is shown as a fold change compared to the Ctrl. Data represent the mean ± SD of three independent cultures. * Significant differences ($p < 0.05$) relative to the Ctrl. This figure was quoted and modified from the literature [28].

2.3.4. LM or casuarinin treatment upregulates *IL-6* mRNA expression in SCs

Skeletal muscle is an endocrine organ that produces and secretes various cytokines called myokines. Among the myokines, IL-6 is the first identified and the most studied myokine [47] and plays a key role in the regulation of muscle homeostasis and SC response [48]. Several studies have shown that IL-6 induces the activation and proliferation of SCs [49–51], and *IL-6* knockout experiments have shown that IL-6 is essential for SC activation and proliferation and subsequent muscle hypertrophy [50].

To investigate whether IL-6 is involved as a signaling molecule in SC activation by LM and casuarinin, I evaluated *IL-6* mRNA expression in SCs *in vitro*. SCs isolated from rats were reseeded to enrich the cell density and incubated with each sample for 16 h, after which *IL-6* mRNA expression levels were quantified. Treatment with LM or casuarinin, which promoted BrdU incorporation into SCs (Figure 3a in Chapter 1 or Figure 6a, respectively), significantly upregulated *IL-6* mRNA expression in SCs compared to the control (both LM and casuarinin: $p < 0.01$) (Figure 8, left panel). In contrast, treatment with hyperin or quercitrin, which had no effect on BrdU incorporation into SCs (Figure 6a), did not affect *IL-6* mRNA expression (Figure 8, right panel). Collectively, these data indicate that SC activation by LM and casuarinin is associated with upregulation of *IL-6* mRNA expression.

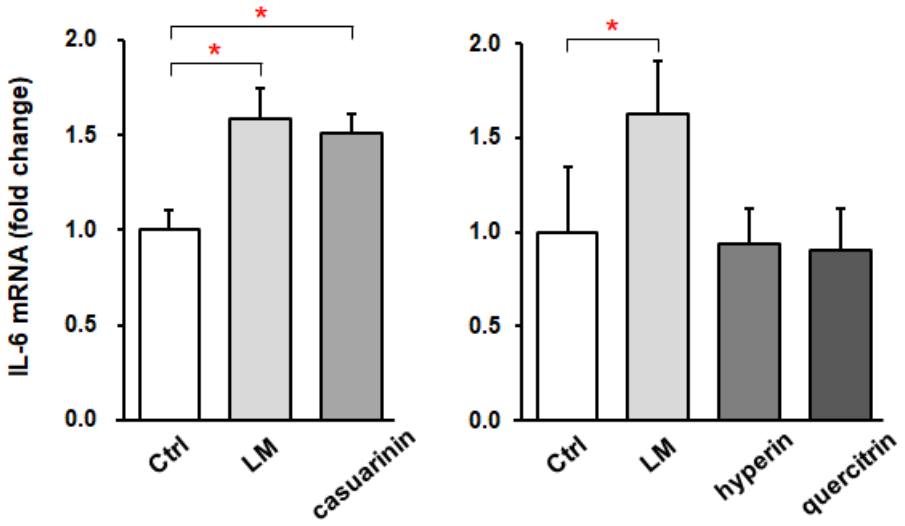

Figure 8. LM and casuarinin upregulate *IL-6* mRNA expression in SCs *in vitro*. SCs were treated with LM (2.5 µg/mL), casuarinin (330 nM), hyperin (220 nM), or quercitrin (220 nM) for 16 h and *IL-6* mRNA expression levels were quantified and normalized to *GAPDH*. Data are shown as fold changes versus Ctrl and represent the mean ± SD of four independent cultures. * Significant differences ($p < 0.05$) relative to the Ctrl. This figure was quoted and modified from the literature [28].

2.3.5. Oral administration of casuarinin activates SCs *in vivo*

To determine whether casuarinin in LM induces SC activation *in vivo* as well, I orally administered LM or casuarinin to rats and evaluated SC activation in rat skeletal muscle. The experimental design is illustrated in Figure 9a. Rats were orally administered water (as the control), LM (250 mg/kg/day), or casuarinin (4 or 8 mg/kg/day) daily for 4 d, followed by intraperitoneal administration of BrdU, then BrdU incorporation into SCs was examined. Compared with the control group, rats administered LM or casuarinin (both doses) significantly increased BrdU incorporation into SCs (LM: $p < 0.01$, casuarinin (4 mg/kg): $p = 0.04$, and casuarinin (8 mg/kg): $p < 0.01$) (Figure 9b). The casuarinin content in the effective dose of LM (250 or 500 mg/kg, Figure 9b or Figure 2b in Chapter 1, respectively) was 4 or 8 mg/kg, which was consistent with the actual effective dose of casuarinin (4 or 8 mg/kg, Figure 9b). Thus, I concluded that casuarinin is the active compound in LM that activates SCs both *in vitro* and *in vivo*.

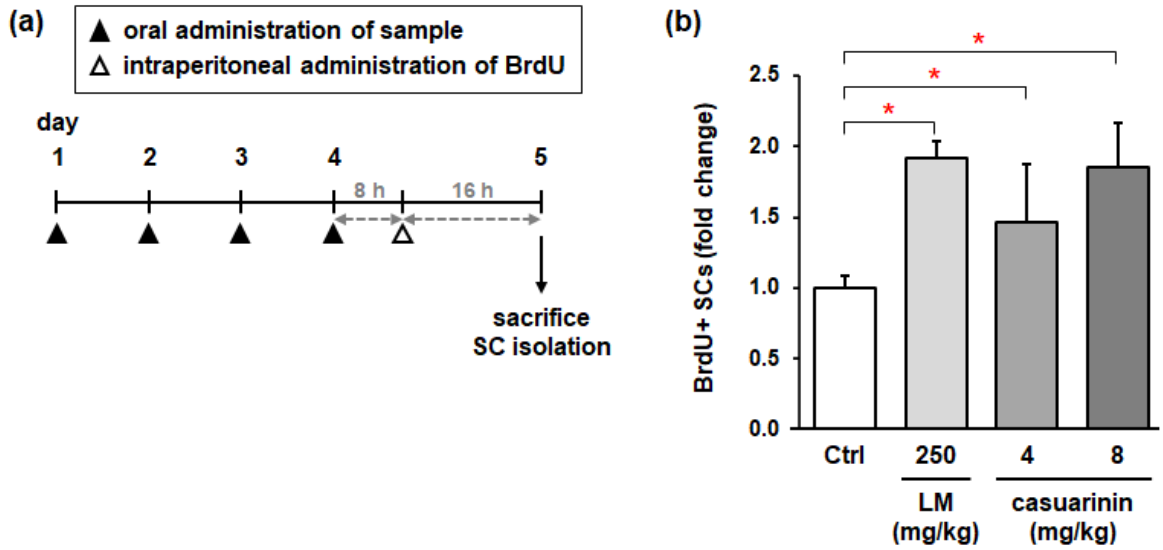

Figure 9. Casuarinin in LM promotes BrdU incorporation into SCs *in vivo*. (**a**) Experimental design of *in vivo* BrdU-incorporation assay. Rats in each group were orally administered water (Ctrl), LM, or casuarinin daily for 4 d. Eight hours after the last administration of each sample, all animals were intraperitoneally administered BrdU (50 mg/kg), and then 16 h later, animals were sacrificed, and SCs were isolated. (**b**) Effects of LM and casuarinin administration on BrdU incorporation into SCs. The daily dose of each sample is shown in the figure. BrdU incorporation into SCs is expressed as $BrdU^+$ SCs per total SCs and is shown as a fold change compared to the Ctrl. Data represent the mean ± SD of each group (three independent cultures per rat). * Significant differences ($p < 0.05$) relative to the Ctrl. This figure was quoted and modified from the literature [28].

2.4. Discussion

In this chapter, I identified five major compounds in LM: gallic acid, casuarinin, myricitrin, hyperin, and quercitrin. Among the five compounds, gallic acid, myricitrin, and hyperin have previously been identified in lemon myrtle [52–54]. To the best of my knowledge, casuarinin and quercitrin have been identified as constituents of lemon myrtle for the first time in this study. Furthermore, I investigated whether each major compound activated SCs or not. Based on three criteria (the effective concentration of LM, content of each compound in LM, and the evaluated concentration of each compound), I showed that only casuarinin activated SCs at the concentration contained in the effective concentration of LM. However, I cannot rule out the possibility that unidentified trace compounds present in LM may also activate SCs and that multiple compounds present in LM collectively regulate SC activation. Therefore, it is unclear whether casuarinin is the only active compound in LM. A comprehensive analysis of compounds present in LM and their activities will provide a deeper understanding of the active compounds present in LM. Interestingly, each of the five major compounds identified in LM, as well as lemon myrtle extract discussed in Chapter 1, has been reported to inhibit cancer cell proliferation [55–62]. In contrast, I showed that only casuarinin activated SCs, whereas the other four compounds did not. Therefore, the effects of LM and casuarinin on SCs may involve different mechanisms than those on other cells, including cancer cells. Evaluating the effects

of each compound present in LM on various cells may help to clarify why LM is thought to activate only SCs.

Casuarinin is a *C*-glycosidic ellagitannin that consists of an open-chain form of glucose, two HHDP groups, and a galloyl group. The molecular structure of casuarinin is not similar to that of the other four major compounds identified in LM, which do not activate SCs. Among the four major compounds, myricitrin, hyperin, and quercitrin are flavonol glycosides and differ from ellagitannins in terms of both their overall and partial structures. In contrast, gallic acid is a partial structure of ellagitannins but does not activate SCs. To examine the relationship between the molecular structure of casuarinin and SC activation, I evaluated whether structurally related compounds of casuarinin could activate SCs. First, ellagic acid, which is a lactonized compound of HHDP and is a partial structure of casuarinin, did not activate SCs. Since neither gallic acid nor ellagic acid induced SC activation, it is likely that the partial structure of casuarinin does not have SC activation properties. Next, casuarictin, which is a structural isomer of casuarinin, did not activate SCs. The differences in molecular structure between casuarinin and casuarictin are the glucose moiety (casuarinin has an open form, whereas casuarictin has a closed form), the number of hydroxyl groups (casuarinin has 16 OH groups, whereas casuarictin has 15), and the position of the galloyl group (in casuarinin, the galloyl group bonds to O-5 of glucose, whereas in casuarictin, it bonds to O-1 of glucose)

(Figure 7a). Plaza et al. have shown that differences in the molecular structures of these isomers affect the strength of their antioxidant capacity [63]. Therefore, it is likely that the differences in the molecular structure between these isomers affect SC activation as well. Finally, castalagin, which is an ellagitannin with a 2,3,5-NHTP group, also did not affect SC activation. The 2,3,5-NHTP group in castalagin is the intramolecular coupling of the 2,3-HHDP and 5-galloyl groups in casuarinin. Casuarinin has a rigid 2,3-HHDP group and a rotatable 5-galloyl group, whereas castalagin has a rigid 2,3,5-NHTP group. Therefore, castalagin has a more rigid structure than casuarinin. Kaneshima et al. have shown that differences in the structural flexibility between casuarinin and castalagin may affect the strength of antioxidant activity [64]. Collectively, the molecular structure of casuarinin may contribute to the activation of SCs. It will be interesting to evaluate SC activation properties of analogs with partially substituted molecular structure of casuarinin.

IL-6 is the first identified and most studied myokine and plays a key role in the regulation of muscle homeostasis and SC response [47,48]. Several studies have shown the effects of IL-6 on SC and myoblast activation and proliferation. Kurosaka et al. reported that IL-6 treatment induced activation and proliferation of SCs *in vitro* [51]. Zhang et al. reported that *in vitro* myoblast activation by IL-6 is counteracted by an IL-6 neutralizing antibody [65]. Moreover, Weigert et al. showed that IL-6 treatment increased *IL-6* mRNA expression in

myoblasts *in vitro* [66]. Collectively, these results suggest that the activation and proliferation of SCs and myoblasts by IL-6 are regulated in an autocrine manner via the upregulation of *IL-6* mRNA expression. Here, I investigated the involvement of IL-6 in SC activation by LM and casuarinin *in vitro*. I showed that LM or casuarinin treatment significantly upregulated *IL-6* mRNA expression in SCs, whereas hyperin or quercitrin treatment, which did not activate SCs, did not change *IL-6* mRNA expression in SCs. These results suggest that the SC activation properties by LM and its major compounds are associated with *IL-6* mRNA expression in SCs. In addition to *in vitro* studies, animal and human studies have shown that IL-6 is involved in skeletal muscle hypertrophy mediated by SCs. Serrano et al. reported that compensatory hypertrophy increased *IL-6* mRNA and IL-6 protein expression in the muscle of normal mice and that *IL-6* knockout mice exhibited blunted muscle hypertrophy and suppressed SC activation and proliferation following compensatory hypertrophy [50]. McKay et al. demonstrated that muscle-lengthening contractions in humans increased the number of SCs, IL-6 protein levels in serum, and *IL-6* mRNA levels in muscle [67]. Begue et al. showed that acute resistance exercise in rats induces SC activation and increases *IL-6* mRNA levels in muscle and that 10 weeks of resistance exercise in rats induces muscle hypertrophy and a persistent increase in *IL-6* mRNA levels in muscle [68]. Taken together, these data suggest that IL-6 is involved in the mechanism of muscle hypertrophy via SC activation. Future studies are

required to evaluate whether IL-6 is involved in the mechanism of SC activation by LM and casuarinin *in vivo* as well.

Finally, I showed that oral administration of casuarinin (4 and 8 mg/kg) to normal rats activated SCs as well as LM. Similar to the *in vitro* data, the effective dose of casuarinin was consistent with the casuarinin content in the effective dose of LM. These results indicate that casuarinin is the active compound in LM *in vivo*. To my knowledge, there are few functional food ingredients that activate SCs in the muscle of normal mice or rats. Oral administration of β-hydroxy-β-methylbutyrate (340 mg/kg) [69] or epigallocatechin-3-gallate (50 mg/kg) [70] to rats activated SCs in the muscle during recovery from atrophy but did not activate SCs in normal muscle. In addition, oral administration of proanthocyanidins (20 mg/kg) to rats [71] or intraperitoneal administration of epicatechin gallate (25 mg/kg) to mice [72] activated SCs in injured muscle, whereas intraperitoneal administration of resveratrol (20 mg/kg) or curcumin (1 mg/kg) to mice induced activation and proliferation of SCs in the atrophied muscle [73]. However, none of these ingredients have been reported to activate SCs in normal muscle. Although intraperitoneal administration of ursolic acid (200 mg/kg) to mice induced SC proliferation in normal muscle [74], it is unclear whether oral administration also activates SCs. Contrary to these studies, oral administration of LM or casuarinin to rats activated SCs in normal muscle. These results suggest that supplementation with LM or casuarinin in healthy

adults may reduce the risk of developing sarcopenia. In addition to the effects on normal muscle, supplementation with LM or casuarinin may improve sarcopenia and disuse atrophy by activating SCs in atrophied muscle and promote muscle regeneration by activating SCs in injured muscle. However, the fate of SCs activated by LM or casuarinin supplementation is unknown; that is, whether these activated SCs further proliferate and differentiate to become new myonuclei or proliferate but return to a quiescent state to maintain the SC pool. Future studies are required to assess the effects of LM or casuarinin supplementation on skeletal muscle hypertrophy as well as SC activation. In addition, exercise induces skeletal muscle hypertrophy through both activation of SCs and stimulation of muscle protein synthesis [19]; therefore, it is important to assess whether LM or casuarinin supplementation also stimulates muscle protein synthesis. Even if supplementation with LM or casuarinin do not affect muscle protein synthesis, supplementation with LM or casuarinin in combination with conventional nutrients that induce muscle protein synthesis, such as protein and leucine, may provide a powerful intervention for improving sarcopenia instead of exercise.

General conclusions

Exercise and nutritional interventions have been conducted to improve sarcopenia. Exercise intervention has much evidence [4] but involves a physical burden depending on the intensity of exercise. In contrast, nutritional interventions are simple and safe methods, but the evidence is inconsistent and more high-quality clinical trials are needed [4]. Kaneka Corporation focused on the skeletal muscle satellite cell (SC) activation, a mechanism of exercise-induced skeletal muscle hypertrophy, and found an extract of lemon myrtle leaves as a candidate ingredient. The objective of this thesis is to elucidate the functionality of lemon myrtle extract as a novel functional food ingredient for skeletal muscle hypertrophy.

In Chapter 1, I examined the general manufacturing method and functional properties of lemon myrtle extract. I showed that the extraction solvent affects the SC activation properties, and that lemon myrtle water extract (LM) activates SCs *in vitro* and *in vivo*. I also showed that LM specifically activates SCs but not myoblast. In Chapter 2, I attempted to elucidate the mechanism by which LM activates SCs. First, I identified casuarinin, an ellagitannin, as the active compound in LM involved in SC activation. Subsequent structure–activity relationship analysis showed that the overall molecular structure of casuarinin is crucial for SC activation. Furthermore, I showed that SC activation by LM and casuarinin was associated with upregulation of IL-6 expression.

The results of present study provide important evidence for establishing a manufacturing method for LM and a product specification for LM with casuarinin as the quality control target. Based on these results, scale-up production should be examined to determine a stable industrial production method for LM. These results also provide fundamental evidence to elucidate the functionality and mechanism of action of LM. Future animal and clinical studies are required to test whether LM supplementation induces skeletal muscle hypertrophy following SC activation. In clinical trials, it is also important to clarify the conditions that influence the efficacy of LM, such as the dose of LM, the duration of intervention, and the age and lifestyle of the subjects. In addition, clarifying the mechanism of action of LM will help distinguish LM supplementation from current nutritional interventions such as protein intake. If LM supplementation and those interventions have different mechanisms, combining LM supplementation with those interventions may augment their efficacy or expand their effective target populations. Thus, I believe that elucidation of the efficacy of LM supplementation in skeletal muscle hypertrophy and its mechanism of action could lead to the commercialization of LM itself and various foods containing LM, and provide a novel intervention for improving sarcopenia through food ingredients.

The functional analysis used in this study is a general approach in the development of functional food ingredients such as plant extracts. However, in probiotics, which have become

popular in recent years, active compounds are usually not identified, and the bacteria themselves are considered functional components. This research approach can be applied to the development of various functional food ingredients such as probiotics as well as plant extracts. Understanding active compounds in functional food ingredients and their structure–activity relationships will help prevent the distribution of substandard and falsified products. Manufacturers have a responsibility to provide consumers with correct product information. I hope to contribute to the extension of healthy life expectancy through the development of various functional food ingredients using this approach.

Acknowledgments

This thesis study has been carried out at the Molecular Biochemistry Laboratory, Unit of Biotechnology, Graduate School of Integrated Sciences for Life, Hiroshima University and at Agri-Bio & Supplement Research Laboratories, Kaneka Corporation.

I am deeply grateful to Professor Seiji Kawamoto (Unit of Biotechnology) for his kind guidance and encouragement throughout this study. I am also very grateful to Professor Masaki Mizunuma (Unit of Biotechnology), Professor Noriyuki Yanaka (Unit of Food and AgriLife Science), and Associate Professor Miyako Nakano (Unit of Biotechnology) for their helpful suggestions and discussions.

I wish to express special thanks to Professor Hidemi Fujino (Kobe University) for his general guidance on skeletal muscle satellite cell (SC) research.

I would like to thank Professor Ryuichi Tatsumi (Kyushu University) for technical guidance on SC isolation and culture, and the late Professor Hidetoshi Yamada (Kwansei Gakuin University) and Assistant Professor Shinnosuke Wakamori (Tokyo University of Agriculture) for providing chemically synthesized casuarinin.

I also thank Ms. Yuko Funabashi (Kaneka Techno Research Corporation) for technical support on LC-MS/MS analysis, Mr. Ryuichi Tanigawa (Kaneka Techno Research Corporation) for technical support on NMR analysis, Ms. Sachie Iida (TechnoPro Inc.) for

assistance with casuarinin isolation, and Dr. Mineko Ogura and Mr. Masanori Kato (Kaneka Corporation) for help with animal experiments.

I would like to express my deep appreciation to Dr. Shinichi Honda, Dr. Shinichi Yokota, Mr. Yuji Tominaga, Mr. Naoaki Taoka, and Dr. Masaru Mitsuda (Kaneka Corporation) for their advice and support throughout this study.

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Nutrients, 14(5), 1078 (2022).