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## Article

# Identification of Petit Vert Leaf-Derived Sinapic Acid Derivatives and Their Enhancement of Glucose Uptake in C2C12 Murine Myoblasts

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**Abstract:** Petit vert (scientific name: *Brassica oleracea* var. *gemmifera* DC. × *Brassica oleracea* var. *acephala* DC.) is a new variety of vegetable created by crossbreeding kale and brussels sprouts (*Brassica oleracea* species). The present study aimed to identify biologically active compounds in extracts of the outer leaves of Petit vert by purification and to examine their biological activities. Dried and powdered outer leaves of Petit vert were extracted, fractionated, and purified to isolate active compounds. Nuclear magnetic resonance and mass spectrometries were performed to elucidate the structures of five compounds (1–5). The compounds isolated from Petit vert leaves were glycosides that contained kaempferol, quercetin (flavonol), or sinapinic acid (phenylpropanoid). Glucose uptake in cultured C2C12 murine myoblasts in the absence of insulin was significantly increased by these compounds, kaempferol, sinapic acid, and ferulic acid, while that in the presence of insulin was also significantly increased by compounds 3 and 4, kaempferol, and sinapic acid. The present study reports for the first time the isolation of five compounds containing sinapic acid from the outer leaves of Petit vert and their stimulation of glucose uptake in cultured C2C12 murine myoblasts. The results obtained herein suggest the potential of these compounds to effectively attenuate hyperglycemia and maintain muscle strength by promoting glucose metabolism in muscle cells.

**Keywords:** Petit vert leaves; sinapic acid; glucose uptake

## 1. Introduction

Polyphenols are phenolic compounds that are present in plants and play a role in defenses against harmful oxidative damage. Among polyphenol compounds, hydroxycinnamic acid and its derivatives are well-known chemical groups that have important biological functions, such as anti-inflammatory and antioxidant activities [1]. The beneficial effects of these compounds as preventive or therapeutic agents have already been demonstrated in various diseases, such as inflammatory damage and cancer [2,3]. Among hydroxycinnamic acids, sinapic acid [(2*E*)-3-(4-hydroxy-3,5-dimethoxyphenyl)prop-2-enoic acid], a natural herb phenolic acid compound, is present in herbs, such as oranges, grapefruits, cranberries, mustard seeds, and rapeseeds [4]. Sinapic acid was previously shown to prevent scopolamine-induced cognitive impairments in memory in a rat model [5,6]. It also exhibited anti-inflammatory activity in carbon tetrachloride-induced acute liver damage and significantly reduced proinflammatory cytokine levels [7,8]. Furthermore, sinapic acid attenuated and prevented oxidative stress, neuroinflammation, and cholinergic deficits [9].

Petit vert (scientific name: *Brassica oleracea* var. *gemmifera* DC. × *Brassica oleracea* var. *acephala* DC.) is a new variety of vegetable created by crossbreeding kale and brussels sprouts (*Brassica oleracea*

species). Its anti-obesity effects were previously demonstrated in mice fed a high-fat diet containing 5% freeze-dried Petit vert [10]; however, the composition and identity of the active compounds present in Petit vert remain unclear. Since only the leafy side shoots of Petit vert are harvested, the outer leaves, which are of high nutritional value, are typically discarded because they contain rough fibers. Therefore, the present study aimed to identify biologically active compounds in extracts of the outer leaves of Petit vert by purification and to examine their biological activities. Dried and powdered outer leaves of Petit vert were extracted, fractionated, and purified to isolate active compounds. Nuclear magnetic resonance (NMR) and mass (MS) spectrometries were performed to elucidate the structures of five compounds. The compounds isolated from Petit vert leaves were glycosides containing either kaempferol, quercetin (flavonol), or sinapinic acid (phenylpropanoid).

## 2. Materials and Methods

### 2.1. Materials

Petit vert (*B. oleracea* var. *gemmifera* DC. × *B. oleracea* var. *acephala* DC.) was cultivated and harvested in a Brassica farm (Kawazu, Shizuoka Japan). Test compounds (**1–5**) were isolated from Petit vert. Quercetin (**6**), kaempferol (**7**), sinapic acid (**8**), ferulic acid (**9**), and caffeic acid (**10**) were purchased from Merck KGaA (Darmstadt, Germany). Penicillin-streptomycin solution, 0.25% trypsin/EDTA solution, cell count reagent SF (WST-8), and Dulbecco's modified Eagle's medium (DMEM) were purchased from Nacalai Tesque Inc. (Kyoto, Japan). Gelatin, type A and Gibco™ Insulin, human recombinant, zinc solution were purchased from MP Biomedicals (Irvine, CA, U.S.A.) and Thermo Fisher Scientific (Waltham, MA, U.S.A.), respectively. Fetal bovine serum (FBS) was obtained from Nichirei Biosciences Inc. (Tokyo, Japan). All other chemicals used were of the highest purity commercially available. All solvents were of high-performance liquid chromatography (HPLC) grade and water was of Milli-Q quality.

### 2.2. Extraction and Isolation

Petit vert leaves were lyophilized and ground to a powder in a domestic blender. Polyphenols were extracted from 400 g of dried Petit vert leaf powder with 6 L of methanol under reflux conditions for 2 h and then filtered. The filtrate was evaporated and the extract was suspended in 1.3 L of 90% aqueous methanol. The suspension was washed three times with 1.3 L of *n*-hexane, and the extract was evaporated and suspended in 1.3 L of distilled water. The extract was washed three times with 1.3 L of ethyl acetate. The extract was again evaporated and then dissolved in 1 L of distilled water. The extract was charged on a porous polymer gel (DIAION HP-20, Mitsubishi Chemical Co., Japan) column and eluted with 3 L of distilled water, 3 L of 50% aqueous methanol, and 3 L of methanol. Extracts were evaporated to obtain the phenolic extract (103.7, 8.4, and 2.4 g, respectively). The 50% aqueous methanol eluate (7.6 g) was charged on a TOYOPEARL HW-40 column (TOSOH, Japan) and then eluted with 10% aqueous ethanol and 50% aqueous ethanol to obtain eight fractions (fractions A–H). Fraction C (705 mg) was purified by preparative HPLC (Inertsil ODS-3, i.d., 30 × 250 mm, GL Sciences Inc., Japan), and compounds **2–5** (**2**; 41.6 mg, **3**; 34.8 mg, **4**; 90.5 mg, **5**; 53.8 mg), which were of sufficient purity and quantity for complete chemical characterization, were isolated. The MeOH eluate (2.4 g) was charged on a TOYOPEARL HW-40 column (TOSOH, Japan) and then eluted with 50% aqueous ethanol and 85% aqueous ethanol to obtain six fractions (fractions A–F). Fraction B (785 mg) was purified by preparative HPLC (Inertsil ODS-3, i.d., 30 × 250 mm, GL Sciences Inc., Japan) and compound **1** (33.4 mg) was isolated.

### 2.3. Identification of Phenolic Compounds by HPLC

To isolate flavonoids and hydroxycinnamic acid derivatives, HPLC consisting of a degasser, quaternary pump, thermostat, a UV detector set at 330 nm, and an injection valve was used. Extracts were analyzed on a Cosmosil 2.5 Cholesterol column (100 × 3 mm, 2.5 μm; Nacalai Tesque, Inc.) at a temperature of 40°C using a gradient of (A) 0.1% trifluoroacetic acid in water and (B) acetonitrile. The

following gradient with a flow rate of 0.75 mL/min was used: 6–11% B (0–20 min), 11–27% B (20–36 min), and 27% B isocratic (36–45 min).

#### 2.4. Structural Elucidation of Phenolic Compounds by NMR and MS

The structures of compounds **1–5** were identified by NMR spectroscopy.  $^1\text{H}$  and  $^{13}\text{C}$  NMR spectra were analyzed on a JEOL lambda-500 spectrometer (JEOL, Tokyo, Japan) (500 MHz for  $^1\text{H}$  and 125 MHz for  $^{13}\text{C}$ ) or a Bruker Avance 700 spectrometer (Bruker, Massachusetts, U.S.A.) (700 MHz for  $^1\text{H}$  and 175 MHz for  $^{13}\text{C}$ ) in  $\text{CD}_3\text{OD}$  or  $\text{DMSO}-d_6$ . High resolution (HR)-electrospray ionization (ESI)-MS data on compounds **2–5** were obtained using a Waters/Micromass Q-TOF micro MS (Waters, Massachusetts, U.S.A.) in the negative ion mode.

#### 2.5. Cell Culture

C2C12 mouse myoblast cells were purchased from the American Type Culture Collection (Manassas, Virginia, U.S.A.). Cells were cultured in DMEM with 25 mM glucose, 10% FBS, and 1% antibiotic solution containing penicillin and streptomycin at 37°C in a humidified atmosphere of 5%  $\text{CO}_2$ –95% air. Cells were plated in a 96-well microplate (100  $\mu\text{l}$  well $^{-1}$ ) at a density of  $4 \times 10^4$  cells well $^{-1}$ . Confluent myoblasts were differentiated to myotubes by lowering serum concentrations to 0.1% and adding 5  $\mu\text{g/ml}$  insulin. Myotubes formed after 4 days of incubation and were used in subsequent experiments.

#### 2.6. Cell Viability Assay

Cell viability assays were performed using cell count reagent SF (WST-8). WST-8 reagent solution (10  $\mu\text{l}$ ) was added to each well of a 96-well microplate containing 100  $\mu\text{l}$  of a cell suspension in culture medium and the test compounds (**1–10**) at various concentrations (1, 10, and 100  $\mu\text{M}$ ). Plates were incubated at 37°C for 24 h. Control wells (0.1% DMSO) containing the same volume of complete culture medium were included in each assay. After being cultured for 24 h, absorbance was measured at 450 nm using a microplate reader (Varioskan Flash, Thermo Fisher Scientific Waltham, MA, U.S.A.). Measurements were performed with triplicate dishes of three different experiments.

#### 2.7. Assay of Glucose Uptake in C2C12 Mouse Myoblast Cells

The glucose uptake rate was measured using the Glucose Uptake-Glo™ Assay kit (Promega Corporation, Madison, Wisconsin, U.S.A) according to the manufacturer's instructions. This assay measures glucose uptake in cells based on the detection of 2-deoxyglucose-6-phosphate. Test compounds (**1–10**) were added at various concentrations (1, 10, and 100  $\mu\text{M}$ ) to each well of a 96-well microplate containing cells in culture medium, and the plate was incubated at 37°C for 24 h. Control wells (0.1% DMSO) containing the same volume of complete culture medium were included in each assay. Before the assay on glucose uptake, cells were placed in serum-free medium for 18 h. C2C12 mouse myoblast cells were then cultured in DMEM with 25 mM glucose and 10% FBS in the presence or absence of insulin and the test substances (1, 10, and 100  $\mu\text{M}$ ) for 1 h. Fluorescence intensity was measured using a fluorescence spectrofluorometer (Varioskan Flash, Thermo Fisher Scientific Waltham, MA, U.S.A.) and used to calculate glucose into cells.

#### 2.8. Statistical Analysis

Data were statistically analyzed using the Student's *t*-test after an analysis of variance. Results are shown as the mean  $\pm$  S.E. unless otherwise stated.

### 3. Results

#### 3.1. Structural Elucidation of Phenolic Compounds by NMR

$^{13}\text{C}$  and  $^1\text{H}$  NMR spectroscopic data on compound **1** showed two characteristic anomeric proton and carbon signals for  $\beta$ -D-glucopyranosyl groups at  $\delta$  91.9, 103.1,  $\delta_{\text{H}}$  5.77 (1H, *d*, *J*=8.0 Hz), 4.20 (1H,



*d*, *J*=8.0 Hz) and two *trans*-sinapoyl groups at  $\delta_c$  164.9, 148.0 $\times$ 2, 145.8, 138.4, 124.3, 113.5, 106.3 $\times$ 2, 56.1 $\times$ 2,  $\delta_H$  7.55 (1H, *d*, *J*=16.0 Hz), 6.99 (1H, *s*)  $\times$ 2, 6.49 (1H, *d*, *J*=16.0 Hz), 3.77 (3H, *s*)  $\times$ 2 and  $\delta_c$  165.6, 148.0 $\times$ 2, 147.3, 138.9, 124.0, 114.6, 106.6 $\times$ 2, 56.1 $\times$ 2,  $\delta_H$  7.53 (1H, *d*, *J*=16.0 Hz), 6.96 (1H, *s*)  $\times$ 2, 6.44 (1H, *d*, *J*=16.0 Hz), 3.78 (3H, *s*)  $\times$ 2. Therefore, compound **1** consisted of two  $\beta$ -D-glucopyranosyl and two *trans*-sinapoyl groups. Consistent with NMR spectroscopic data on compound **1** in the literature, compound **1** was assigned as 6-*O*- $\beta$ -D-glucopyranosyl- $\beta$ -D-[[1-*O*-3,5-dimethoxy-4-hydroxy-(*E*)-cinnamoyl],2-*O*-[3,5-dimethoxy-4-hydroxy-(*E*)-cinnamoyl]]-glucopyranoside [11].

The molecular formula for compound **2**, C<sub>50</sub>H<sub>60</sub>O<sub>31</sub>, was established based on HR-ESI-MS [*m/z*: 1155.3058 [M-H]<sup>−</sup>]. The aglycone of compound **2** was identified as quercetin based on observations of 15 carbon signals, including twelve aromatic carbon signals ( $\delta_c$  164.1, 162.2, 157.3, 149.8, 145.9, 123.3 $\times$ 2, 117.2, 116.1, 107.5, 100.6, and 95.4), two olefin carbons ( $\delta_c$  157.5 and 135.0), and one carbonyl carbon ( $\delta_c$  179.1), and AM-type aromatic protons [ $\delta_H$  6.37 (1H, *d*, *J*=2.0 Hz) and 6.34 (1H, *d*, *J*=2.0 Hz)] derived from the A-ring and AMX-type aromatic proton signals [ $\delta_H$  7.50 (1H, *d*, *J*=2.0 Hz), 7.45 (1H, *dd*, *J*=8.5, 2.0 Hz), and 6.88 (1H, *d*, *J*=8.5 Hz)] derived from the B-ring, in <sup>13</sup>C and <sup>1</sup>H NMR spectroscopic data. Moreover, in the <sup>13</sup>C and <sup>1</sup>H NMR spectra of compound **2**, four anomeric carbon and proton signals of  $\beta$ -D-glucopyranoses were observed at  $\delta_c$  96.9, 98.4, 101.2, 104.5 and  $\delta_H$  6.18 (1H, *d*, *J*=8.0 Hz), 5.24 (1H, *d*, *J*=8.0 Hz), 5.13 (1H, *d*, *J*=8.0 Hz), 4.48 (1H, *d*, *J*=8.0 Hz) together with signals due to one *trans*-sinapoyl group [ $\delta_c$  168.6, 148.7 $\times$ 2, 146.6, 138.7, 125.9, 115.9, 105.5 $\times$ 2, 56.2 $\times$ 2 and  $\delta_H$  7.30 (1H, *d*, *J*=16.0 Hz), 6.23 (1H, *s*) $\times$ 2, 6.09 (1H, *d*, *J*=16.0 Hz), 3.59 (3H, *s*) $\times$ 2]. <sup>13</sup>C and <sup>1</sup>H NMR signals were assigned (Tables 1 and 2) based on the results of two-dimensional (2D)-NMR [<sup>1</sup>H-<sup>1</sup>H COSY, <sup>1</sup>H-detected heteronuclear single-quantum correlation spectroscopy, and <sup>1</sup>H-detected hetero-nuclear multiple-bond connectivity (HMBC)] measurements. In addition, the HMBC experiment provided information on sugar and ester linkages. <sup>3</sup>J<sub>COCHS</sub> were observed between C-3 of the aglycone ( $\delta_c$  135.0) and H-1 of  $\beta$ -D-glucopyranose ( $\delta_H$  6.18), C-2 of  $\beta$ -D-glucopyranose ( $\delta_c$  82.0) and H-1' of  $\beta$ -D-glucopyranose ( $\delta_H$  5.24), C-7 of the aglycone ( $\delta_c$  164.1) and H-1'' of  $\beta$ -D-glucopyranose ( $\delta_H$  5.13), and C-4'' of  $\beta$ -D-glucopyranose ( $\delta_c$  80.1) and H-1''' of  $\beta$ -D-glucopyranose ( $\delta_H$  4.48). Moreover, the acylation shift of the H-2' signal of  $\beta$ -D-glucopyranose ( $\delta_H$  4.93) suggested that the sinapoyl group was attached at C-2' of  $\beta$ -D-glucopyranose, which was supported by the results of the HMBC experiment. Therefore, compound **2** was identified as quercetin 3-*O*- $\beta$ -D-[2-*O*-3,5-dimethoxy-4-hydroxy-(*E*)-cinnamoyl]glycopyranosyl-(1→2)- $\beta$ -D-glycopyranoside-7-*O*- $\beta$ -D-glucopyranosyl-(1→4)- $\beta$ -D-glycopyranoside [12].

**Table 1.** <sup>13</sup>C NMR spectroscopic data of flavonoid glycosides and a cinnamic acid derivative ( $\delta$ /ppm; 175 or 125 MHz instruments).

Compound	1 <sup>a</sup>	2 <sup>b</sup>	3 <sup>b</sup>	4 <sup>b</sup>	5 <sup>b</sup>
Aglycone moiety					
C-2	–	157.5	157.3	157.7	157.5
3	–	135.0	134.9	134.8	134.8
4	–	179.1	179.1	179.1	179.4
5	–	162.2	162.1	162.1	162.2
6	–	100.6	100.6	100.7	100.7
7	–	164.1	164.2	164.1	164.2
8	–	95.4	95.1	95.3	95.3
9	–	157.3	157.5	157.3	157.4
10	–	107.5	107.4	107.5	107.5
1'	–	123.3	123.3	122.9	123.0
2'	–	117.2	117.1	132.2	132.2
3'	–	149.8	149.7	116.2	116.1

4'	–	145.9	145.9	161.4	161.5
5'	–	116.1	116.1	116.2	116.1
6'	–	123.3	123.3	132.2	132.2
Suger moiety					
(3)-O-sugars					
C-1	91.9	96.9	96.9	97.0	97.0
2	73.5	82.0	82.0	82.0	82.1
3	73.8	75.1	75.1	75.1	75.1
4	70.0	71.4	71.3	71.5	71.5
5	76.7	78.3	78.3	78.2	78.3
6	67.8	62.2	62.2	62.2	62.3
C-1'	103.1	98.4	98.4	98.5	98.5
2'	72.6	75.0	75.0	75.0	75.0
3'	76.5	75.8	75.8	75.8	75.9
4'	70.0	71.2	71.1	71.2	71.2
5'	76.8	77.8	77.8	77.8	77.9
6'	61.0	62.2	62.2	62.2	62.2
7-O-sugars					
C-1''	–	101.2	101.4	101.2	101.5
2''	–	74.5	74.8	74.5	74.8
3''	–	76.0	77.7	76.0	77.7
4''	–	80.1	71.4	80.2	71.4
5''	–	76.6	78.1	76.6	78.2
6''	–	61.6	62.5	61.7	62.5
C-1'''	–	104.5	–	104.5	–
2'''	–	74.9	–	74.9	–
3'''	–	77.8	–	78.0	–
4'''	–	71.3	–	71.3	–
5'''	–	78.1	–	77.8	–
6'''	–	62.4	–	62.4	–
Ester moiety					
C-□	164.9	168.6	168.6	168.4	168.4
□	113.5	115.9	115.9	116.0	116.0
□	145.8	146.6	146.6	146.5	146.5
1	124.3	125.9	125.9	126.0	126.0
2	106.3	105.5	105.5	105.6	105.6
3	148.0	148.7	148.7	148.7	148.8
4	138.4	138.7	138.7	138.7	138.8
5	148.0	148.7	148.7	148.7	148.8
6	106.3	105.5	105.5	105.6	105.6
OMe	56.1x2	56.2x2	56.2x2	56.3x2	56.3x2
C-□'	165.6	–	–	–	–

□'	114.6	—	—	—	—
□'	147.3	—	—	—	—
1'	124.0	—	—	—	—
2'	106.6	—	—	—	—
3'	148.0	—	—	—	—
4'	138.9	—	—	—	—
5'	148.0	—	—	—	—
6'	106.6	—	—	—	—
OMe	56.1x2	—	—	—	—

<sup>a</sup>: Measured in DMSO-*d*<sub>6</sub>. <sup>b</sup>: Measured in MeOH-*d*<sub>4</sub>.

**Table 2.** <sup>1</sup>H NMR spectroscopic data of flavonoid glycosides and a cinnamic acid derivative (δ/ppm; 700 or 500 MHz instruments).

Compound 1 <sup>a</sup>		2 <sup>b</sup>	3 <sup>b</sup>	4 <sup>b</sup>	5 <sup>b</sup>
Aglycone moiety					
H-6	—	6.37 ( <i>d</i> , 2.0)	6.37 ( <i>d</i> , 2.0)	6.38 ( <i>s</i> )	6.38 ( <i>d</i> , 2.0)
8	—	6.34 ( <i>d</i> , 2.0)	6.36 ( <i>d</i> , 2.0)	6.38 ( <i>s</i> )	6.40 ( <i>d</i> , 2.0)
2'	—	7.50 ( <i>d</i> , 2.0)	7.51 ( <i>d</i> , 2.0)	7.89 ( <i>d</i> , 8.5)	7.90 ( <i>d</i> , 8.5)
3'	—	—	—	6.90 ( <i>d</i> , 8.5)	6.90 ( <i>d</i> , 8.5)
5'	—	6.88 ( <i>d</i> , 8.5)	6.88 ( <i>d</i> , 8.5)	6.90 ( <i>d</i> , 8.5)	6.90 ( <i>d</i> , 8.5)
6'	—	7.45 ( <i>dd</i> , 8.5, 2.0)		7.46 ( <i>dd</i> , 8.5, 2.0)	7.89 ( <i>d</i> , 8.5)
	7.90 ( <i>d</i> , 8.5)				
Sugar moiety					
(3)- <i>O</i> -Sugar					
H-1	5.77 ( <i>d</i> , 8.0)	6.18 ( <i>d</i> , 8.0)	6.19 ( <i>d</i> , 8.0)	6.16 ( <i>d</i> , 8.0)	6.17 ( <i>d</i> , 8.0)
2	4.91 ( <i>dd</i> 8.5, 8.0)	3.58 *	3.58 *	3.53 *	3.52 *
3	3.59 *	3.84 ( <i>t</i> , 9.0)	3.85 ( <i>t</i> , 9.0)	3.85 ( <i>t</i> , 9.0)	3.84 ( <i>t</i> , 9.0)
4	3.39 ( <i>t</i> , 9.0)	3.34 ( <i>t</i> , 9.0)	3.34 ( <i>t</i> , 9.0)	3.32 ( <i>t</i> , 9.0)	3.31 *
5	3.59 *	3.27 ( <i>m</i> )	3.27 ( <i>m</i> )	3.29 *	3.27 ( <i>m</i> )
6	4.05 ( <i>brd</i> , 9.5)	3.67 ( <i>dd</i> , 12.0, 2.5)		3.67 ( <i>dd</i> , 12.0, 2.0)	3.67 ( <i>brd</i> , 12.0)
6	3.67 ( <i>dd</i> , 12.0, 2.0)				
6	3.67 *	3.51 ( <i>dd</i> , 12.0, 5.5)		3.51 *3.50	( <i>dd</i> , 12.0, 5.5)
	3.49 ( <i>dd</i> , 12.0, 5.5)				
1'	4.20 ( <i>d</i> , 8.0)	5.24 ( <i>d</i> , 8.0)	5.25 ( <i>d</i> , 8.0)	5.25 ( <i>d</i> , 8.0)	5.24 ( <i>d</i> , 8.0)
2'	2.99 ( <i>dd</i> , 9.0, 8.0)		4.93 ( <i>dd</i> , 9.0, 8.0)	4.93 ( <i>dd</i> , 9.0, 8.0)	
	4.95 ( <i>dd</i> , 9.0, 8.0)		4.94 ( <i>dd</i> , 9.0, 8.0)		
3'	3.14 ( <i>t</i> , 9.0)	3.80 ( <i>t</i> , 9.0)	3.80 ( <i>t</i> , 9.0)	3.81 ( <i>t</i> , 9.0)	3.79 ( <i>t</i> , 9.0)
4'	3.08 *	3.53 *	3.54 *	3.53 *	3.52 *
5'	3.08 *	3.53 *	3.52 *	3.53 *	3.52 *
6'	3.67 *	3.93 ( <i>brd</i> , 12.0)	3.93 ( <i>brd</i> , 12.0)	3.91 *	3.93 ( <i>dd</i> , 12.0, 2.0)
6'	3.45 *	3.78 ( <i>dd</i> , 12.0, 4.0)		3.78 ( <i>dd</i> , 12.0, 4.0)	3.78 ( <i>dd</i> , 12.0, 4.0)
	3.77 ( <i>dd</i> , 12.0, 5.5)				

7-O-Sugar					
H-1"	—	5.13 (d, 8.0)	5.10 (d, 8.0)	5.13 (d, 8.0)	5.10 (d, 8.0)
2"	—	3.57 (dd, 9.0, 8.0)		3.51 *3.58	(dd, 9.0, 8.0)
	3.50 *				
3"	—	3.75 (t, 9.0)	3.58 *	3.75 (t, 9.0)	3.58 *
4"	—	3.69 (t, 9.0)	3.42 (t, 9.0)	3.69 (t, 9.0)	3.41 (t, 9.0)
5"	—	3.72 *	3.57 *	3.72 *	3.57 *
6"	—	3.98 (dd, 12.0, 2.2)		3.95 (dd, 12.0, 2.5)	3.99 (brd, 12.0)
	3.94 (dd, 12.0, 2.0)				
6"	—	3.93 (brd, 12.0)	3.74 (dd, 12.0, 6.0)		3.91 * 3.73 (dd, 12.0, 6.0)
H-1'''	—	4.48 (d, 8.0)	—	4.48 (d, 8.0)	—
2'''	—	3.29 (dd, 9.0, 8.0)		—	3.30 * —
3'''	—	3.42 (t, 9.0)	—	3.43 (t, 9.0)	—
4'''	—	3.34 (t, 9.0)	—	3.35 (t, 9.0)	—
5'''	—	3.39 *	—	3.40 *	—
6'''	—	3.92 (dd, 12.0, 2.5)		—	3.91 * —
6'''	—	3.70 *	—	3.70 *	—
Ester moiety					
H-β	6.49 (d, 16.0)	6.09 (d, 16.0)	6.09 (d, 16.0)	6.10 (d, 16.0)	6.10 (d, 16.0)
γ	7.55 (d, 16.0)	7.30 (d, 16.0)	7.30 (d, 16.0)	7.31 (d, 16.0)	7.31 (d, 16.0)
2,6	6.99 (s)	6.23 (s)	6.23 (s)	6.25 (s)	6.25 (s)
OMe	3.77 (s)x2	3.59 (s)x2	3.59 (s)x2	3.59 (s)x2	3.60 (s)x2
H-β'	6.44 (d, 16.0)	—	—	—	—
γ'	7.53 (d, 16.0)	—	—	—	—
2',6'	6.96 (s)	—	—	—	—
OMe'	3.78 (s)x2	—	—	—	—

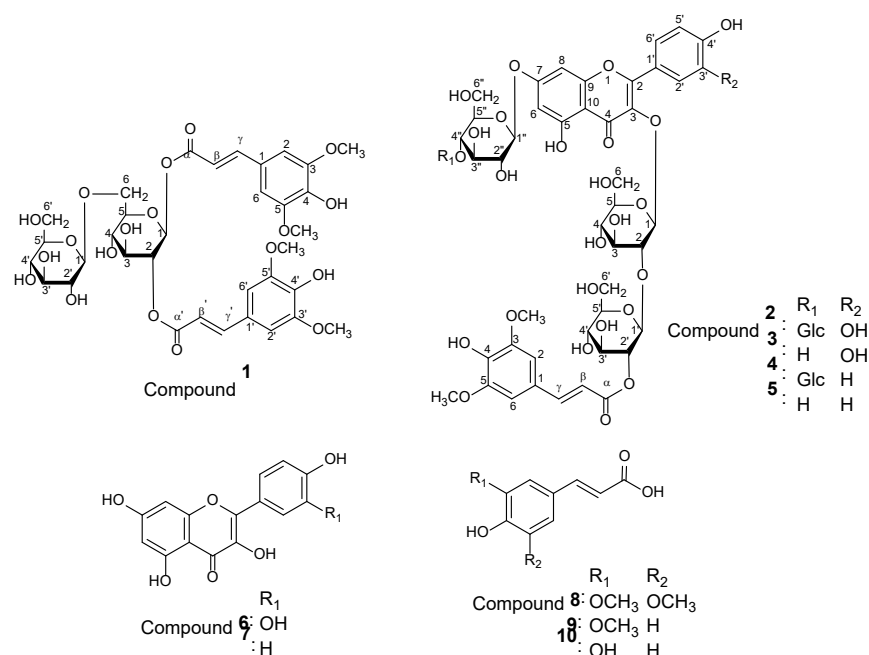
a: Measured in DMSO-*d*<sub>6</sub>. b: Measured in MeOH-*d*<sub>4</sub>. \*: Overlapping with other signals.

Based on HR-ESI-MS, compound **3** had the molecular formula C<sub>44</sub>H<sub>50</sub>O<sub>26</sub>, which was smaller by one glucosyl unit than that of compound **2** [*m/z*: 993.2518 [M-H]<sup>-</sup>]. <sup>13</sup>C- and <sup>1</sup>H-NMR spectroscopic data on the aglycone and 3-O-side chain were consistent with those of compound **2**. However, since the signals of only one β-D-glucopyranosyl group were observed in the 7-O-side chain, this chain consisted of one β-D-glucopyranose. Therefore, compound **3** was identified as quercetin 3-O-β-D-[2-O-3,5-dimethoxy-4-hydroxy-(*E*)-cinnamoyl]glycopyranosyl-(1→2)-β-D-glycopyranoside-7-O-β-D-glucopyranoside [12,13].

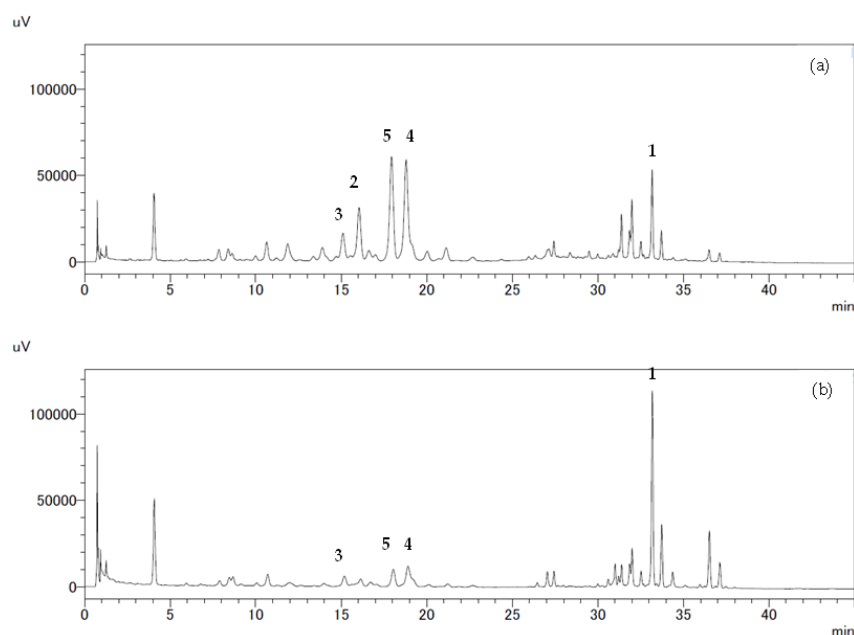
Compounds **4** and **5** were assigned the molecular formulae C<sub>50</sub>H<sub>60</sub>O<sub>30</sub> and C<sub>44</sub>H<sub>50</sub>O<sub>25</sub>, respectively, by HR-ESI-MS [compound **4**: *m/z*: 1139.3077 [M-H]<sup>-</sup>, compound **5**: *m/z*: 977.2565 [M-H]<sup>-</sup>]. The aglycones of compounds **4** and **5** were identified as kaempferol because AA'XX'-type aromatic proton signals derived from the B-ring were observed in each <sup>1</sup>H NMR spectrum [δ<sub>H</sub> 7.89 (1H, *d*, *J*=8.5 Hz)×2, 6.90 (1H, *d*, *J*=8.5 Hz)×2]. NMR spectroscopic data due to the sugar and ester moieties of compounds **4** and **5** were consistent with those of compounds **2** and **3**, respectively. Therefore, the structures of compounds **4** and **5** were elucidated, as shown in Figure 1 [12–15]. In addition to the sinapoyl group containing the compounds reported herein, some flavonoid glycosides containing *p*-cumaroyl, caffeoyl, or feruloyl groups in their molecules were obtained. Since Petit vert is an improved variety of *B*.



*oleracea*, similarities in its composition with that of *B. oleracea* were observed [12,14,15]. The HPLC analysis of Petit vert leaves and side shoots demonstrated that compounds 2–5 with flavonols were more abundant in the leaves than in the side shoots. On the other hand, compound 1, which contained only sinapic acid, was more abundant in the side shoots (Figure 2).



**Figure 1.** Structures of compounds 1–10.



**Figure 2.** HPLC Profiles of MeOH extracts from Petit vert leaf (a) and Petit vert side shoots (b). HPLC conditions [Column: Cosmosil 2.5Cholesterol (2.5  $\mu$ m), i.d. 3.0  $\times$  100 mm (NACALAI TESQUE, INC.); Mobile phase: A 0.1%TFA in water, B CH<sub>3</sub>CN, gradient, 0–20 min: 6–11% B linear, 20–36 min: 11–27% B linear, 36–45 min: 27% B ; Flow rate: 0.75 mL/min; Detection: UV 330 nm; Column temperature: 40°C].

3.2. Effects on Glucose Uptake in Cultured C2C12 Murine Myoblasts

We examined the effects of the following 10 compounds on glucose uptake in cultured C2C12 murine myoblasts: the five compounds isolated from the outer leaves of Petit vert (compounds **1–5**), quercetin, kaempferol (aglycone), and related acyl groups, including sinapic acid, ferulic acid, and caffeic acid. In a cytotoxicity assay, we measured the number of C2C12 cells after a 24-h incubation in differentiation medium supplemented with the test substances (final concentrations: 1, 10, and 100  $\mu$ M). As shown in Table 3, a significant change was observed in the viable cell rate (% of the control) in the presence of compounds **1–5**, quercetin, kaempferol, sinapic acid, ferulic acid, and caffeic acid at 1–100  $\mu$ M, with a significant decrease (20–30%) being noted for 100  $\mu$ M quercetin and kaempferol.

Table 3. Effect of various compounds in the cytotoxicity assay.

Test substance	Control	1	10 (μM)	100
Viable cell rate (% of control)				
<b>1</b>	100.0 ± 0.9	105.0 ± 1.6	101.4 ± 1.1	103.1 ± 0.7
<b>2</b>	100.0 ± 0.6	96.0 ± 0.9	94.9 ± 1.1	96.1 ± 1.0
<b>3</b>	100.0 ± 0.9	97.9 ± 0.4	98.9 ± 1.1	103.8 ± 1.1
<b>4</b>	100.0 ± 1.1	88.6 ± 0.8	93.5 ± 1.8	91.6 ± 0.7
<b>5</b>	100.0 ± 0.2	98.5 ± 2.5	102.0 ± 1.1	99.5 ± 0.8
Quercetin ( <b>6</b> )	100.0 ± 0.6	102.0 ± 0.4	98.0 ± 1.1	76.6 ± 1.2*
Kaempferol ( <b>7</b> )	100.0 ± 1.2	105.7 ± 2.5	111.1 ± 1.7	66.6 ± 1.0*
Sinapic acid ( <b>8</b> )	100.0 ± 1.4	110.0 ± 1.4	107.0 ± 0.9	119.1 ± 2.4
Ferulic acid ( <b>9</b> )	100.0 ± 0.6	102.4 ± 1.2	108.4 ± 1.3	111.5 ± 0.3
Caffeic acid ( <b>10</b> )	100.0 ± 0.1	107.0 ± 1.7	107.8 ± 1.6	101.0 ± 2.4

Each value represents the mean  $\pm$  S.E. for 3 determinations.

\* $P$ <0.05, significantly different from the control (0.1%DMSO).

As described in the “Materials and Methods” section, cultured C2C12 cells were treated with differentiation medium containing the test compounds (1, 10, and 100  $\mu$ M) in the presence or absence of insulin for 1 h, and glucose uptake levels in cells were measured. In comparison with the control, glucose uptake levels significantly increased in the absence of insulin when cells were cultured with compounds **1** (22.2–28.4%), **2** (30.9% at 1  $\mu$ M), **3** (20.1–30.7%), **4** (27.4–45.5%), and **5** (14.9–16.8%) as well as with kaempferol (35.0–44.5%), sinapic acid (27.3–35.8%), and ferulic acid (16.2–20.5%) at 1, 10, and 100  $\mu$ M (Table 4). A significant decrease was observed with compound **2**, quercetin, and kaempferol at 100  $\mu$ M. Caffeic acid did not significantly affect glucose uptake. Furthermore, glucose uptake in the presence of insulin was significantly increased by compounds **3** (1  $\mu$ M) and **4** (100  $\mu$ M), kaempferol (1, 10  $\mu$ M), and sinapic acid (1–100  $\mu$ M) (Table 5). A significant decrease in glucose uptake was noted with compounds **3** (100  $\mu$ M), **4** (1  $\mu$ M), and **5** (100  $\mu$ M), quercetin (1–100  $\mu$ M), and kaempferol (100  $\mu$ M). Ferulic acid and caffeic acid did not significantly affect glucose uptake.

Table 4. Effects of various compounds on glucose uptake by C2C12 skeletal muscle cells in the absence of insulin.

Test compounds	Control	1	10	100
			(μM)	
	Uptake (% of control)			

1	100.0 ± 2.8	128.4 ± 5.5**	125.2 ± 3.2***	122.1 ± 1.4***
2	100.0 ± 2.8	130.9 ± 8.0*	108.7 ± 4.8	88.3 ± 1.8*
3	100.0 ± 3.6	130.7 ± 6.9**	120.1 ± 1.9**	100.8 ± 3.7
4	100.0 ± 3.6	110.4 ± 5.6	127.4 ± 5.0**	145.5 ± 2.8***
5	100.0 ± 3.7	116.8 ± 3.5*	114.9 ± 2.5*	103.0 ± 4.7
Quercetin (6)	100.0 ± 3.7	104.1 ± 3.5	102.3 ± 2.8	25.5 ± 0.7***
Kaempferol (7)	100.0 ± 4.4	135.0 ± 2.4***	144.5 ± 9.5**	39.8 ± 1.4***
Sinapic acid (8)	100.0 ± 4.4	135.8 ± 6.8**	129.7 ± 4.9**	127.3 ± 3.7**
Ferulic acid (9)	100.0 ± 3.7	105.6 ± 2.4	120.5 ± 2.8**	116.2 ± 1.5**
Caffeic acid (10)	100.0 ± 3.7	129.9 ± 11.7	111.9 ± 5.0	108.6 ± 7.1

After treatment with various compounds at the indicated concentration in the absence of insulin, glucose uptake was measured using Promega Glucose Uptake-Glo™ Assay kit.  
Each value represents the mean ± S.E. for 5 determinations.  
\*\*\*  $P<0.001$ , \*\* $P<0.01$ , \* $P<0.05$ , significantly different from the control (0.1%DMSO).

**Table 5.** Effects of various compounds on glucose uptake by C2C12 skeletal muscle cells in the presence of insulin.

Test compounds	Control	1	10	100
	(μM)			
	Uptake (% of control)			
<b>1</b>	100.0 ± 8.4	96.3 ± 4.0	92.1 ± 2.9	81.8 ± 1.8
<b>2</b>	100.0 ± 8.4	84.4 ± 1.9	82.2 ± 3.9	83.8 ± 5.2
<b>3</b>	100.0 ± 3.1	110.6 ± 2.6*	120.5 ± 7.4	81.9 ± 4.6*
<b>4</b>	100.0 ± 3.1	74.2 ± 2.9***	90.5 ± 1.7	123.1 ± 9.4*
<b>5</b>	100.0 ± 3.5	114.5 ± 5.4	103.7 ± 5.7	81.1 ± 4.4*
Quercetin ( <b>6</b> )	100.0 ± 3.5	72.9 ± 5.5**	76.8 ± 3.7**	20.1 ± 1.8***
Kaempferol ( <b>7</b> )	100.0 ± 4.6	148.4 ± 6.8***	132.7 ± 2.3***	31.7 ± 1.0***
Sinapic acid ( <b>8</b> )	100.0 ± 4.6	116.5 ± 1.5*	119.3 ± 2.7*	123.0 ± 3.1**
Ferulic acid ( <b>9</b> )	100.0 ± 8.6	97.0 ± 2.0	118.7 ± 7.3	94.9 ± 2.2
Caffeic acid ( <b>10</b> )	100.0 ± 8.6	94.3 ± 1.9	101.7 ± 5.6	99.5 ± 0.7

After treatment with various compounds at the indicated concentration in the presence of insulin, glucose uptake was measured using Promega Glucose Uptake-Glo™ Assay kit.  
Each value represents the mean ± S.E. for 5 determinations.  
\*\*\*  $P<0.001$ , \*\* $P<0.01$ , \* $P<0.05$ , significantly different from the control (0.1%DMSO).

4. Discussion

In the present study, a Petit vert extract was prepared by extracting its dried and powdered outer leaves with MeOH. The extract was subsequently analyzed by HPLC. We identified several peaks that appeared to correspond to polyphenols with absorption at approximately UV 330 nm. The dried and powdered outer leaves of Petit vert were extracted, fractionated, and purified to isolate five compounds (compounds 1–5). NMR spectroscopy was performed for each of the components to elucidate their structures. Compounds 2–5 isolated from Petit vert leaves were glycosides that contained either kaempferol or quercetin with a sinapoyl group. Compound 1 was previously isolated from broccoli florets [11], compounds 2, 3, and 5 from kale leaves [12], and compounds 4 and 5 from cabbage leaves [14,15]. Compounds 1–5 all contained sinapic acid, which is an acyl group. The

HPLC analysis of Petit vert leaves and side shoots revealed that compounds 2–5 with flavonols were more abundant in the leaves than in the side shoots. On the other hand, compound 1, which contained only sinapic acid, was more abundant in the side shoots.

We investigated the effects of the following 10 compounds on glucose uptake in cultured C2C12 murine myoblasts: five compounds isolated from the outer leaves of Petit vert (compounds 1–5), quercetin, kaempferol, and related acyl groups, including sinapic acid, ferulic acid, and caffeic acid. Glucose uptake in cultured C2C12 murine myoblasts in the absence of insulin was significantly increased by compounds 1–5, kaempferol, sinapic acid, and ferulic acid (Table 4), while that in the presence of insulin was also significantly increased by compounds 3 and 4, kaempferol, and sinapic acid (Table 5). The significant decrease detected in glucose uptake at higher concentrations of quercetin and kaempferol may have been due to a reduction in the viable cell rate (Table 3). The five compounds isolated from the outer leaves of petit vert as well as quercetin, kaempferol, and sinapic acid increased glucose uptake in cultured C2C12 murine myoblasts. These results indicate that the biological activities of the outer leaves of Petit vert, such as increases in glucose uptake, were the summed effects of the phenolic compounds present.

Phenolic acids and their derivatives constitute one of the most common groups of phenolic compounds in plants. These molecules have emerged as high value added products because of their essential role in human nutrition and health [16,17]. Previous studies reported the effects of phenolic compounds, such as sinapic acid, related to those isolated from Petit vert leaves. For example, kaempferol was shown to exert anti-obesity effects because it increased insulin secretion in beta cells [18]. Sinapic acid has attracted increasing attention due to its numerous pharmacological effects [19]. It has been shown to exhibit antihyperglycemic [20], antioxidant [21], anti-inflammatory [22], anti-cancer [23], hepatoprotective [24], cardioprotective [25], renoprotective [26], neuroprotective [9], anti-diabetic [27], and anti-bacterial activities [28].

## 5. Conclusions

The present study reports for the first time the isolation of five compounds containing sinapic acid from the outer leaves of Petit vert and their stimulation of glucose uptake in cultured C2C12 murine myoblasts. The results obtained herein suggest the potential of these compounds to effectively attenuate hyperglycemia and maintain muscle strength by promoting glucose metabolism in muscle cells.

**Authorship Contributions:** Shizuo Yamada: Writing-original draft, Supervision, Investigation, Tsutomu Warashina: Investigation, Data curation, Formal analysis. Osamu Shiota: Investigation, Data curation, Formal analysis. Yoshihisa Kato: Writing draft, Formal analysis. Toshiyuki Fukuda, Investigation, Writing-review & editing, Supervision, Funding acquisition, Conceptualization.

**Declaration of Interest:** The authors declare no competing financial interest or personal relationships that could have appeared to influence the work reported in this paper.

**Data availability Statement:** No data was used for the research described in the article.

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