Identification of functional compounds from aronia juice and their mechanism to improve type 2 diabetes and obesity

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Identification of functional compounds from aronia juice and their mechanism to improve type 2 diabetes and obesity

アロニア果汁からの 2 型糖尿病および肥満を改善する機能性成分の同定とそのメカニズム

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Abstract

Aronia contains many polyphenols including cyanidin-3,5-diglucoside, and these polyphenols have beneficial effects on lifestyle-related diseases such as type 2 diabetes and obesity. Previous studies showed that dipeptidyl peptidase IV (DPP IV) activity was inhibited by cyanidin 3,5-diglucoside from aronia juice. The aim of this study is to investigate the effects of cyanidin 3,5-diglucoside, including aronia juice, on type-2 diabetes through inhibition of DPP IV activity *in vivo*. DPP IV inhibitory activity and increased active GLP-1 level in serum from KKA^{y} mice given cyanidin 3,5-diglucoside were about 50% of those in serum from KKA^{y} mice given aronia juice. This indicated that other DPP IV inhibitors exist in aronia juice. To identify anti-diabetic compounds other than cyanidin 3,5-diglucoside in aronia juice containing association with islet cells and insulin resistance, polyphenols in aronia juice were first separated using flash chromatography on a reversed-phase column. The fraction with α-glucosidase inhibitory activity was further separated using reversed-phase HPLC. Two peak fractions showed inhibitory activity. LC-MS/MS analysis of the fractions indicated that they contained 3-caffeoylquinic acid (3-CQA) and 4-caffeoylquinic acid (4-CQA), respectively. Both CQAs also inhibited DPP IV activity. The results suggest that caffeoylquinic acids in aronia juice are important for the amelioration of type 2 diabetes by the juice. The effects of aronia juice on the white adipose tissues in normal and obesity model mice suggested that aronia juice inhibits lipid accumulation in white adipose tissues by suppressing Arrdc3 mRNA expression in adipocyte.

Keywords: Aronia, DPP IV, HbA1c, α-Glucosidase, Cyanidin 3,5-diglucoside, Caffeoylquinic acids, Arrdc3

I. Introduction

1. *Aronia melanocarpa*

Aronia berries (*Aronia melanocarpa*) have been used in traditional medicine to treat hypertension and atherosclerosis in Eastern European countries[1]. Aronia berries have high contents of phenolic phytochemicals[2,3]. The concentrations of active compounds in aronia berries, including anthocyanins, procyanidins and flavonoids, are over five-fold higher than those in cranberries[4,5]. As Durazzo et al. recently reviewed[6], evidence supporting an association between polyphenol intake and the incidence of human chronic disease has been accumulated. For example, anthocyanin intake is suggested to retard the progression of type-2 diabetes mellitus and to reduce mortality risk of cardiovascular disease. As for aronia berries, recent mice studies have shown that aronia berries have beneficial effects on lifestyle-related diseases such as type-2 diabetes[7-13], hypertension[14], hyperlipidemia[15] and hypercholesterolemia[16]. In human study, aronia juice potently modulated hyperglycemia-related oxidative stress in a beneficial manner[17], and it is also shown that aronia supplementation may lead to an increase in high-density lipoprotein and concomitant reduction in total cholesterol and low-density lipoprotein[18] . Prevention of the onset of lifestyle disease is an important challenge and nutraceuticals play essential roles in proactive medical approach[19]. Nutraceuticals are proposed to have a pharmacological effect in addition to their nutritional value. Importantly, their beneficial health properties must be clinically prove[20]. Aronia juice and the extracts from the juice have a potential to provide nutraceuticals because their various beneficial effects have been shown in animal models.

2. Anti-diabetic effect of Aronia

Type-2 diabetes has become a public health challenge for many countries[21]. Alpha-Glucosidase is an important intestinal enzyme that is responsible for carbohydrates digestion[22]. Inhibitors of this enzyme reduce intestinal glucose absorption to decrease postprandial blood glucose levels. Dipeptidyl peptidase IV (DPP IV) inactivates intestinal peptide glucagon-like peptide 1 (GLP-1) and gastric inhibitory peptide (GIP) [23,24]. Because these peptides stimulate pancreatic insulin secretion after eating, DPP IV

inhibitors enhance the effect of GLP-1 and GIP through retardation of their degradation by DPP IV. Various drugs used for treatment of diabetes mellitus and their action mechanisms are reviewed by Vieira et al[25]. Reduction of glycated hemoglobin (HbA1c) is accepted as a well validated surrogate manner for glycemic control and prevention of microvascular complications[26]. HbA1c levels have become the standard outcome measure in many trials for various diabetes therapies[27].

Aronia berries and their derivatives have an anti-diabetic effect. In an animal model study, hyperglycemia was attenuated in diabetic rats given aronia juice[11]. Fasting blood glucose level was reduced in animals fed aronia extract[9]. Levels of glycemia were significantly decreased in rats with alloxan-induced diabetes that were given aronia juice[8]. Glucose tolerance was improved in rats fed a high-fat diet with aronia juice[7]. In a human intervention study, the fasting glucose level was reduced by aronia juice in patients with non-insulin-dependent diabetes[10]. The postphedidetal blood glucose level was also decreased by drinking aronia juice before meals[13]. Although those studies showed that aronia is very useful for lowering blood glucose level and improving diabetes, the major mechanisms have not been clarified. Cyanidin-3,5-diglucoside, cyanidin 3-o-glucoside, quercetin and cyanidin have been identified as DPP IV inhibitors in aronia juice[28-30]. DPP IV (EC 3.4.14.5) is an exopeptidase[31] and degrades incretins as do glucagon-like peptide 1 (GLP-1) and insulinotropic peptide (GIP). Incretins stimulate insulin secretion from the pancreas to reduce the blood glucose level[32-35]. Therefore, blood glucose level is reduced by DPP IV inhibitors. In this study, to clarify the effect of cyanidin 3,5-diglucoside identified as a DPP IV inhibitor present in aronia juice in our previous study on reduction of blood glucose level, cyanidin 3,5-diglucoside was administered to KKA^y mice. These results indicated that inhibitors other than cyanidin 3,5-diglucoside exist in aronia juice.

On the other hand, α-glucosidase inhibitors have not been isolated from aronia juice. Therefore, we identified anti-diabetic compounds other than cyanidin 3,5-diglucoside in aronia juice and to examine therapeutic effects of aronia juice on type-2 diabetes, including association with islet cells and insulin resistance.

3. Anti-obesity effect of Aronia

Obesity is a serious risk factor in health, and it is caused by a high-fat diet[36]. In obesity, insulin resistance and damage of blood vessels increase, leading to lifestyle-related complications such as type-2 diabetes and cardiovascular diseases[37]. Prevention of obesity is important for life-long health.

Aronia juice has been shown to have beneficial effects on total cholesterol and lipid levels[10,12]. White adipose tissue volumes of rodents increase when they are fed a high-fructose and/or high-fat diet. Importantly, daily drinking of aronia juice attenuates accumulation of fat in these animal models[9,38]. Alpha-Arrestin domain-containing 3 (Arrdc3) was found to prevent β-adrenergic receptor recycling, resulting in decreased responsiveness to adrenergic stimulation[39]. Previously, we showed that Arrdc3 expression increased in the liver of mice fed with a high-fat diet, and that the increase in Arrdc3 mRNA was inhibited by feeding mice aronia berries together with the high-fat diet[15]. Recently, Arrdc3 has been shown to be a regulator of obesity[40]. In white adipose tissue of Arrdc3-null mice, uncoupling protein 1 (Ucp1) expression, cAMP concentration and lipolysis increase[41]. In cancer cells, it has been shown that Arrdc3 expression is suppressed through epigenetic silencing, in which SIRT2, a member of class III histone deacetylases, plays a major role[42].

The aim of this study is to find lipid accumulation inhibitors of aronia juice and determine their inhibition mechanisms. We examined mechanistically the effects of aronia juice on the white adipose tissues in normal and obesity model mice.

II. Examination of anti-diabetic effect of aronia

1. Summary

The aim of this study is to investigate the effects of cyanidin 3,5-diglucoside on type-2 diabetes through inhibition of DPP IV activity *in vivo*. Type-2 diabetes and obesity model KKA^y mice were divided into three groups containing five mice in each group: a control group that was given water, an aronia group that was given aronia juice, and a cy-dg group that was given cyanidin 3,5-diglucoside solution. Although increased blood glucose and hemoglobin A1c (HbA1c) levels were reduced in KKA^y mice administered cyanidin 3,5-diglucoside, the magnitudes of reduction in blood glucose and HbA1c by administration of cyanidin 3,5-diglucoside were about 50% of those by giving aronia juice in KKA^y mice. In a further study, to identify anti-diabetic compounds other than cyanidin 3,5-diglucoside in aronia juice and to examine therapeutic effects of aronia juice on type-2 diabetes, polyphenols in aronia juice were first separated using flash chromatography on a reversed-phase column. The fraction with α-glucosidase inhibitory activity was further separated using reversed-phase HPLC. Two peak fractions showed inhibitory activity. LC-MS/MS analysis of the fractions indicated that they contained 3- and 4-caffeoylquinic acid (CQA), respectively. Both CQAs also inhibited DPP IV activity. The results suggest that CQAs in aronia juice are important for the amelioration of type 2 diabetes by the juice.

2. Materials and methods

1) Materials

Aronia juice was prepared from fresh aronia berries using a press and immediately bottled in Bulgaria. The bottled juice was obtained from Nakagaki Consulting Engineer (Osaka, Japan). 4-Caffeoylquinic acid and 5-caffeoylquinic acid were purchased from Cayman (Michigan, USA). 3-Caffeoylquinic acid was purchased from Nakarai Tesq (Kyoto, Japan). Cyanidin 3,5-diglucoside, and cyanidin 3-O-glucoside were purchased from EXTRASYNTHESE (Lyon, France). Alpha-Glucosidase and pnitrophenyl-α-D-glucopyranoside (PNP-glycoside) were purchased from Sigma-Aldrich (MO, USA). Glycyl-L-proline 4-methylcoumaryl-7-amid (Gly-Pro-MCA) and Gly-Pro-MCA kits

were purchased from Peptide Institute (Osaka, Japan). GLP-1 ELISA kits were purchased from IBL (Gunma, Japan). DPP IV was purified from porcine seminal plasma (Ohkubo, Huang, Ochiai, Takagaki, & Kani, 1994). Cyanidin 3-O-garactoside and cyanidin 3-O-arabinoside were purchased from TOKIWA (Chiba, Japan). HbA1c cartridges and strips for blood glucose levels were provided by SIEMENS (Tokyo, Japan) and Nova Biomedical (Massachusetts, USA), respectively. All other chemicals were of analytical grade and purchased from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan).

2) Animals

KKA^{y} male mice were obtained at 4 weeks of age from CLEA Japan (Tokyo, Japan). All mice were fed a normal diet (CE2 diet, CLEA Japan, Tokyo, Japan). After 4 days, KKA^{y} mice were divided into three groups of five mice in each group: a control group that was given water, an aronia group that was given 100% aronia juice, and a cy-dg group that was given 10 μg/mL solution of cyanidin 3,5-diglucoside (99.7% purity) prepared with water. Aronia juice and cyanidin 3,5-diglucoside solution were given by free intake. At 49 days after starting the diets, the mice were sacrificed by isoflurane anesthesia. Serum, liver, small intestine, kidney and adipose tissues were isolated and weighed.

3) Blood glucose and HbA1c levels

Blood glucose levels were measured using a small blood glucose measurement apparatus, Xpress 900 (Nova Biomedical, Massachusetts, USA). HbA1c levels were measured using DCA Vantage Analyzer (SIEMENS, Tokyo, Japan). Blood was collected from the tail vein.

4) Inhibition of DPP IV activity in mice

Enzyme activity was measured by fluorometrical determination (excitation, 380 nm; emission, 440 nm) of the liberation of 7-amino 4- methylcoumarin at 37 °C in a mixture containing 10 μL of 10 mM substrate Gly-Pro-4-methyl-coumarinamide, 100 μL of 0.5 M Tris - HCl (pH 9.0), 5 μL of enzyme solution and Milli Q water (18 mΩ) in a total volume of 1 mL. After incubation for 30 min, 2 ml of 0.2 M acetic acid was added to the

mixture to terminate the reaction.

5) Enzyme immunoassay for GLP-1 and insulin

The blood level of GLP-1 was measured using a mammalian active GLP-1 measurement kit (IBL, Gunma, Japan).

6) Analysis of liver triglyceride content

Liver triglyceride content was measured as described previously[43]. Briefly, the liver tissue (50 mg) was homogenized with 0.5 mL of 0.1 M acetic acid and 1.8 mL of methanol-chloroform (2:1, v/v) was added. After centrifugation, the lower chloroform layer was collected, and then the residues were washed with 0.5 mL of chloroform. The combined chloroform layer was dried in air. The pellet was dissolved in 0.5 mL of 2-propanol containing 10% Triton X-100. Triglyceride concentrations were measured using Wako Triglyceride E-test (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan).

7) Extraction of anthocyanin from aronia juice

Extraction of anthocyanin was carried out according to the method described by Vieira et al.[44] with some modifications. One g of Wakogel 50C18 (FUJIFILM Wako Pure Chemical Corporation) was put into a small glass column (18 × 144 mm) and washed in the order with 5mL of ethyl acetate, methanol and water, respectively. Five mL of aronia juice was loaded into this pre-conditioned column, and the column was washed with about 5 ml of water and then with about 10 mL of ethyl acetate. The bound anthocyanins were eluted with 90% methanol in water containing 0.1% formic acid. The fraction with 300 μL of aliquote in 1.5 mL tubes was concentrated to near-dryness under vacuum using a centrifugal evaporator. The sample in the tube was redissolved in 300 μL of the equilibration solvent used for HPLC analysis.

8) HPLC analysis of anthocyanin

HPLC analysis of anthocyanins was carried out according to the method described by Gouvêa et al.[45] with some modifications. A Shimadzu HPLC system was used to perform

the chromatographic analysis, and separation was performed on a Cosmosil 3C18-EB column (2.0 mm ID × 150 mm, Nacalai tesque). Solvent A contained 5% formic acid in water, and solvent B contained 5% formic acid, 90% methanol and 5% water. The column was equilibrated with 10%B. A fifty-μL aliquot of the sample and calibration standard solutions (1.25 - 10 μmol/L in 10%B) were loaded onto a 40 μL sample loop. Anthocyanins were eluted using the following time program: 0 - 10 min, 10%B; 10 - 50 min, linear increase in methanol from 10%B to 50%B; and 51 - 59 min, 90%B. The flow rate was 100 μL/min, and the column temperature was at room temperature. The elution of anthocyanins was monitored by measuring absorbance at 520 nm. The anthocyanin contents of aronia juice were shown in Table 1.

Table 1 Anthocyanins content in aronia juice

Anthocyanin	nmol/ mL, juice
Cyanidin 3, 5-diglucoside	0.234 ± 0.013
Cyanidin 3-O-galactoside	2.70 ± 0.05
Cyanidin 3-O-glucoside	0.262 ± 0.039
Cyanidin 3-O-arabinoside	2.79 ± 0.16

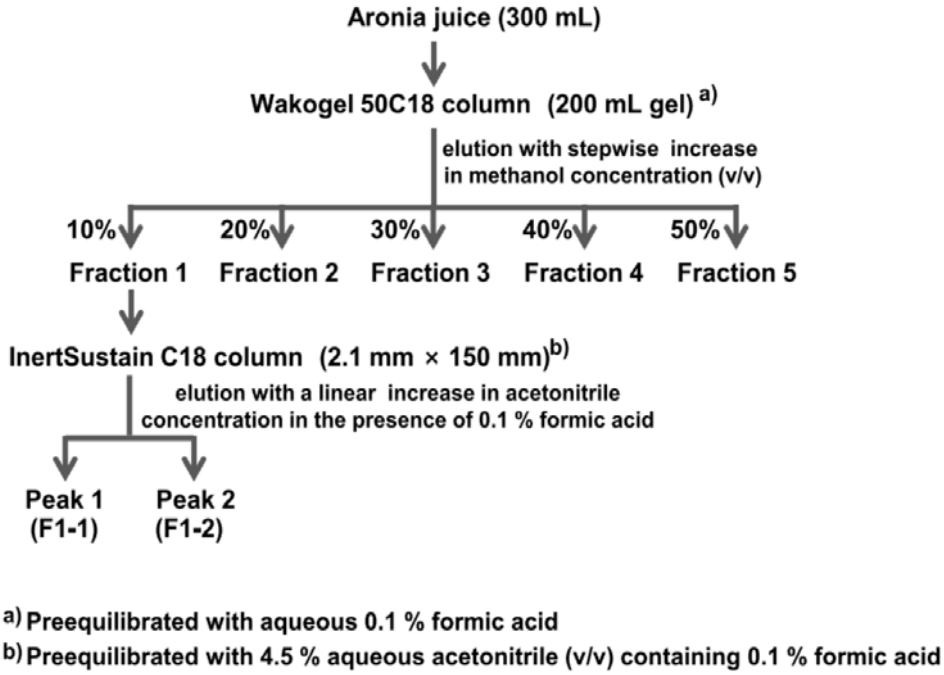
All data is presented as mean ± standard error of five animals.

9) Purification of α-glucosidase inhibitors

Scheme 1 shows the procedure of purification of α-glucosidase inhibitors from aronia juice. Aronia juice (300 mL) was directly applied to a Wakogel 50C18 column (FUJIFILM Wako, 200 mL bed volume) preequilibrated with 0.1% aqueous formic acid (solvent A). The column was washed extensively with solvent A, and then the adsorbates were eluted from the column by a stepwise increase in the methanol concentration (methanol: solvent A = 10, 20, 30, 40 and 50% (v/v)). The eluates were collected and evaporated to dryness (fractions 1 - 5). Each dried fraction was weighed and resolved with 12.5% or 25% aqueous methanol containing 0.1% formic acid to a final concentration of 1 - 10 mg/ mL.

The most active fraction (fraction 1) was further purified using an InertSustain C18 column (2.1 mm × 150 mm, GL Science, Tokyo, Japan). The mobile phase consisted of

solvent A and 90% acetonitrile (10% water) containing 0.1% formic acid (solvent B). The column was developed at the flow rate of 150 μL/min with the following gradient: 0 - 5 min with 5% B, 5 - 15 min with 5 - 30% B, 10–20 min with 30 - 60% B, and 20 - 25 min with 60 - 90% B. The absorbance at 370 nm was monitored and the two major peaks (F1-1 and F1-2) were collected manually.



Scheme 1 Purification of α-glucosidase inhibitors from aronia juice

10) α-Glucosidase assay

Alpha-Glucosidase activity was measured using *p*-nitrophenyl-α-D-glucopyranoside (PNP-glycoside, Sigma-Aldrich, St. Louis, MO, USA) as a substrate. The substrate stock solution (20 mmol/L PNP-glycoside) was prepared with dimethyl sulfoxide. The reaction mixture (300 μL) contained 667 μmol/ L PNP-glycoside, 50 mmol/L sodium phosphate (pH 7.0), and 10 μL of enzyme solution. The reaction was started by the addition of the enzyme solution. After incubation at 37°C for 30 min, enzymatically released *p*-nitrophenol was measured absorbance at 405 nm using a microplate reader (SH-1000 Lab, Corona Electric).

11) DPP IV assay of fraction 1 and caffeoylquinic acids

DPP IV activity was measured using Gly-Pro-MCA as substrate. The reaction mixture (990 μL) consisted with 100 μL of 50 mmol/L Tris-HCl (pH 9.0) and 10 μL of 100 μmol/L Gly-Pro-MCA and 5 μL of enzyme solution was applied. The reaction was started by the addition of 10 μL of the substrate stock solution (10 mmol/L). After incubation at 37°C for 30 min, the reaction was stopped by the addition of 2 mL of 0.2 mol/L acetic acid. The enzymatically released 7-amino 4-methylcoumarin was measured fluorometrically (excitation at 380 nm, emission at 440 nm) using a fluorescent spectrophotometer (F-2500, Hitachi).

12) Liquid chromatography-mass spectrometry (LC-MS)

An aliquot of the peak fractions of F1-1 and F1-2 (5 μL) was injected into an InertSustain C18 column (0.3 mm × 150 mm, GL Science). The mobile phase consisted of 0.1% aqueous formic acid (solvent A) and 90% acetonitrile (10% water) containing 0.1% formic acid (solvent B). The column was preequilibrated with 20%B. The column was developed at the flow rate of 3.0 μL/min with the following gradient: 0 - 5 min with 20%B, 5 - 30 min with 20 - 70%B, and 30 - 35 min with 70 - 90%B. The column temperature was controlled at room temperature. The eluate was equally split into two fused silica capillary tubes (20 μm in internal diameter) and one of them was connected to an electrospray ionization tip (MonoSpray, GL Science). The spray voltage was 2.5 kV and the temperature of the transfer tube was 150°C. A mass spectrum of the eluate was recorded between m/z 150 and 1000 in the positive ion mode. The ion peaks with ion intensity of more than 1000 were data-dependently subjected to MS/MS measurement. A syringe-type HPLC pump (HP 711V Micro-Flow Pump, GL Science) and an ion-trap mass spectrometer (LCQ Fleet, Thermo Fisher Scientific, MA, USA) were used.

13) Statistical analysis

Data are expressed as means ± S.E. of at least three replicates for each sample. Statistical analyses were performed using Statcel4 software (OMS, Tokyo, Japan). The difference between two groups was evaluated using unpaired Student's t-test. For

comparison of multiple samples, the Tukey-Kramer test was used.

14) Ethics statement

All animal experiments were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and the protocols were approved by the Committee for Animal Research at Osaka Prefecture University (permit number: 30–89).

3. Results

1) Reduction of blood glucose and HbA1c levels by cyanidin 3,5- diglucoside

To examine the effects of cyanidin 3,5-diglucoside on blood glucose and HbA1c levels, a solution containing water, aronia juice and cyanidin 3,5-diglucoside was administered orally to mice in the control group, aronia group and cy-dg group respectively. As shown in Fig. 1A, blood glucose levels in the aronia group and cy-dg group were about 39% and 60%, respectively, of that in the control group at 21 days after the start of administration. At forty-nine days, blood glucose levels in the aronia group and cy-dg group were about 58% and 95%, respectively, of that in control group. The blood glucose levels were increased in the cy-dg group at 24 days and in the aronia group at 35 days after the start of administration. HbA1c levels in the aronia group and cy-dg group were about 67% and 84%, respectively, of that in control group at 21 days after the start of administration. HbA1c levels in the aronia group and cy-dg group were about 56% and 77%, respectively, of that in the control group at 49 days after the start of administration (Fig. 1B).

2) Inhibition of DPP IV activity in KKA^{y} mice fed cyanidin 3,5-diglucoside

Forty-nine days after the start of administration, their DPP IV activities were measured. As shown in Fig. 2, serum DPP IV activity in the control group was significantly higher than that in the cy-dg and aronia groups. Inhibition of DPP IV activity was observed in serum of KKA^{y} mice given aronia juice and cyanidin 3,5-diglucoside, and their activities were about 38% and 74%, respectively, of those in the control group.

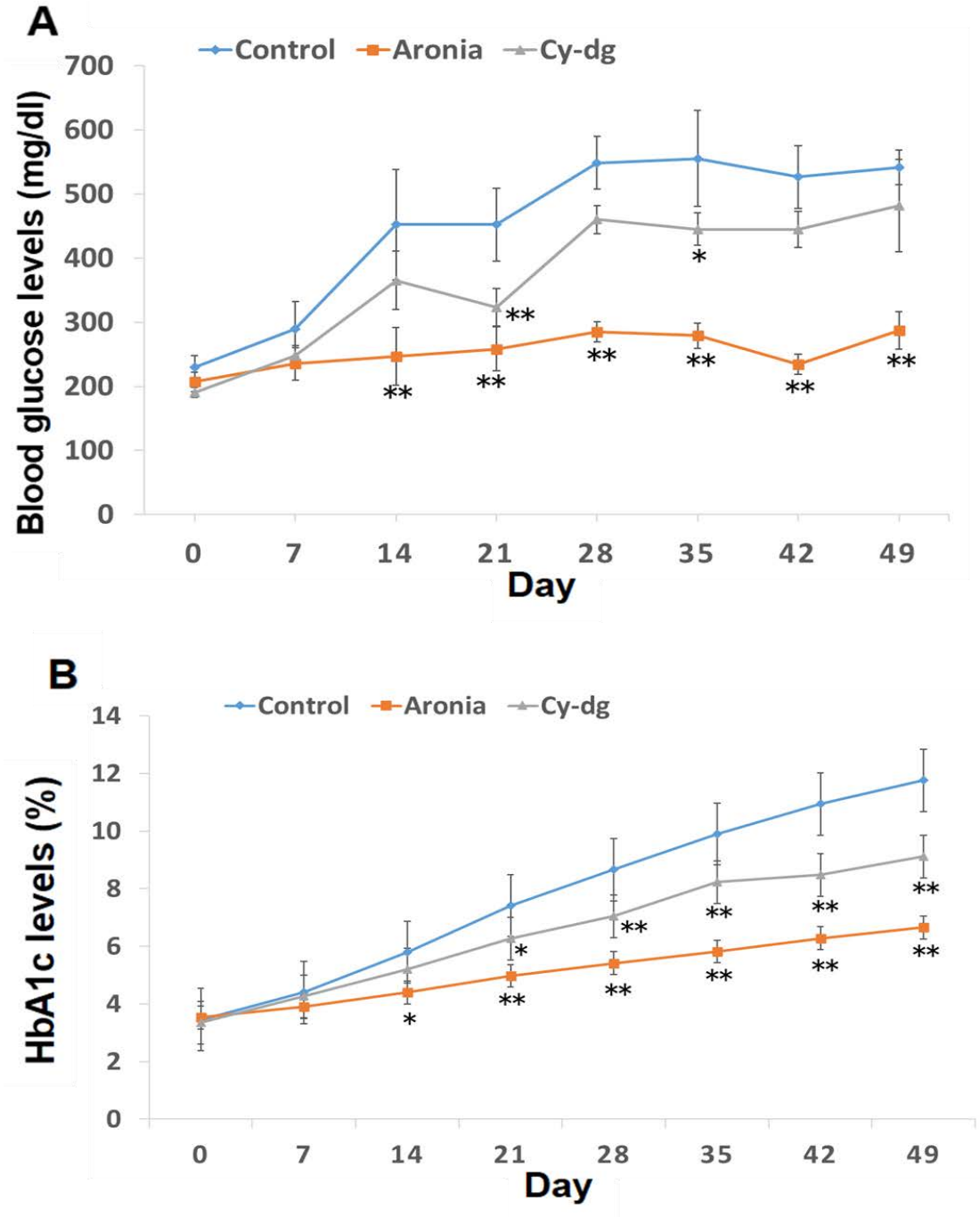


Fig. 1 Differences in blood glucose and HbA1c levels between control and cyanidin-3,5-diglucoside or aronia juice-administered mice. Aronia juice was used as a positive control against cyanidin-3,5-diglucoside. Blood glucose (A) and HbA1c (B) levels in mice were measured every 3 or 4 days for 49 days. There were significant differences between control mice and mice fed cyanidin-3,5-diglucoside or aronia juice. *$p < 0.05$, **$p < 0.01$, $n = 5$.

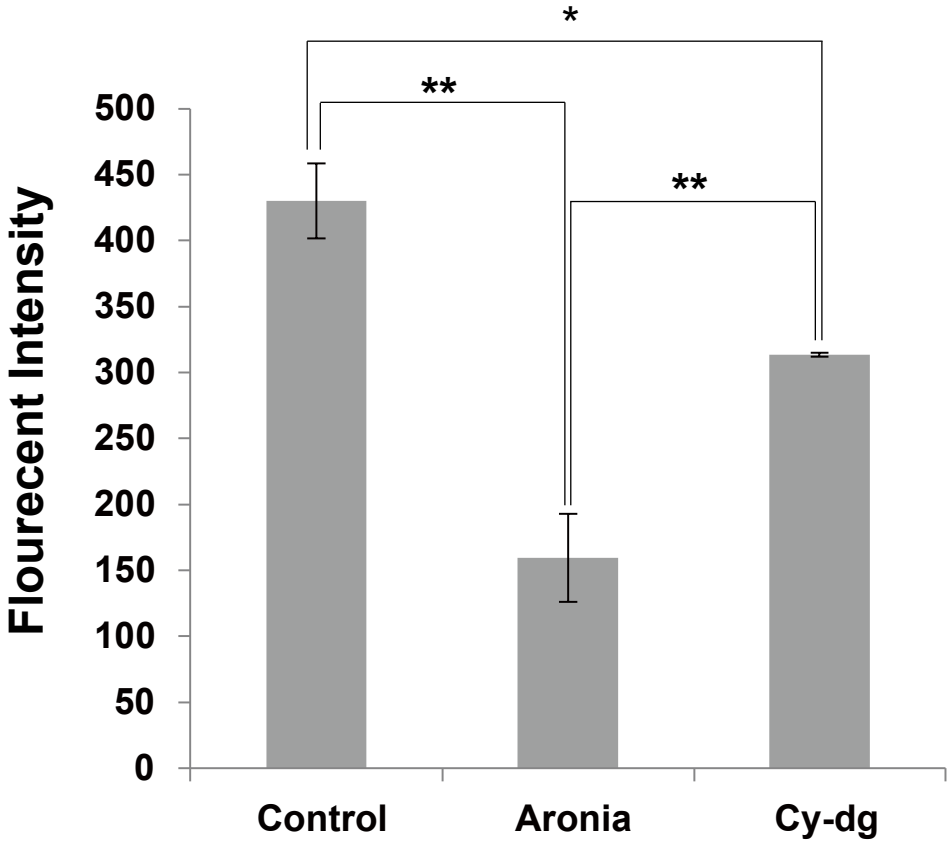


Fig. 2 DPP IV activities in the serum. Water or aronia juice or cyanidin-3,5-diglucoside was administered orally to mice. After 49 days, serum was obtained, and their DPP IV activities were measured. *p < 0.05, **p < 0.01, n = 5.

3) Active GLP-level in serum

Forty-nine days after the start of administration, active GLP-1 levels were measured. As shown in Fig. 3, serum GLP-1 levels in the cy-dg and aronia groups were significantly increased compared to that in the control group. Increased active GLP-1 levels in the serum from KKA^{y} mice given aronia juice and cyanidin 3,5-diglucoside were about 10-fold and 6.4-fold of those in the control group, respectively.

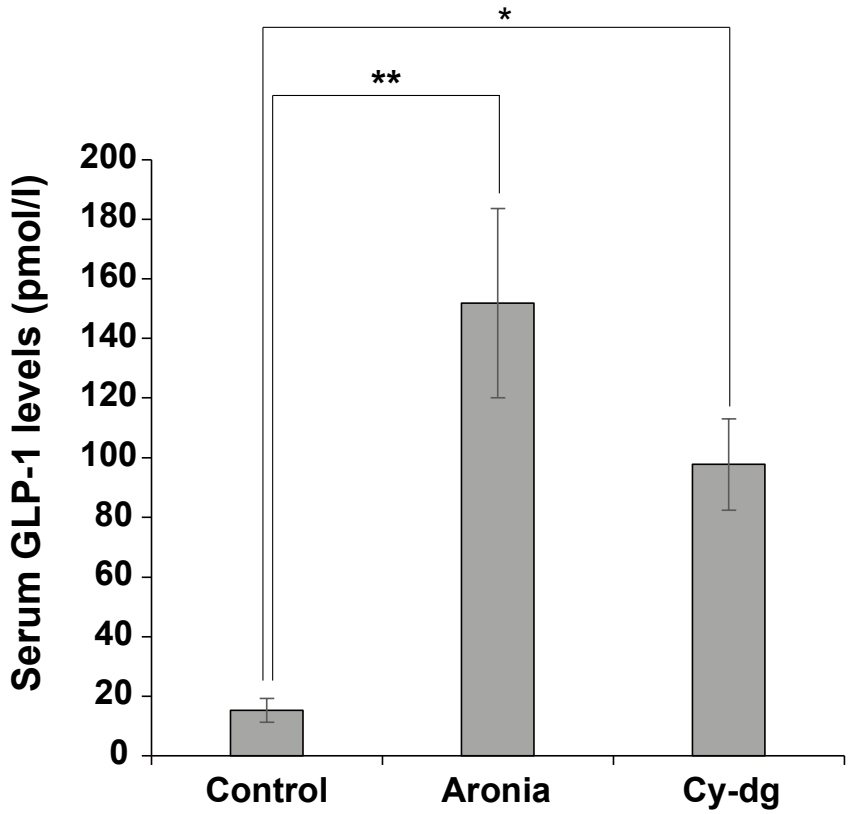


Fig. 3 Active GLP-1 levels in the serum. Protein level of active GLP-1 was examined by ELISA in the serum. *$p < 0.05$, **$p < 0.01$, $n = 5$.

4) Weights of livers and white adipose tissues from KKA^y mice administered cyanidin 3,5-diglucoside

Forty-nine days after the start of administration, livers and white adipose tissues were extracted from the mice and weighed. As shown in Fig. 4, weights of livers and white adipose tissues from KKA^y mice given aronia juice but not cyanidin 3,5-diglucoside were significantly decreased compared to that in the control group.

5) Lipid accumulation in the livers from KKA^y mice fed cyanidin 3,5- diglucoside

Forty-nine days after the start of administration, triglyceride levels in the livers were measured. As shown in Fig. 5, triglyceride levels in the livers from KKA^y mice given aronia juice and cyanidin 3,5-diglucoside were about 50% and 95% of those in the control group, respectively.

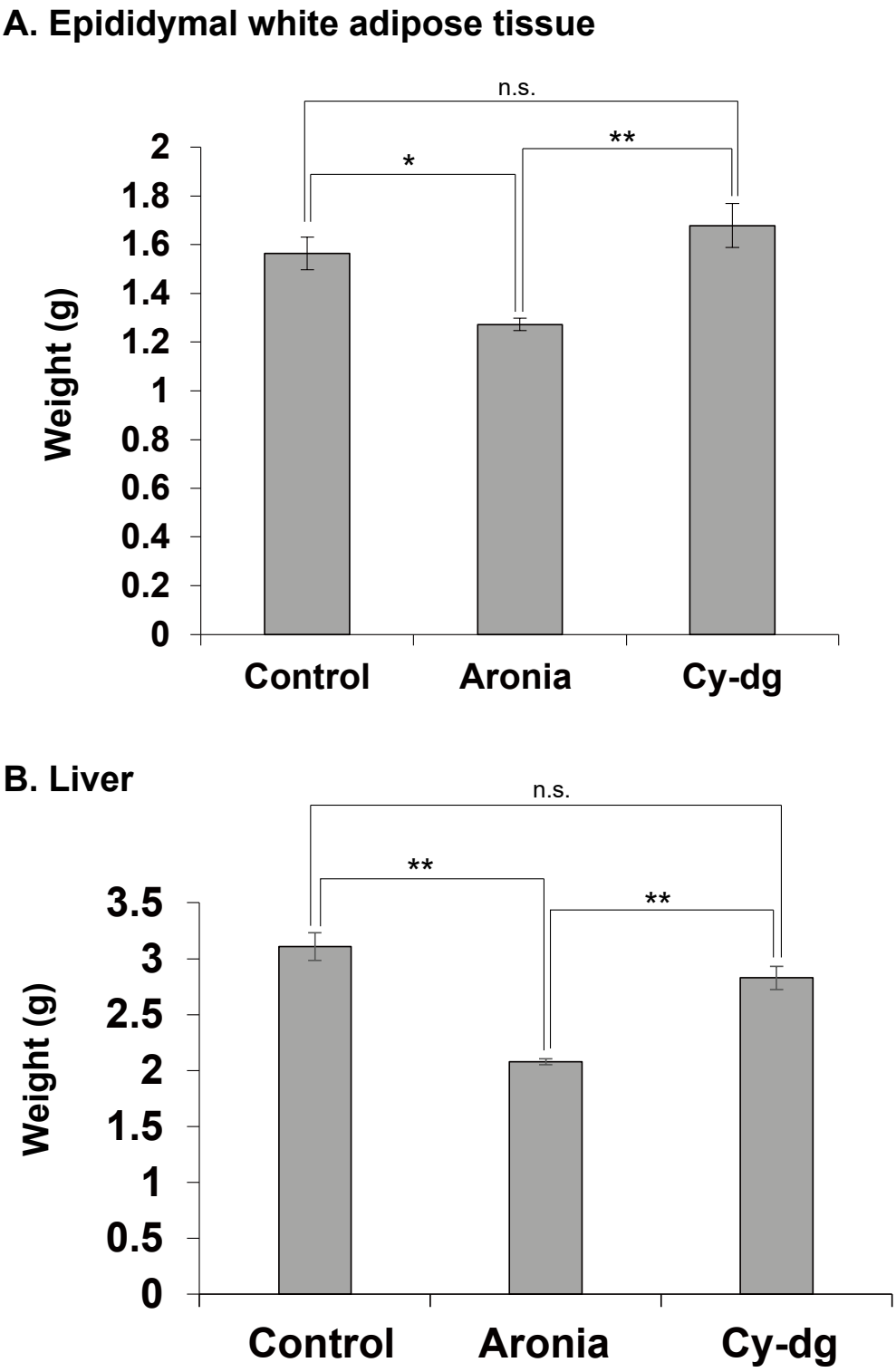


Fig. 4 Reduction of tissue weighs in KKA^{y} mice administered aronia juice but not cyanidin-3,5-diglucoside. Water, cyanidin-3,5-diglucoside or aronia juice was administered orally to mice. After 49 days, epididymal white adipose tissue (A) and the liver were obtained and weighed. *$p < 0.05$, n.s.: not significant. n = 5.

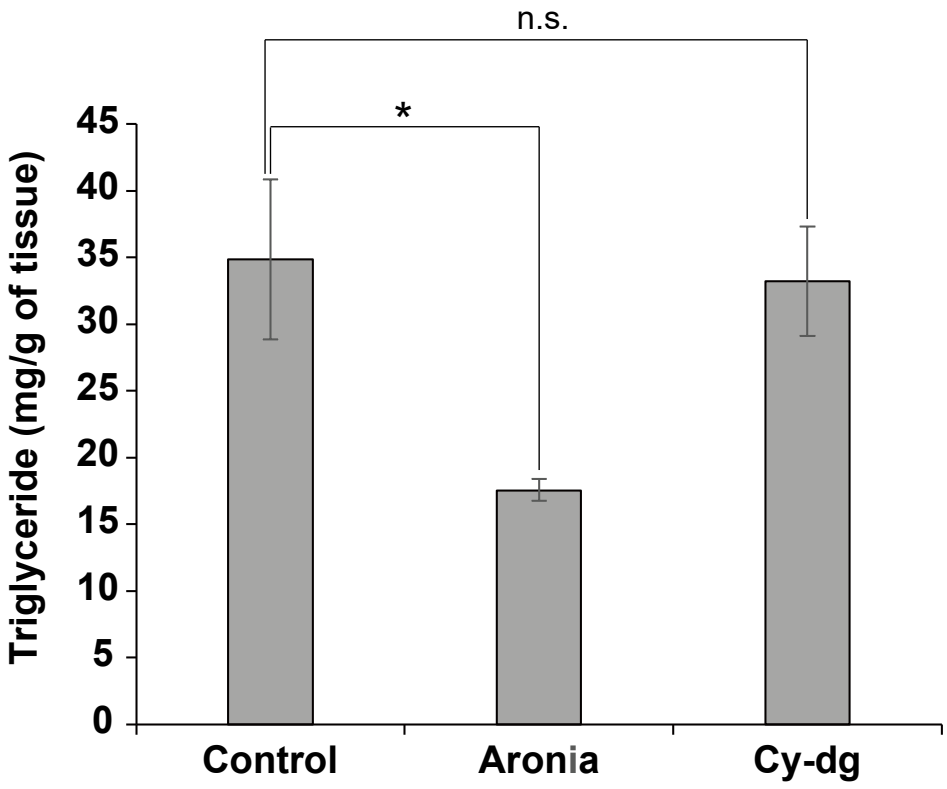


Fig. 5 Changes in the liver triglyceride levels in KKA^{y} mice administered aronia juice but not cyanidin-3,5-diglucoside. Triglyceride levels of the liver in control and aronia juice or cyanidin-3,5-diglucoside-administered mice were measured. *$p < 0.05$, n.s.: not significant. n = 5.

6) Purification of α-glucosidase inhibitors

Aronia juice was first subjected to an open column chromatography on a Wakogel 50C18 column (Scheme 1). Out of five fractions obtained, the fraction eluted with 10% methanol (Fraction 1) showed most significant inhibitory activity against α-glucosidase (Fig. 6A). To further isolate inhibitors, this fraction was applied to a high-performance liquid chromatography column. As shown in Fig. 6B, two major peaks (F1-1 and F1-2) appeared. These two peak fractions showed similar absorption spectra with an absorption peak at 324 nm and a shoulder at 300 nm (Fig. 7D), indicating that the two fractions have the same chromophore.

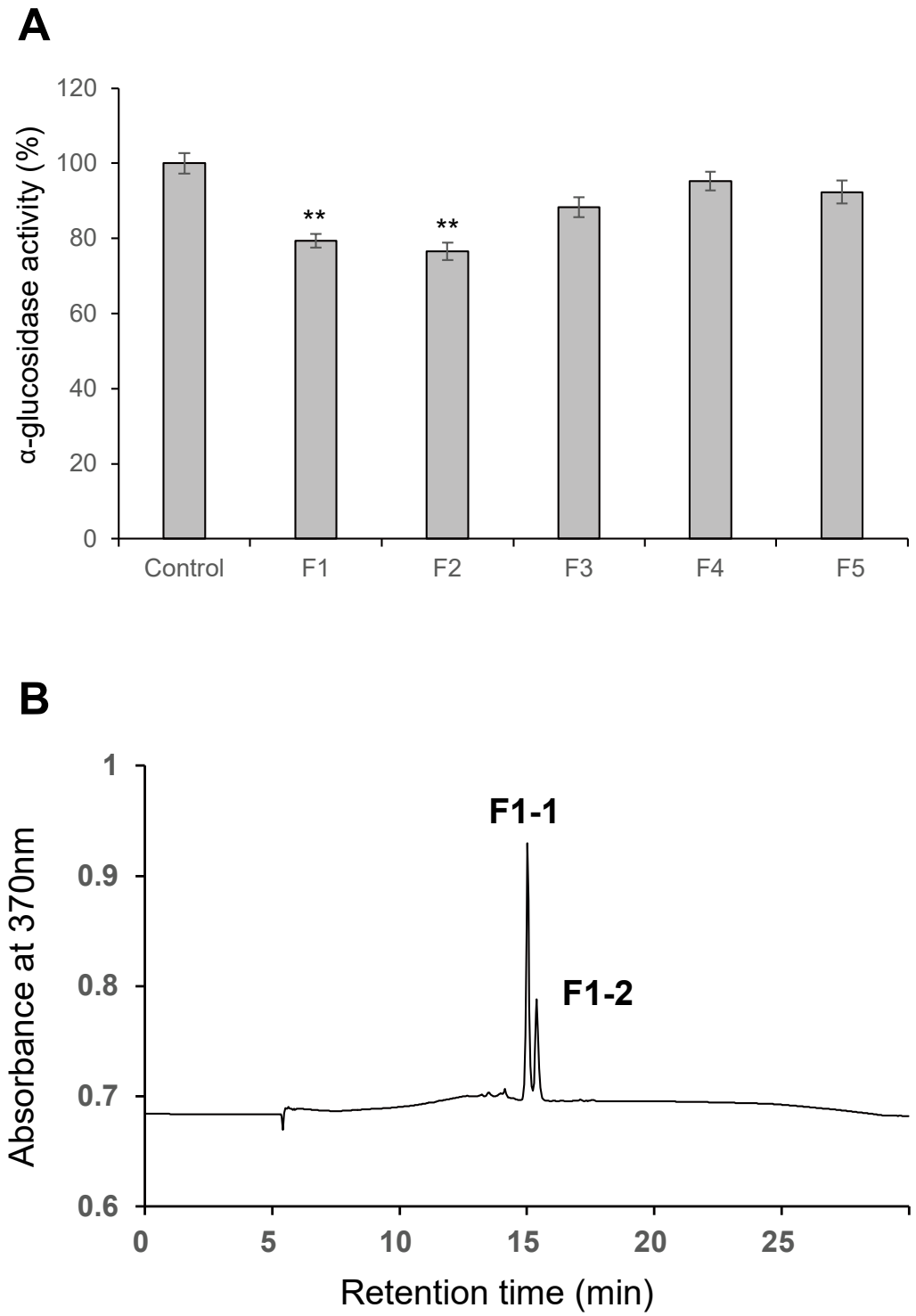


Fig. 6 Purification of α-glucosidase inhibitors from aronia juice. A. Inhibition of α-glucosidase activity. Each five fractions obtained using a Wakogel 50C18 column (Scheme 1) 330 μg was added to the α-glucosidase standard assay solution, respectively. Values are means ± S.E (n = 5). **$p < 0.01$. B. Chromatogram of F1. The fraction showing the inhibitory activity (F1) was applied to an InertSustain C18 column.

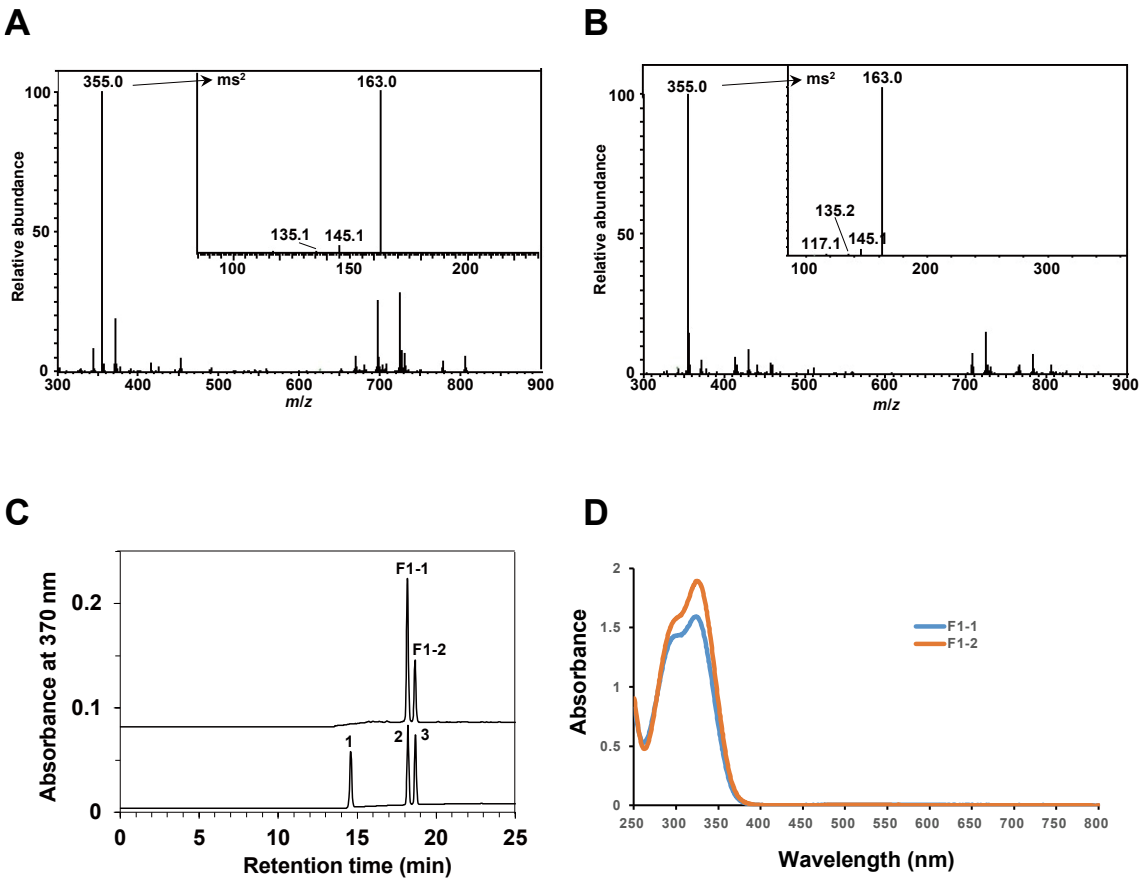


Fig. 7 Characterization of the purified inhibitors (F1-1 and F1-2). A. Mass spectrum of F1-1. The inset shows the MS/MS spectrum of the ion with m/z 355.0. B. Mass spectrum of F1-2. The inset shows the MS/MS spectrum of the ion with m/z 355.0. C. Comparison of the elution profiles of F1-1 and F1-2 with those of authentic samples of 5-CQA (1), 3-CQA (2), 4-CQA (3). D. Absorption spectra of F1-1 and F1-2. After appropriate dilution with 0.1% aqueous formic acid, the spectra were measured using a Shimadzu UV–vis spectrophotometer (UV-2550).

7) Mass spectrometric characterization of the inhibitors

Fig. 7A shows the mass spectrum of F1-1 and the product ion spectrum obtained by collision-induced dissociation (CID) of the ion with m/z 355.0 (the inset of Fig. 7A). The mass spectrum of F1-2 (Fig. 7B) was almost identical to that of F1-1. Since the calculated mass of protonated caffeoylquinic acids ($[M + H]^+$) is 355.10 and fragment ions with m/z 163, 145, and 135 can be produced from the caffeic acid moiety (Fig. 8), the results strongly suggested that the purified inhibitors are caffeoylquinic acids[46-48]. To determine the regio-isomerism of F1-1 and F1-2, retention times of F1-1 and F1-2 were compared to those of authentic 3-CQA, 4-CQA and 5-CQA using an InertSustain C18 column (Fig.

7C). F1-1 and F1-2 were eluted at the same retention times as those of 3-CQA and 4-CQA, respectively. These chemical structures were shown in Fig. 8. The concentrations of 3-CQA and 4-CQA in F1-1 and F1-2 preparations were estimated to be 1.5 and 1.4 mg/mL, respectively.

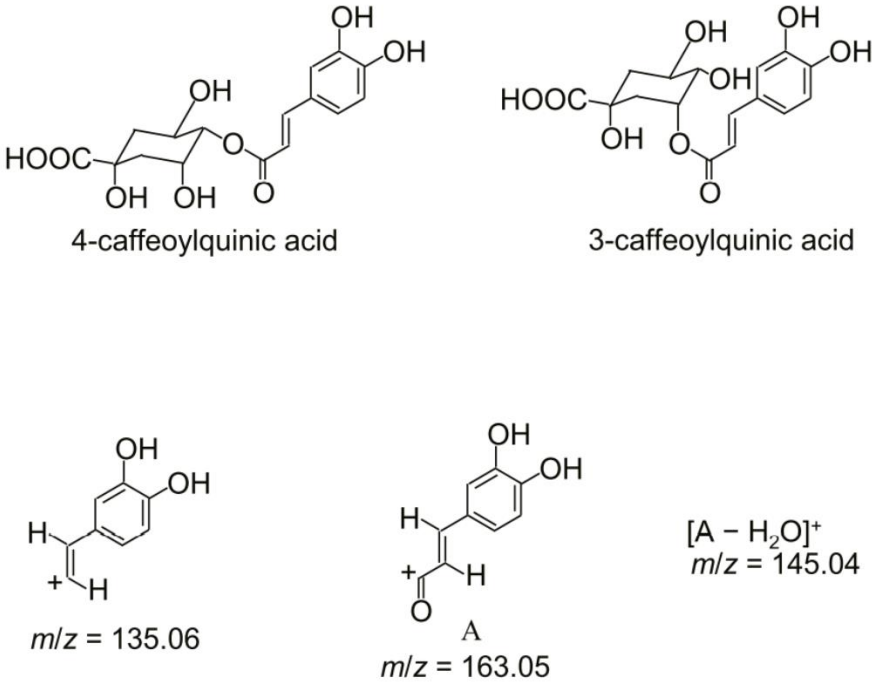


Fig. 8 Chemical structures of 3-CQA and 4-CQA and their fragment ions.

8) DPP IV inhibitory activities of fraction 1 and caffeoylquinic acids

Because aronia juice contains DPP IV inhibitors[12], DPP IV inhibitory activity was examined for Fraction 1 and authentic caffeoylquinic acids (3-CQA and 4-CQA). As shown in Fig. 9A, Fraction 1 inhibited DPP IV activity. In addition, both 3-CQA and 4-CQA inhibited DPP IV activity dose-dependently (Fig. 9B). IC50 values of 3-CQA and 4-CQA were 0.19 and 0.05 μmol/L, respectively.

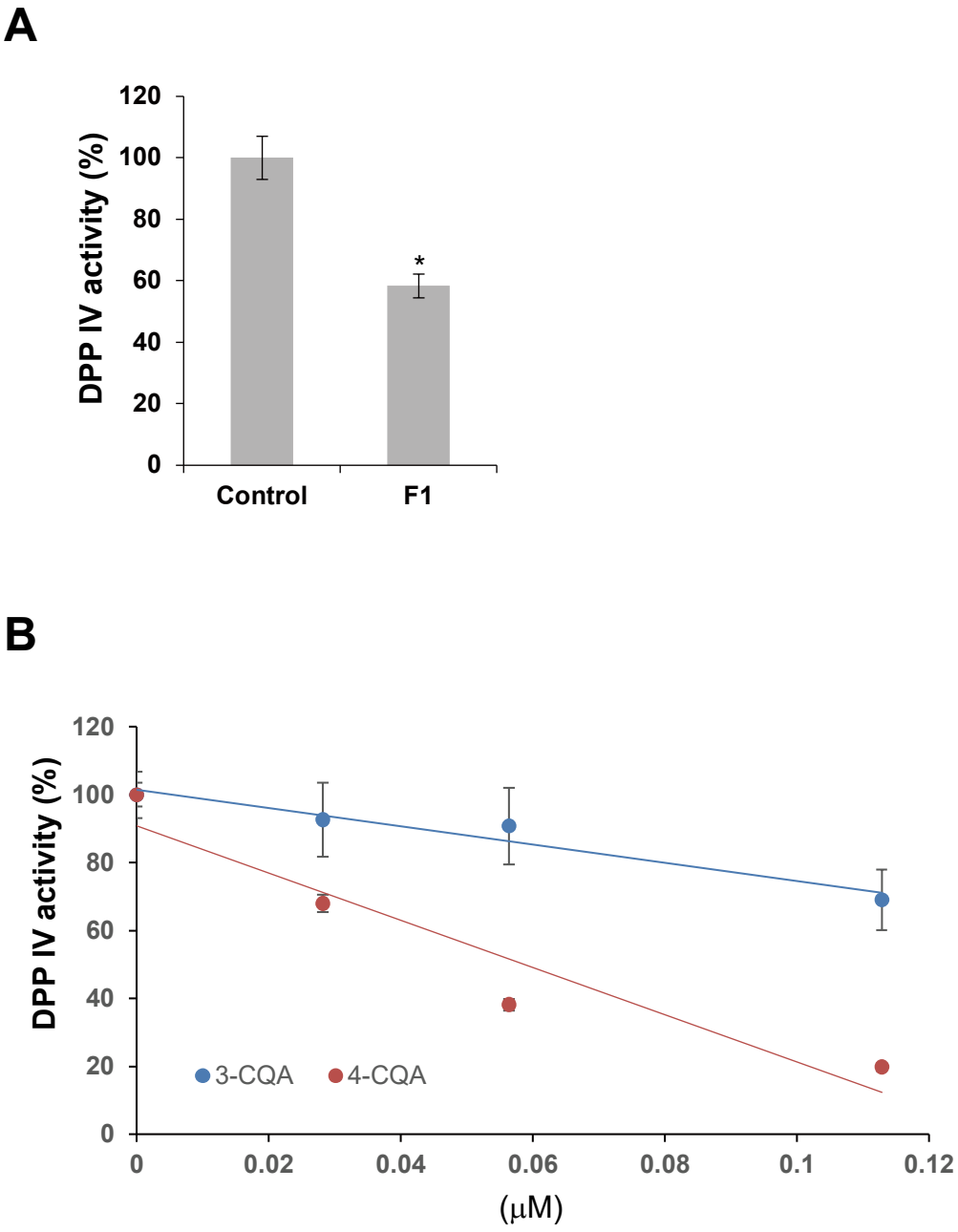


Fig. 9 Inhibition of DPP IV by F1 and CQAs. F1 (A), 3-CQA and 4-CQA (B) were added to the DPP IV standard assay solution, respectively. Values are means ± S.E. (n = 5). *p < 0.05

4. Discussion

The present study revealed that blood glucose and HbA1c levels were reduced by giving aronia juice or cyanidin 3,5-diglucoside. However, the magnitudes of reduction in blood glucose and HbA1c by administration of cyanidin 3,5-diglucoside were about 50% of those by giving aronia juice in KKA^{y} mice. The increase in the level of active GLP-1 in

serum from KKA^y mice by giving cyanidin 3,5-diglucoside was also about 64% of that by giving aronia juice. Although the reduced blood glucose and HbA1c levels in KKA^y mice given aronia juice began to increase slightly from the 21st day, those in KKA^y mice given cyanidin 3,5-diglucoside sharply increased from the same day. These different elevation curves of blood glucose and HbA1c levels suggested that other compounds such as α-glucosidase inhibitors exist in aronia juice. Furthermore, DPP IV inhibitory activity and increased active GLP-1 level in serum from KKA^y mice given cyanidin 3,5-diglucoside were about 50% of those in serum from KKA^y mice given aronia juice. Intake of cyanidin 3,5-diglucoside was also low in aronia group in comparison with cy-dg group (Table 2). These results indicated that other DPP IV inhibitors exist in aronia juice. Alpha-glucosidase activity was also inhibited by giving aronia juice[12] and polyphenols such as anthocyanins have an inhibitory effect on α-glucosidase activity[3]. Since these polyphenols are included in aronia juice, reduction of blood glucose and HbA1c levels by giving aronia juice may occur through inhibition of DPP IV and α-glucosidase activities by the combination of cyanidin 3,5-diglucoside and these polyphenols. Weights of the livers and white adipose tissues from KKA^y mice given aronia juice, but not those from KKA^y mice given cyanidin 3,5-diglucoside, were reduced. The triglyceride level in the liver was reduced by giving aronia juice but not by giving cyanidin 3,5-diglucoside. Since a reduction of white adipose tissue by giving aronia juice has been reported[15], other compounds included in aronia juice may have an effect on white adipose tissue. These results suggest that cyanidin 3,5-diglucoside, in aronia juice, has a beneficial effect on diabetes but not an obesity.

Since aronia juice contained at least cyanidin 3-O-glucoside, cyanidin 3-O-galactoside and cyanidin 3-O-arabinoside, these anthocyanins inhibited DPP IV activity with cyanidin 3,5-diglucoside. It has been reported that many polyphenols are included in aronia berries[49]. Activities of proteases such as DPP IV and angiotensin-converting enzyme were inhibited by these polyphenols from plants including berries[30]. DPP IV activity was inhibited by several polyphenols such as cyanidin 3-O-glucoside, quercetin and cyanidin[30]. DPP IV inhibitory activity of cyanidin 3-O-glucoside was higher than that of cyanidin[28]. DPP IV activity was inhibited by cyanidin 3,5-diglucoside more than by

cyanidin 3-O-glucoside[29]. Therefore, in the present study, we tried to isolate α-glucosidase inhibitors from the juice. Two α-glucosidase inhibitors (F1-1 and F1-2) were purified and they were identified to be 3-CQA and 4-CQA, respectively. Recent comprehensive profiling of phenolic compounds in berry plants by Tian et al[50]. has shown that aronia berries contain 3-CQA and 5- CQA. In the present study, 5-CQA was not detected in our aronia juice. Instead, 4-CQA was isolated as an α-glucosidase inhibitor. Caffeoylquinic acids (CQAs) are abundantly found in coffee and show anti-diabetic effects[51]. In rats fed a standard meal, the elevation of blood glucose level was reduced when caffeoylquinic acids were added to the meal[52]. 3-CQA, 4-CQA and 5-CQA from Kuding Tea (*Ilex kudingcha C.J. Tseng*) inhibit α-glucosidase activity[53]. The IC50 values of 3-CQA, 4-CQA and 5-CQA were reported to be 0.39, 0.34 and 0.30 mg/mL, respectively[53]. Because caffeoylquinic acids have too weak α-glucosidase inhibitory activity to explain their anti-diabetic effects, it is suggested that caffeoylquinic acids has another mechanism to show anti-diabetic effects[54]. Significant but weak inhibitory activity against α-glucosidase was confirmed in the present study (Fig. 6A). Fraction 1 inhibited DPP IV (Fig. 9A) and IC50 of 3-CQA and 4-CQA against the enzyme were determined to be 0.19 and 0.05 μmol/L, respectively (Fig. 9B). Hispidulin, eriodictyol, naringenin and cirsimaritin inhibit DPP IV with respective IC50 of 0.49, 10.9, 2.5 and 0.43 μmol/L[55]. DPP IV inhibitory activities of CQAs, especially 4-CQA, are stronger than those polyphenols. The present results suggest that caffeoylquinic acids contained in aronia juice reduce blood glucose levels through inhibition of both α-glucosidase and DPP IV.

Table 2 Intake contents of cyanidin 3,5-diglucoside.

Groups	Total intake (mL)	Cyanidin 3,5-diglucoside (mg)
Aronia	470 ± 21	0.07 ± 0.01
Cy-dg	578 ± 9	5.78 ± 0.01

All data is presented as mean ± standard error of five animals.

III. Examination of anti-obesity effect of aronia

1. Summary

This study examined mechanistically the effects of aronia juice on the white adipose tissues in normal and obesity model mice. C57BL/6J mice and KKA^{y} mice were fed on a normal diet for 28 days. During this feeding period, the study group was administered with aronia juice via a water bottle, while the control group was administered with tap water. In both C57BL/6J mice and KKA^{y} mice, the size of adipose cells in the study group was significantly smaller than that in the control group. In the study group, expression in the white adipose tissues of Arrdc3 mRNA was suppressed, whereas that of Sirt2 and Ucp1 mRNA was enhanced. To rule out the possibility of a toxic effect of aronia juice on 3T3-L1 cells, aronia juice cytotoxicity was examined by counting viable cells after 24-h culture. Aronia juice did not influence the viability of the 3T3- L1 cells at total polyphenol concentrations up to 16.5 μg/mL. Therefore, effects of aronia juice on the differentiation of 3T3-L1 cells were examined in the culture media containing 4 μg/mL of the total polyphenol. In 3T3-L1 cells, non-cytotoxic levels of aronia juice inhibited the lipid accumulation and Arrdc3 mRNA expression. Using reverse-phase liquid chromatography, compounds suppressing Arrdc3 expression in 3T3-L1 cells were isolated from aronia juice. Mass spectrometric analysis revealed that these compounds were vicenin-2 and quercetin-diglycoside. The results suggest that aronia juice inhibits lipid accumulation in white adipose tissues by suppressing Arrdc3 mRNA expression in adipocyte.

2. Materials and methods

1) Materials

Aronia juice was obtained from Nakagaki Consulting Engineer (Osaka, Japan). The total polyphenol content of the juice was determined by Japan Food Research Laboratories (Tokyo, Japan). PrimeScript RT Master Mix and TB Green Premix Ex Taq II were purchased from Takara (Shiga, Japan). All other chemicals were of analytical grade and were purchased from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan).

2) Animal experiments

C57BL/6J male mice and KKA^{y} male mice (4 weeks old) were obtained from CLEA Japan (Tokyo, Japan). All the mice were fed on a normal diet (CE-2, CLEA Japan, Tokyo, Japan) for 2 weeks before experimentation. Male mice (C57BL/6J or KKA^{y}, six weeks old) were randomized into two groups (n = 5 per group). One group (control group) received tap water by free intake from a bottle, and the other group (study group) received aronia juice instead of tap water. A normal diet (CE-2 diet) was given to both groups of mice. After 28 days, blood was obtained from the mice by venous puncture. The anesthesia was induced by placing the mice in an anesthesia induction chamber (25 × 25 × 30 cm) filled with room air containing 2.5% isoflurane. Epididymal adipose tissues were dissected from the anesthetized mice, and their weights were individually measured. For total RNA isolation, parts of the tissues were immediately mixed with RNA later solution (Thermo Fisher Scientific, CA, USA) and stored at 4℃.

3) Hematoxylin and eosin (HE) staining

The epididymal adipose tissues were fixed in 10% neutrally buffered formalin. The fixed tissues were dehydrated and embedded in paraffin. Paraffin sections (5 μm in thickness) were cut, subjected to deparaffinization, and rehydrated. Hydrated sections were stained with HE and examined using a light microscope (BZ-9000, Keyence, Osaka, Japan). The size of the adipocytes was determined using BZ-II analysis software (Keyence, Osaka, Japan).

4) Cell culture

3T3-L1 cells were purchased from American Type Culture Collection (ATCC). The cells were seeded onto a 6-well plate and cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (10% FBS-DMEM) at 37℃ in a humidified 5% CO_2 atmosphere. Two days after the cells reached confluence (Day 0), the medium was replaced with 10% FBS-DMEM containing 1.7 μM insulin, 0.5 mM 3-isobutyl-1-methylxanthine (IBMX) and 1 μM dexamethasone to induce differentiation. After 2 days (Day 2), the medium was replaced with 10% FBS-DMEM containing 1.7 μM insulin and

aronia juice (4 μg/mL) or fractions isolated from the juice (5 μg/mL). The cells were cultured for 2–8 days. The medium was changed every 2 days.

5) Oil red O staining

The lipid accumulation in 3T3-L1 cells cultured in the presence and absence of aronia juice and fractions isolated from the juice was qualitatively assayed using Oil Red O staining. The cells were fixed with 4% paraformaldehyde for 10 min at room temperature and then incubated with Oil Red O solution for 30 min in the dark. The Oil Red O solution was prepared by dilution of the dye stock solution (0.5% (w/v) in isopropanol, Sigma Aldrich Inc.) with water (60:40, v/v). After staining, the cells were washed three times with water and then examined to identify mature adipocytes using a light microscope.

6) Cell viability assay

A cell viability assay was performed using Cell Counting Kit-8 (Dojindo, Kumamoto, Japan) according to manufacturer's protocol.

7) Purification of Arrdc3 expression-inhibiting compounds from aronia juice

Aronia juice (300 mL) was applied to a Wakogel 50C18 column (200 mL gel) preequilibrated with 0.1% aqueous formic acid (solvent A). The column was washed extensively with solvent A, and then the adsorbed compounds were eluted from the column by a stepwise increase in the methanol concentration (methanol: 10, 20, 30, 40 and 50% (v/v)). Each eluate was collected and evaporated to dryness to give fractions 1 - 5, respectively. The fractions were weighed and resolved with 12.5% or 25% aqueous methanol containing 0.1% formic acid to a final concentration of 1 - 10 mg/mL. The active fractions (fractions 1 and 3) were further purified using an InertSustain C18 column (2.1 mm × 150 mm, GL Science, Tokyo, Japan). The mobile phase consisted of solvent A and 90% acetonitrile (10% water) containing 0.1% formic acid (solvent B). The flow rate was 150 μL/min. In the case of fraction 1, the column was developed with the following gradient: 0 - 5 min, 5% B; 5 - 15 min, 5 - 30% B; 15 - 20 min, 30 - 60% B; 20 - 25 min, 60 - 90% B. In the case of fraction 3, the following time program was used: 0 - 5 min,

30% B; 5 - 40 min, 30 - 67% B; 40 - 46 min, 67 - 90% B. The absorbance at 370 nm of the eluate was monitored. Two major peaks appeared from fraction 1 and they were manually collected (F1-1 and F1-2). Four major peaks (F3-1, F3-2, F3-3 and F3-4) were obtained from fraction 3.

8) Liquid chromatography-mass spectrometry (LC/MS)

Aliquots of F3-2 and F3-4 (each 5 μL) were injected into an InertSustain C18 column (0.3 mm × 150 mm, GL Science) preequilibrated with 20%B (80%A). The column was developed at a flow rate of 3.0 μL/min with the following gradient: 0 - 5 min, 20%B; 5 - 30 min, 20 - 70%B; 30 - 35 min, 70 - 90%B. The column temperature was controlled at room temperature 20℃. The eluate was equally split into two fused silica capillary tubes (20 μm in internal diameter) and one of them was connected to an electrospray ionization tip (MonoSpray, GL Science). The spray voltage was 2.5 kV and the temperature of the transfer tube was 150℃. A mass spectrum of the eluate was recorded between m/z 150 and 1000 in the positive ion mode. The ion peaks with ion intensity of more than 1000 were data-dependently subjected to MS/MS measurement. In some cases, the most intense peak of the MS/MS spectrum was subjected to MS/MS/MS measurement. A syringe-type HPLC pump (HP 711 V Micro-Flow Pump, GL Science) and an ion-trap mass spectrometer (LCQ Fleet, Thermo Fisher Scientific, CA, USA) were used.

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

Total RNA was isolated from the cultured 3T3-L1 cells and the adipose tissue using a RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. cDNA was synthesized using PrimeScript RT Master Mix (Takara Bio, Shiga, Japan) according to manufacturer's protocol. After reactions, PCR products were extracted, separated on 2.0% agarose gels, and stained with ethidium bromide. Real-time PCR analyses were performed with an CFX real-time PCR detection system (Bio-Rad, Hercules, CA, USA) using TB Green Premix Ex Taq II (Takara Bio, Shiga, Japan). Nucleotide sequences of primers and PCR conditions used for RT-PCR and real-time PCR are shown in Table 3. PCR analyses were repeated in triplicate for each sample. The levels

of gene expression were normalized with 18S expression.

Table 3 Nucleotide sequences of primers and PCR conditions.

Gene		Nucleotide sequence	PCR condition
Arrdc3	sense	5'- CGGCTCCAATACTGCCTATACA -3'	95˚C 2 min, 95˚C 10 sec, 60˚C 30 sec, 72˚C 30 sec x 40 cycles
	anti-sense	5'- TGTGGAAGCCTTCTTCAGAATTA -3'	
Sirt2	sense	5'- CTCTGACCCTCTGGAGACCC -3'	95˚C 2 min, 95˚C 10 sec, 60˚C 30 sec, 72˚C 30 sec x 42 cycles
	anti-sense	5'- GCATGTAGCGTGTCACTCCT -3'	
Ucp1	sense	5'- CTGGGCTAGGTAGTGCCAGTG -3'	95˚C 2 min, 95˚C 10 sec, 60˚C 30 sec, 72˚C 30 sec x 40 cycles
	anti-sense	5'- CAACCTTGGCTAGACGCACAG -3'	
18S	sense	5'- TTCTGGCCAACGGTCTAGACAAC -3'	95˚C 2 min, 95˚C 10 sec, 60˚C 30 sec, 72˚C 30 sec x 40 cycles
	anti-sense	5'- CCAGTGGTCTTGGTGTGCTGA -3'	

10) Statistical analysis

Data are expressed as means ± S.E. of at least three replicates for each sample. Statistical analyses were performed using Statcel4 software (OMS, Tokyo, Japan). The difference between two groups was evaluated using unpaired Student's t-test. For comparison of multiple samples, the Tukey-Kramer test was used.

11) Ethics statement

All animal experiments were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and all of the protocols were approved by the Committee for Animal Research at Osaka Prefecture University (permit number 19–163).

3. Results

1) Effects of aronia juice on the weight of body and epididymal white adipose tissues

Table 4 compares the total intake of food and tap water/aronia juice taken by mice in the 28 days of animal experiments. In the case of C57BL/6 J mice, administration of aronia juice significantly reduced the intake of food by 17%. The total amount of aronia juice taken by the mice was also significantly smaller than that of tap water taken by the control mice by 15%. In the case of KKA^y mice, there was no significant difference both in the total intake of food and that of tap water/aronia juice.

As shown in Table 5, administration of aronia juice significantly reduced the body weight of KKA^y mice by 14%, whereas, in the case of C57BL/6 J mice, the intake of aronia juice showed no significant effect on the body weight. Administration of aronia juice significantly reduced the white adipose tissue weight of KKA^y mice by about 19% and that of C57BL/6 J mice by about 48%, respectively (Table 5).

2) Effects of aronia juice on cell size and gene expression of white adipocytes

In both C57BL/6J mice and KKA^y mice, administration of aronia juice significantly inhibited the enlargement of adipocytes in white adipose tissues (Fig. 10). In mice that were given aronia juice, the expression levels of Arrdc3 mRNA were significantly reduced, whereas those of Sirt2 and Ucp1 mRNA were significantly elevated (Fig. 11).

Table 4 Amounts of foods and water/aronia juice taken by mice in 28 days.

mice	Foods (g)	Tap water (g)	Aronina juice (g)
C57BL/6J			
Control	114.3 ± 6.3	175.3 ± 5.6	–
Aronia	97.2 ± 2.7*	–	130.7 ± 4.9***
KKA^y			
Control	204.7 ± 9.8	458.0±43.8	–
Aronia	200.9 ± 9.3	–	422.2±37.3

Control group vs Aronia group: *p<0.05, ***p<0.001

Table 5 Weight of body and epididymal white adipose tissues (WAT) at the 28th day (Day 28).

mice	Body weight (g)	WAT (g)
C57BL/6J		
Control	21.6 ± 0.7	0.23 ± 0.01
Aronia	20.6 ± 0.3	0.12 ± 0.01**
KKA^{y}		
Control	39.1 ± 9.8	1.56 ± 0.06
Aronia	33.8 ± 0.9**	1.27 ± 0.02**

Control group vs Aronia group: *p<0.01

3) Effects of aronia juice and its fractions on lipid accumulation in 3T3-L1 cells

To rule out the possibility of a toxic effect of aronia juice on 3T3-L1 cells, aronia juice cytotoxicity was examined by counting viable cells after 24-h culture. Aronia juice did not influence the viability of the 3T3- L1 cells at total polyphenol concentrations up to 16.5 μg/mL (Fig. 12A). Therefore, effects of aronia juice on the differentiation of 3T3-L1 cells were examined in the culture media containing 4 μg/mL of the total polyphenol. As shown in Fig. 12B, aronia juice reduced the number of Oil Red O-positive cells. The time-dependent increase in Arrdc3 mRNA expression was inhibited in 3T3-L1 cells treated with aronia juice (Fig. 12C). Out of fractions 1 - 5 isolated from aronia juice using a Wakogel 50C18 column, fractions 1 and 3 reduced the number of Oil Red O-positive cells (Fig. 12D).

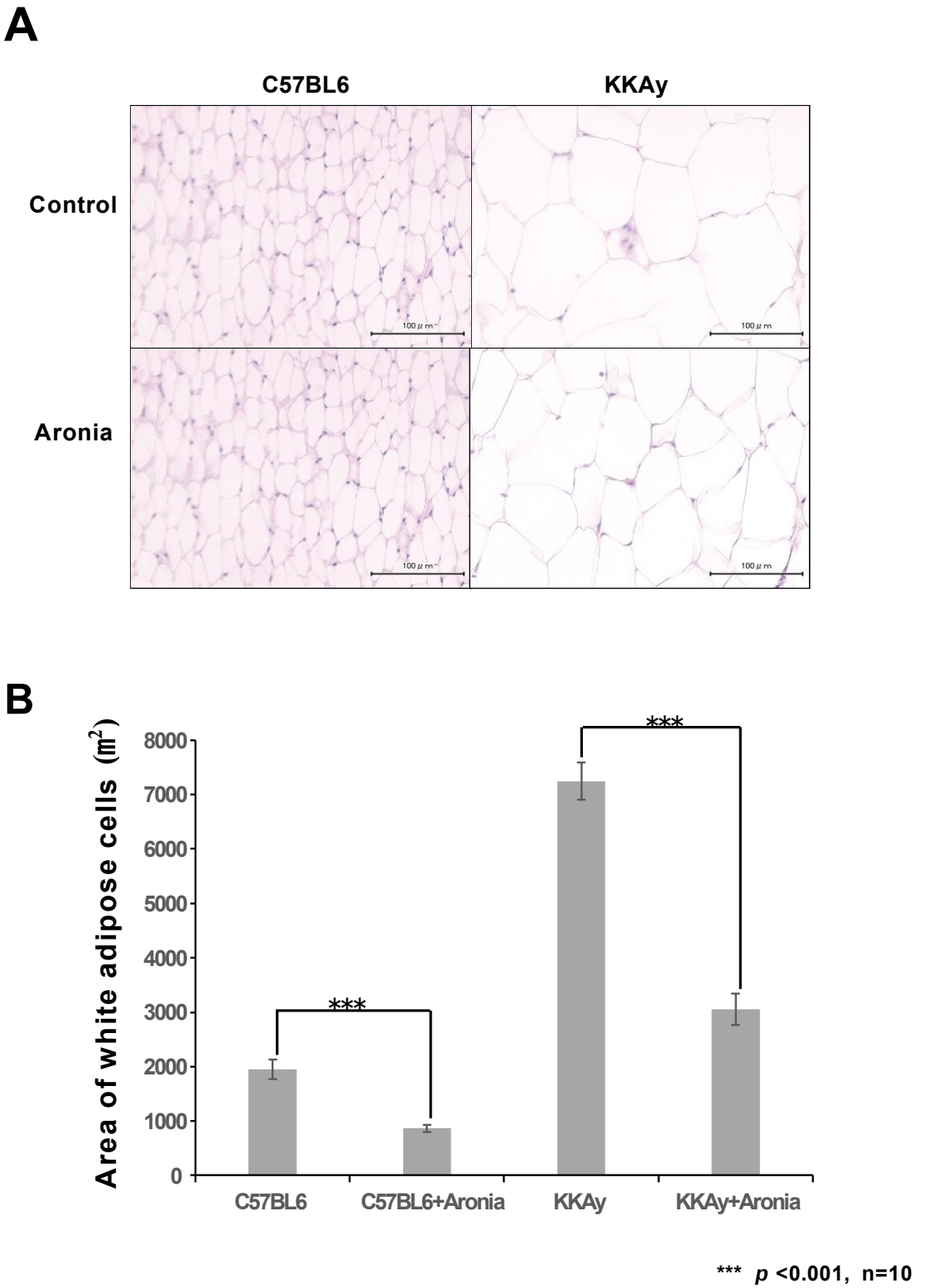


Fig. 10 Effect of aronia juice on the size of white adipocytes. All of the mice were fed a normal diet for 28 days. Throughout the feeding period, aronia juice was given to the aronia group mice, whereas water was given to control mice. (A) Epididymal adipose tissu sections stained with HE. (B) Size of adipocytes. The cell area was calculated as the mean value of an area of 10 adipocytes. Data are means ± SE. ***$p < 0.001$.

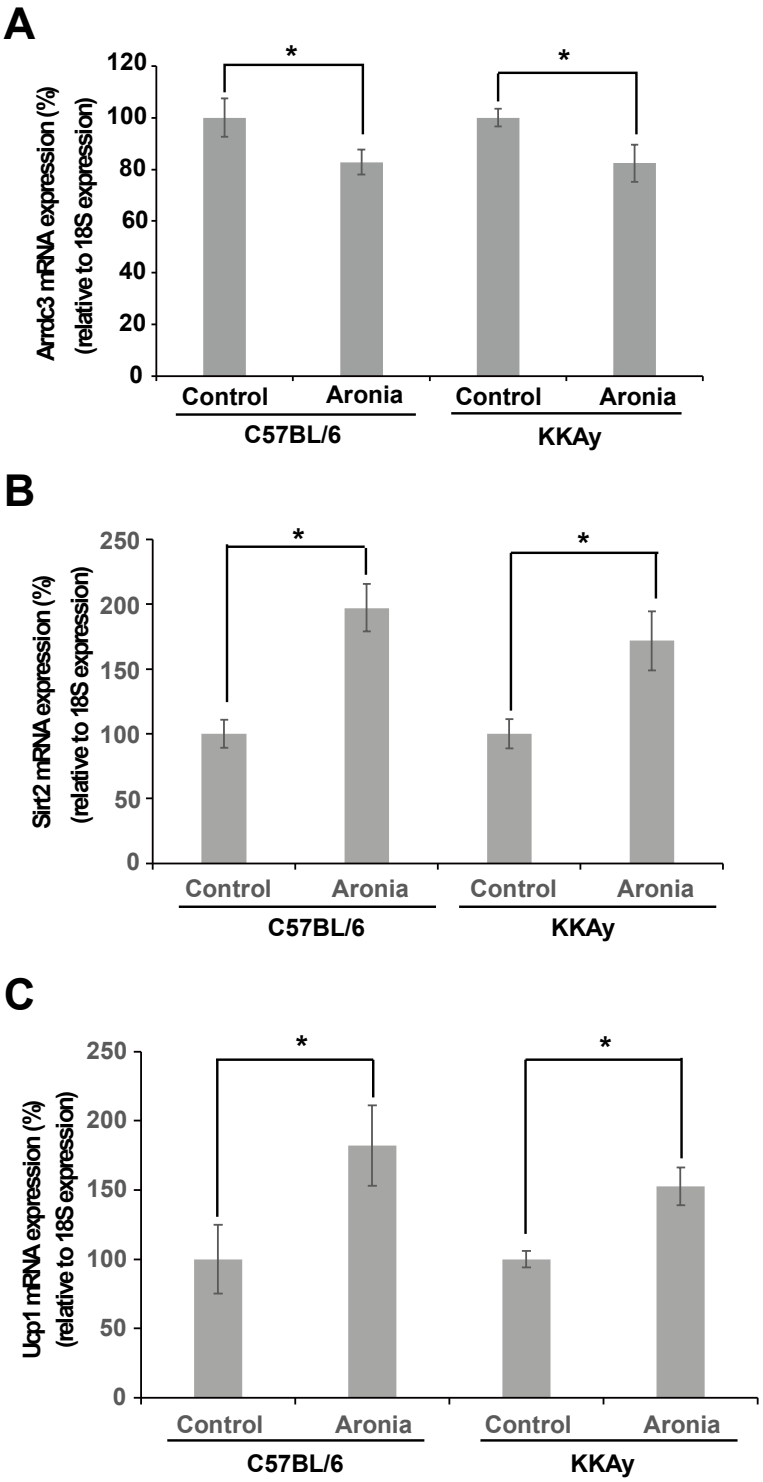


Fig. 11 RT-PCR analysis of Arrdc3 (A), Sirt2 (B), and Ucp1 (C). All of the mice were fed a normal diet for 28 days. Throughout the feeding period, aronia juice was given to the aronia group mice, whereas water was given to control mice. After 28 days of feeding, total RNA was isolated from epididymal adipose tissue. Data are means ± SE (n = 5). *$p < 0.05$.

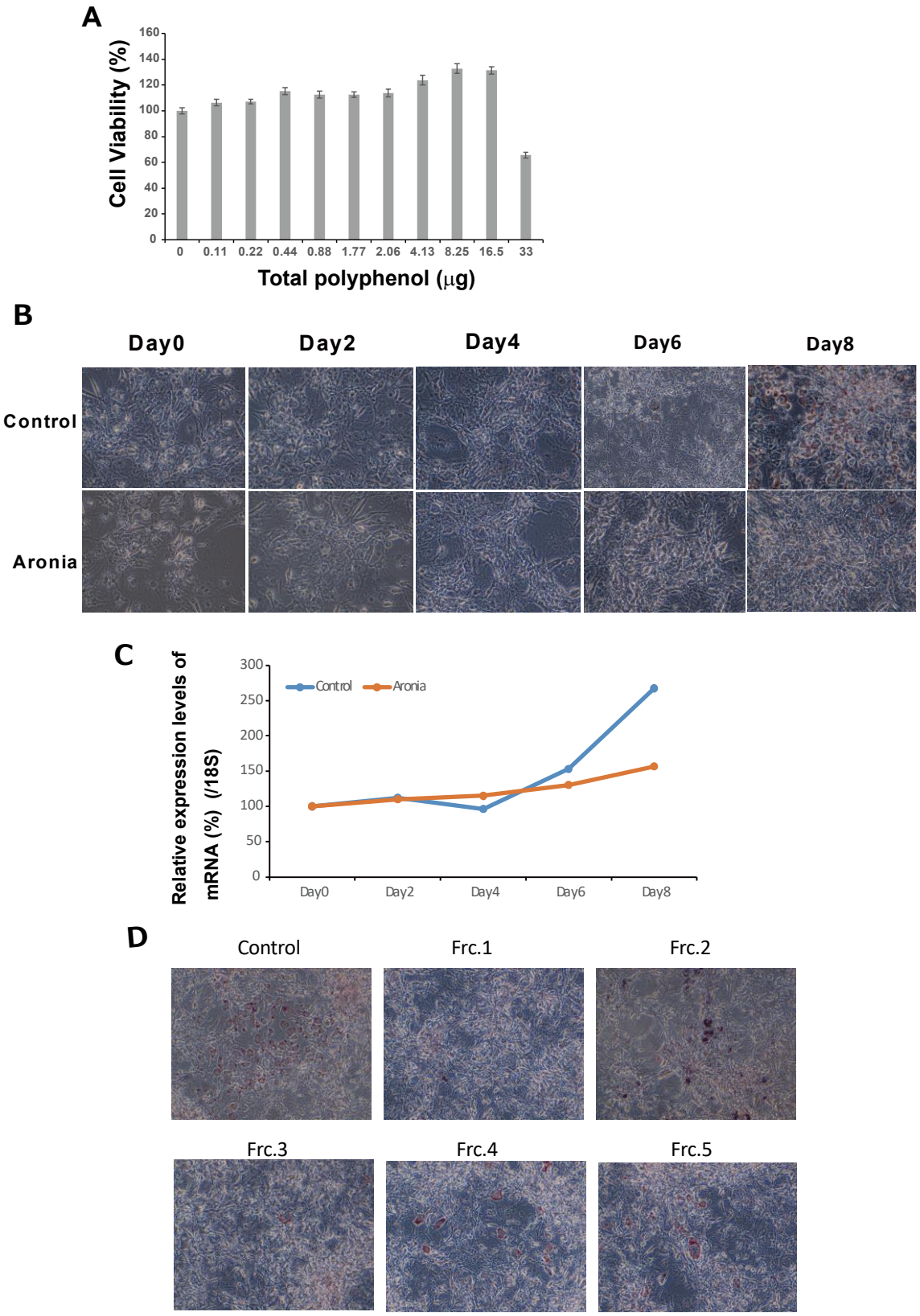


Fig. 12 Effects of aronia juice and its fractions on lipid accumulation in 3T3-L1 cells.
(A) Dose effect of aronia juice on viability of 3T3-L1 cells. Confluent 3T3-L1 cells were induced to differentiate for 2 days, and then the medium was replaced with 10% FBS-DMEM containing

1.7 μM insulin and various concentrations of aronia juice (0 - 33 μg/mL). The concentration of aronia juice is expressed as the total concentration of polyphenols. Oil Red O staining of the 3T3-L1 cells (B) and Arrdc3 expression (C) as a function of culture time. 3T3-L1 cells were cultured in 10% FBS-DMEM containing 1.7 μM insulin and aronia juice (4 μg/mL). Control cells were cultured in the absence of aronia juice. (D) Effects of fractions 1–5 on lipid accumulation in 3T3-L1 cells. Aronia juice was applied to a Wakogel 50C18 column and the adsorbed compounds were eluted with 10%, 20%, 30%, 40% and 50% aqueous methanol (v/v) in the presence of 0.1% formic acid to give fractions 1–5, respectively. 3T3-L1 cells were cultured in 10% FBS DMEM containing 1.7 μM insulin and each of the Wakogel 50C18 fractions (10 μg/mL) for 8 days. Oil Red O staining was performed on the eighth day of culture.

4) Purification of inhibitors of Arrdc3 expression from fractions 1 and 3

To purify inhibitors of Arrdc3 expression from fractions 1 and 3, these fractions were subjected to reverse-phase high-performance liquid chromatography on an InertSustain C18 column. As shown in Fig. 13A, from fraction 1, two compounds designated as F1-1 and F1-2 were obtained as elution peaks 1 and 2. From fraction 3, five compounds (F3-1, F3-2, F3-3, F3-4, and F3-A) were obtained as elution peaks (Fig. 13B). Out of these 7 compounds, F3-2 and F3-4 inhibited Arrdc3 mRNA expression by 45% and 55%, respectively, compared to the control expression (Fig. 13C).

5) Mass spectrometric analysis of F3-2 and F3-4

To examine the chemical structures of F3-2 and F3-4, these two inhibitors were analyzed using LC/MS. As shown in Fig. 14A, the mass spectrum of F3-2 showed a single major ion with m/z 595.2. Fig. 14B shows the mass spectrum of the ions produced from the ion by CID. It is of note that vicenin-2 has a molecular mass of 594.5 Da. The mass spectrum of F3-4 showed a single major peak at m/z of 627.2 (Fig. 15A), and a strong ion with m/z of 303.0 was produced from this ion by CID, with a neutral loss of 324.2 Da. The mass spectrum of the ions produced from the ion with m/z of 303.0 by CID (Fig. 15C) was compared to that of authentic quercetin (Fig. 15D). The results suggested that F3-4 may be quercetin diglucoside.

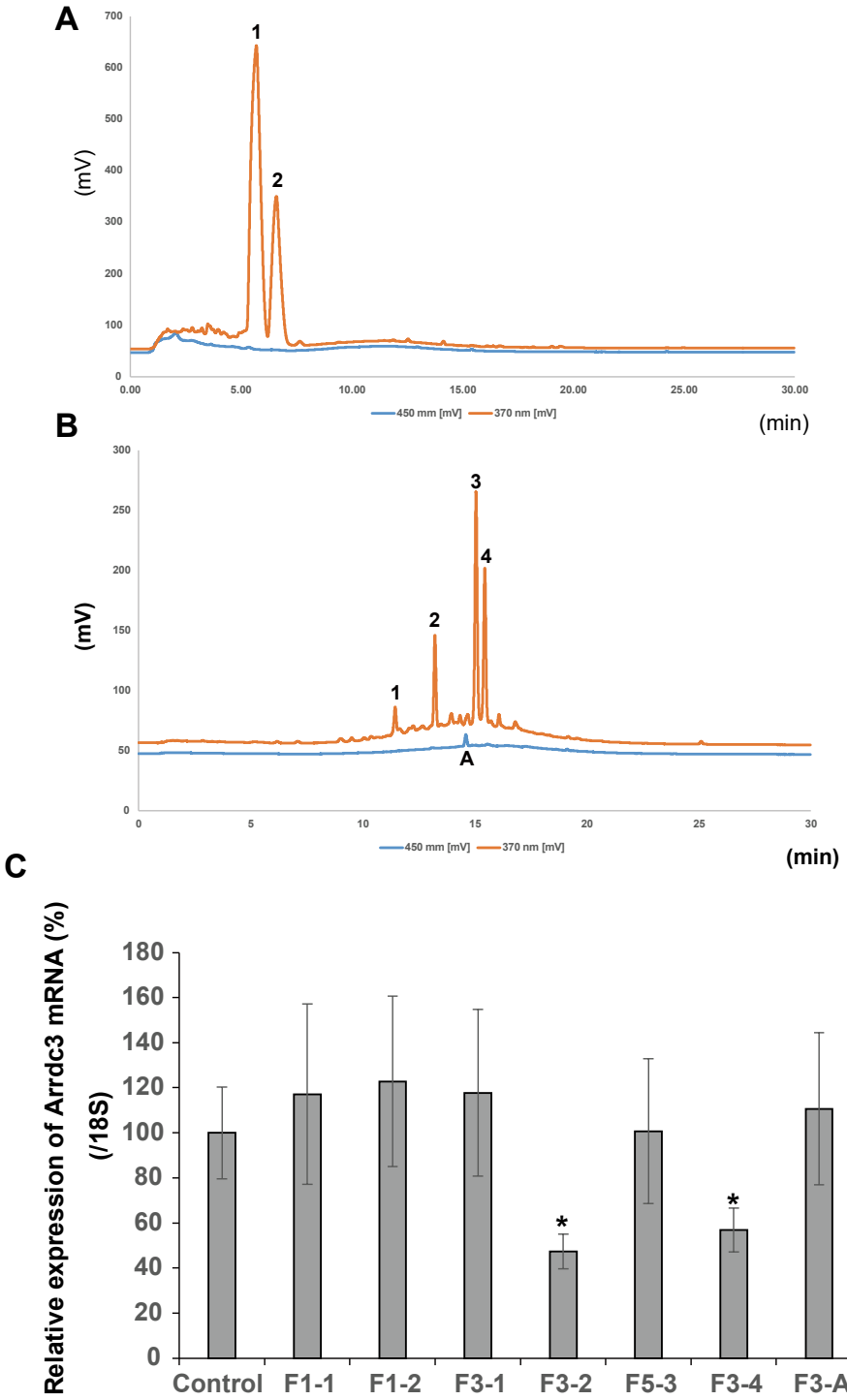


Fig. 13 Reverse-phase chromatographic purification of Arrdc3 expression inhibitors. Inhibitors contained in Wakogel 50C18 fractions 1 and 3 were further purified using an InertSustain C18 column. (A) Chromatogram of fraction 1. Two elution peaks (1 and 2) were separately collected and named F1-1 and F1-2, respectively. Orange line, absorbance at 370 nm; blue line, absorbance at 450 nm. (B) Chromatogram of F3. Five elution peaks (1–4 and A) appeared and were separately collected and named F3-1, F3-2, F3-3, F3-4 and F3-A, respectively. Orange line, absorbance at 370 nm; blue line, absorbance at 450 nm. (C) Effects of the fractions on Arrdc3 expression in 3T3-L1 cells. Data are means ±SE (n = 5). **p < 0.01.

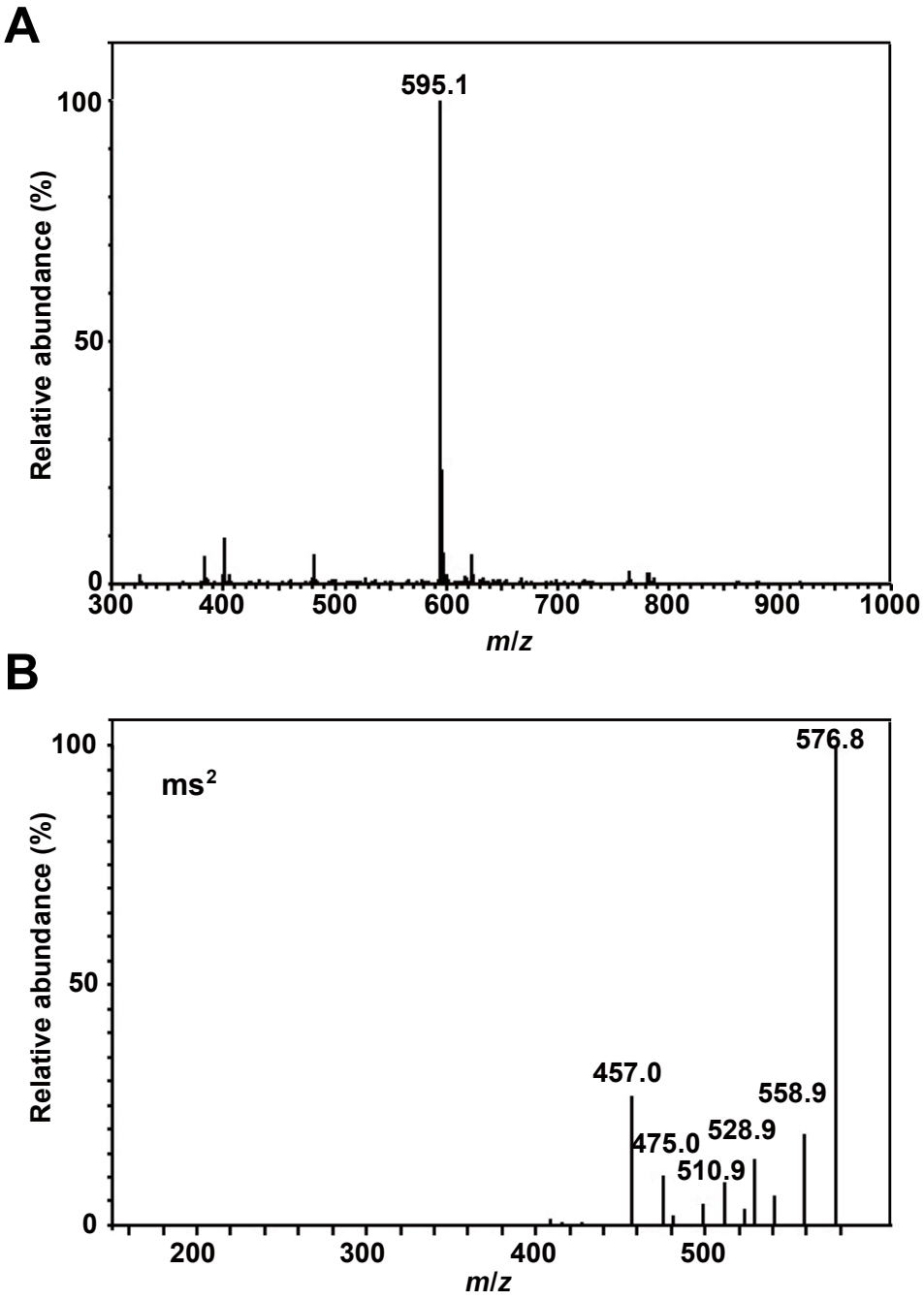


Fig. 14 Mass spectrometric analysis of F3-2. (A) Mass spectrum of F3-2. (B) Product ions produced from the ion with m/z of 595.1 by CID.

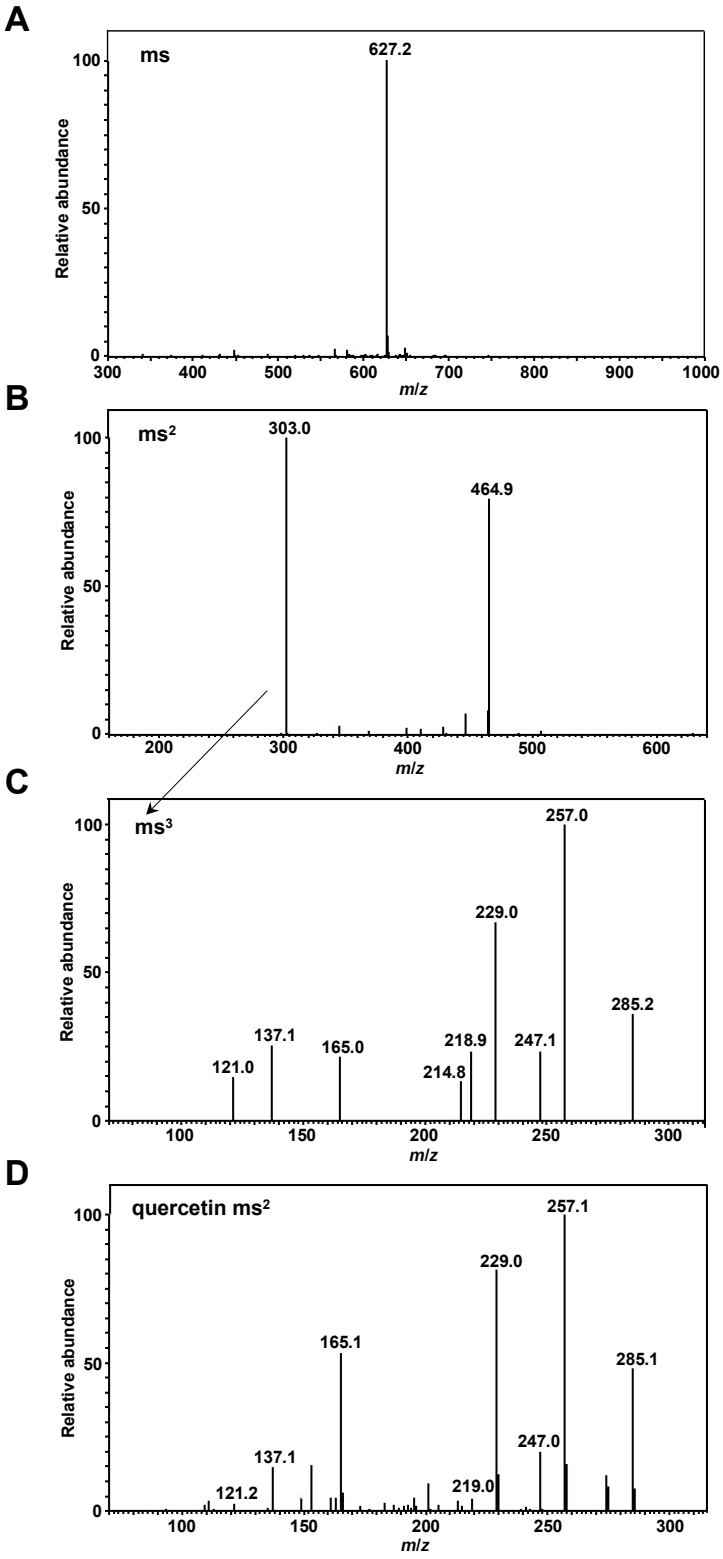


Fig. 15 Mass spectrometric analysis of F3-4 in comparison with that of quercetin. (A) Mass spectrum of F3-4. (B) Product ions produced from the ion with m/z of 627.2 by CID (MS2). (C) Product ions produced from the ion with m/z of 303.0 by CID (MS3). (D) Product ions produced from the authentic quercetin ion (singly protonated quercetin, m/z 303.0) by CID.

4. Discussion

In this study, in both normal mice and obese KKA^{y} mice, daily drinking of aronia juice inhibited the enlargement of adipocyte volume in white adipose tissue (Fig. 10A) and reduced the adipocyte weight, indicating the existence of unidentified inhibitors against obesity in aronia juice. In the white adipose tissue of these mice, the expression of Arrdc3 mRNA was suppressed and that of SIRT2 and Ucp1 mRNA was enhanced (Fig. 11). Furthermore, aronia juice reduced the expression of Arrdc3 mRNA in 3T3-L1 cells and inhibited their differentiation to adipocyte-like cells. These results suggest that inhibition of Arrdc3 mRNA expression by some compounds in aronia juice leads to the inhibition of lipid accumulation through attenuation of the sensitivity of adipocytes to adrenergic stimulation.

Aronia juice drinking significantly reduced the amount of food taken by normal mice, whereas it did not reduce the amount of food taken by obesity model mice. However, the body weight of obesity model mice reduced significantly by drinking aronia juice, whereas the body weight of normal mice did not reduce by drinking aronia juice. These results suggest that daily drinking of aronia juice can be effective to inhibit the progress of obesity by modifying energy metabolism.

In the present animal experiments, aronia juice was given to mice as a drinking water. Therefore, mice in the study group were forced to drink only the juice throughout the 28 days and not allowed to drink ordinary tap water. Taking water and taking a supplement was not separated. Normal mice in the study group ate less food compared to mice in the control group. Concomitantly, the total amount of aronia juice taken by mice in the study group was about 85% of that of tap water taken by mice in the control group, same levels of reduction as in the food intake. The present administration of aronia juice could not control the dairy dose of the juice. Of course, there is a possibility that aronia juice inhibits appetite in some unknown mechanism. In the future study, mice in the study group should be allowed to drink both tap water and aronia juice using two drink bottles and the daily dose of aronia juice is controlled by adjusting the opening and closing of the bottle containing tap water. The present data suggest that aronia juice inhibits the growth of white adipose tissue through the reduction of Arrdc3 gene expression but could not

exclude indirect effects.

Two compounds isolated from aronia juice as fractions F3-2 and F3-4 (Fig. 13B) inhibited the expression of Arrdc3 mRNA in 3T3-L1 cells. Mass spectrometric analysis of these fractions suggested that F3-2 contains vicenin-2 (apigenin 6,8-di-C-β-D-glucopyranoside)[56-58], and that F3-4 contains quercetin diglycoside. Vicenin-2 is a C-glycosyl flavone found in *Cyclopia subternata*, *C. genistoides*, *C.longifolia*, *C.maculata*, *C.intermedia* and *Dendrobium officinale* extracts[59-62]. Vicenin-2 has anti-diabetic[63,64], anti-inflammatory[65-68], and pancreatic lipase inhibitory activities[69]. The present results suggest that vicenin-2 inhibits Arrdc3 mRNA expression. Quercetin prevents obesity induced by a high-fat diet in C57BL/6J mice[70]. Its glycosylated derivative is more potent inhibitor against the differentiation of 3T3-L1 cells than quercetin[71]. Therefore, quercetin diglycoside and vicenin-2 in aronia juice seems to synergistically inhibit Arrdc3 mRNA expression in adipocytes.

Beneficial effects of aronia berries on obesity have been found in animal studies[9,10,38]. In our previous studies, aronia juice showed α-glucosidase and DPP IV inhibitory activities[12], and one of the DPP IV inhibitors was identified to be cyanidin-3,5-diglucoside[29]. However, cyanidin 3,5-diglucoside did not inhibit the increase in white adipose tissue weight of KKA^y mice, which are type-2 diabetes and obesity model mice, although cyanidin 3,5-diglucoside inhibited the elevation of blood glucose and HbA1c levels.

IV. Conclusion

Blood glucose and HbA1c levels were reduced by giving aronia juice or cyanidin 3,5-diglucoside. However, the magnitudes of reduction in blood glucose and HbA1c by administration of cyanidin 3,5-diglucoside were about 50% of those by giving aronia juice in KKA^y mice. Serum DPP IV activity was inhibited in KKA^y mice given aronia juice and cyanidin 3,5-diglucoside for 49 days, and the rate of inhibition of DPP IV activity by giving cyanidin 3,5-diglucoside was about 50% of that by giving aronia juice. The increase in the level of active GLP-1 in serum from KKA^y mice by giving cyanidin 3,5-diglucoside was also about 64% of that by giving aronia juice. Although the reduced blood glucose and HbA1c levels in KKA^y mice given aronia juice began to increase slightly from the 21st day, those in KKA^y mice given cyanidin 3,5-diglucoside sharply increased from the same day. These different elevation curves of blood glucose and HbA1c levels suggested that other compounds such as α-glucosidase inhibitors exist in aronia juice. Furthermore, DPP IV inhibitory activity and increased active GLP-1 level in serum from KKA^y mice given cyanidin 3,5-diglucoside were about 50% of those in serum from KKA^y mice given aronia juice. Intake of cyanidin 3,5-diglucoside was also low in aronia group in comparison with cyanidin 3,5-diglucoside group. These results indicated that other DPP IV inhibitors exist in aronia juice.

To identify these inhibitors in aronia juice, I tried to isolate α-glucosidase inhibitors from the juice, two α-glucosidase inhibitors were purified and they were identified to be 3-CQA and 4-CQA, respectively. 3-CQA and 4-CQA inhibit DPP IV with respective IC_{50} of 0.19 and 0.05 μmol/L, respectively. DPP IV inhibitory activities of CQAs, especially 4-CQA, are stronger than other polyphenols. The results suggest that CQAs contained in aronia juice reduce blood glucose levels through inhibition of both α-glucosidase and DPP IV.

On the other hand, in both normal mice and obese KKA^y mice, daily drinking of aronia juice inhibited the enlargement of adipocyte volume in white adipose tissue and reduced the weight of this tissue, indicating the existence of unidentified inhibitors against obesity in aronia juice. In the white adipose tissue of these mice, the expression of Arrdc3 mRNA was suppressed and that of SIRT2 and Ucp1 mRNA was enhanced. Aronia juice reduced

the expression of Arrdc3 mRNA in 3T3-L1 cells and inhibited their differentiation to adipocyte-like cells. These results suggest that inhibition of Arrdc3 mRNA expression by visenin-2 and quercetin diglucoside in aronia juice leads to the inhibition of lipid accumulation through attenuation of the sensitivity of adipocytes to adrenergic stimulation.

3-CQA, 4-CQA and cyanidin 3,5-diglucoside in aronia juice are inhibitors against type-2 diabetes-related enzymes, have a synergistic effect on the improvement of type-2 diabetes. Since vicenin-2 and quercetin-diglycoside contained in aronia juice have a lipid accumulation inhibitory effect, suggesting aronia juice may be effective in preventing life-style-related diseases.

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