Food Bioscience 34 (2020) 100535

Contents lists available at ScienceDirect

Food Bioscience

journal homepage: www.elsevier.com/locate/fbio

Isolation of HMG-CoA reductase inhibitors from aronia juice

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ARTICLE INFO

Keywords:
Aronia
Aronia melanocarpa
HMG-CoA reductase inhibitor
Petunidin
Delphinidin

ABSTRACT

Daily ingestion of aronia juice is known to reduce serum low density lipoprotein cholesterol levels. However, the mechanism is not clear. In this study, the effect of aronia juice on the activity of human β-hydroxy-β-methylglutaryl-coenzyme A (HMG-CoA) reductase, the enzyme mediating the rate-determining step of cholesterol biosynthesis, was studied. Aronia juice inhibited the reductase activity by 57%. Using reverse-phase chromatography, two fractions (F1 and F2) showing significant inhibitory activity against the reductase were obtained from the juice. Liquid chromatography electrospray ionization tandem mass spectrometry analysis and sugar analysis of these fractions suggested the presence of delphinidin-arabinoside in fraction F1 and petunidin-glycoside-arabinoside in fraction F2, respectively. Since fraction F2 contained only petunidin glycoside as obtained using the total ion chromatogram, petunidin-glycoside-arabinoside seemed to inhibit the activity of HMG-CoA reductase.

1. Introduction

Aronia berries contain polyphenols in high concentrations (Jakobek, Šeruga, Medvidović-Kosanović, & Novak, 2007) and show many functions beneficial for human health such as anti-diabetes (Yamane et al., 2016a), anti-hypertension (Yamane et al., 2016b), anti-hyperlipidemia (Valcheva-Kuzmanova et al., 2007) and anti-hypercholesterolemia (Duchnowicz, Nowicka, Koter-Michalak, & Broncel, 2012). Hypercholesterolemia can lead to atherosclerosis, one of the major causes of cardiovascular diseases (Lu & Daugherty, 2015). Ingestion of aronia berries can result in the reduction of blood low density lipoprotein (LDL)-cholesterol concentration (Ryszawa et al., 2006). However, the mechanism of the cholesterol-reducing effect is unclear.

β-Hydroxy-β-methylglutaryl-coenzyme A (HMG-CoA) reductase is the key enzyme of cholesterol synthesis in the liver (Nicolau, Shefer, Salen, & Mosbach, 1974). Statins have been developed to treat hypercholesterolemia through inhibition of the activity of HMG-CoA reductase (Endo, 1988). Many plant metabolites also inhibit activity of the enzyme. For example, asiatic acid (*Centella asiatica*) (Ramachandran, Saravanan, & Senthilraja, 2014), *n*-octadecanyl-*O*-α-D-glucopyranosyl (6'→1")-*O*-α-D-glucopyranoside (*Ficus virens* bark) (Iqbal et al., 2015), lycopene, chlorogenic acid and naringenin (tomato juice) (Navarro-González, Pérez-Sánchez, Martín-Pozuelo, García-Alonso, & Periago, 2014), epigallocatechin-3-gallate (green tea) (Cuccioloni et al., 2011), and isorhamnetin and piscidic acid (*Opuntia ficus-indica*) (Ressaissi et al., 2017) have been reported to inhibit HMG-CoA reductase. Therefore, it is likely that aronia juice contains secondary metabolites that inhibit HMG-CoA reductase.

To find an inhibitor(s) of HMG-CoA reductase from aronia juice, the metabolites of the juice were separated using reverse-phase chromatography, resulting in two fractions with significant inhibitory activity against the enzyme. Liquid chromatography electrospray ionization tandem mass spectrometry (LC-ESI-MS/MS) and MS^3 analysis and sugar analysis of these two fractions were done to characterize the inhibitors.

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https://doi.org/10.1016/j.fbio.2020.100535
Received 22 March 2019; Received in revised form 22 January 2020; Accepted 22 January 2020
Available online 25 January 2020

2. Materials and methods

2.1. Materials

Aronia berries were harvested in Bulgaria. The fresh juice was bottled and transported to Japan by sea. The bottled aronia juice (brix 21.2) was obtained from Nakagaki Consulting Engineer (Osaka, Japan). The juice was stored at room temperature and used within 6 months after bottling. An HMG-CoA reductase assay kit was purchased from Sigma Aldrich Co. (St. Louis, MO, USA). 3-Methyl-1-phenyl-5-pyrazolne (PMP) was purchased from Nacali Tesque (Kyoto, Japan). D-Glucose, D-galactose, L-rhamnose, D-arabinose, and D-xylose were obtained from Tokyo Chemical Industry (Tokyo, Japan). Petunidin chloride was from Tokiwa Phytochemical (Chiba, Japan). Delphinidine-3-*O*-galactoside chloride was purchased from Extrasynthese (Lyon, France). All other chemicals were of analytical grade and purchased from Wako Pure Chemicals (Osaka, Japan). Pure water (18.2 MΩ cm) was prepared using an ultra-pure water system (RFU685DA, Advantec, Tokyo, Japan) and used throughout the study.

2.2. Chromatographic separation of aronia juice

Aronia juice (300 mL) was applied to a Wakogel 50C18 column (Wako, 200 mL bed volume) preequilibrated with 0.1% aqueous formic acid (solvent A). The column was washed with solvent A extensively, and then the adsorbates were eluted from the column by 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 (Wakogel fractions 1–5). Each dried fraction was weighed and redissolved with 12.5 or 25% aqueous methanol containing 0.1% formic acid to a final concentration of 1–10 mg/mL. Aliquots of the solutions were added to the assay solution for HMG-CoA reductase activity to measure the inhibitory activity.

The most active fraction (Wakogel fraction 4) was further purified using an InertSustain C18 column (2.1 × 150 mm, GL Science, Tokyo, Japan). The mobile phase consisted of solvent A and 90% acetonitrile:10% pure water (v/v) containing 0.1% formic acid (B). The column was developed at a flow rate of 150 μL/min with the following gradient: 0–5 min with 20% B, 5–30 min with 20–60% B, and 30–31 min with 60–90% B. The absorbance at 370 and 450 nm was measured using a multiple wavelength detector (Agilent 1100 series G1315B, Agilent Technologies, Tokyo, Japan). The elution peaks were collected manually and evaporated to dryness (InertSustain fractions 1–12).

2.3. Liquid chromatography mass spectrometry (LC-MS)

The active fractions (InertSustain fractions 8 and 10) were analyzed using LC-MS on the basis of data-dependent MS/MS and MS/MS/MS acquisition. An aliquot of the fraction (5 μL) was injected into an InertSustain C18 column (0.3 × 150 mm) equilibrated with 20% B. The column was developed at a 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 20 ˚C. The eluate was equally split into two fused silica capillary tubes (20 ìm diameter) and one of them was connected to an electrospray ionization tip. The spray voltage was 2.5 kV and the temperature of the transfer tube was 150 ˚C. A mass spectrum of the eluate was first measured between *m/z* 150 and 1000 in the positive ion mode (full scan). Then, the ions with intensity > 1000 (arbitrary units) were subjected to collision-induced dissociation (CID) and the resultant fragment ions were measured (MS/MS scan). Finally, the most intense fragment ion with intensity > 500 was subjected to CID and the fragment ions were measured (MS/MS/MS scan). A syringe-type HPLC pump (HP 711 V Micro-Flow Pump, GL Science) and an ion-trap mass spectrometer (LCQ Fleet, Thermo Fisher Scientific Inc., San Jose, CA, USA) were used. Data-dependent MS^n measurement and the data analysis were done using Xcalibur for LCQ Fleet (Thermo Fisher Scientific Inc.) and default parameters.

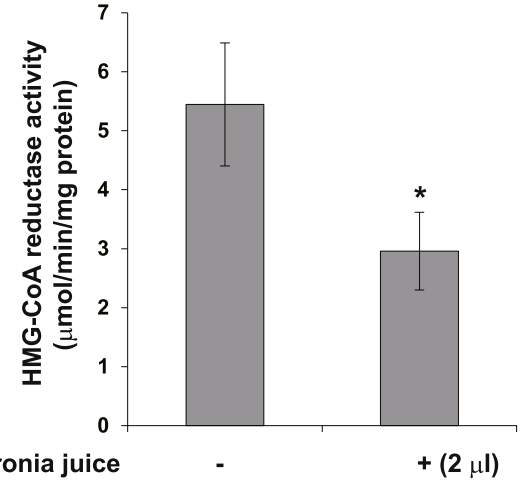

Fig. 1. Effect of aronia juice on HMG-CoA reductase activity. HMG-CoA reductase activity was measured in the presence and absence of aronia juice. Values are means ± S.E (n = 5). Statistically significant: *p < 0.05.

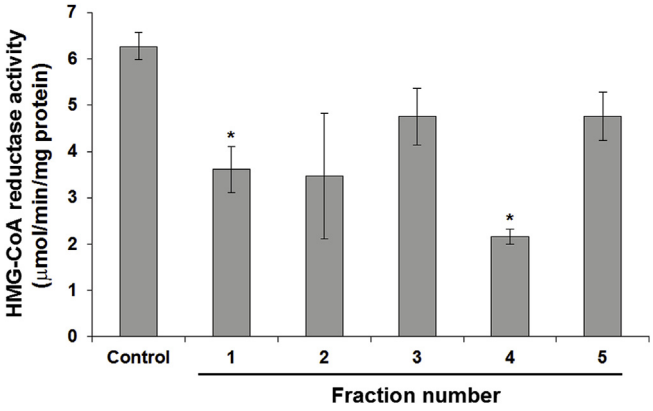

Fig. 2. Inhibitory activity of the chromatographic fractions. Each fraction obtained using Cosmosil 50C18 column chromatography (Wakogel fractions 1–5) was added to the reaction mixture, and the initial velocity was measured. Values are means ± S.E. (n = 5). Statistically significant: *p < 0.05.

2.4. HMG-CoA reductase assay

The inhibitory activity against HMG-CoA reductase was measured using an HMG-CoA reductase assay kit according to the manufacturer's instructions. The enzyme reaction was carried out using a 96-well microplate. Each well contained 100 μL of the reaction mixture with varying amounts of the fractions extracted from aronia juice. The reaction was started by addition of the substrate (HMG-CoA), and the absorbance at 340 nm was measured every 2 min using a microplate reader (SH-1000lab, Corona Electric, Ibaraki, Japan). The initial velocity was calculated using the initial linear portion of the time-dependent decrease in the absorbance at 340 nm and the molar absorption coefficient of $6.22 \times 10^3\ M^{-1}\ cm^{-1}$ (McComb, Bond, Burnett, Keech, & Bowers, 1976).

2.5. Acid hydrolysis of the peak fractions 8 and 10

Acid hydrolysis was carried out according to the method described by Wang, Pu, Wang, Zhang, and Wang (2012). The dried samples, the

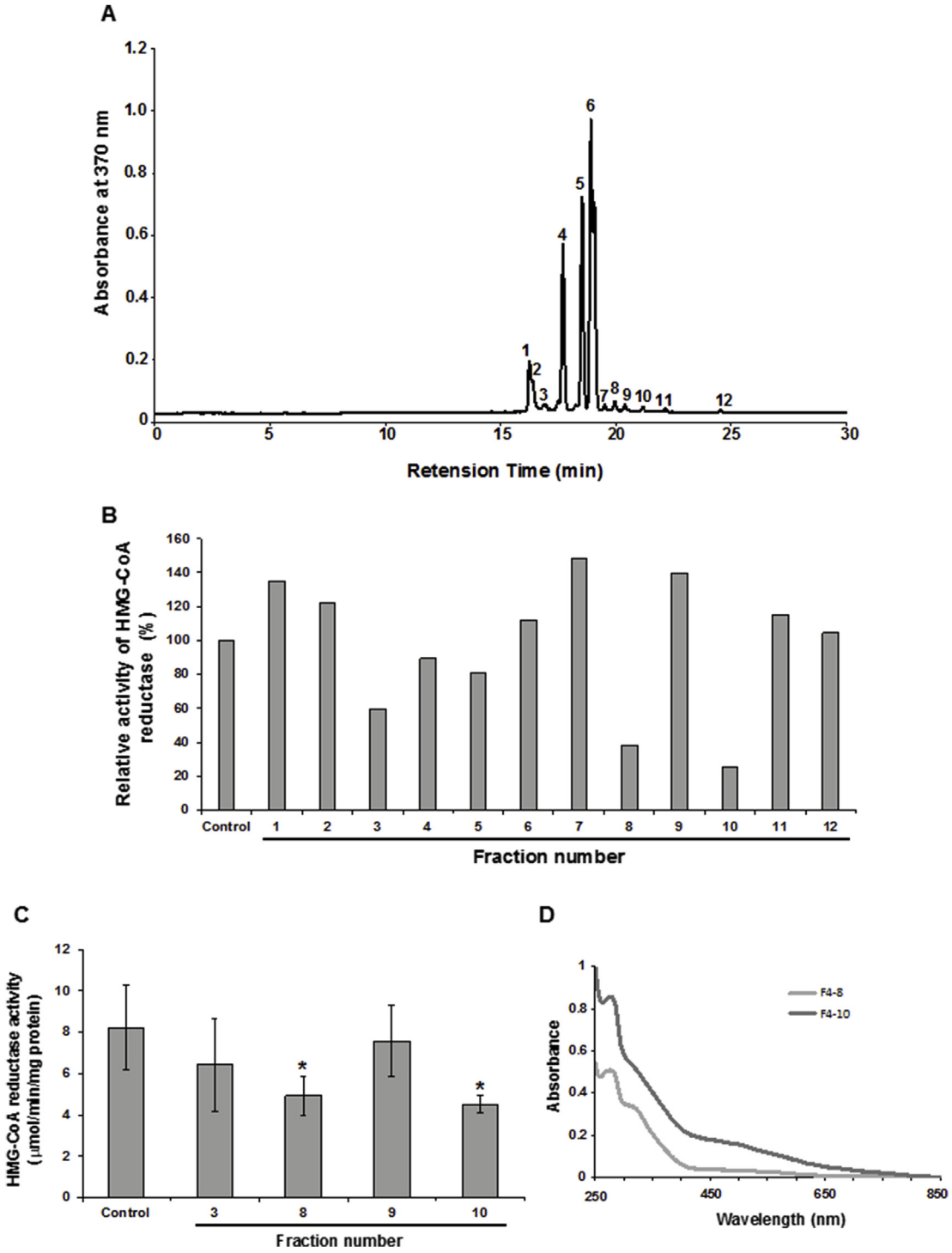

Fig. 3. Chromatographic separation of Wakogel fraction 4 on an InertSustain C18 column. (A) Chromatogram of Wakogel fraction 4. Twelve elution peaks (indicated by numbers) were detected and separately collected (**InertSustain fractions 1–12**). (B) Inhibitory activity against HMG-CoA reductase was assayed for all fractions. The 95% confidence interval of the mean for HMG-CoA reductase activity was calculated to be 90.6–131 (n = 10) except for the activities observed using fractions 8 and 10. The latter two activities were < 62.5, suggesting significant inhibition. (C) Comparison of the inhibitory activities of peak fractions 3, 8, 9 and 10. (D) Absorption spectra of fractions 8 and 10. Each sample was appropriately diluted with 0.1% aqueous formic acid before the measurement. Values are means ± S.E (n = 5). Statistically significant: *p < 0.05.

InertSustain fraction 8 (F1, 300 μg) and the InertSustain fraction 10 (F2, 350 μg), were mixed in a glass vial with 350 μL of 2 M HCl at 90 ˚C for 2 h. After the incubation, ethyl acetate (350 μL) was added to extract aglycon. The water layer was transferred to a new 1.5 mL tube and dried *in vacuo* at room temperature (~25 ˚C).

2.6. Qualitative analysis of monosaccharide composition

Derivatization of saccharides with 3-methyl-1-phenyl-5-pyrazolne (PMP) and reverse-phase HPLC of the derivatives was done according to the method described by Liu et al. (2018) with slight modifications. The dried water layer containing the liberated sugars (see section 2.5) was

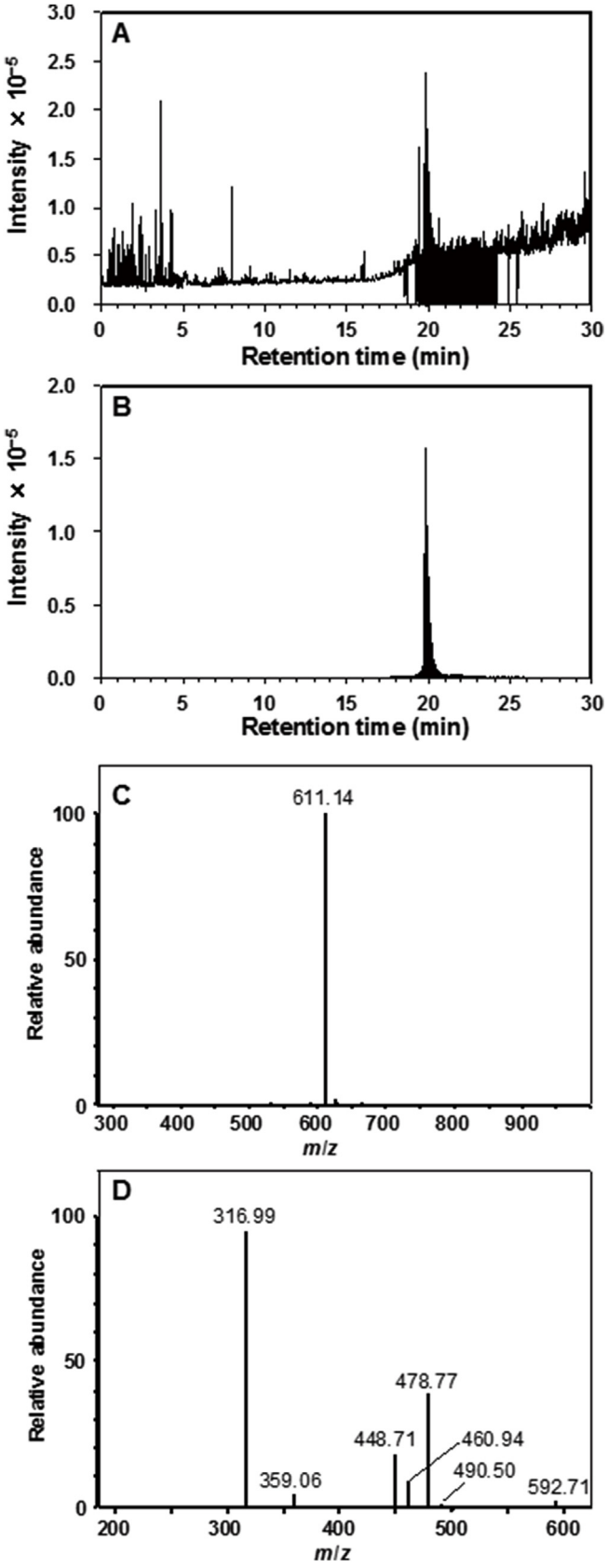

Fig. 4. Running title: Inhibition of HMG-CoA reductase by aronia. (A) Total ion chromatogram. (B) Extracted ion chromatogram of the ion with *m/z* 611.14. (C) Mass spectrum measured at the retention time of 20 min. D. MS/MS spectrum of the ion with *m/z* 611.14.

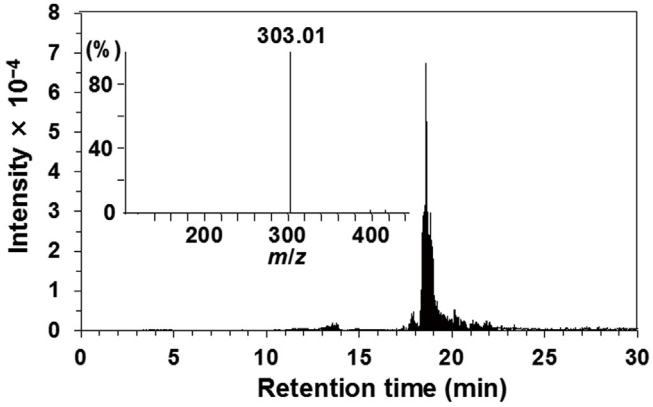

Fig. 5. Chromatogram of the ion with *m/z* 435.0 of F1 (InertSustain fraction 8). The inset shows the MS/MS spectrum of the ion with *m/z* 435.

reconstituted in 100 μL pure water and an aliquot (50 μL) of the solution was mixed with 100 μL of 0.5 M PMP (in methanol) and 50 μL of 0.6 M NaOH. The mixture was incubated at 70 °C for 1 h. After incubation, the mixture was neutralized with 1 M HCl and then 500 μL of chloroform was added to remove the unreacted PMP. This chloroform extraction was repeated three times. The water layer was dried *in vacuo*. The dried PMP derivatives were dissolved in 100 μL pure water. Derivatization of standard monosaccharide solutions (D-Glucose, D-galactose, L-rhamnose, D-arabinose, and D-xylose, 100 μM in pure water) were done as described for the sample.

Analysis of the PMP-labeled sugars was carried out using a Cosmosil 3C18-EB column (2.0 mm ID × 150 mm, Nacali Tesque). The column was developed at a flow rate of 150 μL/min and 20 °C using the following time program: 0–5 min, 19% B; 5–30 min, linear increase in B from 19 to 30%; 31–36 min, 90% B. Solvent A was 50 mM ammonium acetate, and solvent B was 90% acetonitrile:10% water (v/v). The elution of PMP-sugars was monitored by measuring the absorbance at 245 nm. An aliquot of appropriately diluted sample (20 μL in water) was injected into the column.

2.7. UV–vis absorption spectroscopy

UV–vis absorption spectra were measured using a UV–vis spectrophotometer (UV-2550, Shimadzu, Kyoto, Japan) equipped with a micro cell holder.

2.8. Statistical analysis

Data are expressed as means ± S.E. Statistical analyses were done using analysis of variance (one-way ANOVA) followed by the unpaired Student's *t*-test. For comparison of multiple samples, the Tukey-Kramer test was used. Statistical significance was evaluated at $p < 0.05$. Statistical analyses were done using Statcel4 (OMS Publishing Inc., Tokyo, Japan).

3. Results

3.1. Isolation of compounds with inhibitory activities against HMG-CoA reductase from aronia juice

As shown in Fig. 1, aronia juice inhibited HMG-CoA reductase activity by about 57%. Preparative reverse-phase chromatography of the juice resulted in 5 fractions (Wakogel fractions 1–5). Of the 5 fractions, fraction 4 inhibited the enzyme most strongly (63% inhibition, Fig. 2). Wakogel fraction 4 was subjected to HPLC and 12 fractions were obtained (Fig. 3A, InertSustain fractions 1–12). InertSustain fractions 8 and 10 significantly inhibited the enzyme (Fig. 3C). Hereafter, for

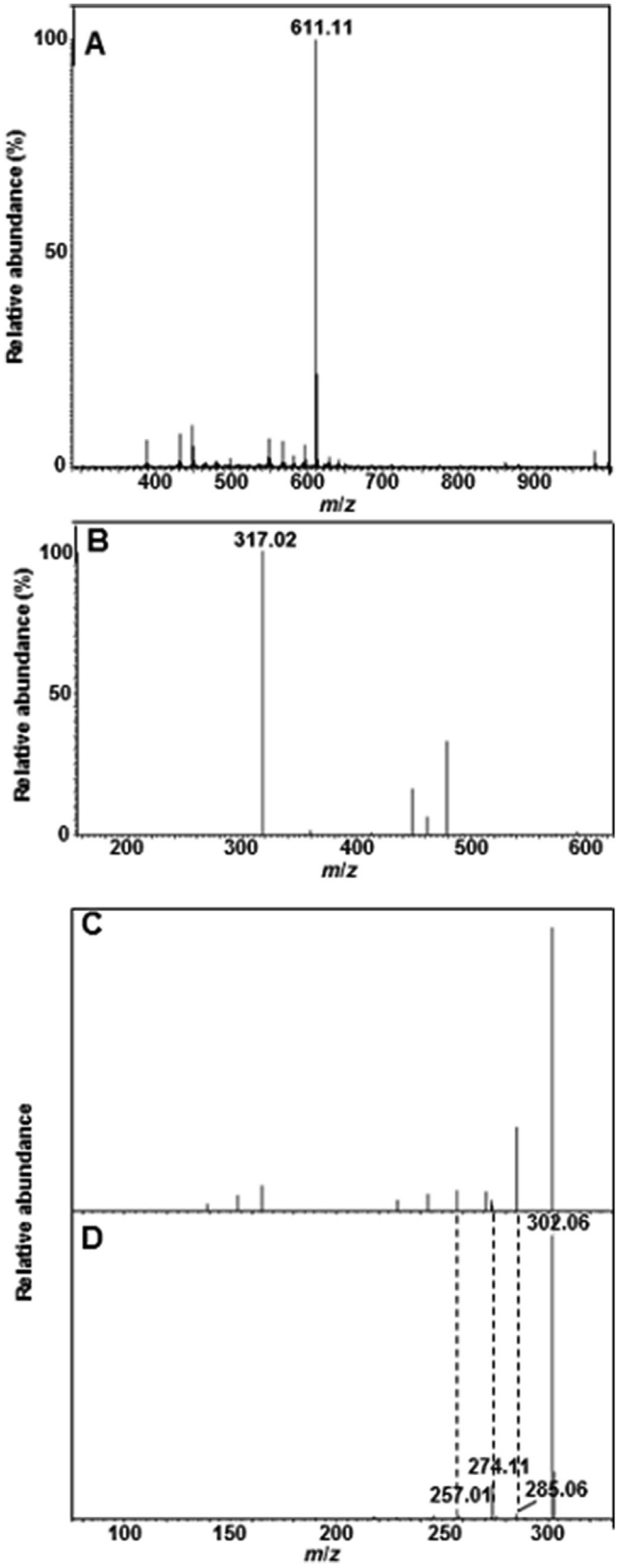

Fig. 6. The positive ESI-CID-MS/MS/MS spectrum of F2 in comparison with the positive ESI-MS/MS spectrum of petunidin. The product ion (*m/z* 317.02, B) obtained using CID from the ion (*m/z* 611, A) detected in F2 was subjected to CID to give the ions shown in C. (D) Positive ESI-MS/MS spectrum of petunidin.

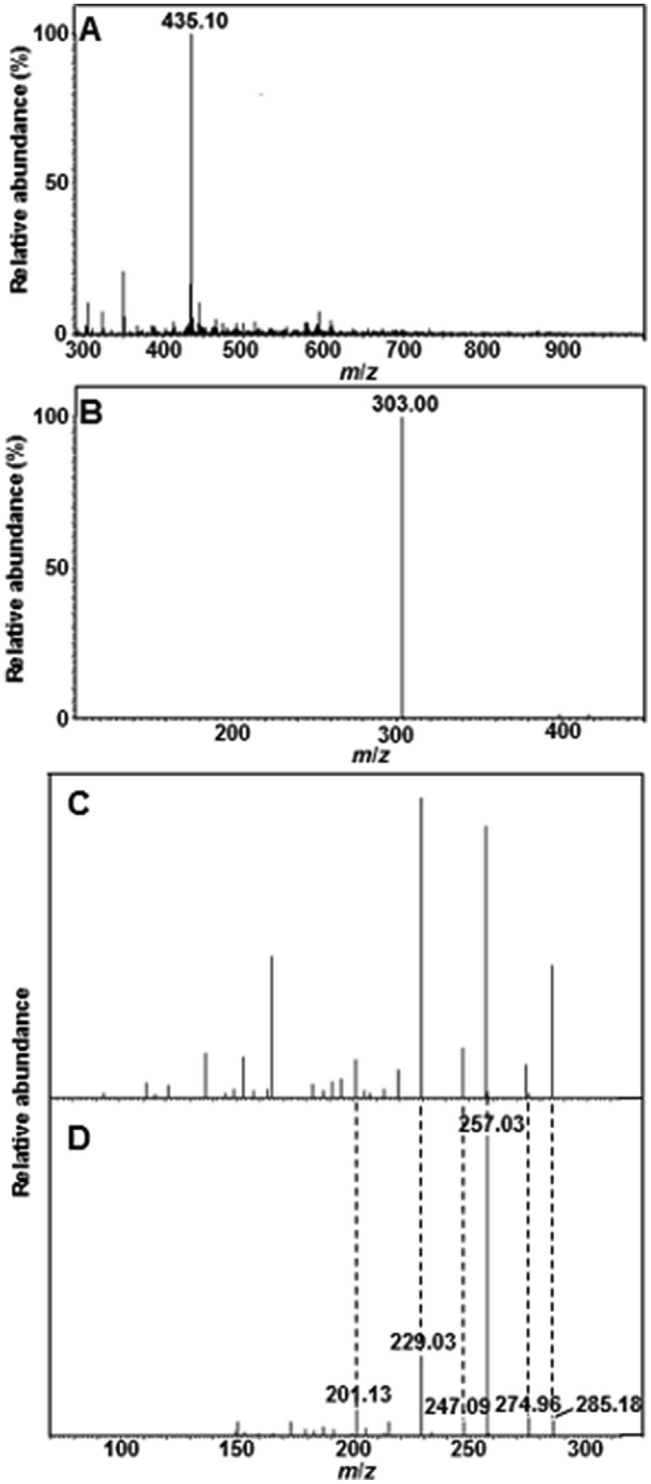

Fig. 7. The positive ESI-CID-MS/MS/MS spectrum of a F1 component in comparison with the positive ESI-MS/MS/MS spectrum of delphinidin 3-O-galactoside. The product ion (*m/z* 303.00, B) obtained using CID from the ion (*m/z* 435, A) detected in F1 was subjected to CID to give the ions shown in C. (D) The product ion (*m/z* 303) obtained using CID of the delphinidin 3-*O*-galactoside ion was subjected to CID.

clarity, the fractions 8 and 10 are called F1 and F2, respectively.

The absorption spectrum of F1 had three peaks at 276, 318 and 494 nm, whereas that of F2 had two peaks at 278 and 494 nm and a shoulder around 320 nm (Fig. 3D).

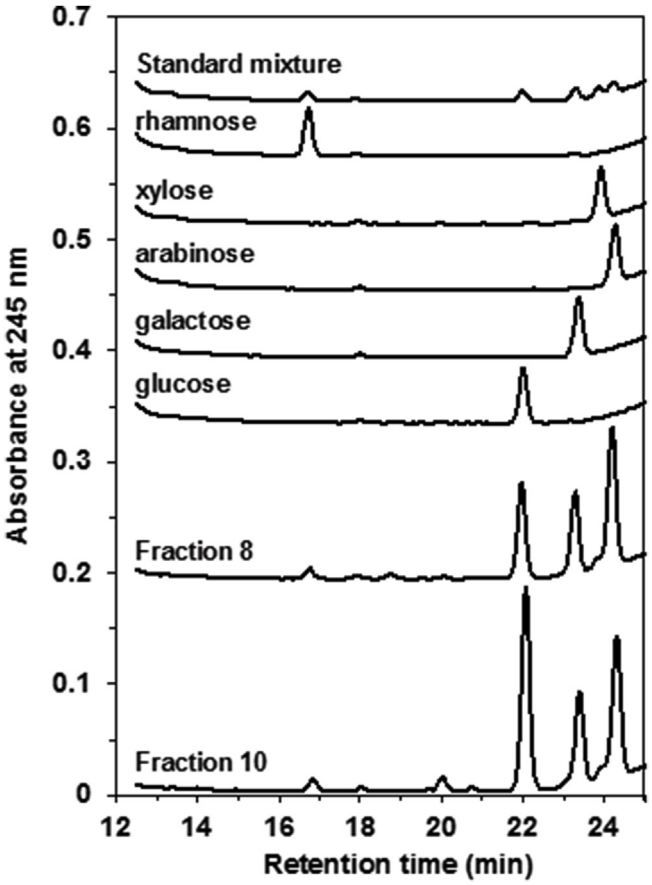

Fig. 8. HPLC profiles of PMP derivatives of standard monosaccharides and the sugars liberated from the peak fractions 8 and 10.

3.2. Mass spectrometric characterization of the inhibitors

F2 was analyzed using LC-MS. The total ion chromatogram and the extracted chromatogram for the ion with *m/z* of 611.14 are shown in Fig. 4A and B, respectively. The MS/MS spectrum of this ion showed a strong ion with *m/z* of 316.99. The difference in the mass of 294 suggested the loss of one hexose and one pentose moieties from the ion with *m/z* of 611.14. There are two known aglycons that can produce the positive ion with *m/z* of 317. One is isorhamnetin and its protonated ion ($[M + H]^+$) has a calculated mass of 317.07. Another is petunidin. Petunidin is cationic and has a calculated mass (M^+) of 317.05. Isorhamnetin forms lightly yellow crystals (Wang et al., 2012) and its solution shows a faintly yellow color. Because the F2 solution showed a strong wine-red color (Fig. 3D), it is unlikely that F2 contains isorhamnetin as an aglycon. Thus, the mass spectrum of the ions produced by collision-induced dissociation (CID) from the F2 aglycon ion with *m/z* of 317 (Fig. 6C) was compared with that from petunidin (Fig. 6D). The fragment ions detected for petunidin were also found in the corresponding spectrum obtained for F2.

As shown in Fig. 5, F1 contained a component with *m/z* of 435, and CID of this ion produced a strong fragment ion with *m/z* of 303.00. The difference in the mass of 132 suggested the loss of one pentose moiety from the ion with *m/z* of 435. Because F1 solution showed a strong red-wine color very similar to that of F2 solution, the fragment ion observed is likely to be delphinidin, which has a calculated mass (M^+) of 303.05. Therefore, the ESI-MS/MS/MS spectrum of this F1 component was compared with that of delphinidin-3-*O*-arabinoside (Fig. 7). The fragment ions detected for delphinidin-3-*O*-arabinoside were also found in the corresponding spectrum obtained for the F1 component.

3.3. Sugar composition of the inhibitors

The sugar species contained in F1 and F2 are shown in Fig. 8. F2 contained glucose and galactose. The amount of glucose was about two-fold greater than that of galactose. F1 contained nearly the same amounts of glucose and galactose. As for pentose, both F1 and F2 only contained arabinose.

4. Discussion

The study showed that aronia juice inhibited HMG-CoA reductase activity. In previous studies, administration of aronia juice was shown to reduce the blood LDL-cholesterol level in humans and mice (Xie et al., 2017; Yamane et al., 2016b). These results and the results of the present study indicated that a compound(s) of aronia juice reduces hypercholesterolemia through inhibition of HMG-CoA reductase activity.

F2 showed a single ion in LC-MS analysis with a mass of 611 and a 317-Da polyphenol moiety. Recent comprehensive profiling of phenolic compounds in berry plants by Tian et al. (2017) has shown the presence of isorhamnetin-hexoside-pentoside and isorhamnetin-pentoside-hexoside in aronia berries. These can produce $[M+H]^+$ ion with m/z of 611 using ESI, and a protonated fragment ion of 317. However, as shown in Fig. 3D, F2 solution showed a strong wine-red color, unlike the faintly yellow color of isorhamnetin. Furthermore, the fragment ions detected for petunidin were also found for the polyphenol fragment of F2 (Fig. 6). Sugar composition analysis showed that F2 contained glucose or galactose and arabinose (Fig. 8). Therefore, the HMG-CoA reductase inhibitor purified from the juice (F2, peak 10 in Fig. 3A) is probably the petunidin-glycoside-arabinoside, although the exact chemical structure of the inhibitor remains to be determined.

Petunidin glycosides are found in Leguminosae (*Pueraria lobata*) (Tatsuzawa, Tanikawa, & Nakayama, 2017) and tomato (*Solanum lycopersicum*) (Ooe et al., 2016). Red grape juice with petunidin glycoside upregulated LDL receptor activity in HepG2 cells (Dávalos et al., 2006).

F1 contained many compounds and most of them could not be identified. However, one compound (Fig. 5) was potentially identified as delphinidin-arabinoside. Delphinidin 3-O-arabinoside is contained in blueberry (*Vaccinium ashei*) wine pomace (He et al., 2016).

5. Conclusions

Aronia juice inhibited HMG-CoA reductase activity and LC-ESI-MS/MS analysis of fractions indicated the presence of petunidin-glycoside-arabinoside and delphinidin-arabinoside as HMG-CoA reductase inhibitors. The mechanism of the cholesterol-reducing effect by HMG-CoA reductase inhibitors of aronia juice might benefit from a study using an obesity model.

CRediT authorship contribution statement

Miyuki Kozuka: Investigation, Writing - original draft. **Takuya Yamane:** Conceptualization, Investigation, Writing - original draft. **Momoko Imai:** Investigation. **Satoshi Handa:** Investigation. **Shigeo Takenaka:** Supervision. **Tatsuji Sakamoto:** Supervision. **Tetsuo Ishida:** Investigation, Writing - review & editing. **Hiroshi Inui:** Supervision. **Yoshio Yamamoto:** Supervision. **Takenori Nakagaki:** Resources. **Yoshihisa Nakano:** Supervision.

Declaration of competing interest

The authors declare no conflicts of interest.

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