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Caffeoylquinic acids from aronia juice inhibit both dipeptidyl peptidase IV and α-glucosidase activities

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ABSTRACT

Aronia (*Aronia melanocarpa*) 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. In type 2 diabetes and obesity model KKA^{y} mice, drinking water supplemented with aronia juice reduced blood glucose levels partly through inhibition of dipeptidyl peptidase IV (DPP IV) activity by cyanidin-3,5-diglucoside. The objective of this study was to find α-glucosidase inhibitors in aronia juice. Polyphenols in aronia juice were first separated using flash chromatography on a reversed-phase column. The fraction with 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.

1. Introduction

Aronia berries (*Aronia melanocarpa*) contain many polyphenols (Jakobek, Šeruga, Medvidović-Kosanović, & Novak, 2007; McDougall, 2017) and the freshly prepared juice contains much higher amounts of sorbitol and polyphenols compared to other berries (Kulling & Rawel, 2008). The chemical composition of aronia berries has been recently reviewed (Sidor & Gramza-Michałowska, 2019).

Aronia berries have been used as a traditional medicine in Russia and eastern Europa (Kokotkiewicz, Jaremicz, & Luczkiewicz, 2010). As Alessandra Durazzo et al. recently reviewed (Durazzo et al., 2019), 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 (Bhaswant, Shafie, Mathai, Mouatt, & Brown, 2017; Oprea, Manolescu, Fărcăşanu, Mladin, & Mihele, 2014; Qin & Anderson, 2012; Simeonov et al., 2002; Valcheva-Kuzmanova, Kuzmanov, Tancheva, & Belcheva, 2007; Yamane et al., 2016a; Yamane et al., 2017a), hypertension (Yamane et al., 2017b), hyperlipidemia (Yamane et al., 2016b) and hypercholesterolemia (Duchnowicz, Nowicka, Koter-Michalak, & Broncel, 2012). In human study, aronia juice potently modulated hyperglycemia-related oxidative stress in a beneficial manner (Banjari et al., 2017), 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 (Rahmani et al., 2019).

Prevention of the onset of lifestyle disease is an important challenge and nutraceuticals play essential roles in proactive medical approach (Santini & Novellino, 2017). Nutraceuticals are proposed to have a pharmacological effect in addition to their nutritional value. Importantly, their beneficial health properties must be clinically proven

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(Santini, Tenore, & Novellino, 2017). 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.

The International Diabetes Federation reported that type 2 diabetes mellitus has become a public challenge for many countries (International Diabetes Federation, 2017). α-Glucosidase is an important intestinal enzyme that is responsible for carbohydrates digestion (Pyner, Nyambe-Silavwe, & Williamson, 2017). Inhibitors of this enzyme reduce intestinal glucose absorption to decrease postprandial blood glucose levels. On the other hand, dipeptidyl peptidase IV (DPP IV) inactivates intestinal peptide glucagon-like peptide 1 (GLP-1) and gastric inhibitory peptide (GIP) (Sterrett. Bragg, & Weart, 2016; Tasyurek, Altunbas, Balci, & Sanlioglu, 2014). 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 Raquel Vieira et al. (Vieira et al., 2019).

Daily ingestion of aronia juice reduced blood glucose and HbA1c levels in KKA^{y} mice, a mouse model of type-2 diabetes and obesity, and that this effect was due to inhibitors against α-glucosidase and DPP IV contained in the juice (Yamane et al., 2016a, 2019). Cyanidin-3,5-diglucoside, cyanidin 3-*O*-glucoside, quercetin and cyanidin have been identified as DPP IV inhibitors in aronia juice (Kalhotra, Chittepu, Osorio-Revilla, & Gallardo-Velázquez, 2018; Kozuka et al., 2015; Yamane, 2018, chap. 8; Yamane et al., 2019). However, α-glucosidase inhibitors have not been isolated from aronia juice.

In this study, two α-glucosidase inhibitors were purified from aronia juice using reverse-phase chromatography. The inhibitors were identified to be 3-caffeoylquinic acid (3-CQA) and 4-caffeoylquinic acid (4-CQA). 3-CQA and 4-CQA also showed DPP IV inhibitory activity.

2. Materials and methods

2.1. 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). Glycyl-L-proline 4-methylcoumaryl-7-amid (Gly-Pro-MCA) was purchased from Peptide Institute (Osaka, Japan). α-Glucosidase and *p*-nitrophenyl-α-D-glucopyranoside (PNP-glycoside) were purchased from Sigma-Aldrich (MO, USA). DPP IV was purified from porcine seminal plasma (Ohkubo, Huang, Ochiai, Takagaki, & Kani, 1994). All other chemicals were of analytical grade and purchased from Wako Pure Chemicals (Osaka, Japan).

2.2. 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 (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 (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.

2.3. α-Glucosidase assay

α-Glucosidase activity was measured using *p*-nitrophenyl-α-D-glucopyranoside (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 using a microplate reader (SH-1000 Lab, Corona Electric) and absorbance at 405 nm.

2.4. DPP IV assay

DPP IV activity was measured using Gly-Pro-MCA as substrate. The reaction mixture (990 μL) contained 50 mmol/L Tris–HCl (pH 9.0), 100 μmol/L Gly-Pro-MCA and 5 μL of enzyme solution. 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).

2.5. 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) preequilibrated with 20% B (80% A). 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 20 °C. The eluate was equally split into two fused silica capillary tubes (20 μmol/L 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.

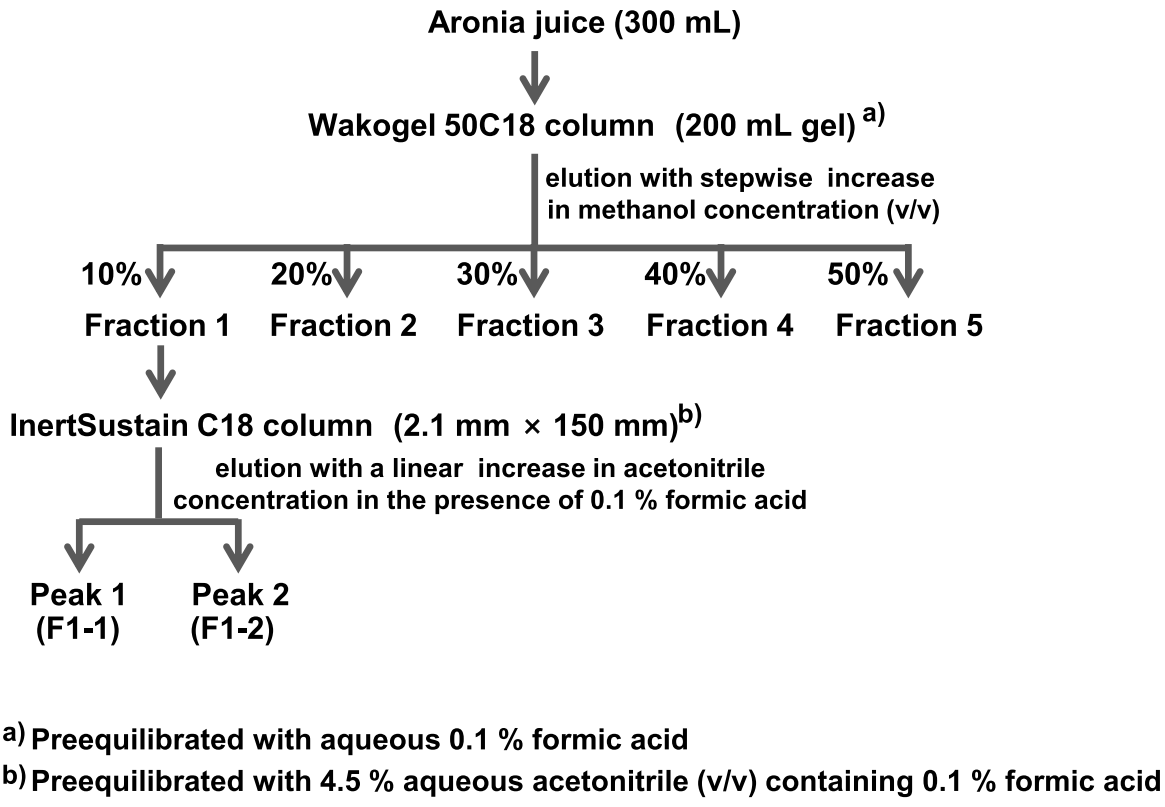
2.6. Statistical analysis

Data are expressed as means ± S.E. Statistical analyses were performed using analysis of variance (one-way ANOVA) followed by unpaired Student's *t*-test. For comparison of multiple samples, the Tukey-Kramer test was used.

3. Results

3.1. 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. 1A). To further isolate inhibitors, this fraction was applied to a semi-preparative high performance liquid chromatography column (Scheme 1). As shown in Fig. 1B, two major peaks (F1-1 and F1-2) appeared. These two peak



Scheme 1. Purification of α-glucosidase inhibitors from aroniajuice

fractions showed similar absorption spectra with an absorption peak at 324 nm and a shoulder at 300 nm (Fig. 2D), indicating that the two fractions have the same chromophore.

3.2. Mass spectrometric characterization of the inhibitors

Fig. 2A 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. 2A). The mass spectrum of F1-2 (Fig. 2B) 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. 3), the results strongly suggested that the purified inhibitors are caffeoylquinic acids (Fang, Yu, & Prior, 2002; Ncube et al., 2014; Xie et al., 2011).

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. 2C). 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. 3. 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.

3.3. DPP IV inhibitory activities of fraction 1 and caffeoylquinic acids

Because aronia juice contains DPP IV inhibitors (Yamane et al., 2016a, 2019), DPP IV inhibitory activity was examined for Fraction 1 and authentic caffeoylquinic acids (3-CQA and 4-CQA). As shown in Fig. 4A, Fraction 1 inhibited DPP IV activity. In addition, both 3-CQA and 4-CQA inhibited DPP IV activity dose-dependently (Fig. 4B). IC_{50} values of 3-CQA and 4-CQA were 0.19 and 0.05 μmol/L, respectively.

4. Discussion

Aronia juice inhibits both α-glucosidase and DPP IV (Yamane et al., 2016a). Cyanidin-3,5-diglucoside has been identified to be a DPP IV inhibitor contained in aronia juice. However, a strong inhibitory effect of aronia juice on elevation of blood glucose and HbA1c levels in model mice could not be attributed to the effect of cyanidin-3,5-diglucoside alone (Yamane et al., 2019). 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. (Tian et al., 2017) 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 (Naveed et al., 2018). In rats fed a standard meal, the elevation of blood glucose level was reduced when caffeoylquinic acids were added to the meal (Tunnicliffe, Eller, Reimer, Hittel, & Shearer, 2011). 3-CQA, 4-CQA and 5-CQA from Kuding Tea (*Ilex kudingcha C.J. Tseng*) inhibit α-glucosidase activity (Xu et al., 2015). 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 (Xu et al., 2015). Because caffeoylquinic acids have too weak α-glucosidase inhibitory activity to explain their anti-diabetic effects, it is suggested that caffeoylquinic acids has another mechanisms to show anti-diabetic effects (Nyambe-Silavwe & Williamson, 2018). Significant but weak inhibitory activity against α-glucosidase was confirmed in the present study (Fig. 1A).

Fraction 1 inhibited DPP IV (Fig. 4A) and IC50 of 3-CQA and 4-CQA against the enzyme were determined to be 0.19 and 0.05 μmol/L, respectively (Fig. 4B). Hispidulin, eriodictyol, naringenin and cirsimaritin inhibit DPP IV with respective IC50 of 0.49, 10.9, 2.5 and 0.43 μmol/L (Bower, Real Hernandez, Berhow, & de Mejia, 2014). DPP IV inhibitory activities of CQAs, especially 4-CQA, are stronger than those

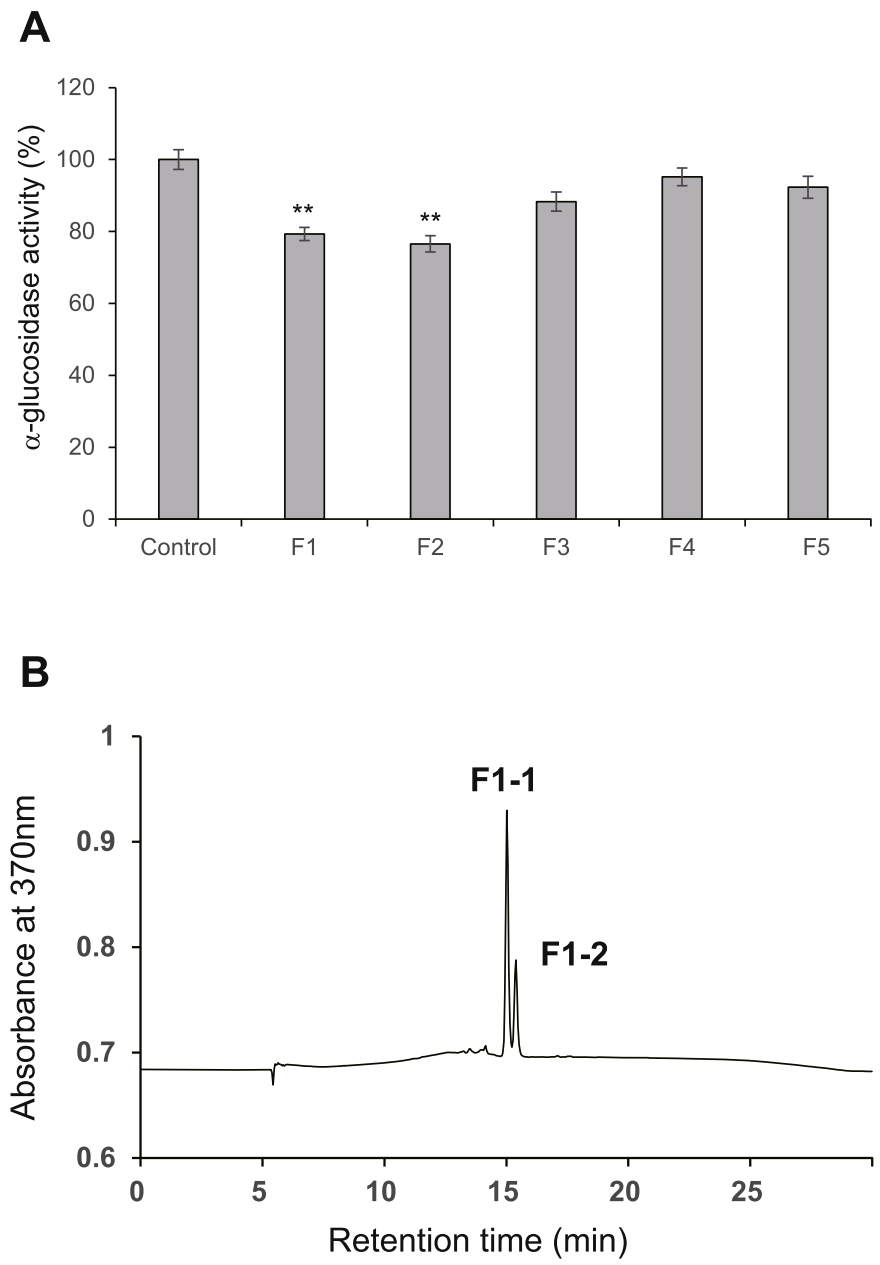


Fig. 1. Purification of α-glucosidase inhibitors from aronia juice.
A. Inhibition of α-glucosidase activity. Each five fractions obtained using a Wakogel 50C18 column (Scheme 1) 330 μg was added to the α-glucosidase standard assay solution, respectively. Values are means ± S.E (n = 5). **p < 0.01. B.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

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.

5. Conclusion

Two α-glucosidase inhibitors were purified from aronia juice. These inhibitors were identified to be 3- and 4-caffeoylquinic acids. Notably, both 3-CQA and 4-CQA strongly inhibited DPP IV. Beneficial effects of aronia juice on type 2 diabetes mellitus are suggested to be due to the inhibition of both α-glucosidase and DPP IV by CQAs contained in the juice.

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CRediT authorship contribution statement

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

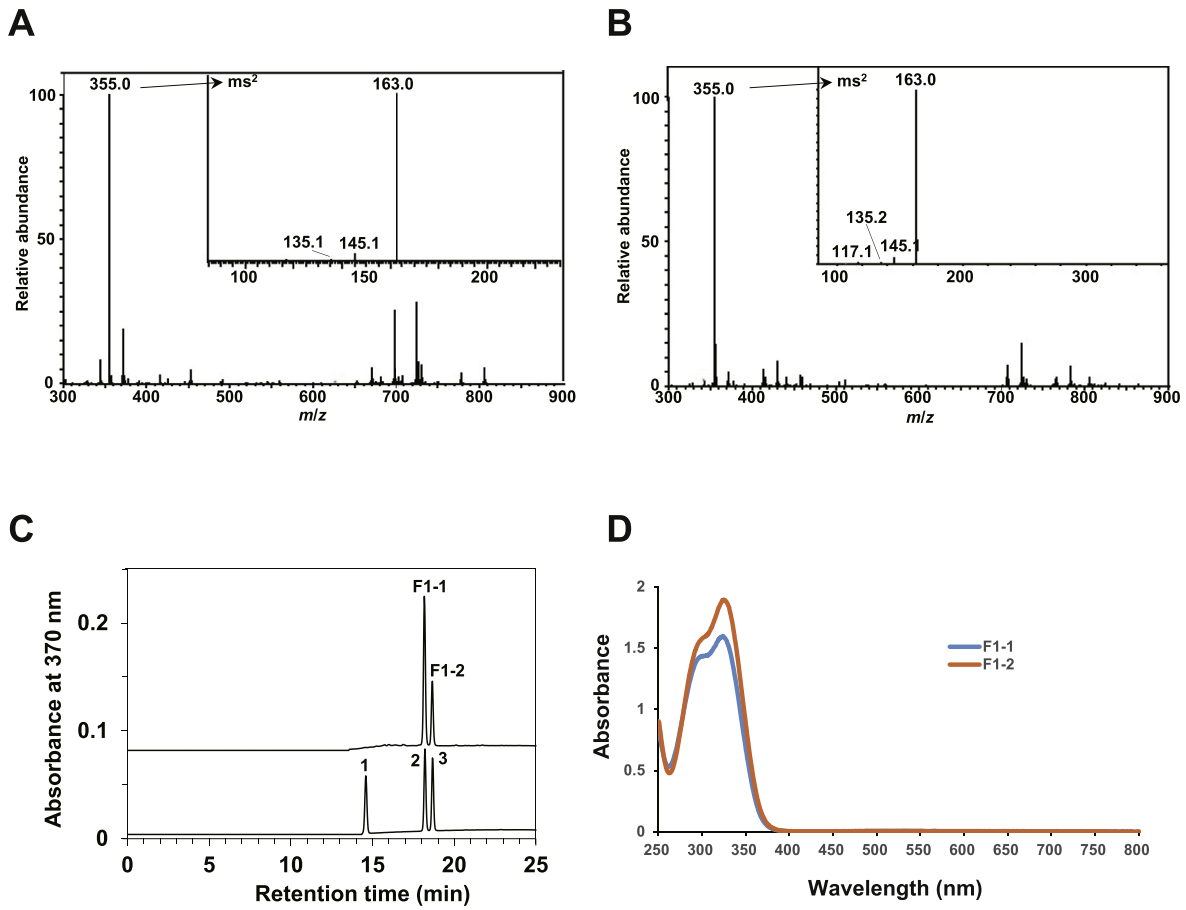

Fig. 2. Characterization of the purified inhibitors (F1-1 and F1-2).
A. Mass spectrum of F1-1. The inset shows the MS/MS spectrum of the ion with *m/z* 355.0. B. Mass spectrum of F1-2. The inset shows the MS/MS spectrum of the ion with *m/z* 355.0. C. Comparison of the elution profiles of F1-1 and F1-2 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).

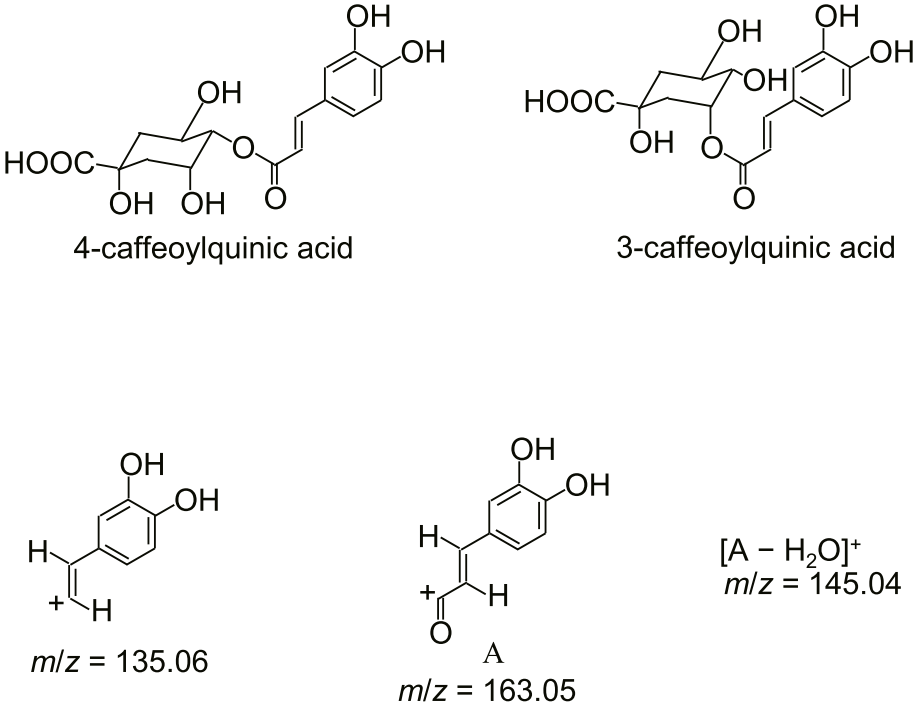

Fig. 3. Chemical structures of 3-CQA and 4-CQA and thei fragment ions.

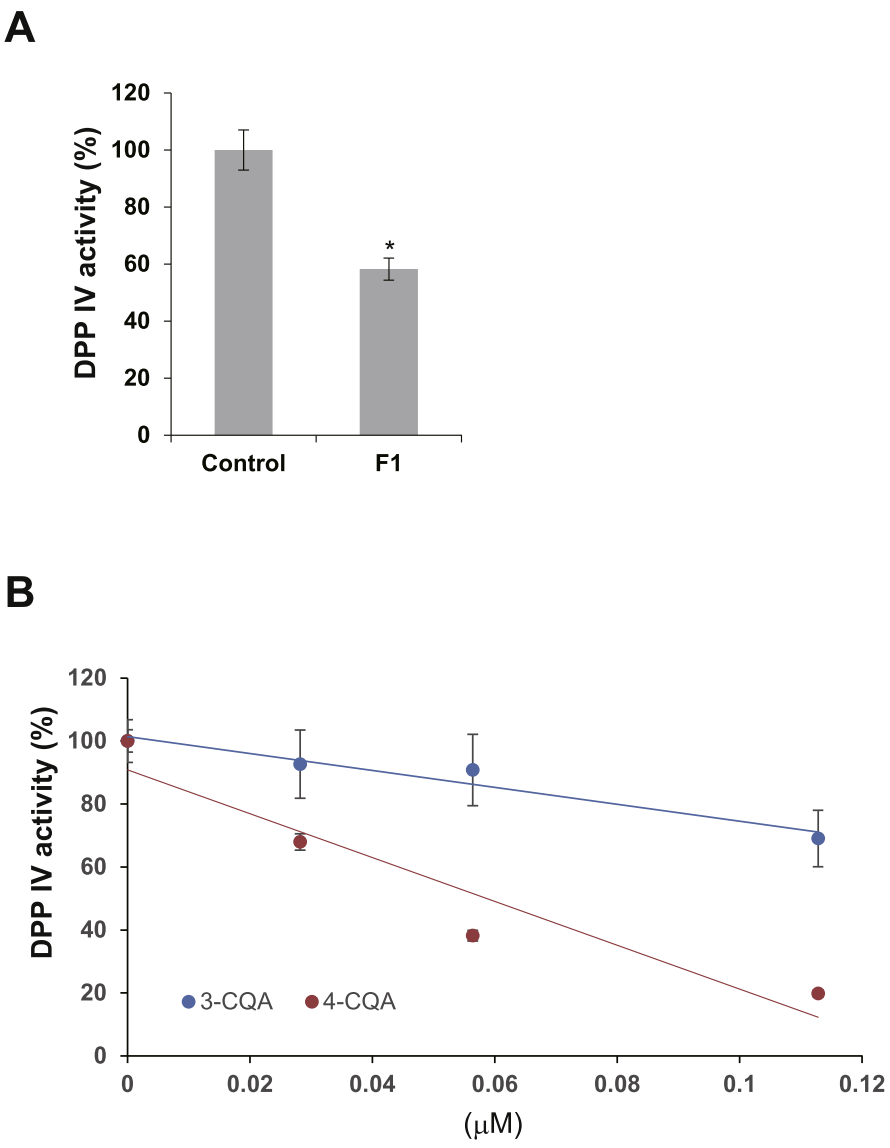

Fig. 4. Inhibition of DPP IV by F1 and CQAs
F1 (A), 3-CQA and 4-CQA (B) were added to the DPP IV standard assay solution, respectively. Values are means ± S.E. (n = 5). *p < 0.05.

Declaration of competing interest

The authors have declared that no competing interests exist.

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