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| Title | Identification and characterization of a dipeptidyl peptidase IV inhibitor from aronia juice |
| Author(s) | Kozuka, Miyuki; Yamane, Takuya; Nakano, Yoshihisa et al. |
| Citation | Biochemical and biophysical research communications, 465(3), 433-436<br>https://doi.org/10.1016/j.bbrc.2015.08.031 |
| Issue Date | 2015-09-25 |
| Doc URL | https://hdl.handle.net/2115/62868 |
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| Type | journal article |
| File Information | manuscript.pdf |

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**Identification and characterization of a dipeptidyl peptidase IV inhibitor from aronia juice**

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**Competing Interests**: The authors have declared that no competing interests exist.

Short title: DPPIV inhibitor from aronia juce

**Abstract**

Aronia berries have many potential effects on health, including an antioxidant effect, effect for antimutagenesis, hepatoprotection and cardioprotection, an antidiabetic effect and inhibition of cancer cell proliferation. Previous human studies have shown that aronia juice may be useful for treatment of obesity disorders. In this study, we found that aronia juice has an inhibitory effect against dipeptidyl peptidase IV (DPP IV) (EC 3.4.14.5). DPP IV is a peptidase that cleaves the N-terminal region of incretins such as glucagon-dependent insulinotropic polypeptide (GIP) and glucagon-like peptide-1 (GLP-1). Inactivation of incretins by DPP IV induces reduction of insulin secretion. Furthermore, we identified that cyanidin 3, 5-diglucoside as the DPP IV inhibitor in aronia juice. DPP IV was inhibited more strongly by cyanidin 3, 5-diglucoside than by cyanidin and cyanidin 3-glucoside. The results suggest that DPP IV is inhibited by cyanidin 3, 5-diglucoside present in aronia juice. The antidiabetic effect of aronia juice may be mediated through DPP IV inhibition by cyanidin 3, 5-diglucoside.

**Key Words**: aronia juice; DPP IV inhibitor; cyanidin 3, 5-diglucoside; antidiabetic effect

## 1. Introduction

Aronia berries have various potential health effects, including an antioxidant effect by radical scavenging activity, antimutagenesis by phenolic compounds, hepatoprotection by anthocyanins, which decrease the toxicity and accumulation of cadmium, cardioprotection in men with mild hypercholesterolaemia, antidiabetic effect, and inhibition of colon cancer cell proliferation [1]. Aronia juice has been shown to have a beneficial effect on plasma glucose level in diabetic humans [2] and rats [3]. However, its mechanism is unknown.

Dipeptidyl peptidase IV (DPP IV) (EC 3.4.14.5) is a serine peptidase [4] that cleaves the N-terminal region of incretins such as glucagon-dependent insulinotropic polypeptide (GIP) and glucagon-like peptide-1 (GLP-1), and reduction of insulin secretion is induced by inactivation of incretin by DPP IV [5-8]. DPP IV inhibitors have beneficial effects on plasma glucose level in diabetic patients [9]. DPP IV inhibitors have also been found in several plants [10].

In this study, we found that aronia juice has an inhibitory effect on DPP IV. Furthermore, we identified cyanidin 3, 5-diglucoside as the DPP IV inhibitor in aronia juice. DPP IV was inhibited more strongly by cyanidin 3, 5-diglucoside than by cyanidin and cyanidin 3-glucoside. The antidiabetic effect of aronia juice may be mediated through DPP IV inhibition by cyanidin 3, 5-diglucoside.

## 2. Materials and Methods

### *2.1. Materials*

Aronia juice was kindly provided by Nakagaki Consulting Engineer (Osaka, Japan). Gly-Pro-MCA was purchased from Peptide Institute (Osaka, Japan). DPP IV was

purified from porcine seminal plasma [4]. Supel Sphere Carbon/$NH_2$ SPE Cartridge, InertSustain C18 column and ACQUITY UPLC M-Class HSS T3 column were obtained from SUPELCO (PA, USA), GL Sciences (Tokyo, Japan) and Waters (MA, USA), respectively. Cyanidin and cyanidin 3-glucoside were purchased from TOKIWA PHYTOCHEMICAL (Chiba, Japan) and EXTRASYNTHESE (Cedex, France), respectively. Cyanidin 3, 5-diglucoside was obtained from Sigma-Aldrich (MO, USA). All other chemicals were of analytical grade and purchased from Wako Pure Chemicals (Osaka, Japan).

*2.2. Assay of proteolytic activity*

Enzyme activity was measured by fluorometrical determination (excitation, 380 nm; emission, 440 nm) of the liberation of AMC at 37°C in a mixture containing 10 μl of 10 mM substrate, 100 μl of 0.5 M Tris-HCl buffer (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.

*2.3. Identification of a DPP IV inhibitor*

All fractionation steps were performed at room temperature unless otherwise specified. At each step, the inhibitory activity of DPP IV was measured in 50 mM Tris-HCl buffer (pH 9.0) using Gly-Pro-MCA as a substrate.

Step 1. Supel Sphere Carbon/$NH_2$ SPE chromatography

Aronia juice was applied at a flow rate of 5 ml/h to a Supel Sphere Carbon/$NH_2$ SPE Cartridge (bed volume: 6 ml) that had been previously equilibrated with 50 mM phosphate buffer (pH 7.0). After pass-through fractions had been collected, the column

was washed extensively with ethanol and then eluted with a stepwise gradient of 50 mM phosphate buffer (pH 7.0) containing 2.0 M NaCl. Fractions with DPP IV inhibitory activity were subjected to the next step.

Step 2. Reversed-phase column chromatography

The sample solutions were subjected to reversed-phase HPLC on an InertSustain C18 column (4.6 x 150 mm) using a 0-100% acetonitrile/0.1% TFA gradient at a flow rate of 1.0 ml/min. Each peak was evaporated, and peaks containing DPP IV inhibitory activity were subjected to the next step.

Step 3. LC-MS/MS analysis

The sample solutions were subjected to an ACQUITY UPLC M-Class HSS T3 column (75 μm x 150 mm) using an ACQUITY UPLC M-Class system at a flow rate of 5 μl/min. MS/MS experiments were performed using a Xevo G2 QTof (Waters, MA, USA) with an ESI source. MassLynx v4.1 software was used for instrument control and data acquisition. The capillary temperature was 120°C, and the capillary voltage was 3 kV. Mass spectra were recorded between m/z 100 and 1000 in the positive ion mode.

### *2.4. 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. DPP IV inhibitory activity in aronia juice*

To examine DPP IV inhibitory effect in aronia juice, DPP IV activity against a synthetic substrate, Gly-Pro-MCA, was examined. As shown Fig.1A, DPP IV inhibitory activity was observed in aronia juice and the rate of reduction in DPP IV activity was about 27% of that by a vehicle control.

*3.2. Isolation of a DPP IV inhibitor from aronia juice*

To isolate a DPP IV inhibitor(s) from aronia juice, aronia juice was fractionated by column chromatography. As shown in Fig.1B, DPP IV inhibitory activity was observed in the eluted fraction but not in the pass-through fraction from the Supel Sphere Carbon/$NH_2$ SPE Cartridge, and the rate of reduction in DPP IV activity was about 28% of that by a vehicle control. Furthermore, fractions with DPP IV inhibitory activity were fractionated by reversed-phase column chromatography, and four fractions were obtained (Fig. 2A). As shown in Fig. 2B, DPP IV inhibitory activity was observed in fraction 2 and its rate of reduction in DPP IV activity was about 81% of that by the control.

*3.3. Identification of a DPP IV inhibitor in aronia juice*

To identify a DPP IV inhibitor(s), the solution with DPP IV inhibitory activity was subjected to UPLC-Xevo G2 QTof. As shown in Fig. 3A, the molecular mass of the main peak was 635.74, and 451.56 and 289.38 m/z fragment peaks were also obtained from MS/MS peaks of the main peak. Since peaks of 451.56 and 289.38 m/z were identified to be cyanidin 3-gulcoside and cyanidin, respectively, 635.74 and 613.74 m/z peaks were identified to be cyanidin 3, 5-digulcoside (Fig. 3B) according to the literature [11-13].

*3.4. Measurement of IC50 of cyanidin 3, 5-diglucoside*

To examine whether DPP IV enzyme activity is inhibited by cyanidin 3, 5-diglucoside, a DPP IV activity assay was carried out using synthetic Gly-Pro-MCA as a substrate. As shown in Fig. 4A, DPP IV was inhibited by 0.5 μM cyanidin 3, 5-diglucoside but not by cyanidin or cyanidin 3-gulcoside. The IC50 value of cyanidin 3, 5-diglucoside was estimated to be 5.5 μM.

## 4. Discussion

Although aronia juice has been shown to have a beneficial effect on plasma glucose level, its mechanism is unknown. Both GLP-1 and GIP are rapidly inactivated by DPP IV [14]. We found that aronia juice has DPP IV inhibitory activity. DPP IV inhibitors have been found in foods and food protein hydrolysates. DPP IV is inhibited by Pterocarpus marsupium Roxb. (Lguminosae), Agonia cretica L (Zygophllaceae) and Hedera nepalensis K.Koch (Araliaceae) [15, 16]. In this study, a new DPP IV inhibitor, cyanidin 3, 5-diglucoside, was identified. Cyanidin 3, 5-diglucoside in aronia juice fraction was found as the main component to act as a DPP IV inhibitor. It has been reported that phenolic compounds such as cyanidin, cyanidin-3-glucocide, malvidin, luteolin, apigenin, quercetin, kaempferol, hesperetin, naringenin, eriocitrin, genistein, resveratrol, gallic acid and caffeic acid are DPP IV inhibitors in berries and citrus fruit [17]. In food protein hydrolysates, DPP IV inhibitors have been reported to be derivative peptides from atlantic salmon skin, tuna cooking juice, Japanese rice bran, gouda cheese and milk protein [18]. Previous studies have also shown that aronia juice contains anthocyanin glycosides including cyanidin 3-galactoside, cyanidin 3-arabinoside, cyanidin 3-gulcoside and cyanidin 3-xyloside [19-24]. Furthermore,

cyanidin and cyanidin 3-glucoside have been reported to be DPP IV inhibitors [17]. We found that DPP IV activity was inhibited more strongly by cyanidin 3, 5-diglucoside than by cyanidin and cyanidin 3-glucoside, which have been reported to be DPP IV inhibitors. Our results and results of previous studies suggest that cyanidin 3, 5-diglucoside is a new inhibitor present in aronia juice.

**Abbreviations**: DPP IV, dipeptidyl peptidase IV; GIP, glucagon-dependent insulinotropic polypeptide; GLP-1, glucagon-like peptide-1; MCA, methylcumarin amide.

**Acknowledgements**

This work was supported by the Scientific Research Fund from Nakagaki Consulting Engineer.

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**Figure legends**

**Fig.1.** Inhibition of DPP IV by aronia juice.

A. DPP IV inhibitory activity by aronia juice was measured using a synthetic substrate, Gly-Pro-MCA. Values are means ± S.E. n=4 experiments. Statistically significant: $^{***}p<0.001$.

B. DPP IV inhibitory activities in fractions from Spelco column chromatography were measured using a synthetic substrate, Gly-Pro-MCA. Values are means ± S.E. n=4 experiments. Statistically significant: $^{***}p<0.001$. Not significant: n.s.

**Fig. 2.** Separation of the DPP IV inhibitory fraction through column chromatography.

A. Separation of a DPP IV inhibitor using RP-HPLC. Four fractions were obtained. B. Enzyme activities the fractions were measured. Fraction number 2 had inhibitory activity against DPP IV, and its visible color was shown to be red. Values are means ± S.E. n=4 experiments. Statistically significant: $^{***}p<0.001$. Not significant: n.s.

**Fig. 3.** Identification of a DPP IV inhibitor.

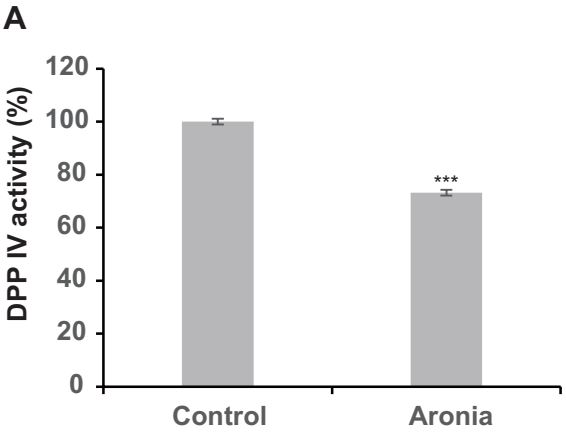
A. LC-MS spectrum of fractions with DPP IV inhibitory activity.

B. MS/MS spectrum of main peaks. Cyanidin 3-glucoside and cyanidin were detected in fragment peaks, and the main peak was identified to be cyanidin 3, 5-diglucoside.

**Fig. 4.** Inhibition of DPP IV by cyanidin, cyanidin 3-glucoside and cyanidin 3, 5-diglucoside.

Inhibitory activities of cyanidin, cyanidin 3-glucoside and cyanidin 3, 5-diglucoside against DPP IV were measured. DPP IV activity was inhibited by about 25% by cyanidin 3, 5-diglucoside but not by cyanidin or cyanidin 3-glucoside. Values are means ± S.E. n=4 experiments. Statistically significant: $^{**}p<0.01$.

Fig.1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100
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60
40
20
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DPP IV activity (%)
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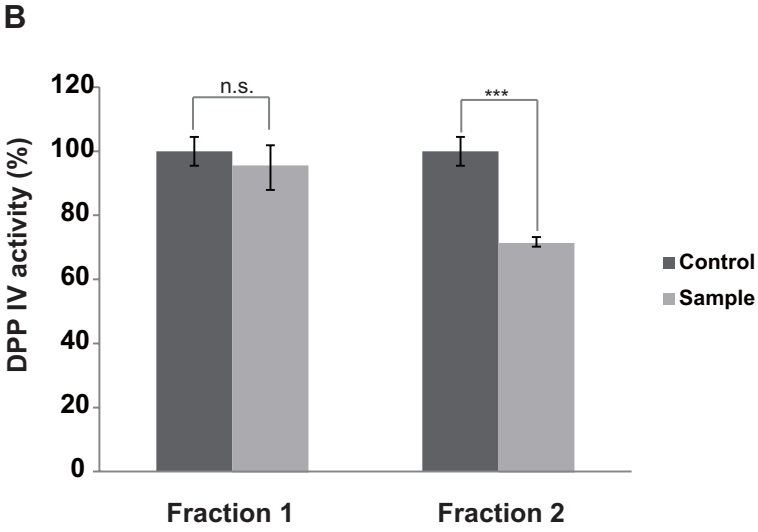
Control
Aronia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20
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DPP IV activity (%)
n.s.
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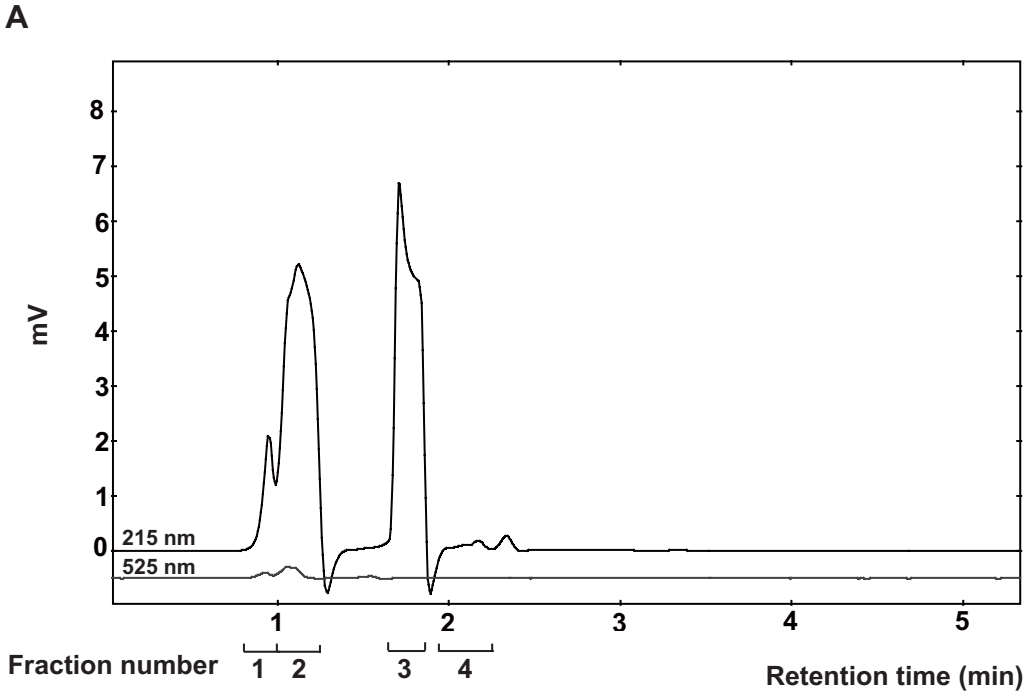
Control
Sample
Fraction 1
Fraction 2

Fig.2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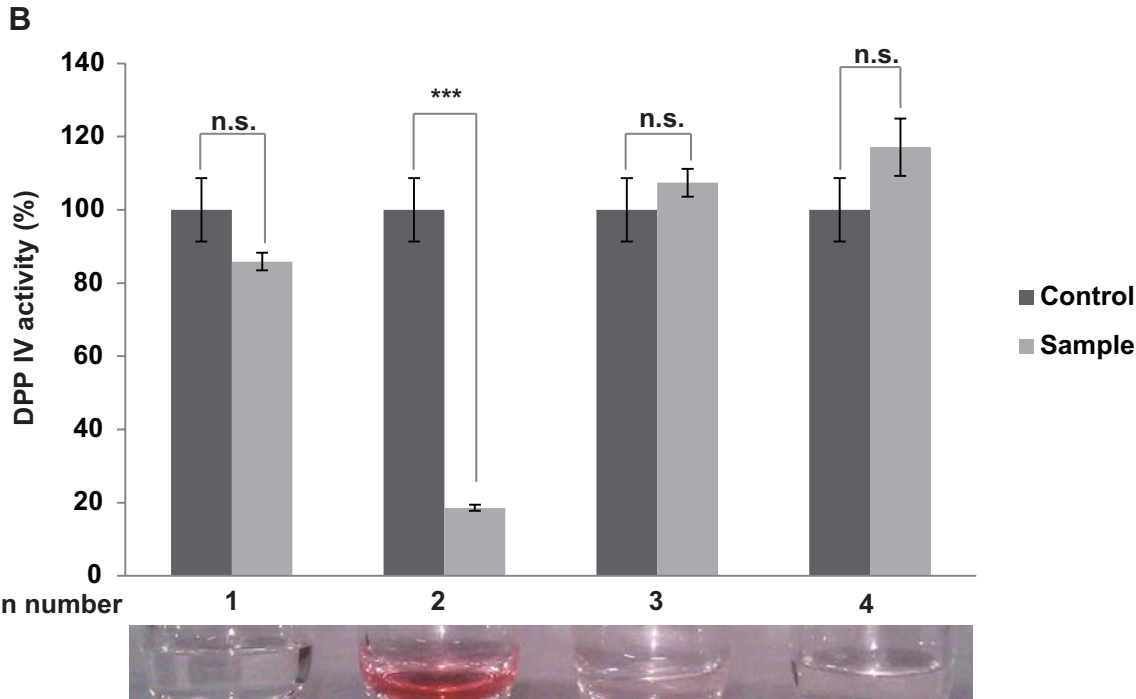
B
DPP IV activity (%)
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120
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Fraction number
1
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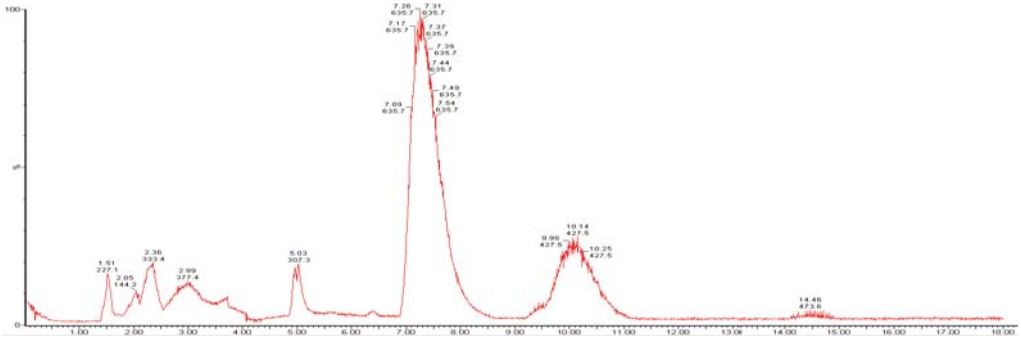
3
4

Fig.3

A



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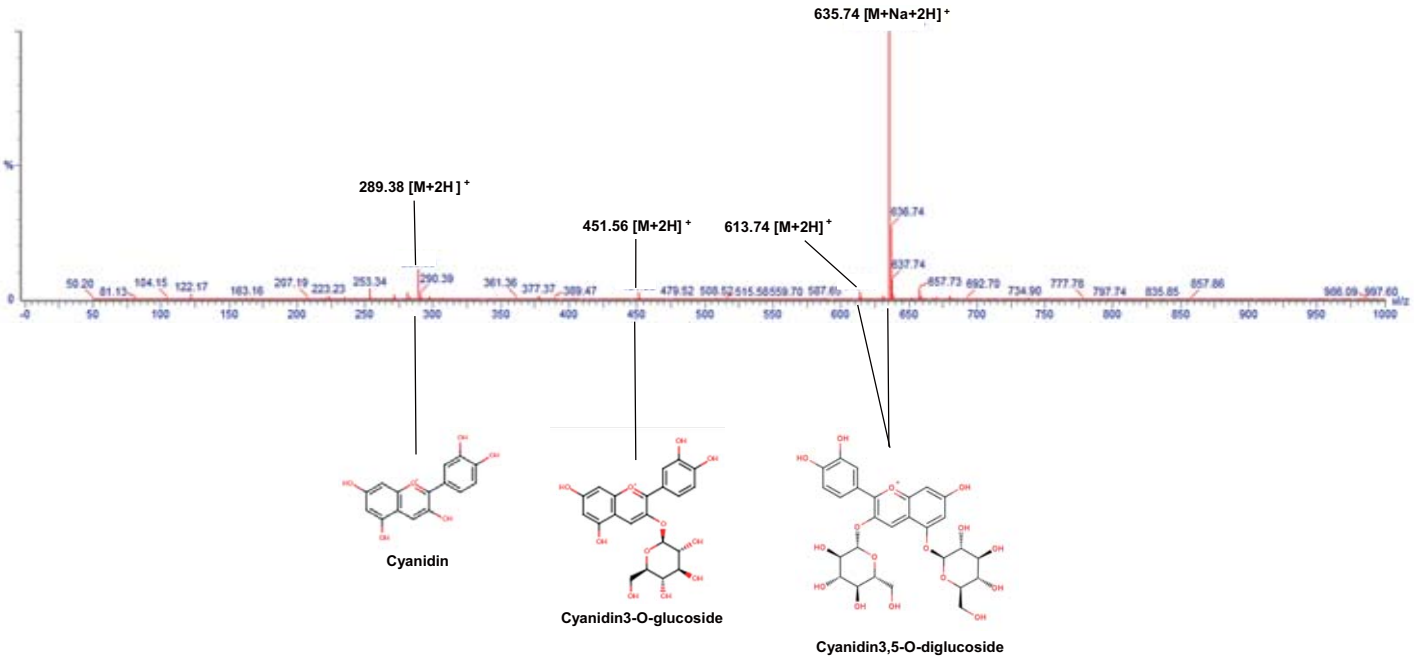
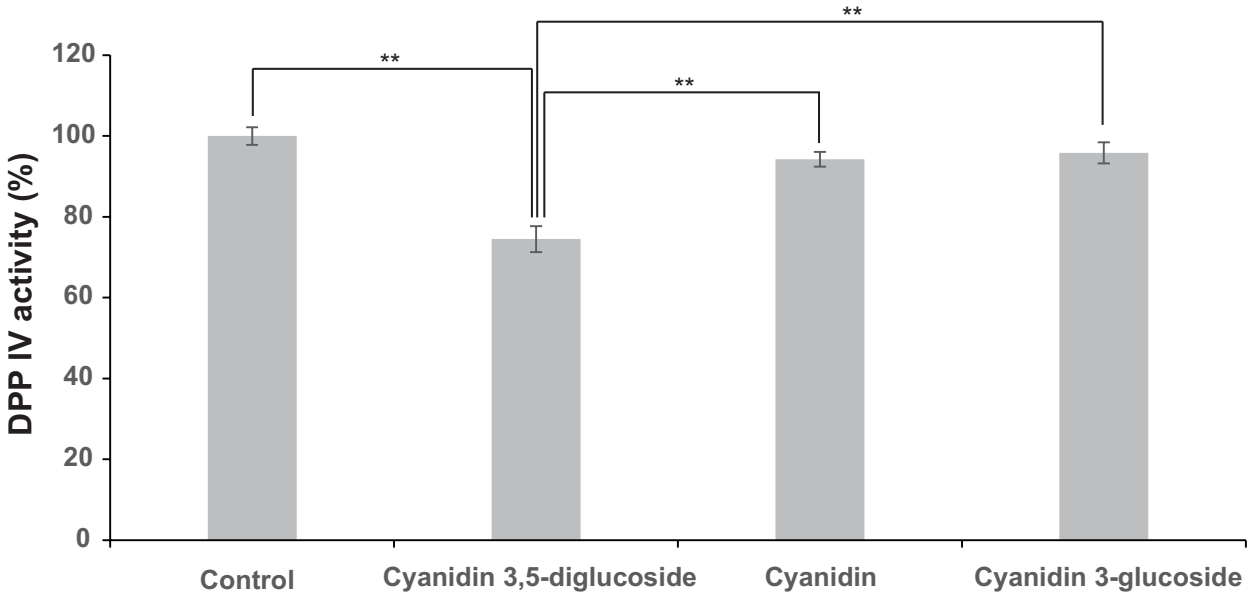

635.74 [M+Na+2H]+
289.38 [M+2H]+
451.56 [M+2H]+
613.74 [M+2H]+
Cyanidin
Cyanidin3-O-glucoside
Cyanidin3,5-O-diglucoside

Fig.4


DPP IV activity (%)
120
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Control
Cyanidin 3,5-diglucoside
Cyanidin
Cyanidin 3-glucoside