BPB Reports

Regular Article

Addition of Malonyl Groups Enhances Intestinal Absorption of Anthocyanins Derived from Edible Red Chrysanthemum (*Dendranthema grandiflorum*) in Rats

Takashi Ichiyanagi,*[,a] Yoshiki Kashiwada,[b] Masayuki Nashimoto,[c] and Norihiko Terahara[d]

[a]*Department of Ophthalmology, Niigata College of Medical Technologies, 5-13-3, Kamishin'ei-cho, Nishi-ku, Niigata 950-2076, Japan;* [b]*Faculty of Pharmaceutical Sciences, Tokushima University, 1-78, Shou-machi, Tokushima 770-8505, Japan;* [c]*Research Institute for Healthy Living, Niigata University of Pharmacy and Applied Life Sciences, 265-1, Higashijima, Akiha-ku, Niigata 956-8603, Japan;* [d]*Department of Food Science and Technology, Faculty of Health & Nutrition, Minami Kyushu University, Kirishima 5-1-2, Miyazaki 880-0032, Japan*

Received December 23, 2021; Accepted February 1, 2022

Petals of red chrysanthemum (*Dendranthema grandiflorum*) are habitually eaten in Japan. In the present study, plasma concentration profiles of two major acylated anthocyanins derived from the petals of red chrysanthemum were evaluated in rats after oral administration of an anthocyanin-rich fraction obtained from red chrysanthemum. The structures of the two major anthocyanins in the petals of red chrysanthemum were determined to be cyanidin 3-*O*-β-D-(3″, 6″-di-*O*-malonyl)-glucopyranoside and cyanidin 3-*O*-β-D-(6″-mono-*O*-malonyl)-glucopyranoside. Both malonyl anthocyanins were quickly absorbed from the gastrointestinal tract and were detected in rat blood plasma in their original acylated forms 15 min after the oral administration of the anthocyanin fraction obtained from the petals of red chrysanthemum. The absorption amounts of anthocyanins evaluated from the area under the plasma concentration curves during 8 h normalized to the orally administered dose were in the following order: cyanidin 3-*O*-β-D-(3″, 6″-di-*O*-malonyl)-glucopyranoside > cyanidin 3-*O*-β-D-(6″-mono-*O*-malonyl)-glucopyranoside > cyanidin 3-*O*-β-D-glucopyranoside. The present results demonstrated that the additional malonylation of the glucopyranosyl moiety of position 3″ and 6″ of cyanidin 3-*O*-β-D-glucopyranoside enhanced the intestinal absorption of anthocyanins.

Key words acylated anthocyanin, red chrysanthemum, cyanidin 3-*O*-β-D-(6″-mono-*O*-malonyl)-glucopyranoside, cyanidin 3-*O*-β-D-(3″, 6″-di-*O*-malonyl)-glucopyranoside, aliphatic acyl moiety, gastrointestinal absorption

INTRODUCTION

Anthocyanins are a family of flavonoids widely distributed in various plant materials.[1)] They present as glycosides or acyl-glycosides of anthocyanidins, and six major anthocyanidins (pelargonidin, cyanidin, delphinidin, petunidin, peonidin, and malvidin) are known as aglycons of anthocyanins.[2)] Acylated anthocyanins are further categorized into two groups depending on whether the attached acyl moieties are of the aliphatic or aromatic type.[3)]

Numerous studies on the health promotional effects of anthocyanins have been reported both *in vitro*[4)] and *in vivo*.[5)] Studies suggest that long-term consumption of anthocyanin-containing food may prevent lifestyle-related diseases such as diabetes.[6)] The disease prevention mechanisms of anthocyanins were also clarified at the molecular level,[7)] indicating that anthocyanins are valuable functional food factors.

Edible flowers such as rose,[8)] hibiscus,[9)] and butterfly pea[10)] contain anthocyanins as plant pigments in the petals. Most of the petals are placed in hot water that is then drunk as tea. In contrast, the petals of chrysanthemum are pickled in vinegar or cooked (boiled or fried), and different cultivars of chrysanthemum are eaten in Japan, especially in northeast regions. For example, yellow cultivars of chrysanthemum, which are rich in biologically active components (e.g., carotenoids,[11)] terpenoids,[12)] flavonols,[13)] and chlorogenic acid derivatives),[14)] are eaten in the north of Japan.

On the other hand, red cultivars of chrysanthemum (*Dendranthema grandiflorum*) are called by different names, *mottenohoka,* and *kakinomoto*, in northeast regions of Japan. The red cultivar of chrysanthemum contains anthocyanins as flower pigments in addition to the functional components in yellow chrysanthemum, except carotenoids. Therefore, the anthocyanins in red chrysanthemum together with other bioactive components may contribute to human health.

In the present study, we isolated two major anthocyanins attaching aliphatic acid as acyl groups from the petals of red chrysanthemum (*D. grandiflorum*; *kakinomoto*) and determined the structure of (I) and (II) to be cyanidin 3-*O*-β-D-(3″, 6″-di-*O*-malonyl)-glucopyranoside (di-mal-Cy3G) and cyanidin 3-*O*-β-D-(6″-mono-*O*-malonyl)-glucopyranoside (mono-mal-Cy3G), respectively, using tandem TOF-MS and NMR spectrometry. Furthermore, the gastrointestinal absorption of the two acylated anthocyanins derived from the petals of red chrysanthemum was evaluated in rats to obtain the information necessary for a discussion of the health promotional effects of

*To whom correspondence should be addressed. e-mail: ichiyanagi@niigata-coll-mt.ac.jp

these components *in vivo* together with the effect of aliphatic acyl groups on the gastrointestinal absorption of anthocyanins.

MATERIALS AND METHODS

Reagents HPLC-grade acetonitrile was obtained from Kanto Chemical Co., Ltd. (Tokyo, Japan). All other reagents, including trifluoroacetic acid (TFA), were purchased from Fuji Film Wako Pure Chemical Industry (Osaka, Japan) and used without further purification. HP–20 resin was purchased from Mitsubishi Chemical Co. (Tokyo, Japan). Sephadex LH–20 (25–100 μm) was obtained from Amersham Biosciences, Inc. (Buckinghamshire, UK).

Plant Material Red chrysanthemum, *kakinomoto* (*D. grandiflorum*) was grown in the Medicinal Botanical Garden of Niigata University of Pharmacy and Applied Life Sciences. The flower petals were collected during September and October 2003, dried at 45°C overnight and stored in a silica gel desiccator until used for extraction.

Preparation of Red Chrysanthemum Anthocyanin Fraction Red chrysanthemum anthocyanin fraction (RCAF) was obtained according to the previous method with modifications.[15,16] Briefly, dried petals of red chrysanthemum (400 g) were immersed in 6 L of 80% aqueous acetone solution overnight at room temperature and filtered. The extract was concentrated to dryness *in vacuo*. The crude extract of the petals of red chrysanthemum containing anthocyanins (prepared above) was adsorbed on an HP–20 resin column (70 × 600 mm) and washed with distilled water, and RCAF was eluted with 60% aqueous methanol solution. The eluent was concentrated to dryness *in vacuo*. The amount of RCAF recovered from 400 g of the petals was 1.4 g. The anthocyanin content in 100 mg of the RCAF used in the administration study was 9.1 mg for (I) and 7.8 mg for (II).

Purification of Major Anthocyanins in Red Chrysanthemum An aliquot (200 mg) of RCAF prepared above was dissolved in distilled water and chromatographed on an MCI column (25 × 500 mm) in the same solvent, and the anthocyanin fraction was eluted with 70% aqueous methanol solution and concentrated to dryness *in vacuo*. The anthocyanin-rich fraction prepared above was dissolved in a 15% acetic acid aqueous solution, and the anthocyanins in the extract were isolated by semi-preparative HPLC using a Develosil ODS-5 column (20 × 250 mm) under isocratic elution conditions.[16] The mobile phase was a mixture of Solvent A (15% acetic acid aqueous solution) and Solvent B (15% acetic acid containing 50% acetonitrile aqueous solution) at various ratios (60%A/40%B to 80%A/20%B (v/v)), with a flow rate of 7 mL/min, and the effluent was monitored at 520 nm. After the eluent was concentrated to dryness, the isolated pigment was dissolved in a small amount of TFA and precipitated with excess diethyl ether to obtain TFA salts as a reddish-purple powder. The purities of isolated (I) and (II) were > 96.2% and > 97.4%, respectively, calculated from the peak area of the HPLC chromatogram.

Structural Elucidation of Anthocyanins The structures of the anthocyanins isolated from RCAF were determined with the previously described methods.[16] Aliquots of (I) and (II) were dissolved in methanol and analyzed by tandem TOF-MS performed using a Q-Tof Ultima (Waters Corp., Milford, MA, USA). A syringe pump (single syringe pump; KD Scientific Inc., USA) was used to provide constant infusion (300 μL/h) of the sample into the MS ion source. The MS parameters used were as follows: 3.2 kV, capillary; 9.1 kV, reflection; argon gas at 11 psi; applied voltage, 24 kV. ^{1}H and ^{13}C NMR spectroscopy was performed using a JEOL-ECA-500 NMR spectrometer (JEOL Ltd., Tokyo, Japan) at magnetic field strengths of 500 and 125 MHz, respectively, in CD_3OD-d_4/TFA-d_1 (9:1), with tetramethylsilane as the internal reference.

HPLC Analysis HPLC conditions were as described in our previous report with modifications.[10] Briefly, aliquots (100 μL) of sample solutions were injected into an HPLC system (Hitachi L-7200) equipped with a Develosil ODS-HG 5 column (1.0 × 150 mm) using 0.5% TFA aqueous solution (Solvent A) and 0.5% TFA containing acetonitrile (Solvent B) in linear gradient elution at 40°C. The gradient conditions were as follows: 87% A/13% B (v/v) to 75% A/25% B (v/v) for 30 min and 60% A/40% B (v/v) for 20 min at a flow rate of 0.1 mL/min. The elution profile was monitored at 520 nm with a UV–VIS detector (Hitachi L-7000).

Animals and Diets SPF male Wistar ST rats (5 weeks old, bodyweight of 160 g) purchased from Japan SLC, Inc. (Hamamatsu, Japan) were individually housed in stainless-steel wire mesh cages at 23 ± 1°C for conditioning under a 12-h light/dark cycle. Rats were allowed *ad libitum* access to tap water and a controlled diet for 7 d before the experiment. Animal experiments were performed in accordance with the Guidelines on the Care and Use of Laboratory Animals issued by Niigata University of Pharmacy and Applied Life Sciences. The protocol was approved by the Committee on the Ethics of Animal Experiments of Niigata University of Pharmacy and Applied Life Sciences (Approval number 17-4). All efforts were made to minimize suffering.

Experimental Design After 7 d of adaptation, five rats were cannulated with a polyethylene tube (PE 50) into a neck vein under anesthesia with a mixed solution of medetomidine hydrochloride (0.15 mg/kg), midazolam (2 mg/kg), and butorphanol tartrate (2.5 mg/kg) by intraperitoneal injection as we described previously with modifications.[10] Briefly, the neck vein was isolated, and a small hole was cut with scissors to insert the PE 50. Once the tube was inserted, the vein and tube were occluded, and the tube was guided out from the back of the rats. After starving for 24 h, 500 mg/kg of body weight of RCAF dissolved in 0.1% aqueous citric acid solution was administered orally to the five rats. The doses of (I) and (II) were 45.5 mg and 39.0 mg, respectively, in 500 mg of RCAF. During the experiment, the rats were allowed to move freely in the cages. Blood samples were collected via the cannulated tube using a heparinized syringe at 0, 15, 30, 60, 120, 240, and 480 min. Donor blood was collected from other healthy rats under anesthesia by venipuncture of the inferior vena cava into a sodium citrate solution (500 μL of 10% sodium citrate per 8 mL of blood). After the blood was withdrawn (600 μL), the same volume of donor blood was injected through the cannulated vein tube. Each blood sample was immediately centrifuged at 3000 × *g*, 4°C for 5 min to prepare plasma samples for HPLC analysis.

Plasma Preparation Anthocyanins were extracted from blood plasma using a Sep-Pak C_{18} Light Cartridge (Waters) as we previously reported.[10] Briefly, plasma (300 μL) samples were applied to the Sep-Pak C_{18} Light Cartridges conditioned with methanol (2 mL) and 3% aqueous TFA solution (2 mL). The cartridges were then washed successively with 2 mL of 3% aqueous TFA solution, dichloromethane, and benzene, and anthocyanins were eluted with 50% aqueous acetonitrile solu-

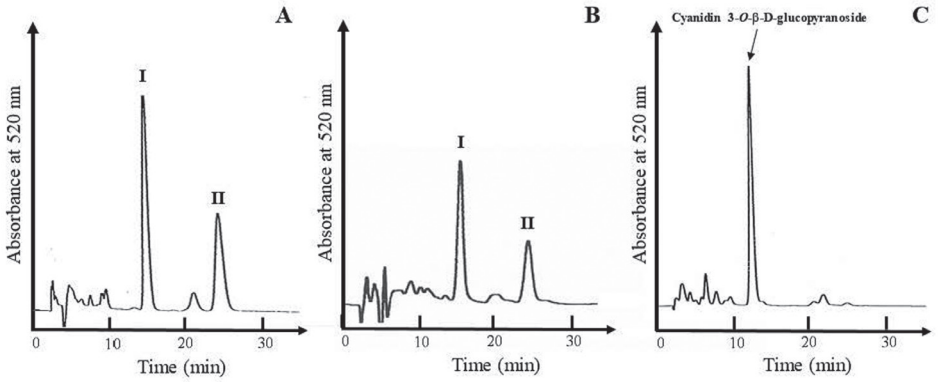

Fig. 1. Typical HPLC Chromatogram of RCAF and Rat Blood Plasma

A: Original RCAF. **B:** Rat blood plasma 15 min after oral administration of RCAF. Peak numbers correspond to the compounds as follows: I, cyanidin 3-*O*-β-D-(3″, 6″-di-*O*-malonyl)-glucopyranoside; II, cyanidin 3-*O*-β-D-(6″-mono-*O*-malonyl)-glucopyranoside. **C:** Rat blood plasma 15 min after oral administration of cyanidin 3-*O*-β-D-glucopyranoside.

tion containing 1% TFA. The eluent was concentrated to dryness *in vacuo* and dissolved in 150 μL of 0.5% aqueous TFA solution. The dissolved sample was passed through a Centricut ultra-membrane filter (0.45 μm; Kurabo Co. Ltd., Osaka, Japan) before HPLC injection. The percentage of anthocyanins recovered by this method was 88.7% for (I) and 89.3% for (II), as verified by spiking purified anthocyanins into the normal blood plasma.

Statistical Analysis Significant differences among parameters of anthocyanins in blood plasma were determined by one-way ANOVA, followed by Tukey's multiple comparison test ($P < 0.05$). All statistical analyses were performed with EZR (Saitama Medical Center, Jichi Medical University, Saitama, Japan), which is a graphical user interface for R (The R Foundation for Statistical Computing, Vienna, Austria, version 4.0.3). More precisely, it is a modified version of R commander (version 2.7-1) containing statistical functions frequently used in biostatistics.

RESULTS

Structural Determination of Anthocyanins in Petals of Red Chrysanthemum Figure 1A shows a typical HPLC chromatogram of RCAF detected at 520 nm, and the two major pigments ((I) and (II)) were observed in RCAF. These components were purified by repeating column chromatography and semi-preparative HPLC. The *m/z* values of the molecular and product ion pairs of (I) (621/287) agreed well with those of di-malonyl-cyanidin-glycoside, whereas those of the molecular and product ion pairs of (II) (535/287) agreed well with those of mono-malonyl-cyanidin-glycoside (Table 1). To determine the malonylation positions of (I) and (II), ^{1}H and ^{13}C NMR spectroscopy was performed, and the summaries are shown in Table 1. Further, 2D NMR analysis revealed that malonyl moieties were attached on both the position 3″ and 6″ of the glucopyranosyl moiety of cyanidin 3-*O*-β-D-glucopyranoside (Cy3G) for (I) and on the position 6″ of the glucopyranosyl moiety of Cy3G for (II). Therefore, the structures of (I) and (II) derived from the petals of red chrysanthemum, *kakinomoto* were determined to be di-mal-Cy3G and mono-mal-Cy3G, as shown in Fig. 2. The amounts of mono-mal-Cy3G and di-mal-Cy3G recovered from 200 mg of RCAF (57 g of dried petals) were 5.8 mg and 6.2 mg, respectively.

Evaluation of Gastrointestinal Absorption of Two Major Acylated Anthocyanins Derived from Petals of Red Chrysanthemum in Rats As shown in Fig. 1B, the two major anthocyanins in RCAF, mono-mal-Cy3G, and di-mal-Cy3G, were directly absorbed from the gastrointestinal tract and present in rat blood plasma in their original acylated forms at 15 min after the oral administration of RCAF at a dose of 500 mg/kg body weight (0.0729 mmol/kg bodyweight for mono-mal-Cy3G and 0.0733 mmol/kg bodyweight for di-mal-Cy3G). Figure 1C shows an HPLC chromatogram of rat blood plasma at 15 min after the oral administration of purified Cy3G. As shown in Fig. 1B, a significant amount of Cy3G was not detected as a de-malonylated metabolite of mono-mal-Cy3G and di-mal-Cy3G in HPLC chromatogram of rat blood plasma after the oral administration of the RCAF. On the other hand, several minor peaks which eluted faster than di-mal-Cy3G were detected in hydrophilic area in HPLC chromatogram of rat blood plasma received RCAF (Fig. 1B), although, structural information of these peaks was not obtained.

Figure 3 shows the plasma concentration profiles of mono-mal-Cy3G and di-mal-Cy3G after the oral administration of RCAF. The maximum plasma concentrations (C_{max}) of mono-mal-Cy3G and di-mal-Cy3G were 0.113 ± 0.009 μM and 0.203 ± 0.016 μM, respectively, at 15 min after oral administration (Table 2). The area under the plasma concentration curve values during the 8 h after oral administration (AUC_{0-8}) obtained from the plasma concentration profile of mono-mal-Cy3G and di-mal-Cy3G was 15.01 ± 1.06 μM·min and 27.67 ± 2.18 μM·min, respectively (Table 2). Because the doses of mono-mal-Cy3G and di-mal-Cy3G in RCAF were different, both the AUC_{0-8} and the C_{max} of mono-mal-Cy3G and di-mal-Cy3G were normalized by an orally administered dose of the corresponding anthocyanins ($AUC_{0-8}/Dose$ and $C_{max}/Dose$) for the comparison of their amounts in blood plasma, and the results are summarized in Table 2 together with those in the previous report of Cy3G.[17] The $AUC_{0-8}/Dose$ values, indicating the bioavailability of the individual anthocyanins, were 205.95 ± 14.54 μM·min/mmol/kg for mono-mal-Cy3G, 377.72 ± 29.86 μM·min/mmol/kg for di-mal-Cy3G, and 108.87 ± 33.65 μM·min/mmol/kg for Cy3G. The $C_{max}/Dose$ values were 1.55 ± 0.12 μM/mmol/kg for mono-mal-Cy3G, 2.78 ± 0.23 μM/mmol/kg for di-mal-Cy3G, and 0.82 ± 0.11 μM/mmol/kg for Cy3G.

Table 1. NMR and Tandem MS Data of Anthocyanins Isolated from Petals of Red Chrysanthemum

	cyanidin 3-*O*-β-D-(3",6"-di-*O*-malonyl)-glucopyranoside (I)		cyanidin 3-*O*-β-D-(6"-*O*-malonyl)-glucopyranoside (II)	
aglycon	δ_H	δ_C	δ_H	δ_C
2		162.2		162.2
3		144.2		144.6
4	8.86 (s)	135.0	8.85 (s)	134.8
5		157.8		157.8
6	6.73 (d, J = 2 Hz)	102.6	6.75 (d, J = 2 Hz)	102.7
7		168.7		168.7
8	6.93 (d, J = 2 Hz)	94.5	6.93 (d, J = 2 Hz)	94.6
4a		112.0		112.1
8a		156.3		156.3
1'		119.8		120.0
2'	7.96 (d, J = 2.5 Hz)	117.6	8.02 (d, J = 2.3 Hz)	117.8
3'		146.5		146.5
4'		154.7		154.7
5'	7.04 (d, J = 8.6 Hz)	117.0	7.06 (d, J = 8.9 Hz)	117.1
6'	8.22 (dd, J = 2.5, 8.6 Hz)	127.4	8.27 (dd, J = 2.3, 8.9 Hz)	127.5
3-*O*-glucosyl				
1	5.59 (d, J = 7.8 Hz)	101.4	5.43 (d, J = 7.8 Hz)	102.1
2	3.75 (dd, J = 7.8, 9.5 Hz)	71.1	3.56 (dd, t = 8 Hz)	73.3
3	5.06 (t, J = 9.5 Hz)	78.3	3.44 (t, J = 8 Hz)	76.5
4	3.52 (t, J = 9.5 Hz)	67.9	3.28 (t, J = 8 Hz)	70.2
5	3.99 (m)	74.2	3.86 (m)	74.7
6	4.42 (br d, J = 12 Hz) 4.19 (dd, J = 7.3, 12 Hz)	64.2	4.49 (br d, J = 12 Hz) 4.16 (dd, J = 7.8, 12 Hz)	64.8
malonyl				
CH_2	3.47 (2H, s) 3.35, 3.39 (each 1H, d, J = 15.8 Hz)	41.5, 41.9	3.38, 3.42 (each 1H, d, J = 16 Hz)	41.6
COO		166.9, 167.3		167.5
COOH		168.3 (2C)		168.4
Molecular and product ion	621/287		535/287	

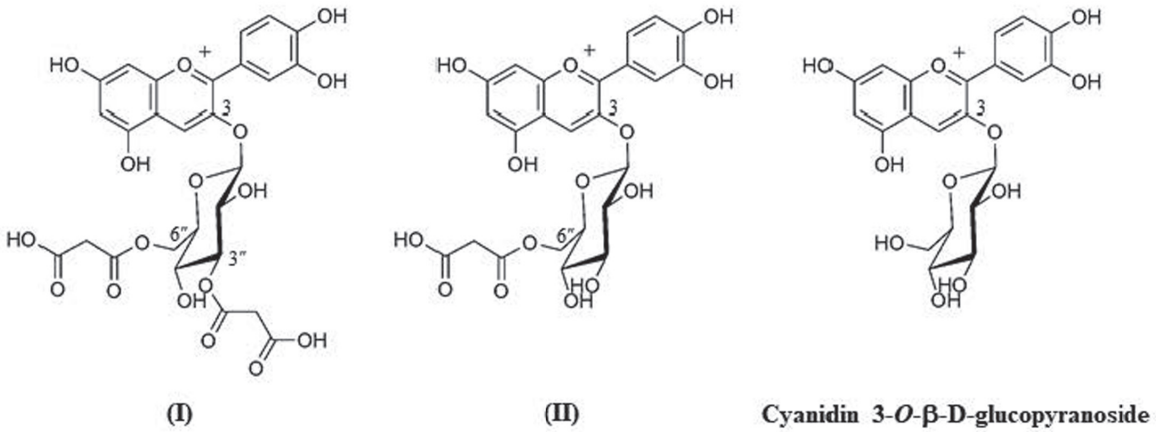

Fig. 2. Structure of Major Acylated Anthocyanins in Petals of Red Chrysanthemum and Cyanidin 3-*O*-β-D-glucopyranoside

(I): cyanidin 3-*O*-β-D-(3″, 6″-di-*O*-malonyl)-glucopyranoside. (II): cyanidin 3-*O*-β-D-(6″-mono-*O*-malonyl)-glucopyranoside.

DISCUSSION

A flower pigment in the petals of red chrysanthemum named chrysanthemin was isolated as a major anthocyanin in 1916, and its structure was determined to be Cy3G.[18] On the other hand, a variety of acylated anthocyanins were later identified as major pigments from the petals of flowers,[19] suggesting that the main original anthocyanin in the petals of red chrysanthemum might not be Cy3G. Major pigments have been isolated from one of the cultivars of red chrysanthemum (*akane*) grown in the western region of Japan, and their structures were elucidated to be acylated anthocyanins attaching malonic acid as acyl groups.[20,21] Because the aliphatic acyl groups of acylated anthocyanins are easily hydrolyzed during long-term exposure in acidic conditions, such as extraction and isolation procedures, anthocyanins were extracted from the dried petals of red chrysanthemum with 80% aqueous acetone solution to prevent the acid-catalyzed hydrolysis of malonyl groups.[15] Two

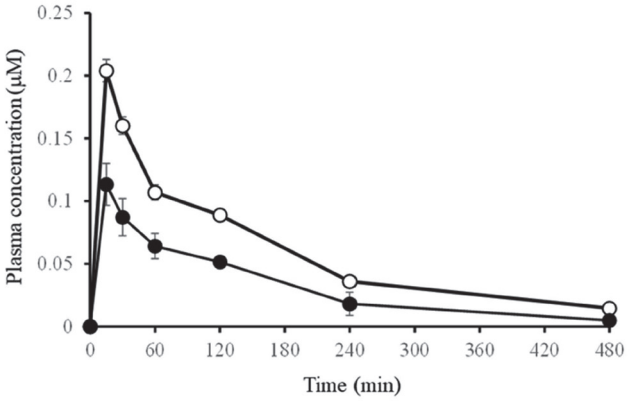

Fig. 3. Plasma Concentration Profiles of Acylated Anthocyanins After Oral Administration of RCAF

Open circles and closed circles indicate cyanidin 3-*O*-β-D-(3″, 6″-di-*O*-malonyl)-glucopyranoside and cyanidin 3-*O*-β-D-(6″-mono-*O*-malonyl)-glucopyranoside, respectively. Values are the means ± SEM of five rats.

anthocyanins were successfully isolated from the petals of one of the cultivars of red chrysanthemum (*kakinomoto*), and their structures were determined using tandem TOF-MS and NMR spectrometry. The *m/z* values of the molecular ion of 535 and 621 showed good agreement with those of mono-mal-Cy3G and di-mal-Cy3G. Similar to the previous reports,[20,21] the fragment ion of 535 (mono-malonyl cyanidin-glycoside) was not observed for di-mal-Cy3G, and that of 449 (cyanidin-glycoside) was not observed for either mono-mal-Cy3G or di-mal-Cy3G, although, the fragment ion of 287 (cyanidin) was observed for mono-mal-Cy3G and di-mal-Cy3G. The ^{1}H NMR data for mono-mal-Cy3G and di-mal-Cy3G reported here show good agreement with those for DMSO-d_6/TFA-d_1 (9:1) previously reported.[20,21] The positions of malonylation were confirmed with 2D NMR analysis (^{1}H-^{1}H COSY and ^{1}H-^{13}C COSY). The structures of the two major anthocyanins isolated from the petals of red chrysanthemum were finally determined to be mono-mal-Cy3G and di-mal-Cy3G.

The two malonyl anthocyanins, mono-mal-Cy3G and di-mal-Cy3G have been reported in the various cultivars of red to purple chrysanthemum as major pigments and the profile of both anthocyanins in chrysanthemum was well correlated with flower color.[22] Various types of malonyl anthocyanins also have been reported in the petals of other flowers.[19] Furthermore, anthocyanin malonyl transferase was isolated from the petals of flowers such as salvia[23] and dahlia,[24] and the gene encoding of anthocyanin malonyl transferase was also elucidated.[23] Therefore, malonylation is a general metabolic route for flavonoids-glycosides including anthocyanins in plant cells.

Biological functions of acylated anthocyanins, such as antioxidant activity have been extensively reported *in vitro*.[25–27] Results indicate that attached acyl groups are critical factors for enhancing the biological activity of acylated anthocyanins.[25–27] However, studies on the bioavailability of acylated anthocyanins are necessary to clarify whether acylated anthocyanins are possessed their functions in biological systems. In the present study, the gastrointestinal absorption of mono-mal-Cy3G and di-mal-Cy3G was evaluated after the oral administration of RCAF as the anthocyanin source. The results demonstrated that both anthocyanins were quickly absorbed from the gastrointestinal tract and detected in rat blood plasma in their original acylated forms at 15 min after the oral administration of RCAF, similar to the case of other acylated anthocyanins.[10,25,28]

On the other hand, a significant amount of Cy3G was not detected in the HPLC chromatogram of rat blood plasma after the oral administration of the RCAF. This indicates de-malonylation of mono-mal-Cy3G was presumed to be a minor metabolism. This is partially supported by the result that acid-catalyzed cleavage of the malonyl groups of mono-mal-Cy3G and di-mal-Cy3G was not observed when both anthocyanins were incubated in 0.1 M HCl at 37°C for 2 h (data not shown). Also, it was proved that the intestinal hydrolysis of acyl groups did not occur by a direct visualization technique guided with matrix-assisted laser desorption ionization MS after the oral administration of acylated cyanidin-glucoside attaching aromatic acyl groups derived from black carrot.[29] Similarly, de-malonylation of di-mal-Cy3G to mono-mal-Cy3G was presumed to be a minor metabolic route. Thus, it was concluded that the hydrolysis of the malonyl moieties of mono-mal-Cy3G and di-mal-Cy3G is a minor metabolism in the gastrointestinal tract, although absorption studies using purified mono-mal-Cy3G and di-mal-Cy3G were necessary to clarify this point.

As the intravenous administration of anthocyanins was not performed, both AUC_{0-8} and C_{max} values were normalized to the orally administered dose (0.0729 mmol/kg bodyweight mono-mal-Cy3G and 0.0733 mmol/kg bodyweight for di-mal-Cy3G) to evaluate the gastrointestinal absorption of the two major acylated anthocyanins derived from the petals of red chrysanthemum, and the results were compared with those of the previous reports of various anthocyanins including Cy3G.[10,17,25,28,30] We have previously reported that acylated anthocyanins attaching aromatic acyl groups, such as nasunin[25] (delphinidin 3-*O*-(6-*O*-(4-*O*-*p*-coumaroyl-α-L-rhamnopyranosyl)-β-D-glucopyranoside)-5-*O*-β-D-glucopyranoside) derived from eggplant (*Solanum melongena*) and ternatins[10] (malonylated delphinidin 3,3′,5′-triglucoside, with 3′ and 5′ side chains containing replacing glucosyl and *p*-coumaroyl units) derived from the petals of butterfly pea (*Clitoria ternatea* L.), showed higher absorption, despite their large molecular weight, than that of a non-acylated anthocy-

Table 2. Orally Administered Dose and Plasma Parameters of Anthocyanins

Anthocyanin	C_{max} (μM)	t_{max} (min)	AUC_{0-8} (μM min)	*Dose* (mmol/kg)	C_{max}/*Dose* (μM/mmol/kg)	AUC_{0-8}/*Dose* (μM min/mmol/kg)
di-mal-Cy3G	0.203 ± 0.016	15	27.67 ± 2.18	0.0733	2.78 ± 0.23^{a,b}	377.72 ± 29.86^{d,e}
mono-mal-Cy3G	0.113 ± 0.009	15	15.01 ± 1.06	0.0729	1.55 ± 0.12^{a,c}	205.95 ± 14.54^{d}
Cy3G	0.183 ± 0.025	15	24.25 ± 7.50	0.2227	0.82 ± 0.11^{b,c}	108.87 ± 33.65^{e}

Values are the means ± SEM of five rats for di-mal-Cy3G and mono-mal-Cy3G and four rats for Cy3G.
C_{max}, maximum plasma concentration; t_{max}, time at which maximum plasma concentration was reached; AUC_{0-8}, area under the plasma concentration curve during the 8-h period after oral administration.
Values of Cy3G were cited from reference 17 of our previous report for the comparison.
The same lowercase letters on the values indicate a significant difference ($P < 0.05$).

anin, delphinidin 3-*O*-β-D-glucopyranoside (Dp3G).[30] The AUC_{0-8}/*Dose* value of di-mal-Cy3G (377.72 μM·min/mmol/kg) was higher than those in the previous results of acylated anthocyanins comprising delphinidin as the aglycon: total ternatin (311.15 μM·min/mmol/kg)[10] and nasunin (280.9 μM·min/mmol/kg),[25] and no structural correlations were observed. In contrast, mono-mal-Cy3G showed a relatively low AUC_{0-8}/*Dose* value (205.95 μM·min/mmol/kg).

When the AUC_{0-8}/*Dose* values were compared among anthocyanins comprising cyanidin as the aglycon, the results were in the following order with significance ($0.05 < P$): di-mal-Cy3G (377.72 ± 29.86 μM·min/mmol/kg) > mono-mal-Cy3G (205.95 ± 14.54 μM·min/mmol/kg) > Cy3G (108.87 ± 33.65 μM·min/mmol/kg).[17] The C_{max}/*Dose* values also showed a similar trend with significance ($0.05 < P$): di-mal-Cy3G (2.78 ± 0.23 μM/mmol/kg) > mono-mal-Cy3G (1.55 ± 0.12 μM/mmol/kg) > Cy3G (0.82 ± 0.11 μM/mmol/kg).[17] Higher absorption of mono-mal-Cy3G than Cy3G was previously reported in rats after the oral administration of red-orange juice as the anthocyanin source.[31] Here, for the first time, we revealed that the additional malonylation of the glucopyranosyl moiety of mono-mal-Cy3G enhanced the gastrointestinal absorption of anthocyanin. Both C_{max}/*Dose* and AUC_{0-8}/*Dose* levels increased twofold when the number of malonylation sites increased, suggesting that additional acylation with other types of aliphatic acyl groups increases the gastrointestinal absorption of anthocyanin. Further studies on the intestinal absorption of acylated anthocyanins attaching other aliphatic acyl groups, such as oxalic, succinic, or malic acid, are required to clarify this point.

Considering both the present and previous results,[10,25] the acyl moieties of acylated anthocyanins enhanced the gastrointestinal absorption of anthocyanins independent of aliphatic or aromatic type. However, certain types of acylated anthocyanins attaching aromatic acyl groups, for example, peonidin 3-*O*-β-(6″-*O*-caffeoyl)-sophoroside-5-*O*-β-D-glucopyranoside derived from purple sweet potato (*Ipomoea batatas* L.), showed exceptionally poor AUC_{0-8}/*Dose* values (72.34 μM·min/mmol/kg)[28] compared with that of large molecular, heavenly blue anthocyanin (peonidin 3-*O*-(2″-*O*-(6‴-*O*-(3-*O*-(β-D-glucopyranosyl)-caffeoyl)-β-D-glucopyranosyl)-6″-*O*-(4-*O*-(6-*O*-(3-*O*-(β-D-glucopyranosyl)-caffeoyl)-β-D-glucopyranosyl)-caffeoyl)-β-D-glucopyranoside)-5-*O*-β-D-glucopyranoside) (409.51 μM·min/mmol/kg)[32] isolated from the petals of morning glory (*Pharbitis nil* L.). Therefore, the structural factors modulating the absorption of acylated anthocyanins, such as combinations of organic acids and sugars and attached positions of acyl groups on sugar moieties, should be considered.

Focusing on the metabolism of anthocyanins, traces of *O*-methyl metabolites were observed in rat blood plasma after the oral administration of anthocyanins carrying catechol or pyrogallol groups such as Cy3G[17] and Dp3G.[30] *O*-methyl metabolites of mono-mal-Cy3G and di-mal-Cy3G were not observed in HPLC chromatograms of rat blood plasma during the 8 h after the oral administration of RCAF. Because *O*-methyl metabolites were the main forms distributed in tissues,[33] *O*-methyl metabolites of mono-mal-Cy3G and di-mal-Cy3G may be produced by hepatic metabolism and further excreted in rat urine after the administration of RCAF. The excretion of *O*-methyl metabolites of mono-mal-Cy3G was observed in rat urine after the administration of red-orange juice,[31] indicating that the mono malonylation of the glucosyl moiety did not inhibit the *O*-methyl metabolism of anthocyanins. This contrasts with the finding that acylated anthocyanins attaching aromatic acyl groups (e.g., nasunin) could not be the substrate for *O*-methyl metabolism.[25] The effect of the additional malonylation of the glucosyl moiety on *O*-methyl metabolism remains unclear.

Glucuronides of anthocyanidin (anthocyanidin-glucuronides) and glucuronides of anthocyanin (anthocyanidin-glycoside-glucuronides) are known as other predominant metabolites of non-acylated anthocyanins.[17,34–37] In the present study, trace amounts of new peaks which eluted faster than di-mal-Cy3G were detected in rat blood plasma. These peaks might be glucuronyl metabolites of mono-mal-Cy3G or di-mal-Cy3G (glucuronide of mono-mal-Cy3G or di-mal-Cy3G),[36,37] although, further structural information is necessary. Contrary to this, intestinal hydrolysis of the glucosyl moiety of Cy3G is required for the production of cyanidin-glucuronide.[17] However, significant cyanidin-glucuronide (anthocyanidin-glucuronide) and cyanidin (anthocyanidin) were not observed in the HPLC chromatograms of rat blood plasma detected at 520 nm after the administration of the RCAF. The present result suggests that the malonylation of glucopyranose inhibits the intestinal hydrolysis of the glucosyl linkage of acylated anthocyanins. The same results were obtained for acylated anthocyanins attaching aromatic acyl groups in which anthocyanidin-glucuronide was not detected in rat blood plasma.[25] Therefore, it could be concluded hydrolysis of the glucosyl linkage of acylated anthocyanins was a minor metabolism for acylated anthocyanins.

Collectively, the present and previous results on the metabolism of acylated anthocyanins indicate that glucuronidation is not the major metabolic pathway for acylated anthocyanins attaching aromatic and aliphatic groups, although *O*-methylation could occur for mono-acylated anthocyanins attaching aliphatic acyl groups.

It was reported that a typical non-acylate anthocyanin, Cy3G, is transported via intestinal glucose transporter 2 (GLUT-2)[38] and is in part transported via organic anion transporting polypeptide (OATP).[29] In contrast, acylated anthocyanins attaching aromatic acid as acyl groups (e.g., cyanidin 3-*O*-(6-*O*-(6-*O*-feruloyl-β-D-glucopyranosyl)-(2-*O*-β-D-xylopyranosyl))-β-D-galactopyranoside) are transported via OATP alone.[29] Because the terminal feruloyl moiety of arabinoxylan oligosaccharide inhibits its GLUT-2-mediated transport,[39] acylated anthocyanins attaching the feruloyl moiety or other aromatic acyl groups may similarly inhibit the GLUT-2-mediated self-transport of acylated anthocyanins. In contrast, as relatively small malonyl parts do not inhibit the intestinal GLUT-2-mediated transport, mono-mal-Cy3G and di-mal-Cy3G may be absorbed similarly to Cy3G via the GLUT-2 route. Furthermore, it was presumed di-mal-Cy3G attaching two malonyl groups on position 3″ and 6″ could be strongly recognized by OATP compared with mono-mal-Cy3G attaching sole malonyl group on position 6″, leading to the higher intestinal absorption of di-mal-Cy3G than mono-mal-Cy3G.

In conclusion, this is the first report to show that additional malonylation enhances the absorption of acylated anthocyanins from the gastrointestinal tract in their original acylated forms. Further studies on the absorption mechanism of acylated anthocyanins attaching aliphatic acyl groups from the gastrointestinal tract via various transporters, such as OATP and GLUT-2, are required.

Acknowledgment We are grateful to Dr. Y. Shida (Mass Laboratory of Tokyo University of Pharmacy and Life Sciences) for the tandem TOF-MS analysis. We also thank Dr. J. Shirasaki (Medicinal Botanical Garden of Niigata University of Pharmacy and Applied Life Sciences) for providing the red chrysanthemum, *kakinomoto*.

Conflict of interest The authors declare no conflict of interest.

REFERENCES

1) Markakis P, Jurd L. Anthocyanins and their stability in foods. *Crit. Rev. Food Sci. Nutr.*, **4**, 437–456 (1974).
2) Strack D, Wray V. *"The Flavonoids" advances in research since 1986.* (Harborne JB ed.) Chapman and Hall/CRC, London (1994).
3) Harborne JB. Plant polyphenols XI: the structure of acylated anthocyanins. *Phytochemistry*, **3**, 151–160 (1964).
4) Kalt W, Cassidy A, Howard LR, Krikorian R, Stull AJ, Tremblay F, Zamora-Ros R. Recent research on the health benefits of blueberries and their anthocyanins. *Adv. Nutr.*, **11**, 224–236 (2020).
5) Ma Z, Du B, Li J, Yang Y, Zhu F. An insight into anti-inflammatory activities and inflammation related diseases of anthocyanins: A review of both *in vivo* and *in vitro* investigations. *Int. J. Mol. Sci.*, **22**, 11076 (2021).
6) Hou J, Chen L, Zhang Y, Szkudelski T, Delmas D, Daglia M, Xiao J. Dietary polyphenols and type 2 diabetes: human study and clinical trial. *Crit. Rev. Food Sci. Nutr.*, **59**, 3371–3379 (2019).
7) Les F, Cásedas G, Gómez C, Moliner C, Valero MS, López V. The role of anthocyanins as antidiabetic agents: from molecular mechanisms to *in vivo* and human studies. *J. Physiol. Biochem.*, **77**, 109–131 (2021).
8) Shikov V, Kammerer DR, Mihalev K, Mollov P, Carle R. Heat stability of strawberry anthocyanins in model solutions containing natural copigments extracted from rose (*Rosa damascena* mill.) petals. *J. Agric. Food Chem.*, **56**, 8521–8526 (2008).
9) Kim JH, Nonaka G, Fujieda K, Uemoto S. Anthocyanidin malonylglucosides in flowers of Hibiscus syriacus. *Phytochemistry*, **28**, 1503–1506 (1989).
10) Ichiyanagi T, Nashimoto N, Terahara N. Gastrointestinal absorption of ternatins, polyacylated anthocyanins derived from butterfly pea (*Clitoria ternatea* L.) petals in rats. *BPB Reports*, **4**, 136–141 (2021).
11) Kishimoto S, Maoka T, Nakayama M, Ohmiya A. Carotenoid composition in petals of chrysanthemum (*Dendranthema grandiflorum* (Ramat.) Kitamura). *Phytochemistry*, **65**, 2781–2787 (2004).
12) Uchio Y, Tomosue K, Nakayama M, Yamamura A, Wakia T. Constituents of the essential oils from three tetraploid species of Chrysanthemum. *Phytochemistry*, **20**, 2691–2693 (1981).
13) Harborne JB, Heywood VH, Saleh NAM. Chemosystematics of the composite: flavonoid patterns in the chrysanthemum complex of the tribe Anthemideae. *Phytochemistry*, **9**, 2011–2017 (1970).
14) Leiss KA, Maltese F, Choi YH, Verpoorte R, Klinkhamer PGL. Identification of chlorogenic acid as a resistance factor for thrips in *chrysanthemum. Plant Physiol.*, **150**, 1567–1575 (2009).
15) Garcia-Viguera C, Zafrilla P, Tomás-Barberán FA. The use of acetone as an extraction solvent for anthocyanins from strawberry fruit. *Phytochem. Anal.*, **9**, 274–277 (1998).
16) Ichiyanagi T, Kashiwada Y, Nashimoto N. Large-scale isolation of three *O*-methyl anthocyanins from bilberry extract. *Chem. Pharm. Bull. (Tokyo)*, **68**, 1113–1116 (2020).
17) Ichiyanagi T, Shida Y, Rahma MM, Hatano Y, Matsumoto H, Hirayama M, Konishi T. Metabolic pathway of cyanidin 3-*O*-β-D-glucopyranoside in rats. *J. Agric. Food Chem.*, **53**, 145–150 (2005).
18) Willstatter R, Bltono EK. Untersuchungen über die anthocyane. XI. über das anthocyan der rotblünden salviaarten. *Justus Liebigs Ann. Chem.*, **412**, 113–136 (1916).
19) Iwashina T. Contribution to flower colors of flavonoids including anthocyanins: A review. *Nat. Prod. Commun.*, **10**, 1934578X1501000335 (2015).
20) Saito N, Toki K, Honda T, Kawase K. Cyanidin 3-malonylglucuronylglucoside in bellis and cyanidin 3-malonylglucoside in *Dendranthema. Phytochemistry*, **27**, 2963–2966 (1988).
21) Nakayama M, Koshioka M, Shibata M, Hiradate S, Sugie H, Yamaguchi MA. Identification of cyanidin 3-*O*-(3″, 6″-*O*-dimalonyl-β-glucopyranoside) as a flower pigment of chrysanthemum (*Dendranthema grandiflorum*). *Biosci. Biotechnol. Biochem.*, **61**, 1607–1608 (1997).
22) Chenfei L, Yanfei L, Jiaying W, Jiaping Q, Yi C, Xiaoyan C, He H, Silan D. Flower color classification and correlation between color space values with pigments in potted multiflora chrysanthemum. *Sci. Horticul.*, **283**, 110082 (2021).
23) Suzuki H, Sawada S, Watanabe K, Nagae S, Yamaguchi MA, Nakayama T, Nishino T. Identification and characterization of a novel anthocyanin malonyltransferase from scarlet sage (*Salvia splendens*) flowers: an enzyme that is phylogenetically separated from other anthocyanin acyltransferases. *Plant J.*, **38**, 994–1003 (2004).
24) Suzuki H, Nakayama T, Yonekura-Sakakibara K, Fukui Y, Nakamura N, Yamaguchi MA, Tanaka Y, Kusumi T, Nishino T. cDNA cloning, heterologous expressions, and functional characterization of malonyl-coenzyme A: anthocyanidin 3-*O*-glucoside-6"-*O*-malonyltransferase from dahlia flowers. *Plant Physiol.*, **130**, 2142–2151 (2002).
25) Ichiyanagi T, Terahara N, Rahman MM, Konishi T. Gastrointestinal uptake of nasunin, acylated anthocyanin in eggplant. *J. Agric. Food Chem.*, **54**, 5306–5312 (2006).
26) Moriya C, Hosoya T, Agawa S, Sugiyama Y, Kozone I, Shin-Ya K, Terahara N, Kumazawa S. New acylated anthocyanins from purple yam and their antioxidant activity. *Biosci. Biotechnol. Biochem.*, **79**, 1484–1492 (2015).
27) Azuma K, Ohyama A, Ippoushi K, Ichiyanagi T, Takeuchi A, Saito T, Fukuoka H. Structures and antioxidant activity of anthocyanins in many accessions of eggplant and its related species. *J. Agric. Food Chem.*, **56**, 10154–10159 (2008).
28) Suda I, Oki T, Masuda M, Nishiba Y, Furuta S, Matsugano K, Sugita K, Terahara N. Direct absorption of acylated anthocyanin in purple-fleshed sweet potato into rats. *J. Agric. Food Chem.*, **50**, 1672–1676 (2002).
29) Hahm TH, Tanaka M, Nguyen HN, Tsutsumi A, Aizawa K, Matsui T. Matrix-assisted laser desorption/ionization mass spectrometry-guided visualization analysis of intestinal absorption of acylated anthocyanins in Sprague-Dawley rats. *Food Chem.*, **334**, 127586 (2021).
30) Ichiyanagi T, Rahman MM, Kashiwada Y, Ikeshiro Y, Shida Y, Hatano Y, Matsumoto H, Hirayama M, Tsuda T, Konishi T. Absorption and metabolism of delphinidin 3-*O*-β-D-glucopyranoside in rats. *Free Radic. Biol. Med.*, **36**, 930–937 (2004).
31) Felgines C, Talavera S, Texier O, Besson C, Fogliano V, Lamaison JL, Fauci L, Galvano G, Remesy C, Galvano F. Absorption and metabolism of red orange juice anthocyanins in rats. *Br. J. Nutr.*, **95**, 898–904 (2006).
32) Ichiyanagi T, Nashimoto M, Terahara N. Effect of acylated sugar chain terminals on the intestinal absorption of large molecular heavenly blue anthocyanin in rats. *BPB Reports*, **4**, 166–169 (2021).
33) Ichiyanagi T, Shida Y, Rahman MM, Hatano Y, Konishi T. Bioavailability and tissue distribution of anthocyanins in bilberry (*Vaccinium myrtillus* L.) extract in rats. *J. Agric. Food Chem.*, **54**, 6578–6587 (2006).
34) Kay CD, Mazza G, Holub BJ, Wang J. Anthocyanin metabolites in human urine and serum. *Br. J. Nutr.*, **91**, 933–942 (2004).
35) Ichiyanagi T, Kashiwada Y, Shida Y, Sekiya M, Hatano Y, Takaishi Y, Ikeshiro Y. Structural elucidation and biological fate of two glucuronyl metabolites of pelargonidin 3-*O*-β-D-glucopyranoside in rats. *J. Agric. Food Chem.*, **61**, 569–578 (2013).
36) Ichiyanagi T, Shida Y, Rahman MM, Hatano Y, Konishi T. Extended glucuronidation is another major path of cyanidin 3-*O*-β-D-glucopyranoside metabolism in rats. *J. Agric. Food Chem.*, **53**, 7312–7319 (2005).
37) Ichiyanagi T, Shida Y, Rahman MM, Sekiya M, Hatano Y, Matsumoto H, Hirayama M, Konishi T, Ikeshiro Y. Effect on both aglycone and sugar moiety towards Phase II metabolism of anthocyanins. *Food Chem.*, **110**, 493–500 (2008).
38) Alzaid F, Cheung H, Preedy V, Sharp P. Regulation of glucose transporter expression in human intestinal caco-2 cells following exposure to and anthocyanin-rich berry extract. *PLoS One*, **8**, e78932 (2013).
39) Malunga L, Eck P, Beta T. Inhibition of intestinal α-glucosidase and glucose absorption by feruloylated arabinoxylan mono- and oligosaccharides from corn bran and wheat aleurone. *J. Nutr. Mebatol.*, 1932532 (2016).