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Phenolic compounds and *in vitro* biological activities of nonanthocyanin fractions from mulberry fruits harvested at different maturity stages

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Summary

Mulberry fruits contain substances involved in physiological activities beneficial to human health. To explore potential utilization of mulberry fruits as functional foods, functional compounds and *in vitro* biological activities of their extracts were determined at different maturity stages. As maturity progressed, nonanthocyanin fraction (NAF) decreased, while anthocyanin fraction (AF) increased. Main phenolic acid contained in the NAF was chlorogenic acid, which decreased during ripening. Main flavonols present in the NAF were rutin, isoquercetin, and morin, which also decreased during ripening. The NAF and AF exhibited anti-inflammatory properties by lowering nitric oxide production in Raw264.7 cells. The NAF of immature mulberry fruits promoted hexokinase activity in HepG2 cells and inhibited α-glucosidase, indicating its possible hypoglycemic effect. It is suggested that immature mulberry fruits that are rich in nonanthocyanin phenolics might be a potential functional food source.

Keywords: mulberry, maturity, phenolic compound, nonanthocyanin, hypoglycemic effect

Introduction

Mulberry (*Morus alba* L.) is widely cultivated from the tropics to the subarctic (Machii, Koyama, and Yamanouchi, 2000) and high in phytochemicals having health promoting effects. Several studies revealed that mulberry fruits possess a wide range of biological activities attributed to the high content of phenolic compounds. Most of the studies on mulberry fruits have concentrated on anthocyanins and bioactive compounds in mature fruits. Mulberry extracts and anthocyanins in them have been reported to be associated with anti-inflammatory (Chen et al., 2016; Qian et al., 2015), anti-cancer (Chen et al., 2006) and anti-diabetes effects (Wang et al., 2013).

Mulberry fruits are commonly consumed fresh, but they are prone to bruising during storage and distribution because of their soft tissue. Mature mulberries are more prone to mold growth than immature ones, making it easier to deteriorate in quality (Park et al., 2013). Mature mulberries are often processed and consumed as juice, jam, or liquor. Processing, however, can degrade the bioactive compounds leading to a decrease in their bio-accessibility (Cavalcanti, Santos, and Meireles, 2011). In terms of the potential availability of mulberry fruits, it is necessary to retain their bioactive compounds associated with biological activity.

Immature and mature mulberry fruits are used in traditional medicine as tonics, antidiabetics, and immunostimulants (KFDA, 2012; Kim et al., 2013; Tang and Eisenbrand, 2011). Our previous study revealed that 1-deoxynojirimycin, γ-aminobutyric acid, and amino acids, as well as nonanthocyanin phenolics including phenolic acids and rutin, were higher in immature mulberry fruits than in mature ones. Additionally, immature fruits had an advantage retaining their physicochemical characteristics during storage (Lee and Hwang, 2017). With this result, immature mulberries are likely to be a promising resource of functional food; However, only a few studies on biological activity of immature mulberries have been reported (Lin et al., 2013; Oki et al., 2006).

Anthocyanins and nonanthocyanin phenolics, including phenolic acids and flavonoids, demonstrated strong biological effects such as antioxidant (Zhang et al., 2008), antidiabetic (Wang et al., 2013), and α-glucosidase inhibitory effects. Chlorogenic acids, rutin, and isoquercetin are known to be the main nonanthocyanins in mulberry fruits (Yang et al., 2016; Zhang et al., 2008) and are more abundant in immature mulberry fruits than in mature ones (Lee and Hwang, 2017). Nonanthocyanin phenolics in mulberry fruits presented a higher antioxidant activity than Trolox (Zhang et al., 2008). Phenolic compounds (polyphenol-rich extracts or isolated phenolic compounds) affect inhibitory activity on digestive enzymes including α-amylase and α-glucosidase, which contribute to preventing type 2 diabetes (Posedek et al., 2014). Ethyl acetate extract (nonanthocyanin fraction) of mulberry fruits had a lowering effect on fasting blood glucose level in hyperglycemic mice and higher α-glucosidase inhibitory effect than ethanol, hexane, dichloromethane, butanol, and water extracts (Wang et al., 2013). Furthermore, rutin and quercetin in mulberry fruits contributed to promoting viability of pancreatic β-cells (Li et al., 2017).

In this study, therefore, we investigated phenolic compounds and *in vitro* biological activities in nonanthocyanin and anthocyanin fractions from mulberry fruits at different maturity stages (MS) and evaluated *in vitro* biological activities of nonanthocyanin phenolics in mulberry fruits in order to enhance the potential availability of mulberry as a functional food source.

Materials and methods

Materials and chemicals

Freeze-dried mulberry powders obtained from seven different MS (MS1-7) as described in the materials and methods section of the previous study (Lee and Hwang, 2017) were used. Mulberry fruits (*Morus alba* L.) were hand-harvested at seven different MS(1-7). MS were set by the color and sizes. The fruits turned green into red, purple, and black as ripening progressed: MS-1 and 2 were reddish green, MS-3 was red, MS-4 was dark red, MS-5 was dark purple, and MS-6, 7 were black.

Methanol and acetonitrile were from Merck Chemicals (Darmstadt, Germany). Ethanol, n-hexane, and ethyl acetate were from Fisher Scientific Korea Ltd. (Seoul, Korea). Acetic acid (LC-MS grade) was from Fisher Chemical Co. (Leicestershire, UK). Dulbecco's modified eagle medium (DMEM), DMEM/F-12, 10% fetal bovine serum (FBS), 2% 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 1% penicillin/streptomycin, and trypsin were from Gibco (Grand Island, NY). Phosphate buffered saline (PBS) was from Bio-Rad Laboratories (Hercules, CA). Phosphoric acid, hydrochloric acid, sulfuric acid, sodium hydroxide, and dimethyl sulfoxide (DMSO) were from Samchun Pure Chemical Co. (Seoul, Korea). Perchloric

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acid and naphtylethylenediamine dihydrochloride were from Wako Pure Chemical Industries (Osaka, Japan). Hexokinase assay kit was from Abcam (Cambridge, UK). Protocatechuic acid (reference standard grade) was from HWI Analytik GmbH (Ruelzheim, Germany). All the other reagents and standard materials were purchased from Sigma-Aldrich Co. (St. Louis, MO).

Analysis of total cyanides in mulberry fruits

Total cyanides were determined by the method of BRADBURY, EGAN, and LYNCH (1991) with some modification. Mulberry powder (0.3 g) was vigorously mixed with 10 mL 0.1 M phosphoric acid, followed by centrifugation (1,900 × g) at 4 °C. The residue was re-extracted. The supernatants were filled into a 20 mL volumetric flask with 0.1 M phosphoric acid. The extract (1.0 mL) was hydrolyzed with 1 mL 4 M sulfuric acid at 100 °C for 50 min and neutralized with 2.5 mL 3.6 M sodium hydroxide after cooling. 100 μL of the solution was reacted with 700 μL 2 M phosphate buffer (pH 6.0) and 40 μL 0.5% (w/v) chloramine-T for 5 min. In 60 min after adding 160 μL barbituric acid/pyridine solution absorbance was measured at 600 nm with a microplate reader (SpectraMax 190, Sunnyvale, CA). Cyanide standard was used to quantify total cyanides.

Analysis of proanthocyanidins in mulberry fruits

Proanthocyanidin content was determined by the method of PRIOR et al. (2010). 80% (v/v) ethanol extracts of mulberry powder at different MS and blank (70 μL) were reacted with 0.1% (w/v) 4-dimethylaminocinnamaldehyde reagent solution (210 μL) at room temperature for 10 min. Absorbance was measured using the microplate reader at 640 nm. Catechin was used as standard, and proanthocyanidin content was expressed as mg catechin equivalent (eq)/100 g on dry weight basis (dw).

Preparation of nonanthocyanin fraction (NAF) and anthocyanin fraction (AF) from mulberry fruits

Freeze-dried mulberry powder (approximately 1.5-2.0 g) was defatted with n-hexane (3 × 20 mL) and extracted with acidified 80% ethanol (pH 2.25) (3 × 20 mL) in a shaking water bath for 1 h at 40 °C. According to the method described by KAMMERER et al. (2004), the extract was suspended in 10 mL distilled water (pH 3.0) after evaporation of the solvent. 5 mL aliquot of the extract solution was made up to 20 mL with distilled water and pH was adjusted to 1.5, followed by partitioning with 50 mL ethyl acetate. The ethyl acetate layer was entirely evaporated to obtain NAF, which was dissolved in 10 mL distilled water for analysis of nonanthocyanin phenolics or 2 mL PBS for biological assay. The water layer was further fractionated by a C18 SPE cartridge (Waters Co., Milford, MA) to collect AF according to the method described by KAMMERER et al. (2004). After the cartridge was activated with 3 mL methanol and cleaned with 10 mL distilled water, the AF was eluted from the water layer with 10 mL methanol with 0.01% hydrochloric acid (v/v). The AF was concentrated in vacuo and then dissolved in 2 mL PBS.

HPLC-MS/MS analysis of nonanthocyanin phenolics

Nonanthocyanin phenolics were eluted from the NAF using the SPE cartridge according to the method described by KAMMERER et al. (2004). After the cartridge was activated with 3 mL methanol and cleaned with 10 mL distilled water, phenolic acids were eluted from the NAF with 10 mL distilled water and 10 mL 0.01% (v/v) hydrochloric acid (pH 2.65). Flavonols were subsequently eluted with 20 mL ethyl acetate. The eluates were concentrated in vacuo. The concentrated eluates of phenolic acids and flavonols were dissolved in 2 mL 2% acetic acid and 2 mL methanol, respectively.

Identification and quantification of nonanthocyanin phenolics in the NAF were carried out by HPLC (Agilent 1200 Series, Agilent Technologies) coupled with an API 4000 triple quadrupole mass spectrometer (Applied Biosystem, Foster City, CA) with an electrospray interface. Separation was operated on an Atlantis T3 column (3 μm, 2.1 mm × 100 mm; Waters Co.) with a guard column (XDB C18, 5 μm, 4.6 mm × 12.5 mm; Agilent Technologies) at 30 °C. Mobile phases consisted of deionized water with 2% (v/v) acetic acid (solvent A) and acetonitrile (solvent B). Flow rate was 0.5 mL/min with a gradient elution program as follows: 0-3 min, 5% B; 3-5 min linear gradient from 5 to 10% B; 5-7 min 10% B; 7-10 min linear gradient from 10 to 15% B; 10-13 min 15% B; 13-16 min linear gradient from 15 to 20% B; 16-20 min 20% B; 20-25 min linear gradient from 20 to 25% B; 25-30 min 25% B; 30-35 min linear gradient from 25 to 30% B; 35-40 min 30% B; 40-45 min linear gradient from 30 to 95% B; 45-50 min 95% B; 50-51 min linear gradient from 95 to 5% B; and 4 min reconditioning. Injection volume was 5 μL. Mass spectroscopy was performed in negative mode using multiple reaction monitoring with the optimal conditions obtained by direct infusion of the standard solution. The optimum conditions were -4500 V of ion spray voltage and 50 psi for ion source gas 1 and gas 2. Standard compounds used for identification and quantification were purchased from Sigma-Aldrich Co. (chlorogenic acid, protocatechuic acid, caffeic acid, 4-hydroxybenzoic acid, ferulic acid, rutin, isoquercetin, taxifolin, and scopoletin) and Wako Pure Chemicals Co. (morin).

Cell culture

Mouse macrophage Raw264.7 and human hepatoma HepG2 cells obtained from Korea Cell Bank (Seoul, Korea) were cultured in DMEM supplemented with 10% FBS, 2% HEPES, and 1% penicillin/streptomycin. All the cells were maintained at 37 °C in a humidified atmosphere of 5% CO_2. The cells were harvested with trypsin and subcultured when they reached 80 % confluence.

Determination of cell viability and nitric oxide (NO) assay

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (MOSMANN, 1983) was conducted to measure cytotoxicity of the AF and NAF on Raw264.7 and HepG2 cells. Raw264.7 or HepG2 cells were plated in a 96-well plate at a density of 2.0×10^4 cells/well and incubated at 37 °C for 24 h. After removing the medium, the cells were incubated with serum-free medium including the AF and NAF at a concentration range of 25-200 μg/mL for 24 h and then added to a serum-free medium with MTT solution (5 mg/mL in PBS). After incubating for 2 h, 100 μL DMSO was added to dissolve formazan. Absorbance was measured at 540 nm with the microplate reader.

The level of NO production was determined by measuring accumulation of nitrile (IZUMI, OHNO, and YADOMAE, 1997). Raw264.7 cells were plated in a 96-well plate at a density of 1.0×10^5 cells/well and incubated at 37 °C for 24 h. After the medium was removed, the cells were incubated with serum-free medium including the AF and NAF at a concentration range of 25-200 μg/mL for 4 h, and then added with 100 μg/L lipopolysaccharides (LPS). After incubation for 20 h, the supernatant of the wells was reacted with Griess reagent (0.5% (w/v) sulphanilamide with 2.5% phosphoric acid and 0.05% (w/v) naphthylethylenediamine dihydrochloride) at room temperature for 5 min. Absorbance was measured at 540 nm with the microplate reader and NO production (%) was expressed as the ratio of absorbance of the control and the sample.

Hexokinase activity of HepG2 cells

HepG2 cells were plated in a 96-well plate at a density of 1.0×10^5 cells/well and incubated at 37 °C for 24 h. After removing the me-

dium, the cells were treated with the NAF, AF (50 μg/mL), or metformin (positive control) and then incubated with high glucose DMEM (5 μg/mL of insulin) for an additional 24 h. After removing the medium again and washing with PBS, 200 μL assay buffer was added to the wells. Supernatant was obtained by centrifuging (Smart R17, Micro refrigerated centrifuge, Hanil Science Industrial Co., Incheon, Korea) the lysate at 13,475 × g. Hexokinase activity was determined using hexokinase assay kit and measured at 450 nm with the microplate reader. Hexokinase activity (%) was calculated as the ratio of absorbance of the control and the sample for which the protein content was corrected. The protein concentration was determined using the Bradford assay (BRADFORD, 1976).

α-Glucosidase inhibition assay

A modified bioassay method described by Sigma-Aldrich (KAPUSTKA, ANNALA, and SWANSON, 1981) was used to determine α-glucosidase inhibitory effect. Twelve g maltose was dissolved in 300 mL 50 mM sodium acetate buffer to prepare a 4% maltose solution. α-Glucosidase enzyme (EC 3.2.1.20) solution was prepared at 1 unit/mL using ice-cold distilled water. *o*-Dianisidine (DIAN) solution and peroxidase/glucose (PGO) system-color reagent solution which are both colorimetric reagents were prepared by dissolving one DIAN tablet and one PGO capsule in 25 mL and 100 mL of ice-cold distilled water, respectively. Acarbose was obtained from Sigma-Aldrich Co., and acarbose solution (10 μg/mL) was used as positive control. In the first step, 20 μL of sample, distilled water (control) or acarbose was mixed with 20 μL 4% maltose solution, left in an incubator at 37 °C for 5 min. Reaction started after adding 50 μL of the α-glucosidase solution, and the mixture was left in an incubator at 37 °C for 30 min. After that, 10 μL 4.2% (v/v) perchloric acid was added to terminate the reaction. In the second step, 50 μL of supernatant obtained by centrifuging (13,475 × g) the reaction tube was reacted with DIAN and PGO solutions, left in an incubator at 37 °C for 30 min. Absorbance of the reactant solution was measured at 500 nm with the microplate reader. α-Glucosidase inhibition was expressed as IC_{50} value (μg/mL), representing the concentration of a compound that causes 50% enzyme loss.

Statistical analysis

Statistical analysis was performed using SPSS program (SPSS version 20.0, Chicago, IL). Data were presented as means ± standard deviations (SD) of three independent experiments. To compare differences among the experiment groups, the data were analyzed by analysis of variance (ANOVA) and t-test ($P<0.05$). Regression analysis was also calculated between nonanthocyanin phenolic compounds and α-glucosidase inhibitory effect.

Results and discussion

Total cyanides and proanthocyanidins in mulberry fruits, and yield of mulberry extract

Since seeds of some immature fruits may have higher amount of cyanides, the total cyanides in mulberry fruits were analyzed for the different MS. Total cyanides in the fruits of the MS1-5 ranged from 4.1 to 7.7 mg/100 g dw, and those of the MS6 and 7 were not detectable (Tab. 1). Cyanides released from a large number of plants and fruit seeds such as apricots and peaches are lethal at a dose of 0.5-3.5 mg/kg body weight (BRADBURY, EGAN, and LYNCH, 1991). It is, therefore, suggested that intake of immature or mature mulberry fruits may not cause a risk of cyanide toxicity.

As shown in Tab. 1, proanthocyanidins in immature mulberry fruits (MS2-4, 20.3-35.3 catechin eq mg/100 g dw) were significantly ($P<0.05$) higher than those of mature fruits (MS6 and 7, 4.1 and 8.9 mg/100 g, respectively). Proanthocyanidins function as a plant defense against predation by accumulating in different tissues and organs. Proanthocyanidins, as strong antioxidants, also have potential to protect against cancers, cardiovascular disease, and Alzheimer's disease (SHARMA et al., 2011).

The yield of the total extract rapidly increased after the MS5, changing from 26.1% (w/w, dw) at the MS1 to 76.2% at the MS7. AF also increased from 3.1% at the MS4 to 4.9% at the MS7 whereas NAF decreased from 6.2% at the MS3 to 2.8% at the MS7. It is assumed that as fruits matured, sugars and anthocyanins increased, affecting the yield of the extracts.

Nonanthocyanin phenolics in the NAF

Nonanthocyanin phenolics in the NAF analyzed by LC-MS/MS are shown in Tab. 2. According to total ion chromatogram (not shown) of the NAF, three high peaks observed at 6.6, 10.0, and 12.8 minutes were identified as chlorogenic acid (CGA) and their isomers (353 *m/z*, precursor ion; 191, 85 *m/z*, product ion). CGA is 3-caffeoylquinic acid, of which neochlorogenic acid (neo-CGA, 5-caffeoylquinic acid) and cryptochlorogenic acid (crypto-CGA, 4-caffeoylquinic acid) are isomers. Several studies demonstrated that CGA and crypto-CGA (ZHANG et al., 2008), neo-CGA and crypto-CGA (OKI et al., 2006), and all the 3 CGA isomers (IZABELLE et al., 2008; LEE et al., 2016) are present in mulberry fruits. Phenolic acid contained in the NAF was mostly CGA (Tab. 2); CGA content dropped drastically (12,813-2,251 mg/100 g dw) as the fruits matured. This observation corresponded to previous studies reporting that CGA in mulberry fruits decreased during ripening (LEE et al., 2016; OKI et al., 2006; YANG et al., 2016). Neo-CGA was the most abundant among the CGA isomers in agreement with the results in a previous study (YANG et al., 2016).

Tab. 1: Changes in total cyanides and proanthocyanidins in mulberry fruits and yield of extracts from mulberry fruits

	Total cyanides (mg/100 g, dw)	Proanthocyanidins (catechin eq mg/100 g, dw)	Yield of mulberry extract (%, dw)		
			Total extract	NAF	AF
MS-1	5.6±2.8	11.3±5.1 cd	26.1±1.9 e	5.2±0.5 a	-
MS-2	5.6±1.8	21.2±8.9 b	27.9±4.3 de	5.9±0.6 a	-
MS-3	7.4±3.0	35.3±3.5 a	32.4±0.8 d	6.2±1.6 a	-
MS-4	7.7±2.5	20.0±5.3 bc	40.9±3.5 c	5.4±0.8 a	3.1±1.2 a
MS-5	4.1±0.7	11.3±2.7 cd	51.7±1.8 b	4.7±0.7 a	3.5±1.2 a
MS-6	nd	4.1±1.0 d	74.2±2.3 a	2.8±0.5 b	4.2±0.5 a
MS-7	nd	8.9±2.9 d	76.2±2.2 a	2.9±0.6 b	4.9±0.6 a

MS, maturity stage; eq, equivalent; dw, dry weight basis; NAF, nonanthocyanin fraction; AF, anthocyanin fraction; Values are means ± standard deviations (n=3); Values with different lower case letters within the same column are significantly different by Duncan's test at $P<0.05$; nd, not detected.

Tab. 2: Phenolic compounds in nonanthocyanin fractions (NAF) from mulberry fruits, measured by LC-MS/MS

	Precursor ion	Product ion	tR (min)	Phenolic compounds (mg/100 g, dw) in NAF						
				MS-1	MS-2	MS-3	MS-4	MS-5	MS-6	MS-7
Total CGA	-	-	-	12812.9	11141.6	5796.5	6400.0	5059.8	3008.7	2251.5
CGA	353	191, 85	6.6	2917.1	2456.0	1284.0	1210.5	859.2	251.0	254.2
Neo-CGA	353	191, 85	10.0	7669.2	6672.6	3780.3	4179.5	3542.7	1650.5	1785.3
Crypto-CGA	353	191, 85	12.8	2226.6	2013.0	732.2	1010.0	657.9	264.4	212.0
PCA	153	129, 91	5.4	14.0	14.0	11.1	17.7	21.9	78.5	57.4
Caffeic acid	179	134, 89	11.6	22.3	17.3	8.0	9.5	5.8	7.8	8.2
4-HBA	137	95, 65	9.0	2.4	2.2	1.4	1.7	1.4	5.0	3.4
Ferulic acid	193	137, 178	17.9	1.8	1.7	1.0	1.0	1.1	2.2	3.7
Rutin	609	300, 271	19.4	693.1	594.9	342.3	491.2	483.9	455.7	401.1
Isoquercetin	463	300, 271	20.0	622.8	533.3	275.8	390.8	326.4	235.2	162.6
Morin	301	151, 149	28.8	320.4	273.7	171.5	253.0	237.9	263.6	181.1
Taxifolin	303	285, 125	19.0	14.2	13.1	13.8	27.9	36.5	44.0	36.4
Scopoletin	191	176, 105	17.4	13.1	10.4	7.8	4.3	5.9	4.8	3.8

MS, maturity stage; dw, dry weight basis; CGA, chlorogenic acid; PCA, protocatechuic acid; 4-HBA, 4-hydroxybenzoic acid; t_R, retention time of each compound in chromatogram; Values are means (n=2).

Two major NAF flavonols, rutin and isoquercetin, decreased with ripening from 693.1 to 401.1 mg/100 g dw and from 622.8 to 160.6 mg/100 g, respectively (Tab. 2). Morin ranged from 171.5 to 320.4 mg/100 g. Previous studies reported that morin content was 15.7 mg/100 g dw in dried mulberry fruits (YANG, YANG, and ZHENG, 2010) and in a range from 0.6 to 1.6 mg/100 g dw in six mulberry cultivars (LEE et al., 2004). Scopoletin, one of coumarin derivatives, decreased from 13.1 to 3.8 mg/100 g dw, whereas taxifolin increased from 14.2 to 36.4 mg/100 g during ripening. Previous studies reported similar trends that main flavonols present in mulberry fruits were rutin and isoquercetin (LIN and LAY, 2013; YANG, YANG, and ZHENG, 2010; ZHANG et al., 2008), and rutin was present in higher concentrations than isoquercetin (PAWLOSKA, OLESZEK, and BRACA, 2008; YANG et al., 2016). Taxifolin level was 0.7 and 3.1 mg/100 g fresh weight in two cultivars of mulberry fruits (ZHANG et al., 2008), slightly increasing (0.01-0.04 mg/100 g) with ripening (YANG et al., 2016). LIN and LAY (2013) also reported that scopoletin decreased from 22.1 to 5.3 mg/100 g dw with progressing maturity. It is well known that the content of phenolic compounds varies by genetic diversity and environmental conditions although the previous reports demonstrated some similar results to this study.

BASSOLI et al. (2008) reported that chlorogenic acid with 0.5-1.0 mM inhibited glucose-6-phosphatase activity in liver cells of rats by 40% and glucose transport capability of brush-border membrane vesicles separated from small intestine of rats by 80%. In addition, chlorogenic acid and rutin were dominant nonanthocyanin phenolics separated from mulberry fruits using ethyl acetate (ZHANG et al., 2008). Ethyl acetate fraction of mulberry fruits demonstrated higher α-glucosidase inhibitory and radical scavenging activities than ethanol, hexane, chloroform, butanol, and water-soluble fractions (WANG et al., 2013). The ethyl acetate fraction could also significantly decrease fasting blood glucose and glycosylated serum protein levels and increase antioxidant enzymatic activities in streptozotocin-induced diabetic mice (WANG et al., 2013). It has been reported that formation and coagulation of islet amyloid polypeptide worsened symptoms of type 2 diabetes due to apoptosis of pancreatic β-cells (Clark et al., 1987), and morin hydrate with 32 μM (10.2 mg/mL) was effective in breaking down islet amyloid polypeptide fiber decomposition (NOOR, CAO, and RALEIGH, 2011).

NO production in LPS-stimulated Raw264.7 cells

Conventional MTT assay was conducted to determine cytotoxicity of anthocyanin (AF4-7) and nonanthocyanin (NAF1-7) fractions on Raw264.7 and the HepG2 cells. The viabilities of all the cells treated with different concentrations (50-400 μg) of all the fractions were higher than 80% (data not shown).

Fig. 1 shows the amounts of NO generated when the extracts were treated in Raw264.7 macrophage cells stimulated by LPS. The cells treated with the NAF from the fruits of all the MS except MS2 and

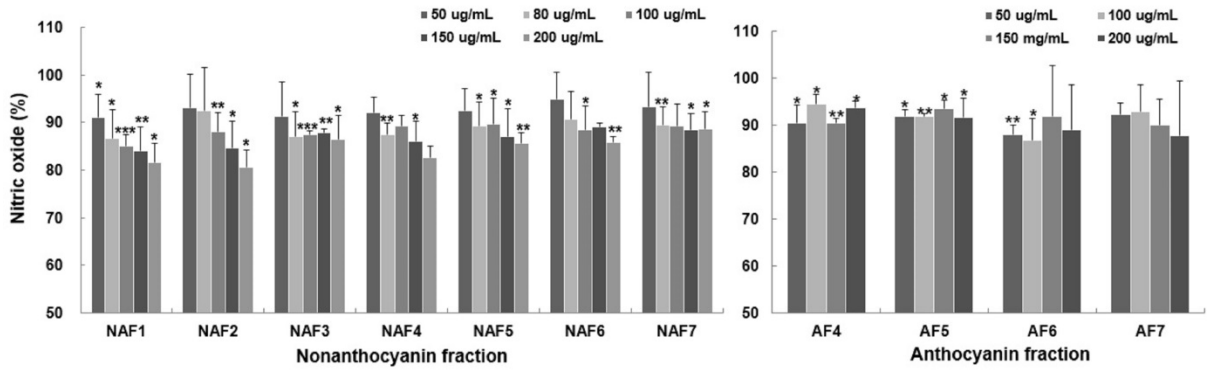

Fig. 1: Nitric oxide production of Raw264.7 cells treated with nonanthocyanin fraction (NAF, maturity stage 1-7) and anthocyanin fraction (AF, maturity stage 4-7). Untreated control cells were set at 100. Bars represent means ± standard deviations (n=3). Statistical significance is based on the difference when compared with the cells without treating extracts (*P<0.05, **P<0.01, and ***P<0.001).

6 had less NO than those with the control at concentrations above 80 μg/mL and tended to inhibit NO generation dependent on concentration. All the concentrations of the AF from the fruits of the MS4 and 5 retarded production of NO compared to the control, whereas the AF of the MS6 only showed inhibition effect at concentrations of 50 and 100 μg/mL. Qian et al. (2015) observed that nitrite generation was inhibited in Raw264.7 macrophage cells treated with mulberry extract at 25-200 μg/mL. Cuevas-Rodriquez et al. (2010) reported that antioxidant activity and NO production inhibition were significantly correlated with polyphenol contents in blackberry extracts. Therefore, the abundant phenolic compounds in the mulberry extract may contribute to the inhibition of inflammation in Raw264.7 macrophage cells stimulated by LPS.

Hexokinase activity and α-glucosidase inhibition of mulberry extracts

Hexokinase, an enzyme involved in glucose metabolism in numerous organisms, produces hexose phosphate by phosphorylating hexoses. There are four isoforms of hexokinase, and one of them is glucokinase, which facilitates glycolysis by phosphorylating glucose and producing glucose-6-phosphate in liver cells of animals and humans. It is known that glucokinase is less active in a large number of type 2 diabetes patients (Caro et al., 1995).

The effect of the mulberry extract fractions (NAF and AF) at different maturity stages (MS1-7) on hexokinase activity is shown in Fig. 2. Hexokinase activity is expressed as a percentage of the untreated group (control). In the assay containing 25 mM glucose, insulin, and NAF5, hexokinase activity was significantly higher than in the control (NAF5 114.8% compared to control) and in the same range as the assay containing metformin (120.3% compared to control). Significantly higher hexokinase activities could also be observed in several NAF from immature fruits (MS2-5) when compared to AF from mature fruits (MS6-7).

Primary side effects of currently used α-glucosidase inhibiting drugs such as acarbose include flatulence, abdominal distention, and diarrhea (Bischoff, 1994). α-Glucosidase is a digestive enzyme located in the brush border of the small intestine and degrades carbohydrates such as disaccharides and oligosaccharides into monosaccharides (Balfour and McTavish, 1993). Reduction of postprandial hyperglycemia by inhibition of α-glucosidase is essential for prevention and control of type 2 diabetes. In this study, α-glucosidase inhibitory ability of the mulberry fractions was determined and presented in Fig. 3. As maturity progressed, IC_{50} of the NAF (MS1-7) increased from 178.0 to 428.3 μg/mL, whereas that of the AF (MS4-7) decreased from more than 500.0 to 276.9 μg/mL, indicating α-glucosidase inhibitory activity decreased in the NAF but increased in the AF. All the fractions except the NAF of mature fruits (MS6 and 7) and the

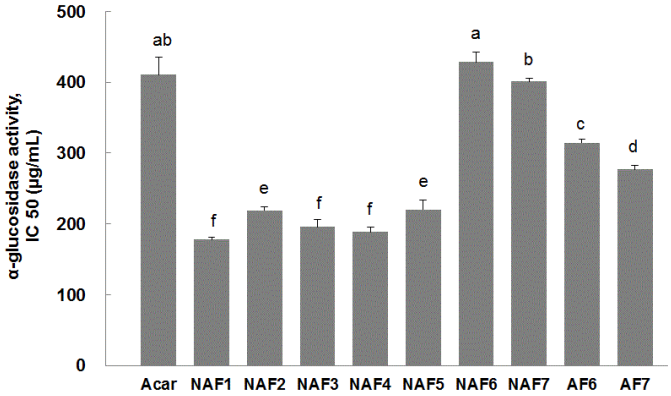

Fig. 3: Effect of mulberry extracts on α-glucosidase activity. IC_{50} values (μg/mL) of compounds represent the concentrations causing 50% enzyme activity loss. Acar, acarbose; NAF, nonanthocyanin fraction (maturity stage1-7); and AF, anthocyanin fraction (maturity stage4-7). IC_{50} of AF4 and 5 are more than 500 μg/mL. Bars represent means ± standard deviations (n=3). Different letters (a-f) above the bars indicate significant differences by Duncan's test at $P<0.05$.

AF of immature fruits (MS4 and 5) had a significantly ($P<0.05$) higher activity than acarbose (410.9 μg/mL). Also, the NAF (MS1-4) showed higher inhibitory effect than the AF (MS4-7), suggesting that nonanthocyanin phenolics in immature mulberry fruits might have stronger hypoglycemic effect than anthocyanins in mature mulberry fruits. Wang et al. (2013) reported that ethyl acetate extract of mulberry fruits showed a decreasing effect on fasting blood glucose in hyperglycemic mice and higher α-glucosidase inhibitory activity than ethanol, hexane, dichloromethane, butanol, and water extracts.

Regression analysis was performed with total amount of nonanthocyanin phenolic compounds (NAF1-7, 27329.0-5360.7 mg/100 g dw) and IC_{50} value expressing α-glucosidase inhibitory effect (NAF1-7, 178.0-401.3 μg/mL). Log-transformed total amount of nonanthocyanin phenolic compounds significantly correlated with IC_{50} value ($P=0.016$). A negative linear relationship ($R^2=0.719$) was shown between nonanthocyanin phenolic compounds and IC_{50} value, indicating that phenolic compounds including chlorogenic acid, rutin, isoquercetin, and morin, which were abundantly found in the NAF, were likely to contribute to the biological activity.

Conclusion

The present study investigated phenolic compounds and *in vitro* biological activities of mulberry extracts at seven different MS in order to explore if mulberry fruits could be a potential source of functional

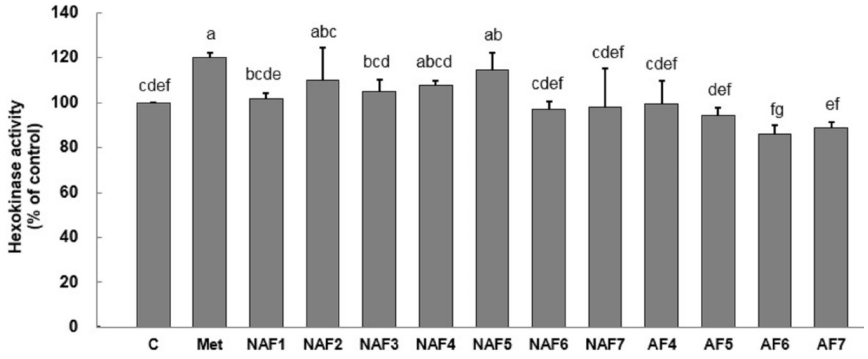

Fig. 2: Effect of mulberry extracts on hexokinase activity of HepG2 cells. C, control; MET, metformin; NAF, nonanthocyanin fraction (maturity stage 1-7); and AF, anthocyanin fraction (maturity stage 4-7). Bars represent means ± standard deviations (n=3). Different letters (a-g) above the bars indicate significant differences by Duncan's test at $P<0.05$.

foods. As maturity progressed, NAF decreased but AF increased. Main phenolic acid contained in the NAF was CGA, which decreased during ripening. Furthermore, main flavonols present in the NAF were rutin, isoquercetin, and morin, which also decreased during ripening. The NAF and AF had anti-inflammatory effects by lowering NO production in Raw264.7 cells. The NAF of immature mulberry fruits promoted hexokinase activity in HepG2 cells and inhibited α-glucosidase, suggesting that the NAF might have a hypoglycemic effect. CGA, rutin, isoquercetin, and morin present in the NAF might contribute to biological activities of mulberry fruits. It is suggested that immature mulberry fruits that are rich in nonanthocyanin phenolics might be a potential functional food source.

Conflict of interest

No potential conflict of interest was reported by the authors.

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