EVALUATION OF YAK-KONG SOYBEAN (*GLYCINE MAX*) FOR ITS PHENOLICS AND METABOLITES WITH A FOCUS ON ATHEROSCLEROSIS PREVENTION

A Dissertation

Presented to the Faculty of the Graduate School

of Cornell University

In Partial Fulfillment of the Requirements for the Degree of

Doctor of Philosophy

by

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May 2017

EVALUATION OF YAK-KONG SOYBEAN (*GLYCINE MAX*) FOR ITS PHENOLICS AND METABOLITES WITH A FOCUS ON ATHEROSCLEROSIS PREVENTION

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Cornell University 2017

Increasing intake of healthy or functional foods is the promising way to prevent noncommunicable diseases especially cardiovascular diseases (CVDs) which have been the number one cause of death in globe. Soybean (*Glycine max*) has been supported by significant scientific evidence particularly on the risk reduction of CVDs. With the aim of seeking a competitive functional soybean ingredient, a small black soybean cultivar with a green embryo commonly called Yak-Kong (YK) in Korea was chosen based on its physical characteristics and historical background and was evaluated its phenolics and their metabolites with a focus on prevention of CVDs.

First, antioxidant activities and phenolics of YK were analyzed. In comparison to common yellow and black soybean cultivars, YK contained significantly higher concentrations of antioxidants, particularly in its seed coat. A comprehensive phenolic composition analysis revealed that proanthocyanidins and isoflavones were the major phenolic groups in YK.

Potential effect of proanthocyanidins and isoflavones on prevention of CVDs was further evaluated with their prospective metabolites using a monocyte-endothelial cell adhesion *in vitro* assay. The present study found that 5-(3',4'-dihydroxyphenyl)-γ-

valerolactone (DHPV), one of the previously reported major human microbial metabolites of proanthocyanidins, strongly attenuated adhesion of THP-1 monocytes to tumor necrosis factor (TNF)-α-stimulated human umbilical vein endothelial cells (HUVECs). DHPV downregulated TNF-α-stimulated expressions of vascular cell adhesion molecule-1 (VCAM-1), monocyte chemotactic protein-1 (MCP-1) and activation of nuclear factor-κB (NF-κB), which all are associated with atherosclerosis.

Additionally, several hydroxylated metabolites of isoflavones in addition to their precursors were target-analyzed in the plasma of rat fed with YK embryo extract. 7,8,4'-trihydroxyisoflavone (7,8,4'-THI), a hydroxylated daidzein metabolite, was exclusively detected with the highest amount (average 5.1 μM) and average 574.3 ± 112.8 area under the curve within 240 min. In contrast to daidzein and daidzin, 7,8,4'-THI significantly prevented adhesion of THP-1 monocytes to TNF-α-stimulated HUVECs via downregulating expressions of VCAM-1, MCP-1 and phosphorylation of I Kappa B Kinase (IKK) in NF-κB signaling pathway.

In sum, YK has promising potential for further development as a functional food source targeted at atherosclerosis prevention.

BIOGRAPHICAL SKETCH

Charles Changwon Lee was born on February 20th, 1987 in New York, USA. In 1990, his family decided to move back to their home country, Republic of Korea. He received his early education in Republic of Korea and came back to USA for his bachelor's degree. In 2008, he joined the Food Science program in the University of Wisconsin-Madison and obtained his B.S. in 2011. While he was an undergraduate, he connected with a number of scientists studying health benefits of phytochemicals in foods. In Prof. Kirk Parkin's food science laboratory, University of Wisconsin-Madison and the Hormel Institute, University of Minnesota, he studied anti-cancer and anti-inflammatory bioactivities of phytochemicals.

After graduating with his B.S. degree, Charles desired to pursue further studies on the health benefits of foods and their application. In 2012, he joined a Ph.D. program in Food Science and Technology at Cornell University. At Cornell, he majored in Food Chemistry under the supervision of Dr. Chang Yong Lee and minored in Food Processing Waste Technology and Food and Beverage Management under the guidance of Dr. Olga I. Padilla-Zakour and Dr. Mary Tabacchi, respectively. During his Ph.D. program, he developed his interest in product development and processing. He, as a team member and a leader, won multiple national food product development competitions. With his experience and passion, he established a food startup MacroFuel Food, Inc. with his colleagues. After graduating from Cornell, he wish to continue to develop his interest in studying the functionality of plant-derived ingredients, the application of valuable food by-products and the development of functional health products with glocalization and personalization strategies.

To my beloved family and friends

ACKNOWLEDGMENTS

First and foremost I would like to thank God for his blessings over my life.

I would like to express my deepest appreciation to my advisor Dr. Chang Yong Lee for his guidance and support throughout my PhD program at Cornell. Not only has he served as an academic advisor, he has also served as my mentor. He provided insightful feedback in all of my work and gave me a lot of inspiration on how to become a better scientist and person.

I would also like to express my sincere thanks to Dr. Ki Won Lee for his continuous support and encouragement. Without his great help, I could not have pursued my PhD degree and finish this dissertation. His enthusiasm always has motivated me. He has been an incredible resource on numerous levels from carrying out my research to professional development.

I would like to thank my minor advisors, Dr. Olga Padilla-Zakour and Dr. Mary Tabacchi, for their great help on developing my career interests in product development and management. I could not be more grateful to have both extraordinary advisors who have continuously helped me to gain knowledge and experience in my minor fields of study. I thank Dr. Olga Padilla-Zakour for her valuable advice in career development. If someone asks me who my career role model is, I do not hesitate to say it is her. Thanks to Dr. Mary Tabacchi for her warm encouragement throughout my PhD program.

I express my gratitude to Dr. Myoung Ok Kim and Dr. Sung Keun Jung who taught me all the essential experiments that were needed for molecular biology studies in my PhD work and the time/multi-task management skills. I also thank the people

from the Food-Medicine Genomics lab in Seoul National University, Republic of Korea. I thank Prof. Jung Han Yoon Park, Dr. Jong Hun Kim, Dr. Tae Gyu Lim and Dr. Jong Eun Kim for their friendship and research support. Especially I thank Hee Yang, Si Young Lee, Hee Joo Kim, “Yong Soo Cheol” and Ji Seung Kim for all the great memories we had. We went through both good and tough times together and shared laughter and tears. I also express special thanks to the people from the Institute of Nutrition and Functional Foods, University Laval, Canada. Thanks to Prof. Yves Desjardins, Dr. Stephanie Dudonne and Pascal Dube for their collaboration and great help in my PhD research.

Most importantly, I am deeply grateful to my family. I thank my parents, my sister and my relatives for their unconditional love, care and prayer. They all mean everything to me. I could not go this far without them.

I thank my best friend and girlfriend Vanessa for her love, care, support and trust. Thank you for making me laugh and smile throughout all those tough times. I also would like to thank my best friends, Tokito and Ddolddori, for their special friendship. Lastly, I thank all my friends who encouraged me with their faith in me.

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CHAPTER 1

INTRODUCTION

Cardiovascular diseases and healthy diet

According to the World Health Organization (WHO, 2014), more than 50% of global deaths are due to noncommunicable diseases (NCDs) also known as chronic diseases that can be preventable. The number one cause of NCD deaths has been cardiovascular diseases (CVDs). CVDs killed 17.5 million people in 2012, representing more than 45% of NCD deaths (WHO, 2014). Among different types of CVDs such as coronary heart disease (CHD), cerebrovascular disease (CBD), hypertension, and peripheral arterial disease, CHD has been the number one cause of death for both men and women globally (WHO, 2011). It has been reported that the underlying process of CVDs, especially CHD and CBD, is atherosclerosis, a complex pathological process that develops plaques over time in walls of blood vessels (WHO, 2011). The most important factors contributing to the development of atherosclerosis are unhealthy diets (rich in salt, fat, and calories), tobacco use, physical inactivity, and harmful use of alcohol (WHO, 2011). Therefore, the main prevention strategy for atherosclerosis is lifestyle changes such as increasing intake of fruits, vegetables, and fish but decreasing salt, saturated fat, and trans-fat cholesterol in diets (WHO, 2011). Application of healthy diets and functional foods is a promising way to prevent NCDs, including CVDs and atherosclerosis.

Atherosclerosis and antioxidative phenolics

Previously, it has been elucidated that atherosclerosis is not a simple lipid-

storage disease, but a chronic inflammatory disease (Ross, 1999). Atherosclerosis starts once vascular endothelial cells are dysfunctional (Plutzky, Viberti & Haffner, 2002). Several risk factors including smoking, intake of diets rich in saturated fat, salt and calorie, and other stress inducers can damage innate functions of vascular endothelial cells such as regulation of endothelium permeability and adhesiveness of leukocytes to the arterial wall (WHO, 2011; Libby, 2006). Some other disease conditions such as obesity, insulin resistance, hyperglycemia, hypertension, and infection can also cause endothelial dysfunction (Libby, Ridker & Maseri, 2002). These risk factors can potentially induce oxidation of low-density lipoprotein and elevation of reactive oxygen species (ROS) levels or reduce antioxidants/antioxidant synthase (Libby, 2006; Plutzky et al., 2002). ROSs such as $O_2^{\cdot -}$, $^1O_2$, $HO^{\cdot}$, $NO^{\cdot}$, $ONOO^{-}$, HOCL, $RO(O)^{\cdot}$, and $LO(O)^{\cdot}$ are either radicals or reactive non-radical compounds that are capable of oxidizing biomolecules (Prior, Wu & Schaich, 2005). Antioxidants can scavenge ROSs and free radicals by donating electrons or hydrogens (Halliwell & Gutteridge, 1995). Numerous studies support that antioxidants and polyphenols from fruits and vegetables can potentially lower the risk of mortality from CVDs (Wang et al., 2014).

Potential health benefits of naturally occurring antioxidants and polyphenols have been studied extensively in the past two decades. Their structures have been characterized to have at least one aromatic ring with hydroxyl groups (El Gharras, 2009). Polyphenols can be divided into four different classes: phenolic acids, flavonoids, stilbenes, and lignans (El Gharras, 2009). Flavonoids are the largest class of polyphenols that share a common structure consisting of two aromatic rings bound

together with three carbon atoms, forming an oxygenated heterocycle (C ring) (RiceEvans, Miller & Paganga, 1996). It has been found that the antioxidant activity of flavonoids such as delocalization of unpaired electrons is derived from their ortho-dihydroxy structure in the B ring, 2 & 3 double bond conjugated with a 4-oxo group in the C ring, and 3- & 5-OH groups with 4-oxo group in A and C rings (RiceEvans et al., 1996). Based on the type of heterocycle function (Figure 1.1), flavonoids can be further divided into six subclasses: flavones, flavanones, flavonols, flavanonols, flavan-3-ols, anthocyanidins, and isoflavonoids (El Gharras, 2009).

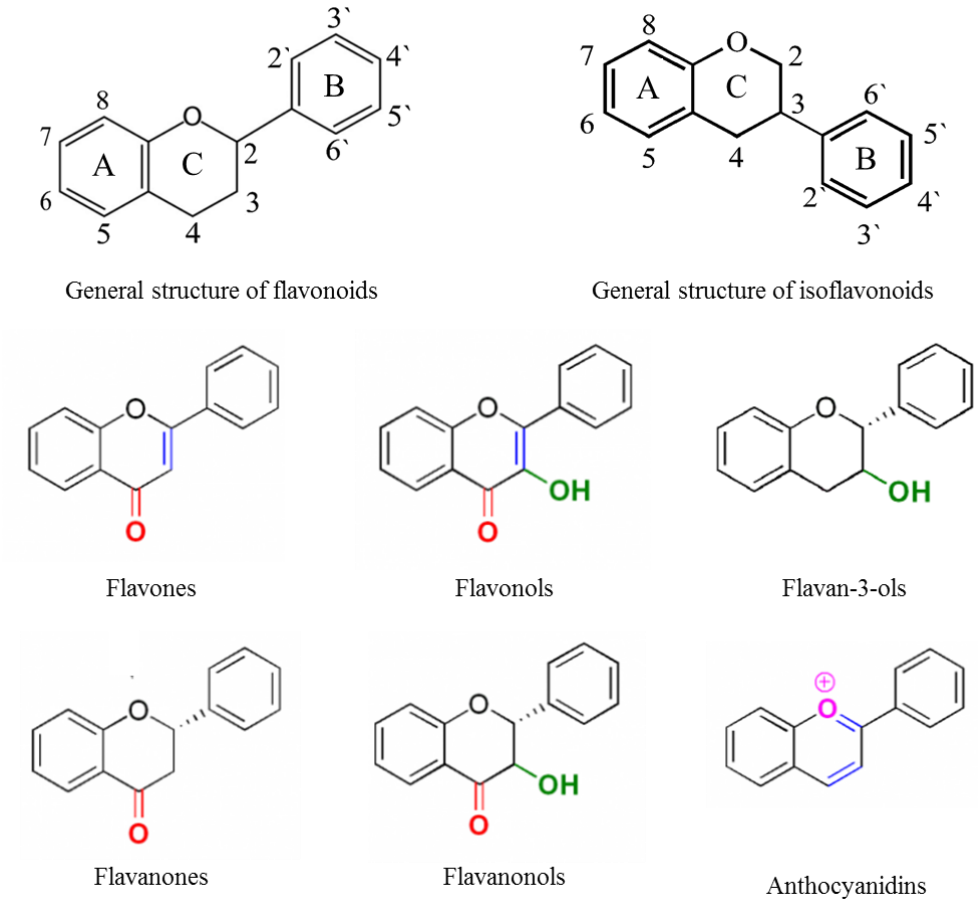

Figure 1.1. Structures of flavonoids.

Soybean as a CHD preventive food ingredient

Soybean (*Glycine max*) is a popular food source for health-conscious consumers. It has numerous health benefits (United Soybean Board, 2014). High consumption of soy protein has been associated with reduced incidence of CHD (Anderson & Bush, 2011). In contrast to other members of the Leguminosaea family including *Phaseolus* (pinto bean, kidney bean), *Lens* (lentil), and *Vigna* (blackeyed pea, mung bean), only *Glycine max* (soybean) protein as an ingredient has been granted a health claim by the U.S. Food and Drug Administration (FDA) (1999). FDA (1999) has approved the health claim that consumption of 25 grams soy protein a day as part of a diet low in saturated fat and cholesterol may reduce the risk of CHD. In addition to soy protein, other soybean phytochemicals such as isoflavones have been widely studied for their potential to lower the risks of chronic diseases through their antioxidant, anti-inflammatory, and other bioactive properties (Boye & Ribereau, 2011; O'Bryan et al., 2014).

Phenolics of black soybean

Several studies have reported that black soybean has a higher antioxidant capacity than yellow soybean (Takahashi, Ohmori, Kiyose, Momiyama, Ohsuzu & Kondo, 2005; Xu et al., 2008), and black soybean extract may prevent the development of chronic diseases including diabetes, obesity (Kanamoto et al., 2011) and thrombotic disease (Kim et al., 2011). Unlike yellow soybean, black soybean contains additional phytochemicals besides isoflavones, such as anthocyanins and proanthocyanidins in its seed coat (Todd & Vodkin, 1993).

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CHAPTER 2

COMPREHENSIVE PHENOLIC COMPOSITION ANALYSIS AND EVALUATION OF YAK-KONG SOYBEAN (*GLYCINE MAX*) FOR THE PREVENTION OF ATHEROSCLEROSIS

Abstract

With the aim of seeking a competitive soybean ingredient for prevention of cardiovascular diseases (CVDs), a small black soybean cultivar with a green embryo (*Glycine max*) commonly called Yak-Kong (YK) in Korea was chosen among the many black soybean cultivars based on its physical characteristics and historical background. We evaluated the potential of YK as a functional food by comparing its total antioxidant activity to the common yellow soybean (YS) and black soybean (BS) cultivars. In comparison to YS and BS, YK contains significantly higher concentrations of phenolics, particularly in its seed coat. In addition, we conducted a comprehensive analysis of the phenolic compounds present in YK and evaluated them with a focus on prevention of CVDs. It revealed that proanthocyanidins and isoflavonoids are the major phenolic groups in each seed coat and embryo of YK respectively. In addition, YK was a good source of flavan-3-ols, cyanidin-3-glucoside, protocatechuic acid, coumestrol and phloridzin. We demonstrated that both YK seed coat and embryo are important potential sources of CVD-preventive phenolics.

Introduction

Among the few health claims for functional foods supported by significant scientific evidence, the U.S. Food and Drug Administration (FDA) (1999) has approved the claim of coronary heart disease (CHD) risk reduction for protein present in soybean (*Glycine max*). Additionally, a meta-analysis conducted by Zhan & Ho, 2005 demonstrated that consumption of soy protein together with a higher dosage of isoflavones exerts stronger effects on CHD risk reduction than soy protein intake alone. Proanthocyanidins and anthocyanins have also shown potential health benefits for risk reduction of cardiovascular diseases (CVDs) including CHD (Hooper et al., 2008; McCullough, Peterson, Patel, Jacques, Shah & Dwyer, 2012; Mink et al., 2007). A meta-analysis of 133 randomized controlled trials showed that proanthocyanidins- and flavan-3-ol-rich foods such as chocolate, cocoa and black tea are linked to the reduction of several CVDs risk factors (Hooper et al., 2008). Additionally, several large prospective cohort studies have shown that anthocyanin-rich foods have a positive effect on CVDs risk reduction (McCullough et al., 2012; Mink et al., 2007). These findings were the basis for our hypothesis that black soybean is more effective functional soybean for CVDs prevention than yellow soybean.

Numerous soybean varieties are found in Korea, China and Japan, as soybean originated from East Asia and has been cultivated since 7050-3050 BC (Lee, Crawford, Liu, Sasaki & Chen, 2011). Historical records show that black soybean was particularly favored as a herbal medicine (Xu & Chang, 2008). The various black soybean cultivars can be distinguished by their size and embryo color. In Korea, a small black soybean with green embryo commonly called Yak-Kong (YK) (*Glycine*

max) is being recognized for its medicinal properties, and particularly for improving blood circulation and kidney health. People have consumed Yak-Kong in soup and fermented alcohol to treat nervous disorders and as a detoxification agent against food poison. However, there is a lack of scientific research to support these claims. In this study, we evaluated the potential of YK as a functional food by comparing its total antioxidant activity to the common yellow soybean (YS) and black soybean (BS) cultivars. In addition, we conducted a comprehensive analysis of the phenolic compounds present in YK and evaluated them with a focus on prevention of CVDs.

Materials and methods

Chemicals and Reagents. Food-grade ethanol (95%) was purchased from Daehan Ethanol Life (Seoul, Republic of Korea). Liquid chromatography-grade solvents including acetone and acetonitrile were purchased from EMD Millipore Chemicals (Billerica, MA, USA), while glacial acetic acid, formic acid and phosphoric acid were obtained from Anachemia (Quebec, Canada). Phenolic standards were purchased from Sigma-Aldrich (St. Louis, MO, USA). All other chemicals were purchased from Merck KGaA (Darmstad, Germany).

Soybean Materials. Three different soybeans (Glycine max) were used in this study. Standardized YK soybean (Registration number: 01-0003-2013-3) was provided by the Rural Development Administration, Republic of Korea. The remaining two (YS and BS) were purchased from local suppliers in Danyang and Boeun, Republic of Korea in 2016.

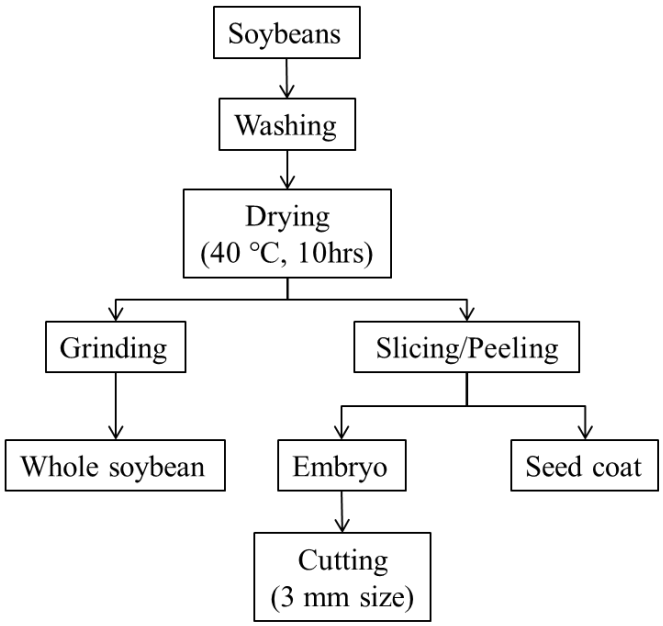
Preparations of Soybeans. The physical characteristics of the three soybeans

are presented in Figure 2.1. Soybean size differences were characterized by the numbers of seeds on a per-weight basis. Weight proportions for the seed coats and embryos in whole soybean were also calculated. Soybean sample preparations and the extraction procedure are shown in Figure 2.2A & B. Briefly, whole ground soybeans were extracted with 50% ethanol at 25 ℃ for 3 h. The peeled seed coats were extracted with 50% ethanol at 75 ℃ for 1.5 h. The 3 mm cut embryos were extracted with 70% ethanol at 75 ℃ for 3 h. YK seed coat and embryo extracts were then freeze-dried as powders.

	2 g of whole soybean	Embryo	Seed coat
Yellow soybean (YS)			
Weight proportion	100%	91%	9%
Black soybean (BS)			
Weight proportion	100%	92%	8%
Yak-Kong (YK)			
Weight proportion	100%	88%	12%

Figure 2.1. Physical characteristics of the three soybean samples.

A.



B.

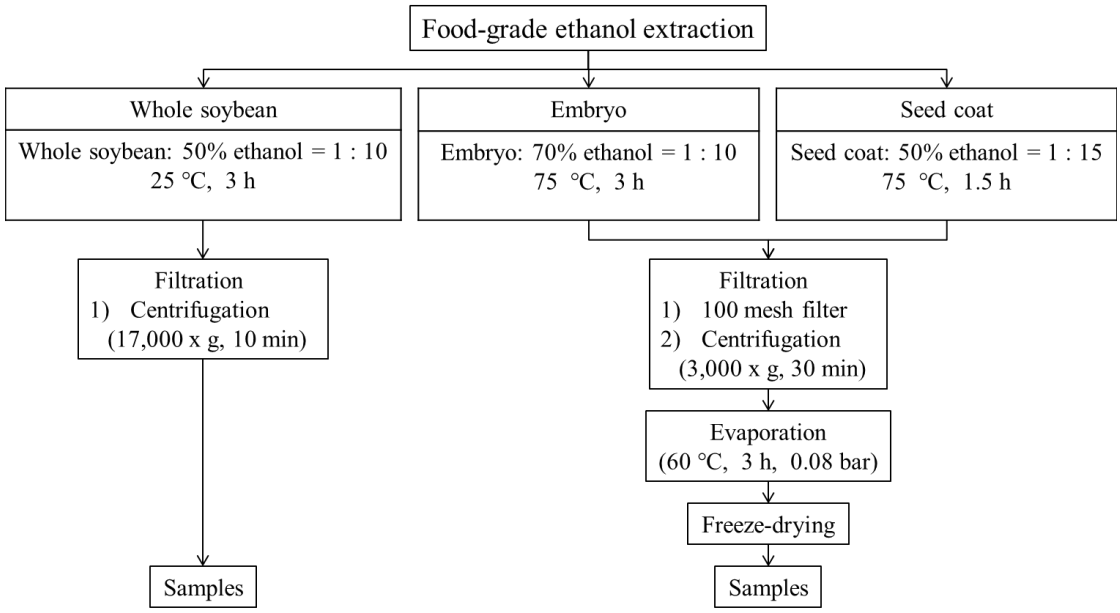


Figure 2.2. Soybean sample preparations (A) and extractions (B).

Determination of Total Antioxidant Activity. Total antioxidant activity was measured using the method developed by Blois (1958) with some modifications. Briefly, the samples were mixed with 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical solution and incubated in 96-well plates. The absorbance was measured at 517 nm, and the results were expressed as milligrams of ascorbic acid equivalent (ACE) per gram of whole soybean or soybean parts.

Determination of Total Phenolic Content. Total phenolic content was determined using the Folin-Ciocalteu (FC) assay (Singleton & Rossi, 1965) with some modifications. Briefly, the samples were mixed with FC phenol reagent, 7% Na_2CO_3 and ddH_2O in 96-well plates with an appropriate incubation time. The absorbance was compared against ddH_2O at 750 nm, and the results were expressed as milligrams of gallic acid equivalents (GAE) per gram of whole soybean.

Determination of Total Flavonoid Content. Total flavonoid content was measured using the method initially proposed by Christ and Muller (1960) with some modifications. The samples were mixed with 5% $NaNO_2$, 10% $AlCl_36H_2O$, 1 M NaOH and ddH_2O in 96-well plates with an appropriate incubation time. The absorbance was read against ddH_2O at 510 nm, and the results were expressed as milligrams of catechin equivalents (CE) per gram of whole soybean.

Determination of Total Anthocyanin Content. Total anthocyanin content was measured using the pH differential method (Lee, Durst & Wrolstad, 2005) with some modifications. In 96-well plates, 0.025 M KCl (pH 1.0) and 0.4 M CH_3COONa (pH 4.5) were added to the samples with appropriate dilution factors. The absorbance was measured against ddH_2O at 510 nm ($\lambda_{vis\text{-}max}$ of Cyanidin-3-glucoside (C3G)) and

700 nm. The total anthocyanins (mg/g whole soybean) were calculated based on Lambert-Beer's Law: (A x MW x DF x 1000)/(ε x 1). (A: Absorbance = $(A_{\lambda vis\text{-}max} - A_{700nm})_{pH1.0} - (A_{\lambda vis\text{-}max} - A_{700nm})_{pH4.5}$, DF: Dilution factor, ε: C3G molar absorptivity coefficient = 26900 M^{-1} cm^{-1}, MW: C3G molecular weight = 449.2)

Determination of Phenolic Composition.

Ultra-High Performance Liquid Chromatography-photodiode Array (UHPLC–PDA) Analysis. Isoflavonoids were analyzed by reversed phase UHPLC using a Waters Acquity UPLC coupled to a PDA detector (Milford, MA, USA). The compounds were separated at 30℃ on a Waters Acquity HSS Cyano column (2.1 mm x 50 mm, 1.8 µm) using a mobile phase consisting of 0.1% formic acid in water (solvent A) and 0.1% formic acid in acetonitrile (solvent B) mixed using the following gradient: 0–0.36 min, 10% B; 0.36–3.6 min, 10–30% B; 3.6–3.96 min, 30% B; 3.96–4 min, 10% B; 4–6 min, 10% B, with a flow rate of 0.58 ml/min and an injection volume of 1 µl. Chromatographic data were acquired at 260 nm. Daidzein, glycitein and their derivatives were quantified using calibration curves prepared with a daidzein standard, while genistein and coumestrol were quantified using their corresponding standards.

HPLC–Fluorescence Analysis. Proanthocyanidins were analyzed by normal-phase analytical HPLC with fluorescence detection as previously described by Dudonné et al (2014). The fluorescence was monitored at excitation and emission wavelengths of 230 and 321 nm respectively. Proanthocyanidins with degrees of polymerization (DP) from 1 to >10 were quantified using an external calibration curve of epicatechin, applying a correction factor according to their respective responses in

fluorescence.

UHPLC–Tandem Quadrupole Detector-Mass Spectrometer (TQD–MS) and UHPLC–Quadrupole Time of Flight (QToF–MS) Analyses. YK seed coat extract was further analyzed by reverse-phase UHPLC coupled to tandem mass spectrometry as previously published by Dudonné et al. (2014) using a Waters Acquity HSS T3 column (2.1 x 150 mm, 1.8 μm), tracking specific transitions of A- and B-type dimers of procyanidins (577 > 289 and 575 > 289 respectively) through multiple reaction monitoring (MRM) mode. The presence of A-type dimers of procyanidins in YK seed coat extract was confirmed using UHPLC–QToF–MS. Briefly, the analysis was conducted on an Agilent 6560 instrument coupled to an LC 1290 Infinity II system (Santa Clara, CA, USA) with a Waters Acquity HSS T3 column (2.1 x 100 mm, 1.8 μm). The elution was performed at 40 °C with a flow rate of 0.4 ml/min using a linear gradient for 30 min which changed from 98% A (0.1% formic acid in water) to 100% B (0.1% formic acid in acetonitrile). The QToF–MS instrument was operated with an electrospray ion source ESI–Agilent Jet Stream Technology in negative ionization mode. The MS scan data were collected at a rate of 1 spectra/s in the range of m/z 100–1,000 in high resolution mode. A collision cell with nitrogen as collision gas and collision energy 0–40 eV was used. A-type procyanidin dimers were identified using MassHunter Qualitative Analysis B.06.00 with Service Pack 1 software and were confirmed with their exact molecular mass.

Anthocyanins were analyzed using a Waters Acquity UPLC H-Class system equipped with a quaternary pump system. A Supelco Titan C18 column (100 mm x 2.1 mm id, 2.0 mm particle size) was used. The anthocyanins were separated with a

mobile phase that consisted of 10% acetic acid (eluent A) and acetonitrile (eluent B). The flow-rate was 0.4 ml/min and the gradient elution was 0–10 min, 5–35% B; 10–10.10 min, 35–80% B; 10.10–11.00 min, isocratic 80% B; 11.0–11.10 min, 80–5% B. The MS analyses were carried out on a Waters Xevo TQD mass spectrometer equipped with a Z-spray electrospray interface. The analysis was performed in positive mode and the data was acquired through MRM, tracking transitions of 40 glycosylated anthocyanins. The ionization source parameters were capillary voltage, 2.50 kV; source temperature, 150 ℃; cone gas flow rate, 50 l/h and desolvation gas flow rate, 800 l/h; desolvation temperature, 400 ℃. Nitrogen (99% purity) and argon (99% purity) were used as nebulizing and collision gases, respectively.

UHPLC–Tandem Mass Spectrometer (MS/MS) Analysis. Phenolic acids and all other phenolics were analyzed by UHPLC–MS/MS using the same parameters as in the proanthocyanidin analysis. Data were acquired in negative ionization mode through MRM. All phenolic compounds were quantified as GAE.

Statistics. The results were expressed as means ± SD and compared using SPSS (Statistical Analysis System Institute, 2010). The significance between mean values was determined by Tukey's Honest Significant Difference test at $P < 0.05$.

Results

Comparison of Physical Characteristics, Total Antioxidant Activity and Total Phenolic Content between BS, YS and YK. Physical characteristics of the three soybean types are shown in Figure 2.1. YK was the smallest as it had more seeds per gram, with an average of 5, 8 and 18 seeds per gram were recorded for BS, YS and

YK, respectively. As seed size decreased, the proportion of seed coat in the whole soybean increased. The seed coat weight proportion averaged 8%, 9% and 12% for BS, YS and YK, respectively.

Analyses of the total antioxidant activity, total phenolics, total flavonoids and total anthocyanins between the three whole soybeans (Figure 2.3A-D) revealed that one gram of YK showed higher total antioxidant activity (4.44 ± 0.30 mg ACE), total phenolic content (6.14 ± 0.18 mg GAE), total flavonoid content (1.50 ± 0.07 mg CE) and total anthocyanin content (0.51 ± 0.01 mg C3G equivalent) than the other two soybean types ($P < 0.05$).

When seed coats and embryos of the three soybeans were isolated and compared for antioxidant activity (Figure 2.3E & F), both YK seed coat and embryo showed higher total antioxidant activity ($P < 0.05$). One gram of YK seed coat harbored 55.92 ± 1.64 mg ACE, which was approximately 30 times higher than for YS embryo (1.76 ± 0.41 mg ACE).

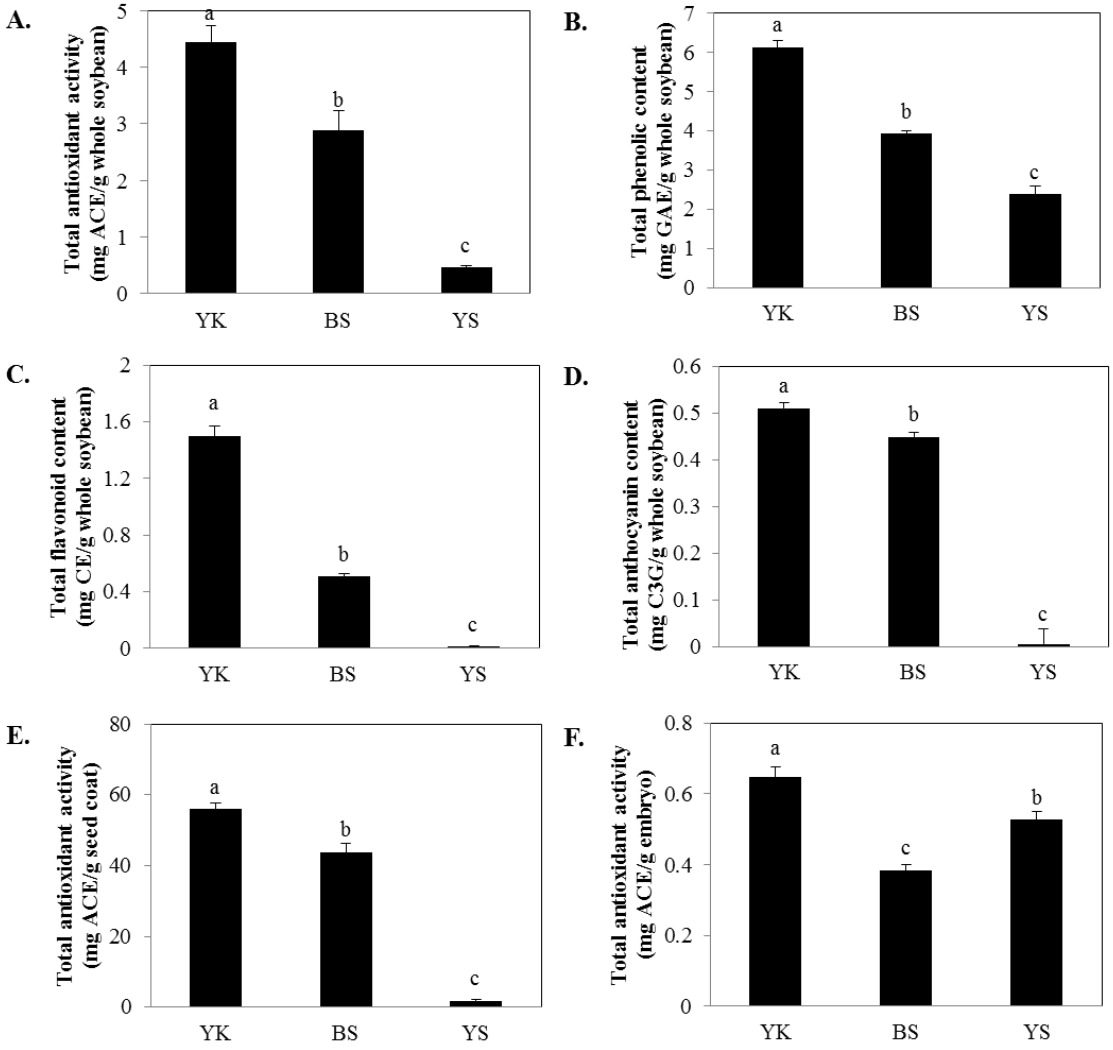


Figure 2.3. Comparison of antioxidant profiles among soybean cultivars. (A) Total antioxidant activity, (B) total phenolic content, (C) total flavonoid content and (D) total anthocyanin content in whole soybeans. (E) Total antioxidant activity in separated seed coats and (F) embryos. Data are expressed as means ± SD (n = 3). Different letters denote significant differences ($P < 0.05$); values bearing the same letters are not significantly different from each other. YK, Yak-Kong; BS, black soybean; YS, yellow soybean; ACE, ascorbic acid equivalents; GAE, gallic acid equivalents; CE, catechin equivalents; and C3G, cyanidin-3-glucose.

Comprehensive Phenolic Composition of YK. Comprehensive phenolic composition analysis of the YK seed coat and embryo was conducted for isoflavonoids, proanthocyanidins, anthocyanins, phenolic acids and other phenolics (Figure 2.4).

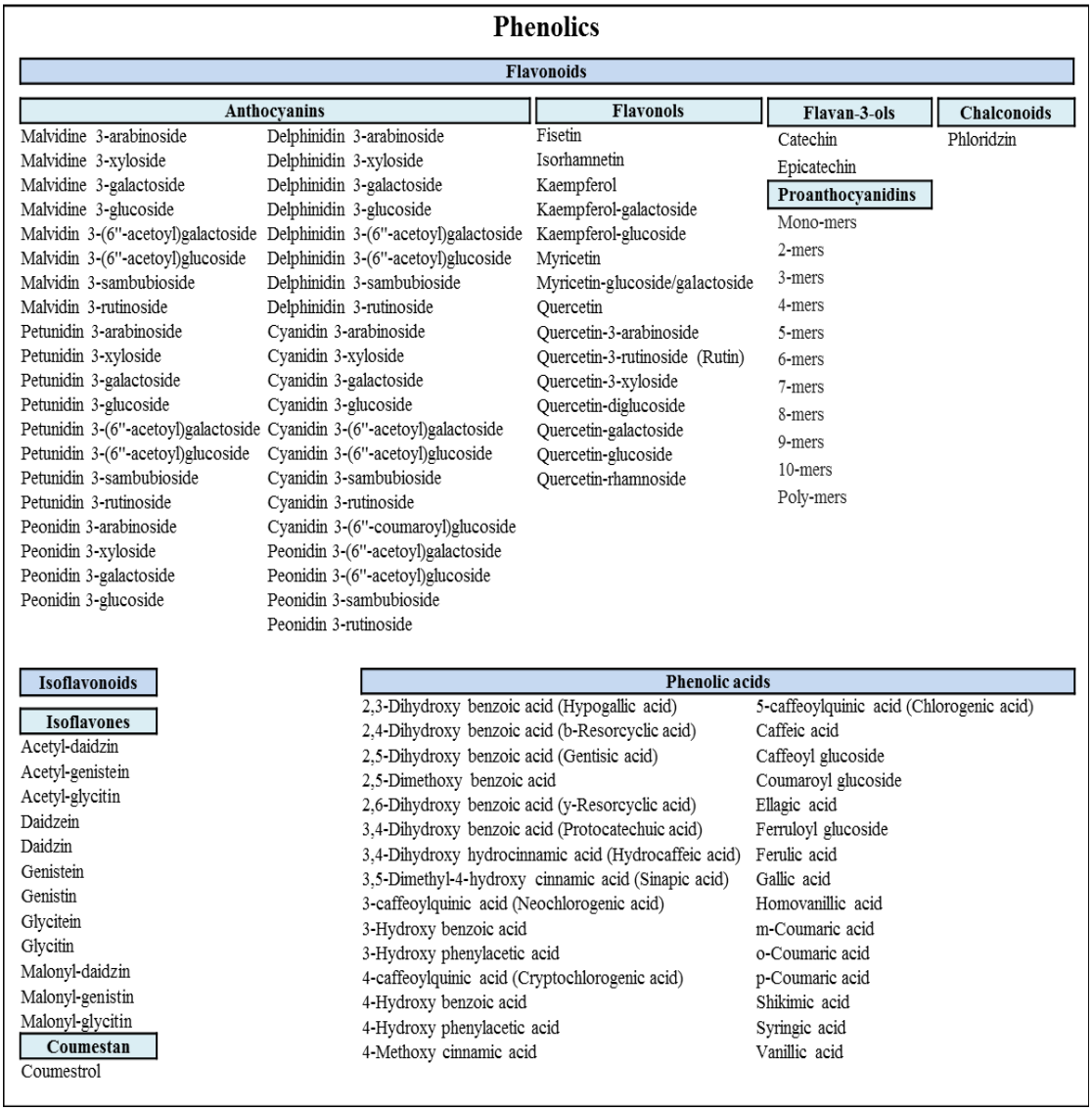


Figure 2.4. List of analyzed phenolics.

Compounds with higher than the average 0.1 mg/100 g whole YK were selected and are shown in Table 2.1. YK seed coat contained 1867.05 ± 29.92 mg phenolics per 100 g, a value which was on average 8 times higher than for YK embryo (225.22 ± 1.91 mg/100 g). Phenolic content in whole YK (422.42 ± 5.2 mg/100 g) was calculated based on the % weight ratio of seed coat to embryo (12:88). Among the phenolic groups determined for whole YK, proanthocyanidin was the major group comprising more than 70% of the total phenolic composition. YK seed coat contributed more than 50% of the total phenolic composition for whole YK, however more than 90% of the isoflavonoids in whole YK was contained in the embryo.

Table 2.2. YK phenolic composition[a]

Compounds	Seed coat		Embryo		Whole[b]		
	mg/100g	%[c]	mg/100g	%	mg/100g	%	% Distribution[d] (Seed coat: Embryo)
Phenolics	**1867.05 ± 29.92**	**100**	**225.22 ± 1.91**	**100**	**422.24 ± 5.23**	**100**	**53:47**
Flavonoids	**1814.88 ± 26.99**	**97.21**	**223.22 ± 2.05**	**99.11**	**414.21 ± 4.97**	**98.10**	**53:47**
Total proanthocyanidins	**1522.97 ± 21.77**	**81.57**	**133.83 ± 1.40**	59.42	**300.53 ± 3.81**	**71.17**	**61:39**
Monomers	646.53 ± 12.91	34.63	12.23 ± 1.21	5.43	88.34 ± 1.74	20.92	88:12
Dimers	415.03 ± 2.52	22.23	ND[e]	-	49.80 ± 0.30	11.80	100:0
Trimers	176.23 ± 4.36	9.44	ND	-	21.15 ± 0.52	5.01	100:0
Ttetramers	88.17 ± 0.64	4.72	ND	-	10.58 ± 0.08	2.51	100:0
Pentamers	47.92 ± 2.79	2.57	ND	-	5.75 ± 0.34	1.36	100:0
Hexamers	33.16 ± 2.71	1.78	ND	-	3.98 ± 0.32	0.94	100:0
Heptamers	12.55 ± 1.66	0.67	ND	-	1.51 ± 0.20	0.36	100:0
Octamers	7.93 ± 0.70	0.42	ND	-	0.95 ± 0.08	0.23	100:0
Nonamers	7.25 ± 2.53	0.39	ND	-	0.87 ± 0.30	0.21	100:0
Polymers (DP>10)	88.21 ± 1.72	4.72	121.61 ± 1.35	53.99	117.60 ± 0.98	27.85	9:91
Isoflavonoids	**44.94 ± 1.34**	**2.41**	**76.55 ± 1.48**	**37.94**	**72.75 ± 1.27**	**19.09**	**7:93**
Coumestrol	ND	-	8.91 ± 0.61	3.96	7.84 ± 0.54	1.86	0:100
Daidzein derivatives	**21.58 ± 1.24**	**1.16**	**24.38 ± 0.54**	**10.82**	**24.04 ± 0.55**	**5.69**	**11:89**
Daidzin	11.03 ± 0.01	0.59	14.50 ± 0.41	6.44	14.08 ± 0.36	3.33	9:91
Acetyl daidzin	0.52 ± 0.01	0.03	1.14 ± 0.01	0.51	1.07 ± 0.00	0.25	6:94
Malonyl daidzin	9.90 ± 1.22	0.53	8.50 ± 0.11	3.78	8.67 ± 0.20	2.05	14:86
Daidzein	0.12 ± 0.02	0.01	0.23 ± 0.02	0.10	0.22 ± 0.02	0.05	7:93
Genistein derivatives	**9.18 ± 0.47**	**0.49**	**39.47 ± 0.68**	**17.53**	**35.84 ± 0.58**	**8.49**	**3:97**
Genistin	2.08 ± 0.17	0.11	22.32 ± 0.47	9.91	19.89 ± 0.42	4.71	1:99
Acetyl genistin	0.54 ± 0.01	0.03	1.82 ± 0.01	0.81	1.67 ± 0.01	0.39	4:96
Malonyl genistin	6.32 ± 0.40	0.34	14.29 ± 0.22	6.34	13.33 ± 0.15	3.16	6:94
Gnistein	0.23 ± 0.02	0.01	1.04 ± 0.01	0.46	0.94 ± 0.01	0.22	3:97
Glycitein derivatives	**14.19 ± 0.33**	**0.76**	**12.70 ± 0.51**	5.64	**12.88 ± 0.47**	**3.05**	**13:87**

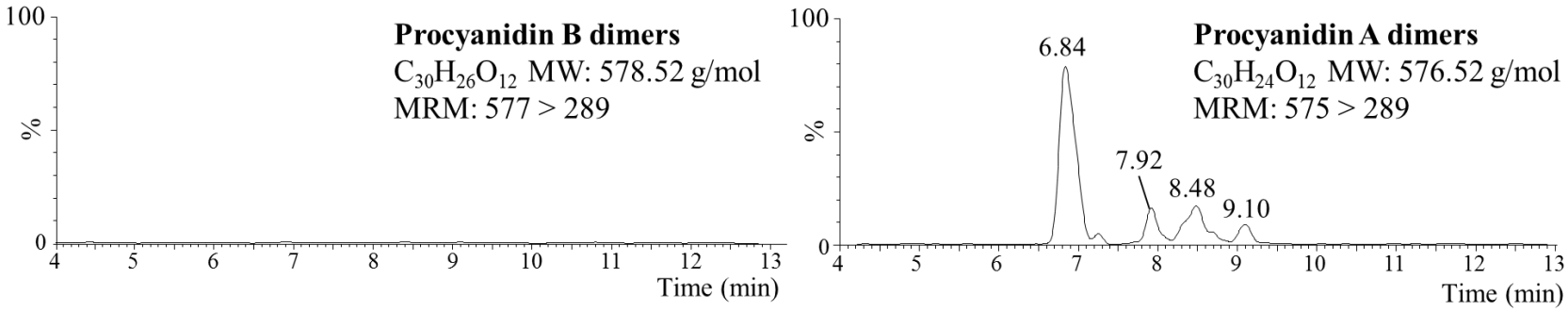
Table 2.1 (Continued)

Glycitin	5.07 ± 0.37	0.27	7.02 ± 0.43	3.12	6.79 ± 0.39	1.61	9:91
Acetyl glycitin`	0.49 ± 0.03	0.03	2.06 ± 0.34	0.91	1.87 ± 0.30	0.44	3:97
Malonyl glycitin	6.46 ± 0.11	0.35	3.26 ± 0.29	1.45	3.64 ± 0.24	0.86	21:79
Glycitein	2.16 ± 0.09	0.12	0.36 ± 0.00	0.16	0.58 ± 0.01	0.14	45:55
Flavan-3-ols	**129.14 ± 3.92**	**6.92**	**2.65 ± 0.69**	**1.18**	**17.83 ± 1.06**	**4.22**	**87:13**
Catechin	46.34 ± 1.60	2.48	0.37 ± 0.04	0.16	5.88 ± 0.16	1.39	95:5
Epicatechin	82.80 ± 3.68	4.43	2.28 ± 0.73	1.01	11.94 ± 1.00	2.83	83:17
Flavonols	**83.46 ± 4.86**	**4.47**	**0.62 ± 0.14**	**0.28**	**10.57 ± 0.47**	**2.50**	**95:5**
Quercetin	3.44 ± 0.75	0.18	0.18 ± 0.12	0.08	0.57 ± 0.06	0.14	72:28
Quercetin-3-arabinoside	0.25 ± 0.04	0.01	ND	-	0.03 ± 0.00	0.01	100:0
Quercetin-diglucoside	3.44 ± 0.75	0.18	0.18 ± 0.12	0.08	0.57 ± 0.06	0.14	72:28
Quercetin-galactoside	22.01 ± 1.12	1.18	0.04 ± 0.03	0.02	2.68 ± 0.12	0.63	99:1
Quercetin-glucoside	28.40 ± 1.65	1.52	0.14 ± 0.06	0.06	3.53 ± 0.17	0.84	97:3
Quercetin-3-rutinoside	25.86 ± 2.62	1.38	0.08 ± 0.07	0.04	3.17 ± 0.37	0.75	98:2
Total anthocyanins	**32.17 ± 1.88**	**1.72**	**0.65 ± 0.01**	**0.29**	**4.43 ± 0.22**	**1.05**	**87:13**
Cyanidin 3-glucoside	30.65 ± 1.85	1.64	0.61 ± 0.01	0.27	4.21 ± 0.21	1.00	87:13
Chalconoids Phloridzin	**2.20 ± 0.70**	**0.12**	**ND**	**-**	**0.26 ± 0.08**	**0.06**	**100:0**
Phenolic acids	**52.17 ± 3.21**	**2.79**	**2.01 ± 0.17**	**0.89**	**8.03 ± 0.26**	**1.90**	**78:22**
p-Coumaric acid	0.42 ± 0.27	0.02	1.12 ± 0.15	0.50	1.04 ± 0.15	0.25	5:95
4-Hydroxybenzoic acid	0.55 ± 0.04	0.03	0.19 ± 0.05	0.08	0.23 ± 0.04	0.05	29:71
Protocatechuic acid	50.22 ± 3.16	2.69	0.57 ± 0.04	0.25	6.52 ± 0.41	1.55	92:8

[a] Data are expressed as means ± SD (n = 3) on a dry weight basis. [b] Phenolics in whole YK [= (0.12 x seed coat value) + (0.88 x embryo value)]. [c] % Phenolic composition [= 100 x (each value/total phenolics measured in each part)]. [d] % Phenolic distribution ratio of seed coat to embryo in whole YK [= 100 x (0.12 x seed coat value)/(whole value):100 x (0.88 x embryo value)/(whole value)]. [e] ND, not detectable.

Proanthocyanidins of various DP were measured in YK. YK seed coat contained significantly higher concentrations of proanthocyanidins (1522.97 ± 21.77 mg/100 g) ($P < 0.05$) with various DP compared to YK embryo (133.83 ± 1.40 mg/100 g) which only comprised monomers and polymers (DP ≥ 10). The proanthocyanidin dimer types were further characterized by UHPLC–TQD–MS (Figure 2.5A). Several peaks were exclusively detected at the molecular mass of A-type procyanidin dimers (MW: 576.52 g/mol) rather than at the molecular mass of B-type procyanidin dimers (MW: 578.52 g/mol). The presence of A-type procyanidins was validated using UHPLC–QToF–MS (Figure 2.5B) with a mass obtained from the system at 0.3 ppm error of 575.5044 (M-H) ($C_{30}H_{24}O_{12}$) and a 93% isotope match.

A.



B.

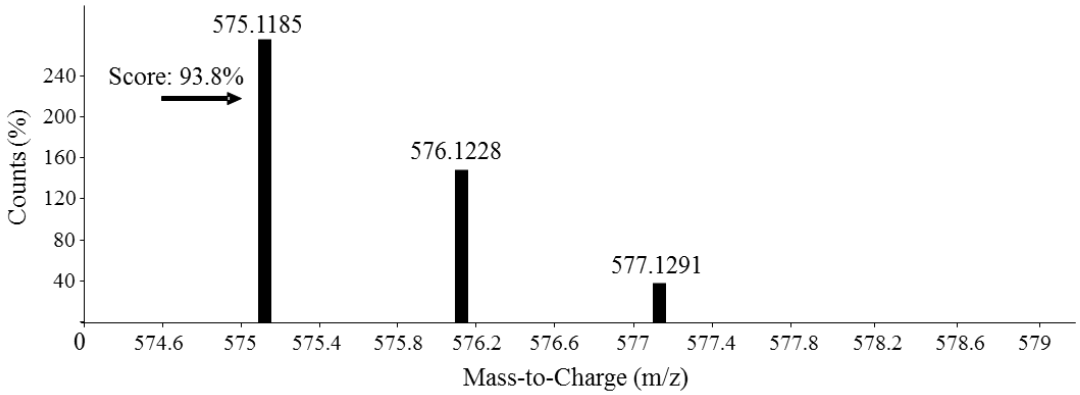


Figure 2.5. Identification of A type procyanidin dimers in YK seed coat extract by (A) UHPLC–TQD–MS and (B) UHPLC-QToF-MS. Specific A-type procyanidin dimers were detected by TQD-MS tracking MRM transitions of A- and B-type procyanidin dimers (577 > 289 and 575 > 289 respectively) (A), and confirmed by QToF–MS (B).

Significant concentrations of isoflavonoids were found in whole YK (72.75 ± 1.27 mg/100 g). Daidzein and genistein derivatives including their acetyl, malonyl and aglycon forms were the major isoflavones present at 1.16% and 17.53% of the total phenolic composition in YK seed coat and embryo, respectively. Among the aglycones, a high concentration of glycitein was found in YK seed coat (2.16 ± 0.09 mg/100 g). Except glycitein, more than 75% of all other YK isoflavones were located in the embryo. In particular, the high concentration of coumestrol in whole YK (7.84 ± 0.54 mg/100 g) was exclusively derived from its embryo.

Less than 10 % of phenolics in whole YK were comprised of flavan-3-ols, flavonols, anthocyanins and phenolic acids. Cyanidin-3-glucosid and protocatechuic acid were the main anthocyanins and phenolic acids, respectively. More than 70% of these four phenolic subclasses in whole YK were derived from its seed coat. YK seed coat also exclusively contained phloridzin (2.20 ± 0.70 mg/100 g).

Discussion

In the present study, we compared antioxidant profiles among the three soybeans and conducted a comprehensive analysis of the composition of the phenolic compounds present in YK. We found that YK contains higher concentrations of total phenolics including flavonoids and anthocyanins ($P < 0.05$) and exhibits significantly higher antioxidant activity than YS and BS. Phenolic compound profiling of YK as analyzed by UHPLC–MS/MS showed that proanthocyanidins and isoflavonoids were the major phenolic groups, representing more than 90% of the total phenolic composition. YK seed coat contained particularly higher concentrations of phenolic

compounds than the embryo.

A previous study reported that black soybean contains phenolics such as protocatechuic acid that have not been identified in other soybean types (Kanamoto et al., 2011). We therefore conducted additional phenolic composition analyses for YK and found several new phenolics, most of which were in the seed coat. YK seed coat harbored significant concentrations of phloridzin. Phloridzin has been predominantly found in apple and is used as a pharmaceutical for the treatment of diabetes mellitus, as it functions as an inhibitor of the sodium-glucose transporter (Ehrenkranz, Lewis, Kahn & Roth, 2005). Phloridzin was also found to exist in strawberry (Hilt et al., 2003), however this marks the first report of its presence in soybean. In addition to phloridzin, YK seed coat was found to contain several isomers of A-type procyanidins potentially linkage between catechin or epicatechin units (Sarnoski, Johnson, Reed, Tanko & O'Keefe, 2012). In contrast to an abundance of B-type proanthocyanidin-containing foods such as cocoa, grape seed, red wine and berries, only a small number of foods are known to contain A-type proanthocyanidins, including cranberry, plum, cinnamon and peanut skin (Bhagwat & Haytowitz, 2015). This marks the first report of A-type proanthocyanidins found in soybean (*Glycine max*), a completely different genus to peanut (*Arachis hypogaea*), which is also in the Leguminosae family.

It was reported that A-type proanthocyanidins can be absorbed in their native forms with higher efficiency than B-type proanthocyanidins (Appeldoorn, Vincken, Gruppen & Hollman, 2009). In addition, more than 50% of proanthocyanidins in YK were of low DP (DP $\leq$3), which are more easily absorbed than highly polymerized proanthocyanidins (Deprez, Mila, Huneau, Tome & Scalbert, 2001). This is in contrast

to most known foods containing proanthocyanidins as polymers (DP ≥ 10) (Bhagwat et al., 2015). When the present results were compared to those in the U.S. Department of Agriculture (USDA) database, which used the same proanthocyanidin quantifying method as our study, whole YK was found to contain higher concentrations of proanthocyanidin dimers than other legumes and nuts including black-eyed pea, peanut and berries, such as cranberry (Bhagwat et al., 2015). YK seed coat as a food ingredient also contained higher concentrations of proanthocyanidin dimers than other food ingredients including grape skin, dried cocoa powder and cinnamon powder (Bhagwat et al., 2015). Taken together, these results indicate that YK seed coat is an excellent source of bioavailable proanthocyanidins.

YK embryo is a good source of isoflavonoids, particularly coumestrol. The total quantity of isoflavones in YK was comparable to various other soybeans common in different countries (Kim, Ro, Kim, Kim & Chung, 2012; Xu et al., 2008). In the case of coumestrol, it is known that only a few foods contain considerable amounts, such as red clover, clover sprouts and Kala Chana (Bhagwat, Haytowitz & Holden, 2008). In contrast to the USDA database which reports small concentrations of coumestrol in beans and bean sprouts (Bhagwat et al., 2008), significant amounts of coumestrol were found in YK. Coumestrol has been shown to have higher low density lipoprotein-antioxidant activity compared to genistein and daidzein (Lee et al., 2006), however there has been no direct evidence yet whether it has CVDs preventive bioactivity.

YK seed coat was found to have a very similar phenolic composition to pycnogenol, which is the dietary supplement derived from pine bark and standardized

to contain 65–75% proanthocyanidin and other phenolics such as (epi)catechins and phenolic acids, and are alleged to prevent or treat cardiovascular disorders (Kim, Kim, Kang, Sung & Kang, 2010). And as mentioned earlier, meta-analyses conducted by other research groups have shown strong correlations between CVDs prevention and the consumption of proanthocyanidin- or anthocyanin-rich foods and soy protein together with isoflavones (Zhan et al., 2005; Hooper et al., 2008; Mink et al., 2007; McCullough et al., 2012). Several randomized controlled trials have also demonstrated that the consumption of cranberry products, which are rich in A type proanthocyanidins may provide cardioprotective benefits such as improvement of lipid profile and blood pressure and the reduction of lipid peroxidation biomarkers expression and cell adhesion molecules (Blumberg et al., 2016). In addition, a number of *in vivo* studies have shown that phloridzin may provide protective effects against diabetic cardiomyopathy or atherosclerosis triggered by a high glucose diet (Azuma et al., 2006; Cai et al., 2013) and increase the absorption efficacy of isoflavone (Andlauer, Kolb & Furst, 2004). Taken together, the present results suggest that YK could be an effective functional soybean ingredient for atherosclerosis prevention.

Conclusion

Soybean appears to be a complex food material harboring various types of phenolic compounds, not only limited to isoflavones. Depending on the soybean cultivar and cultivation conditions, harvest time and processing methods, the phytochemical profiles of soybean are likely to be varied. Therefore, comprehensive phenolic composition analyses are necessary to characterize the different soybeans,

particularly their seed coat. YK was found to be a competitive functional soybean in regards to higher concentrations of antioxidants than YS and BS and phenolics that are potentially beneficial for atherosclerosis prevention. YK was a good source of not only isoflavonoids, particularly coumestrol in its embryo but also bioavailable proanthocyanidins (DP $\leq$ 3) with A-type dimers, flavan-3-ols, cyanidin-3-glucoside, protocatechuic acid and phloridzin in its seed coat. We demonstrated that both YK seed coat and embryo are important potential sources of CVDs-preventive phenolics. The higher consumption of YK as a whole may therefore potentially impart numerous health benefits including atherosclerosis prevention.

Acknowledgements

The authors thank the Rural Development Administration, Republic of Korea, for providing a small black soybean cultivar (Registration number: 01-0003-2013-3). This work was carried out with the support of "Cooperative Research Program for Agriculture Science and Technology Development (Project No. PJ01133403)" Rural Development Administration, Republic of Korea. Also this work was supported by the Korea Institute of Planning and Evaluation for Technology in Food, Agriculture, Forestry and Fisheries (IPET) through Agri-Bio industry Technology Development Program, funded by Ministry of Agriculture, Food and Rural Affairs (MAFRA) (514004), Republic of Korea.

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CHAPTER 3

5-(3', 4' DIHYDROXYPHENYL-γ-VALEROLACTONE), A MAJOR MICROBIAL METABOLITE OF PROCYANIDIN, ATTENUATES MONOCYTE-ENDOTHELIAL ADHESION

Abstract

The major phenolic group of Yak-Kong (*Glycine max*) especially its seed coat is found to be proanthocyanidin. Although clinical studies have shown that intake of flavan-3-ol-rich foods is associated with reduced risk of cardiovascular diseases (CVDs), it remains unclear which of the metabolic derivatives are responsible for the effect. Several metabolomics studies focusing on polymeric flavan-3-ols have reported that procyanidins are extensively catabolized by gut microbiota, with 5-(3',4'-dihydroxyphenyl)-γ-valerolactone (DHPV) identified as a major microbial metabolite. We observed that DHPV has a more potent preventive effect against TNF-α-induced THP-1 adhesion to human umbilical vein endothelial cells in comparison to its known precursors, including procyanidin A1, A2, B1 and B2, (+)catechin, (-)epicatechin and its microbial metabolites 3-(3,4-dihydroxyphenyl)propionic acid and 2-(3,4-dihydroxyphenyl)acetic acid. Further investigation revealed that DHPV prevents monocyte-endothelial cell adhesion by downregulating TNF-α-stimulated expression of two biomarkers of atherosclerosis, suggesting that DHPV has relatively high potency for the prevention of atherosclerosis compared to other procyanidin metabolites.

Introduction

Retrospective and interventional studies have found evidence for the beneficial effects of high flavonoid intake on the prevention of cardiovascular diseases (CVDs) (Kay, Hooper, Kroon, Rimm & Cassidy, 2012; McCullough, Peterson, Patel, Jacques, Shah & Dwyer, 2012; Wang, Ouyang, Liu & Zhao, 2014). A meta-analysis of human randomized controlled trials conducted by Hooper et al. (2008) found that the consumption of flavonoid-rich foods strongly correlates with a reduced risk of CVDs (Hooper et al., 2008). Specific foods like chocolate, cocoa and black tea are known to be a rich source of flavan-3-ols comprising monomeric and polymeric compounds (Hooper et al., 2008). Despite promising findings in clinical studies, it remains unclear exactly which bioavailable compounds derived from flavan-3-ols exert the most potent preventive effects against CVDs.

The major phenolic group of Yak-Kong (*Glycine max*) especially its seed coat is found to be proanthocyanidin polymeric flavan-3-ols. Flavan-3-ols are subject to several metabolic pathways within the human body (Monagas et al., 2010). The bioavailability of flavan-3-ols is highly dependent on their degree of polymerization (DP) (Monagas et al., 2010). Polymeric flavan-3-ols (also known as proanthocyanidins) have a large molecular size and increased hydrophilicity, thereby rendering it difficult for them to penetrate the lipid bilayer of the small intestine (Monagas et al., 2010). Proanthocyanidins therefore primarily undergo microbial biotransformation in the colon (Ou & Gu, 2014). Among the proanthocyanidin subclasses, procyanidin has been widely studied, as it is the most common type found in foods including cocoa, chocolate, pine bark extract and grape seed extract (Ou et al.,

2014; Uhlenhut & Hogger, 2012). Procyanidin is exclusively composed of (epi)catechins and is further classified into A and B types, depending on the presence of an additional ether bond (Ou et al., 2014). Although metabolites of A and B type procyanidins derived from same microbial fermentation show slightly different profiles (Sanchez-Patan et al., 2015), metabolomics analyses of both types have identified common metabolites such as (epi)catechins, 3-(3',4'-dihydroxyphenyl)-propionic acid (DHPA) and 3,4-dihydroxyphenyl-acetic acid (DHAA) (Appeldoorn, Vincken, Aura, Hollman & Gruppen, 2009; Sanchez-Patan et al., 2015). 5-(3',4'-dihydroxyphenyl)-γ-valerolactone (DHPV) has been recognized as the major microbial metabolite of procyanidins and (epi)catechins (Appeldoorn et al., 2009; Unno, Tamemoto, Yayabe & Kakuda, 2003). In this study, we sought to investigate DHPV in comparison with procyanidin derivatives including A and B type procyanidin dimers and other major microbial metabolites for their preventive effect on CVDs using a monocyte-endothelial cell adhesion model.

Materials and methods

Chemicals and reagents. 5-(3',4'-dihydroxyphenyl)-γ-valerolactone (DHPV) was purchased from Chemieliva Pharmaceutical (Chondquing, China). Procyanidin A1 (Pro A1) was purchased from Phytolab (Vestenbergsgreuth, Germany). Procyanidin A2 (Pro A2) and Procyanidin B1 (Pro B1) were purchased from Extrasynthese (Genay, France). Procyanidin B2 (Pro B2) was purchased from Funakoshi (Tokyo, Japan). (+)catechin, (-)epicatechin, 3-(3,4-dihydroxyphenyl)propionic acid (DHPA), 2-(3,4-dihydroxyphenyl)acetic acid

(DHAA), fetal bovine serum (FBS), medium 199 (M199), hydrocortisone, 2-mercaptoethanol, puromycin and calcein AM solution were purchased from Sigma-Aldrich (St. Louis, MO, USA). RPMI 1640 medium was purchased from Welgene (Daegu, Republic of Korea). Recombinant human epidermal growth factor (hEGF), basic fibroblast growth factor (bFGF) and L-glutamine were purchased from Gibco (Grand Island, NY, USA). Recombinant human TNF-α (TNF-α) was purchased from PeproTech Korea (Seoul, Republic of Korea). Penicillin (10,000 units/ml)-streptomycin (10,000 μg/ml) (P/S) was purchased from Corning (Corning, NY, USA). Antibodies against vascular cell adhesion protein-1 (VCAM-1), β-actin and total I kappa-B kinase (IKK)α/β were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Phosphorylated IKKα/β and IκBα and total IκBα were purchased from Cell Signaling Biotechnology (Danvers, MA, USA). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide tetrazolium salt (MTT) solution was purchased from USB Corporation (Cleveland, OH, USA).

Cell culture. Human umbilical vein endothelial cells (HUVECs) were purchased from Lonza (Walkersville, MD, USA) and grown in M199 supplemented with 25 mM HEPES containing 10% (v/v) FBS (Gibco), 2 mM L-glutamine, 1 ng/ml hydrocortisone, 1% (v/v) P/S and the two growth factors hEGF (1 ng/ml) and bFGF (2 ng/ml). Stably-transfected HUVECs harboring NF-kB luciferase reporter plasmids were cultured with M199 containing 1 μg/ml puromycin. HUVECs between passages 7 and 12 were used. THP-1 cells, which consist of monocyte-like cells derived from a leukemia line, were purchased from the Korean Cell Line Bank and cultured in RPMI 1640 media supplemented with 10% (v/v) FBS (Sigma-Aldrich), 50 μM 2-

mercaptoethanol and 1% (v/v) P/S. Subculturing occurred when the density reached between 2 x 10^5 and 1 x 10^6/ml.

Cell viability assay. In 96-well plates, confluent HUVECs were starved with serum-free M199 medium containing 2 mM L-glutamine for 4 h. The starved HUVECs were treated with various concentrations of procyanidin derivatives dissolved in dimethylsulfoxide (DMSO). After 22 h incubation, MTT solution was added to the medium at 0.5 mg/ml. At 24 h, 200 µl DMSO was added, and the absorbance at 570 nm was measured after 30 min incubation.

Monocyte adhesion assay. In 96-well plates, confluent HUVECs were starved with serum free M199 containing 2 mM L-glutamine for 4 h. The starved HUVECs were treated with proanthocyanidin derivatives in M199 supplemented with 2 mM L-glutamine and 10% (v/v) FBS for 1 h and then stimulated with 10 ng/ml TNF-α for 5 h. THP-1 cells were stained with calcein AM and added to the HUVECs at 5 x 10^5 cells/well in M199. After 1 h incubation, non-adhered THP-1 cells were washed off with phosphate-buffered saline (PBS), and the adhered cells were measured its florescence using an Infinite 200 PRO (Tecan group Ltd., Männedorf, Switzerland) at excitation and emission wavelengths of 485 nm and 538 nm.

Western blot assay. Confluent HUVECs in 6-well plates or 6 cm dishes were starved and treated with DHPV at up to 30 µM for 1 h. Pre-treated cells were stimulated with 10 ng/ml TNF-α and harvested with RIPA lysis buffer after cold PBS washing. Quantified protein lysate samples were separated in 10% sodium dodecyl sulfate-polyacrylamide gels and transferred onto polyvinylidene difluoride membranes. The membranes were blocked and incubated with specific primary

antibodies at 4 ℃ overnight. Horseradish peroxidase-conjugated secondary antibodies were added for 1 h, and the protein bands were then visualized using an enhanced chemiluminescence detection kit (GE Healthcare, London, UK).

The enzyme-linked immunosorbent assay for MCP-1. Following DHPV pretreatment and TNF-α-stimulation, protein levels of MCP-1 in the HUVEC culture supernatant were measured using Human MCP-1/CCL2 ELISA MAX Deluxe Sets (BioLegend, San Diego, CA, USA). Briefly, 100 μl of standard cytokines or diluted culture supernatants were added to each well of an MCP-1 antibody-coated 96-well plate and incubated for 2 h at room temperature. Each well was incubated with the detection antibody for 1 h followed by Avidin-HRP solution together with the substrate solution for 30 min and 20 min, respectively. Appropriate washing was performed between each addition of solution. The optical density of each well was determined using a microplate reader at 450 nm and 570 nm. A standard curve for the cytokine was generated, and linear regression analysis was performed.

Real-time quantitative PCR. Total RNA was extracted from HUVECs using Trizol and RNA iso Plus (Takara Bio Inc., Shiga, Japan), and quantified using a NanoDrop ND-2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). cDNAs were synthesized from a total of 1 μg/μl RNA using a PrimeScript^{TM} 1st strand cDNA Synthesis Kit (Takara Bio Inc., Shiga, Japan). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was selected as an internal standard. The cDNAs were probed using the following primers (Bioneer, Daejeon, Korea): human VCAM-1 forward (5’- CCC TCC CAG GCA CAC ACA -3’); human VCAM-1 reverse (5’- GAT CAC GAC CAT CTT CCC AGG -3’); human MCP-1 forward (5’- TCG CCT

CCA GCA TGA AAG TC -3'); human MCP-1 reverse (5'- GGC ATT GAT TGC ATC TGG CT -3'); human GAPDH forward (5'- CAG GGC TGC TTT TAA CTC TGG TAA A -3'); human GAPDH reverse (5'- GGG TGG AAT CAT ATT GGA ACA TGT AA -3'). For quantitative real-time PCR, iQ™ SYBR Green® Supermix and a CFX Connect™ Real-time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA) were used.

Luciferase Assay for NF-kB transactivation. The lentiviral expression vector pGF1-NF-kB-EF1-Puro (System Biosciences, Palo Alto, CA, USA) was transfected into HEK293T cells with the packaging vectors pMD2.G and psPAX2 (Addgene, Cambridge, MA, USA) using JetPEI DNA transfection reagent (Polyplus-transfection, New York, NY, USA). HUVECs were transfected using filtered transfection medium harvested from the HEK293T cell culture and 10 μg/ml polybrene (EMD Millipore, Billerica, MA, USA). After selection with 1 μg/ml puromycin (InvivoGen, San Diego, CA, USA) stably-transfected HUVECs were seeded at 1 x 10^4 cells/well in 96-well plates. After 4 h starvation, the cells were pretreated with DHPV at up to 30 μM for 1 h before stimulation with 10 ng/ml TNF-α. After 10 h stimulation, the cells were disrupted with lysis buffer (0.1 M pH 7.8 PBS, 1% Triton X-100, 1 mM DTT and 2 mM EDTA), and luciferase activity was measured using a Luminoskan Ascent (Thermo Electron, Helsinki, Finland).

Statistical analysis. Statistical analyses were performed using SPSS (Statistical Analysis System Institute, 2010). Significant differences between the means were determined using Tukey's Honest Significant Difference test at $P < 0.05$.

Results

DHPV exerts superior preventive effects against TNF-α stimulated monocyte-endothelial adhesion compared to other procyanidin derivatives. Major procyanidin derivatives were selected based on previous studies (Figure 3.1). To investigate the preventive effect of the compounds against atherosclerosis, we pretreated HUVECs with 30 μM of procyanidin dimers (Pro A1, Pro A2, Pro B1 and Pro B2), monomers ((-) epicatechin and (+)-catechin) or their microbial metabolites including DHPV, DHPA and DHAA for 1 h prior to TNF-α stimulation. Compared to the selected procyanidin derivatives, DHPV significantly prevented THP-1 adhesion to TNF-α-stimulated HUVECs in a dose-dependent manner (Figure 3.2A). Concentrations of the procyanidin derivatives up to 30 μM did not compromise cell viability (Figure 3.2B).

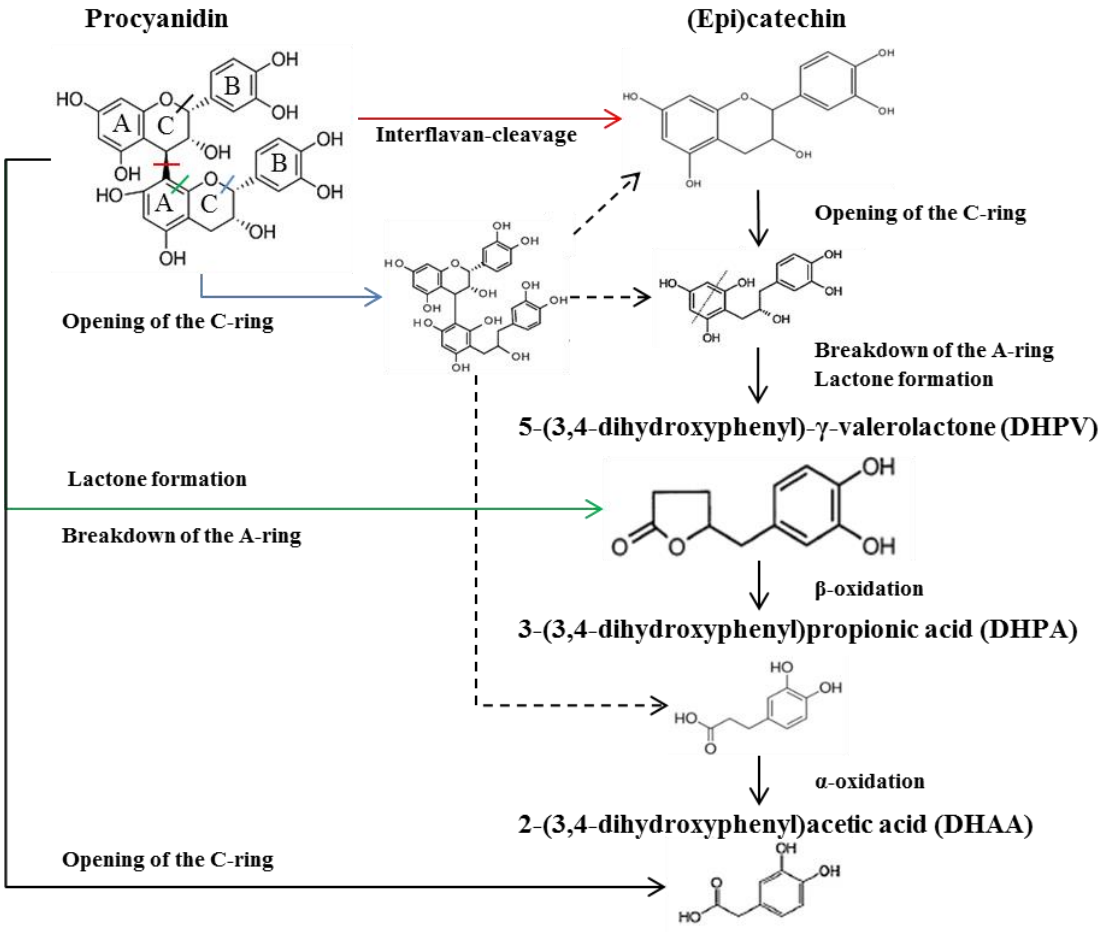
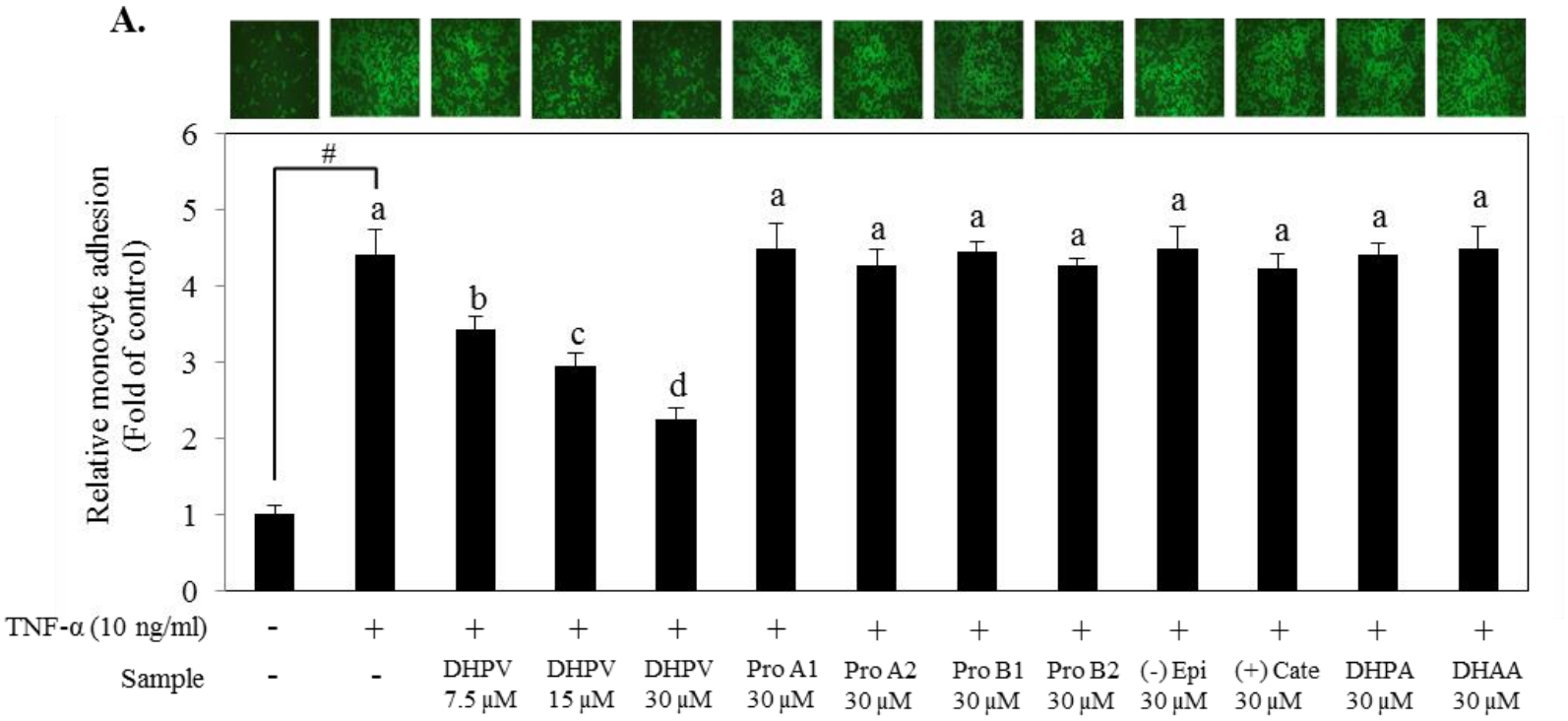


Figure 3.1. Procyanidin microbial catabolism.


A.
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TNF-α (10 ng/ml) - + + + + + + + + + + + +
Sample - - DHPV 7.5 μM DHPV 15 μM DHPV 30 μM Pro A1 30 μM Pro A2 30 μM Pro B1 30 μM Pro B2 30 μM (-) Epi 30 μM (+) Cate 30 μM DHPA 30 μM DHAA 30 μM

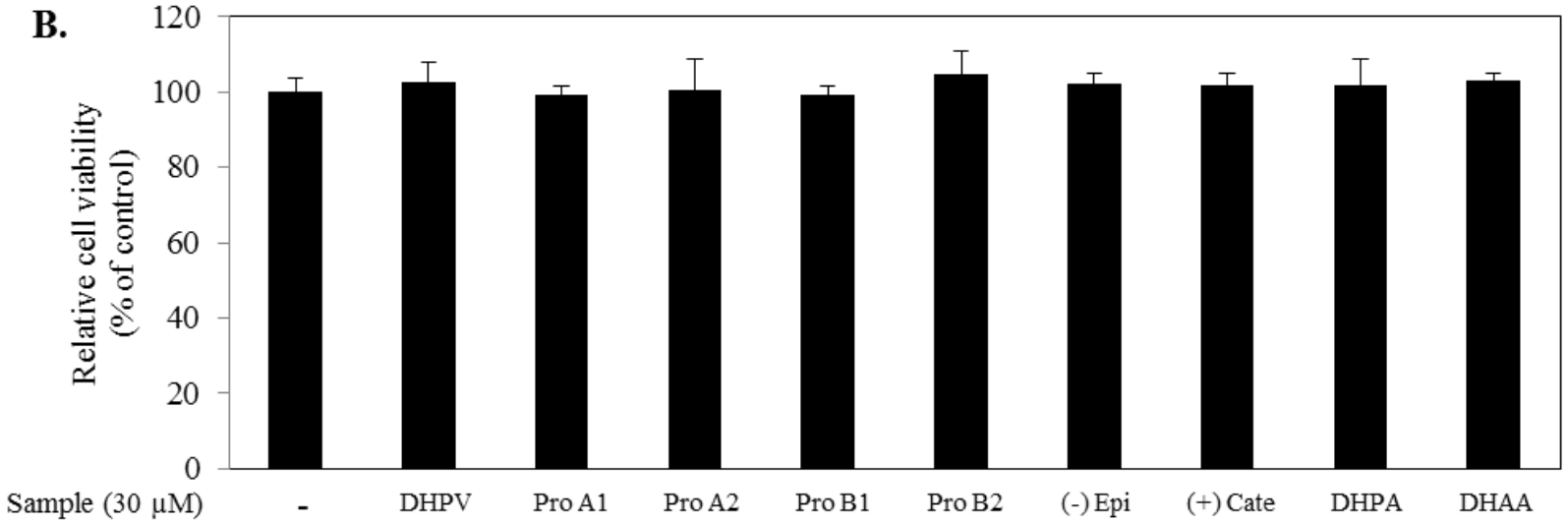

Figure 3.2. Comparison of monocyte-endothelial adhesion inhibitory effects between DHPV and other procyanidin microbial metabolites. (A) Following pretreatment of procyanidin derivatives at the indicated concentrations for 1 h, HUVECs were exposed to TNF-α for a further 5 h. Calcein AM-labeled THP-1 was then added to the HUVEC medium and for 1 h to allow for adherence. The number of adhered THP-1 cells was determined with a fluorescence microplate reader at excitation and emission wavelengths of 485 and 535 nm, respectively. Images were captured with a fluorescence microscope. (B) Varying concentrations of DHPV and 30 μM of various other procyanidin microbial metabolites were tested for cytotoxicity. Data are expressed as means ± SD (n = 4). Different letters denote significant differences ($P < 0.05$); values bearing the same letters are not significantly different from each other.

DHPV attenuates TNF-α-stimulated upregulation of VCAM-1 mRNA and protein expression. We sought to evaluate whether DHPV may be exerting preventive effects on TNF-α-stimulated monocyte-endothelial adhesion through the suppression VCAM-1. Western blot analysis and RT-qPCR showed that DHPV prevented VCAM-1 expression at both the mRNA and protein levels in TNF-α-stimulated HUVECs in a dose-dependent manner (Figure 3.3A & B).

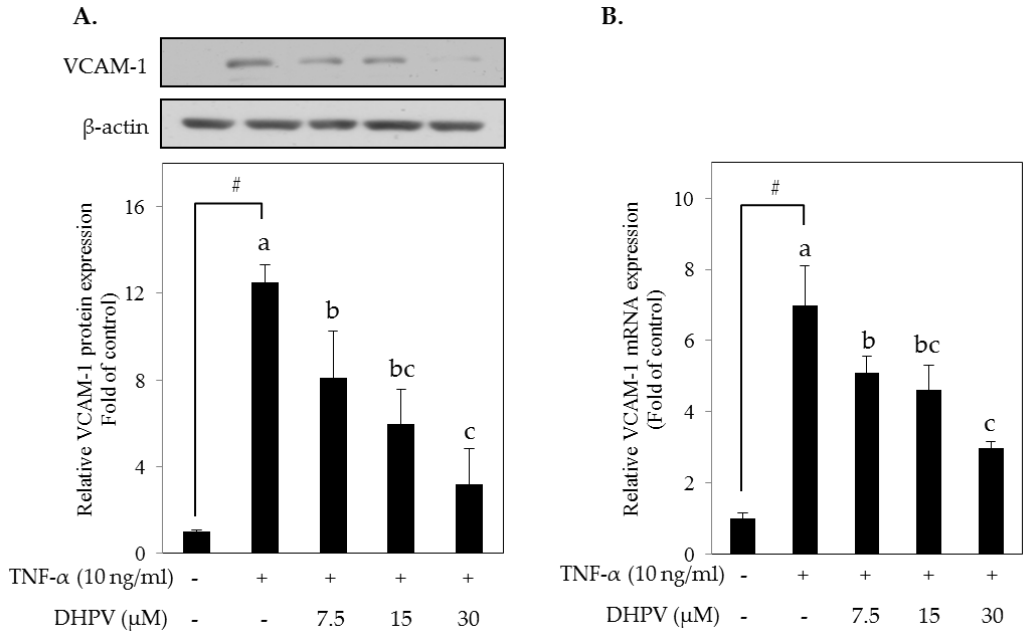

Figure 3.3. Effect of DHPV on VCAM-1 expression in TNF-α-stimulated HUVECs. Preventive effect of DHPV on TNF-α-stimulated (A) VCAM-1 protein expression as assessed by Western blot. Data are expressed as means ± SD (n = 3) (B) VCAM-1 mRNA expression was measured by RT-qPCR. Data are expressed as means ± SD (n = 4). Different letters denote significant differences ($P < 0.05$); values bearing the same letters are not significantly different from each other.

DHPV attenuates TNF-α-stimulated upregulation of MCP-1 at mRNA and protein levels. To investigate whether DHPV treatment can prevent monocytes from migrating into the vascular intima while they are adhered to the vascular endothelium, the secretion of MCP-1 protein was quantified in TNF-α-stimulated HUVEC culture supernatants by ELISA. DHPV pretreatment attenuated TNF-α-stimulated MCP-1 protein secretion as well as mRNA expression in these cells (Fig. 3.4A & B).

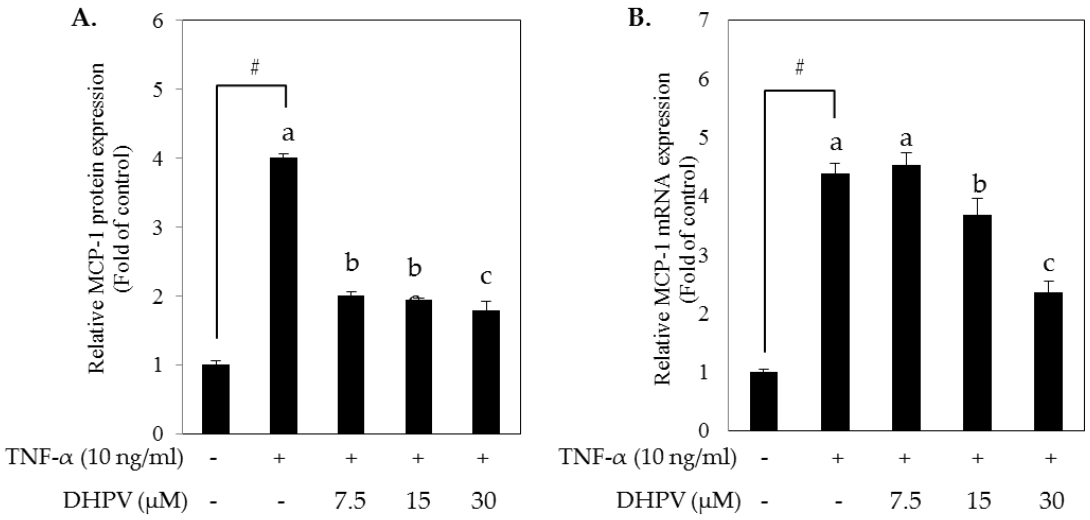

Figure 3.4. Effect of DHPV on MCP-1 expression in TNF-α-stimulated HUVECs. Preventive effect of DHPV on TNF-α-stimulated (A) MCP-1 protein secretion as assessed by ELISA. (B) MCP-1 mRNA expression was analyzed by RT-qPCR. Data are expressed as means ± SD (n = 4). Different letters denote significant differences ($P < 0.05$); values bearing the same letters are not significantly different from each other.

DHPV suppresses TNF-α-stimulated activation of NF-κB in HUVECs. To determine whether the preventive effect of DHPV on TNF-α-stimulated expression of VCAM-1 and MCP-1 is associated with the inhibition of NF-κB, we analyzed NF-κB transactivation in HUVECs stably transfected with NF-κB luciferase reporter plasmids. Pretreatment with DHPV significantly suppressed TNF-α-stimulated transcriptional activation of NF-κB (Fig. 3.5) in a dose-dependent manner.

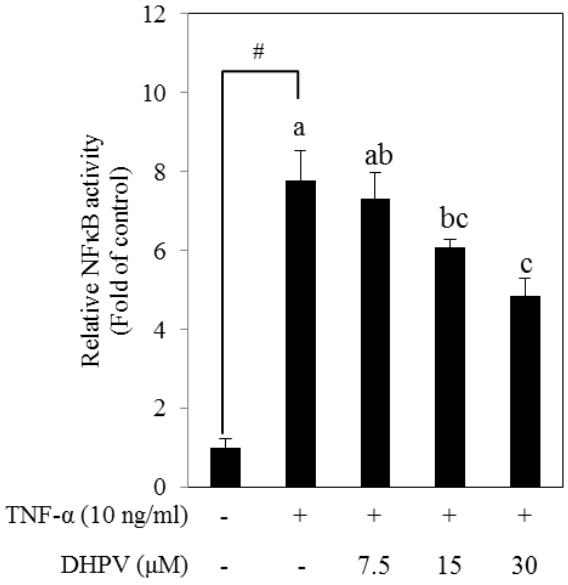

Figure 3.5. Effect of DHPV on TNF-α-induced NF-κB transcriptional activity. Following pretreatment with DHPV and TNF-α-stimulation, NF-κB transcriptional activity was measured in HUVECs transfected with NF-κB luciferase reporter plasmids. Data are expressed as means ± SD (n = 4). Different letters denote significant differences ($P < 0.05$); values bearing the same letters are not significantly different from each other.

DHPV suppresses TNF-α-stimulated phosphorylation of proteins involved in NF-kB signaling in HUVECs. To further elucidate the manner in which DHPV modulates NF-κB activity, we examined the preventive effect of DHPV on TNF-α-induced phosphorylation of IKK and IκBα, two key regulators of NF-κB activation. We observed that DHPV downregulated TNF-α-stimulated phosphorylation of IKK in a concentration-dependent manner as well as IκBα, a downstream effector of IKK (Fig. 3.6). DHPV also prevented degradation of IκBα following TNF-α-stimulation (Fig. 3.6).

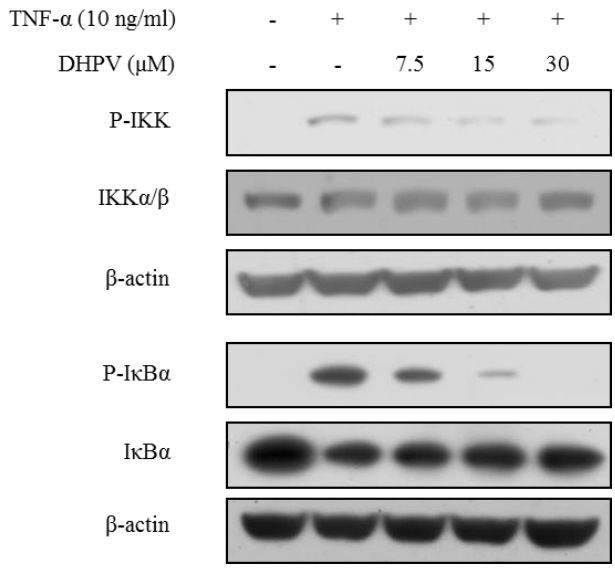

Figure 3.6. Effect of DHPV on TNF-α-stimulated NF-κB signaling. Expression of phosphorylated and unphosphorylated proteins comprising the NF-κB signaling pathway as determined by Western blot.

Discussion

Several metabolomics studies have shed light on the potential metabolic pathways responsible for the microbial catabolism of procyanidins (Fig. 3.1) (Appeldoorn et al., 2009). Gut microflora can cleave the strong interflavan bonds within procyanidins to produce (epi)catechin-monomer building blocks (Bittner, Kemme, Peters, Kersten, Danicke & Humpf, 2014) with a slow reaction rate (Appeldoorn et al., 2009; Stoupi, Williamson, Drynan, Barron & Clifford, 2010). It appears that the A- & C-rings in (epi)catechins and procyanidins are primarily targeted by microbiota (Appeldoorn et al., 2009; Ou et al., 2014). Two human intestinal bacterial species, *Eggerthella lent*a rK3 and *Eubacterium (E.) sp.* Strain SDG-2, were found to cleave C-rings in (epi)catechins (Kutschera, Engst, Blaut & Braune, 2011; Wang et al., 2001). In addition, *Flavonifractor plautii* aK2 further converts the C-ring-cleaved intermediate into DHPV via A-ring breakdown and lactone formation (Kutschera et al., 2011). DHPV may also be directly derived from breakdown of the A-ring in procyanidins (Appeldoorn et al., 2009). DHPV is further degraded into DHPA and DHAA via β- and α-oxidation, respectively (Ou et al., 2014). These metabolites have been found in human biological fluids, and procyanidin dimers such as A2 (Zampariello, Mckay, Dolnikowski, Blumberg & Chen, 2012), B1 (Sano, Yamakoshi, Tokutake, Tobe, Kubota & Kikuchi, 2003) and B2 (Holt et al., 2002), (epi)catechins (Richelle, Tavazzi, Enslen & Offord, 1999), DHPV (Urpi-Sarda et al., 2009) and DHAA (Bartolome et al., 2010; Saura-Calixto et al., 2010) have been identified in human blood plasma after intake of procyanidin-rich foods. In contrast to DHPA and DHAA, which can be derived from the microbial catabolism of other

flavonoid subclasses (Serra, Macia, Romero, Reguant, Ortega & Motilva, 2012), DHPV has been exclusively detected in human biological fluids after consumption of (epi)catechin-rich green tea (Li et al., 2000), proanthocynidin-rich cocoa (Urpi-Sarda et al., 2009; Urpi-Sarda et al., 2009), and pycnogenol (Grimm et al., 2006). Despite the importance of DHPV in procyanidin catabolism, little research has been focused on the properties of DHPV, such as its anti-inflammatory activity. DHPV exhibits stronger inhibitory activity than its precursor catechin on LPS-induced nitric oxide production in RAW 264.7 macrophages and the key enzymes of inflammatory and degenerative disorders, such as the matrix metalloproteinases (Grimm, Schafer & Hogger, 2004; Uhlenhut et al., 2012). In addition, DHPV exhibits more potent radical scavenging and antioxidative activities than catechin and vitamin C (Grimm et al., 2004). The present study marks the first report of the potential cardioprotective effects of DHPV.

Atherosclerosis is a major cause of CVDs and is thought to be highly preventable, particularly through adherence to a healthy diet (Mendis, Puska, Norrving, World Health Organization, World Heart Federation, & World Stroke Organization, 2011). In the early stages of atherosclerosis, vascular endothelial cells under inflammatory conditions begin to attract leukocytes such as monocytes (Libby, 2006; Libby, Ridker & Maseri, 2002; Ross, 1999). It has been well established that VCAM-1 and MCP-1 in the vascular endothelial cells are the key drivers of monocyte-endothelial adhesion (Tedgui & Mallat, 2006). VCAM-1 integrin is reported to be the critical mediator that strongly binds monocytes (Libby, 2006). After integrin-monocyte binding, inflammatory conditions continue to activate the

chemokine MCP-1, which attracts further monocytes into the vascular intima (Libby, 2006). Proinflammatory cytokines such as TNF-α stimulate a phosphorylation cascade within the IκB/NF-κB pathway, and the translocation of NF-κB into the nucleus activates transcription of numerous genes, including pro-inflammatory cytokines, adhesion molecules and chemokines (Libby, 2006; Libby et al., 2002; Tedgui et al., 2006). It has been previously reported that frequent consumption of procyanidin-rich cocoa improves vascular endothelial function and reduces levels of soluble adhesion molecules in the plasma of individuals who are at a high-risk of CVDs (Monagas et al., 2009; Wang-Polagruto et al., 2006). Several other *in vivo* and *in vitro* studies have shown that procyanidin-rich extracts reduce levels of VCAM-1 and MCP-1 under inflammatory conditions (Mohana, Navin, Jamuna, Sadullah & Devaraj, 2015; Zhang, Li & Li, 2016) (Sen & Bagchi, 2001). Other studies focusing on (epi)catechin metabolites have reported that both epicatechin phase II metabolites (Claude et al., 2014) and rat plasma after intake of (+)catechin (Koga & Meydani, 2001) inhibits monocyte-endothelial adhesion. In the case of DHPV, a single study has shown that the plasma of human volunteers containing DHPV after intake of pycnogenol inhibits NF-κB activation and MMP-9 secretion in activated human monocytes (Grimm et al., 2006). The present study marks the first report showing that DHPV has the most potent preventive effects on TNF-α-stimulated monocyte-endothelial adhesion among the procyanidin derivatives, downregulates two prominent biomarkers of atherosclerosis (VCAM-1 and MCP-1), activates NF-κB transcription, and phosphorylates IKK and IκBα. Further kinase targets of DHPV need to be identified in order to elucidate its full effects on atherosclerosis prevention.

Conclusion

Metabolomics studies have supported the notion that DHPV is a biologically important microbial metabolite exclusively derived from flavan-3-ols. Several DHPV-producing gut microbiota have been identified as well. Our findings suggest that DHPV may be a primary contributor toward the preventive effects of procyanidins on CVDs that have been observed in clinical studies. Further studies on the reduced risk of CVDs from chronic procyanidin intake and the presence of DHPV-producing microbiota, correlating with bioavailable DHPV levels in plasma are warranted.

Acknowledgements

This work was carried out with the support of "Cooperative Research Program for Agriculture Science and Technology Development (Project No. PJ01133403)" Rural Development Administration, Republic of Korea. Also this work was supported by the Korea Institute of Planning and Evaluation for Technology in Food, Agriculture, Forestry and Fisheries (IPET) through Agri-Bio industry Technology Development Program, funded by Ministry of Agriculture, Food and Rural Affairs (MAFRA) (514004), Republic of Korea.

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CHAPTER 4

A MAJOR DAIDZEIN METABOLITE 7,8,4'-TRIHYDROXYISOFLAVONE FOUND IN THE PLASMA OF YAK-KONG EMBRYO EXTRACT-FED RATS ATTENUATES MONOCYTE-ENDOTHELIAL CELL ADHESION

Abstract

Among many functional foods and their phytochemicals, ingestion of soybean is highly correlated to reduced risk of cardiovascular diseases (CVDs). Validation of potential health benefits of functional foods requires information about the bioavailability and metabolism of bioactive compounds. In this context, hydroxylated metabolites of isoflavones were target-analyzed in the plasma of rats acutely supplemented with Yak-Kong embryo (YKE) extract. A daidzein metabolite, 7,8,4'-trihydroxyisoflavone (7,8,4'-THI), was detected at the highest concentration (average 5.1 μM). Therefore, its potential prevention effect on atherosclerosis was investigated using monocyte-endothelial cell adhesion assay. Different from its precursor daidzein or daidzin, 7,8,4'-THI attenuated adhesion of THP-1 monocytes to TNF-α stimulated human umbilical vein endothelial cells. In addition, 7,8,4'-THI significantly downregulated the expression of vascular cell adhesion molecule-1 and monocyte chemotactic protein-1 and phosphorylation of IκB kinase and IκBα involved in the initiation of atherosclerosis. Therefore, 7,8,4'-THI, a highly bioavailable hydroxylated isoflavone metabolite, has potential anti-atherosclerotic effect via inhibiting monocyte-endothelial adhesion.

Introduction

Among many functional foods and their phytochemicals, consumption of soybean (*Glycine max*), especially its two functional constituents (protein and isoflavones), is highly correlated with reduced risk of cardiovascular diseases (CVDs) (Zhan & Ho, 2005). However, it is currently unclear which isoflavone is highly bioavailable in our body after soybean intake to exert the bioactivity for CVD prevention. Validation of potential health benefits of functional foods requires information about the bioavailability and metabolism of bioactive compounds because native compounds found in plants are recognized as xenobiotics in human body. They go through structural changes via several metabolic reactions such as phase I and II metabolisms (Cassidy & Minihane, 2017). Most dietary flavonoids are first subjected to enzymatic hydrolysis since their glycoside conjugates are too hydrophilic to be absorbed by the small intestine (Chen, Zheng, Li & Jiang, 2014). In enterocytes, absorbed aglycones will mainly undergo phase II reactions such as methylation, sulfation, and glucuronidation involving catechol-*O*-methyltransferases, sulfotransferases and uridine-5'-diphosphate glucuronosyltransferases, respectively (Chen et al., 2014). Absorbed conjugates or aglycones are then transported to the liver via portal vein for further phase II metabolism (Cassidy et al., 2017; Chen et al., 2014). In addition to phase II metabolism, hydrolyzed flavonoids after absorption will undergo phase I metabolism such as hydroxylation and demethylation involving cytochrome P450 (Cassidy et al., 2017; Chen et al., 2014). Some metabolites can be effluxed back to the intestinal lumen directly or through bile (Cassidy et al., 2017; Chen et al., 2014). Flavonoids that cannot be absorbed in the small intestine and

effluxed metabolites will reach the large intestine where they are extensively metabolized by gut microbiota (Cassidy et al., 2017; Chen et al., 2014).

For many years, researches in metabolomics of isoflavones have been focused on aglycones, mostly genistein and daidzein, and their reductive metabolites such as equol (Manach, Williamson, Morand, Scalbert & Remesy, 2005). Among the reported bioavailable metabolites of isoflavones, hydroxylated isoflavones have shown stronger antioxidant activity against lipid peroxidation than its precursors (Esaki, Onozaki, Morimitsu, Kawakishi & Osawa, 1998). In this study, isoflavone aglycones and their phase I metabolites especially hydroxylated metabolites were target-analyzed in the plasma of rats fed with Yak-Kong embryo (YKE) extract and the potential anti-atherosclerotic effect of major circulating metabolite was compared to that of its precursors.

Materials and methods

Chemicals and reagents. 7,8,4'-trihydroxyisoflavone (7,8,4'-THI) was purchased from Indofine Chemical Company (Hillsborough, NJ, USA). Isoflavone standards (daidzin, daidzein, genistein, and coumestrol), medium199 (M199), hydrocortisone, fetal bovine serum (FBS), and calcein AM dye were purchased from Sigma-Aldrich (St. Louis, MO, USA). RPMI 1640 medium was purchased from Welgene (Daegu, Republic of Korea). L-glutamine, basic fibroblast growth factor (bFGF), and recombinant human epidermal growth factor (hEGF) were purchased from Gibco (Grand Island, NY, USA). Penicillin (10,000 units/ml)-streptomycin (10,000 μg/ml) (P/S) was purchased from Corning (Corning, NY, USA). Tumor

necrosis factor-alpha (TNF-α) was purchased from PeproTech Korea (Seoul, Republic of Korea). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide tetrazolium salt (MTT) solution was purchased from USB Corporation (Cleveland, OH, USA). Dimethylsulfoxide (DMSO) was purchased from Duksan Pure Chemicals (Ansan, Republic of Korea). Antibodies against vascular cell adhesion molecule-1 (VCAM-1) and β-actin were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Phosphorylated and basal IκB kinase (IKK) and IκBα were purchased from Cell Signaling Biotechnology (Danvers, MA, USA). Liquid chromatography grade solvents such as acetone and acetonitrile were purchased from EMD Millipore Chemicals (Billerica, MA, USA). Glacial acetic acid, formic acid, and phosphoric acid were obtained from Anachemia (Lachine, QC, Canada). All other analytical chemicals were purchased from Merck KGaA (Darmstad, Germany).

Animal study and plasma preparation. Twenty male Wistar rats (Charles River, St. Constant, QC, Canada) were placed in temperature- and humidity-controlled rooms (21 ± 2 ℃, 35−40%) with a daily light-dark cycle of 12 h−12 h. Animal facilities met the guidelines of the Canadian Council on Animal Care. Protocols were approved by the Animal Care Committee of Laval University (reference CPAUL 2015-137-1). Animals were acclimated to their environment for a minimum of 5 days. They were provided *ad libitum* access to nonpurified rodent diet (rodent chow no. 2918, Harlan Teklad, Madison, WI, USA). All animals had continuous access to tap water. After the acclimation period, rats were fed a soybean excluded diet (AIN-76A, Envigo Teklad Diets, Madison, WI, USA) for 3 days. The day before the test, rats were fasted for 12 h and randomly allocated to two groups (10 rats per group). The

YKE group was administered a single dose of YKE extract with an average intake of 12 mg/kg body weight (BW) of isoflavones while the control group was only administered vehicle (water) by intragastric gavage. Blood samples were collected from the saphenous vein using EDTA-containing syringes (Sarstedt Microvettes) at time 0 (pre-ingestion) and at 30, 60, 120, and 240 min post-ingestion. Plasma samples were obtained by centrifugation (1,100 x g, 10 min at 4 ℃) and stored at −80 ℃ until analysis.

Isoflavones metabolites were extracted from plasma as previously described by Dudonné et al. with slight modifications. Briefly, Waters OASIS HLB micro-elution plates 2 mg–30 mm were preconditioned using 250 µl of methanol and 250 µl of 0.2% acetic acid. Plasma samples were mixed with 4% phosphoric acid in ultrapure water (v/v) to disrupt phenol-protein binding before loading into plates. Loaded plates were washed with 200 µl of ultrapure water and 200 µl of 0.2% acetic acid. Retained phenolic compounds were eluted with 75 µl of acetone/ultrapure water/acetic acid solution (70/29.5/0.5, v/v/v). Eluted phenolic metabolites were then directly analyzed by UHPLC−MS/MS.

Targeted analysis of isoflavone metabolites in plasma. Isoflavone metabolites were analyzed by reverse-phase UHPLC coupled to tandem mass spectrometry. Separation was achieved at 45 ℃ with a Waters Acquity HSS T3 column (2.1 mm x 100 mm, 1.8 µm) at a flow rate of 0.45 ml/min and an injection volume of 2.5 µl. Gradient elution was performed with a mobile phase consisted of 0.2% acetic acid in ultrapure water and acetonitrile (solvent A and B, respectively) using the following conditions: 0–0.5 min, 5% B; 0.5–1 min, 5–25% B; 1–3 min, 25–

45% B; 3–4.5 min, 45–55% B; 4.5–4.7 min, 55–100% B. MS/MS analyses were carried out in negative mode using electrospray source parameters as follows: electrospray capillary voltage, 1.3 kV; source temperature, 150 ℃; desolvation temperature, 400 ℃; cone and desolvation gas flows, 50 and 800 l/h, respectively. Data were acquired at multiple reaction monitoring (MRM) mode to track specific parent-product ion transition of the following compounds: genistein, daidzein, glycitein, orobol, 6,7,4'-THI, 7,3',4-THI and 7,8,4'-THI. Cone voltage and collision energy were optimized for each compound.

The identification of metabolites was validated by comparing their retention times and molecular ions with those of available standards or with fragmentation information described in literatures otherwise. External quantification was achieved using calibration curves constructed with available standards. Parent/product ion pairs (MRM transitions) and standards used for the quantification of identified metabolites are summarized in Table 4.1.

Table 4.1. Targeted analysis of isoflavone metabolites in plasma of rats supplemented with YKE extract.[a]

Isoflavones metabolites	MRM transitions	Standard for quantification	Concentrations in plasma (AUC)	
			Control	Embryo
Genistein (5,7,4'-trihydroxyisoflavone)	269 > 169	-	ND[b]	ND
Daidzein (7,4'-dihydroxyisoflavone)	253 > 91	Daidzein	ND	42.5 ± 6.7*
Glycitein (7,4'-dihydroxyisoflavone-6-methoxyisoflavone)	283 > 184	-	ND	ND
Orobol (5,7,3',4'-tetrahydroxyisoflavone)	285 > 148	-	ND	ND
6,7,4'-trihydroxyisoflavone	269 > 169	-	ND	ND
7,3',4'-trihydroxyisoflavone	269 > 169	-	ND	ND
7,8,4'-trihydroxyisoflavone	269 > 169	7,8,4'-trihydroxyisoflavone	ND	574.3 ± 112.8*

[a] Data are displayed as means of replicates ± SEM (n=10). [b] ND = not detected. *, $P < 0.005$.

Cell culture. Human umbilical vein endothelial cells (HUVECs) were purchased from Lonza (Walkersville, MD, USA). HUVECs (passage between 7 and 14) were grown in M199 with 25 mM HEPES containing 10% FBS, 2 mM L-glutamine, 1 ng/ml hydrocortisone, 1% (v/v) P/S, 1 ng/ml hEGF and 2 ng/ml bFGF. THP-1 cells, monocyte-like leukemia derived cells, were purchased from the Korean Cell Line Bank. THP-1 cells were grown in RPMI1640 supplemented with 10% FBS, 50 μM 2-mercaptoethanol and 1% (v/v) P/S. THP-1 cell subculture was conducted when its density reached between 2 x 10^5 and 1 x 10^6 cells/ml. For *in vitro* cell experiments, stock solutions were prepared in DMSO.

Cell viability assay. Confluent HUVECs were starved with serum free M199 containing 2 mM L-glutamine for 4 h and were incubated with different concentrations of isoflavone metabolites. After 22 h incubation, MTT solution was added. After additional 2 h incubation, DMSO was added, and the absorbance was measured at 570 nm.

Monocyte-endothelial cell adhesion assay. Starved HUVECs were pretreated with a non-toxic dosage of isoflavone metabolites for 1 h and then stimulated with 10 ng/ml TNF-α for 5 h. Calcein AM stained-THP-1 cells (5 x 10^5/well) were then layered over the HUVEC monolayers. After 1 h incubation, the non-adhered cells were washed off with PBS, and the fluorescence was measured using an Infinite 200 PRO system (Tecan group Ltd., Männedorf, Switzerland) at excitation and emission wavelengths of 485 nm and 538 nm.

Western blot assay. Confluent HUVECs were starved and treated with isoflavones for 1 h and stimulated with 10 ng/ml TNF-α. At various time points, cells

were scraped and disrupted with lysis buffer to obtain cell lysates. Equal amounts of proteins in lysate samples were subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to polyvibylidene difluoride membrane. After incubating the membrane with specific primary antibodies at 4 ℃ overnight and horseradish peroxidase-conjugated secondary antibodies, protein bands were visualized using enhanced chemiluminescence detection kit (Amersham Pharmacia Biotech, Piscataway, NJ, USA).

Enzyme-linked immunosorbent assay. Levels of monocyte chemoattractant protein-1 (MCP-1) in culture supernatant were determined using Human MCP-1/CCL2 ELISA MAX Deluxe Sets (BioLegend, San Diego, CA, USA) according to the manufacturer's protocol.

Real-time quantitative PCR. Total RNA was extracted from HUVECs and quantified. Primers of human VCAM-1 forward (5'-CCC TCC CAG GCA CAC ACA-3'), human VCAM-1 reverse (5'-GAT CAC GAC CAT CTT CCC AGG-3'), human MCP-1 forward (5'-TCG CCT CCA GCA TGA AAG TC-3'), human MCP-1 reverse (5'-GGC ATT GAT TGC ATC TGG CT-3'), human GAPDH forward (5'-CAG GGC TGC TTT TAA CTC TGG TAA A-3'), human GAPDH reverse (5'-GGG TGG AAT CAT ATT GGA ACA TGT AA-3') were used for running a quantitative real-time PCR. The amount of target gene expression was calculated as a ratio of the target transcript relative to GAPDH in each sample.

Statistical analyses. Area under plasma concentration (μM) time (min) curve (AUC) of isoflavone metabolites was calculated according to linear trapezoidal rule. Plasma concentrations of isoflavone metabolites detected in supplemented rats

(expressed as AUC) were compared to those of control rats using Welch's t-test (correcting for unequal variance) when data were normally distributed or Mann–Whitney test otherwise (GraphPad Prism 6.05, La Jolla, CA, USA). Biological results were compared using SPSS (Statistical Analysis System Institute, 2010). Significance of difference among means was determined by Tukey's Honest Significant Difference test. Statistical significance was set at $P < 0.05$.

Results

7,8,4'-THI was detected as the major isoflavone metabolite in rat plasma after intake of YKE extract. Isoflavone compositions of YKE extract are shown in Chapter II. Table 2.1. Among derivatives of genistin, daidzin, and glycitin, genistin derivatives including its acetyl, malonyl, and aglycon forms were the main isoflavones, accounting for more than 50% of isoflavones found in the YKE extract.

Targeted metabolomics analysis of the plasma of rats following their administration of around 12 mg of isoflavone content/kg BW revealed the presence of daidzein and 7,8,4'-THI (Table 4.1). 7,8,4'-THI was found to have the highest average AUC value (574.3 ± 112.8), which was at least 10 times higher than its precursor daidzein (average AUC 42.5 ± 6.7). Kinetic profiles of 7,8,4'-THI and daidzein are shown in Figure 4.1. 7,8,4-THI was detected in micro molar concentration ranges, with the highest average 5.1 µM within 30–60 min. Plasma levels of this metabolite started to decrease after it reached the highest peak within 60 min.

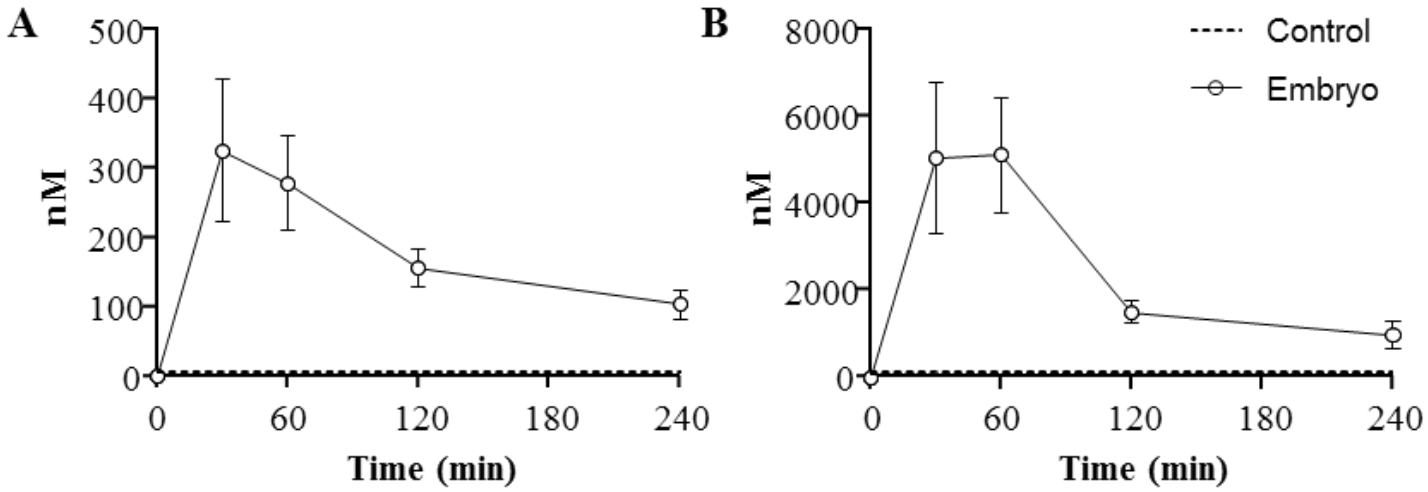

Figure 4.1. Plasma kinetic profile of daidzein (A) and its hydroxylated metabolite 7,8,4'-THI (B) in rats acutely treated with SE extract. Data are displayed as means ± SEM (n = 10).

7,8,4'-THI exerts stronger inhibitory effects on cell adhesion of THP-1 to TNF-α-stimulated HUVECs in comparison with its precursor daidzein or daidzin. Prevention effect of 7,8,4'-THI on atherosclerosis was evaluated *in vitro* using THP-1-HUVEC co-culture system. One hour before TNF-α stimulation, HUVECs were pretreated with 7,8,4'-THI or its precursor daidzein or daidzin for comparison (Figure 4.2A). Different from daidzein or daidzin, 7,8,4'-THI showed inhibitory effect on adhesion of THP-1 to TNF-α stimulated HUVECs in a dose-dependent manner (Fig. 4.2B). In addition, treatment concentrations of each compound did not show any cytotoxicity to HUVECs within 24 h (Figure 4.2C).

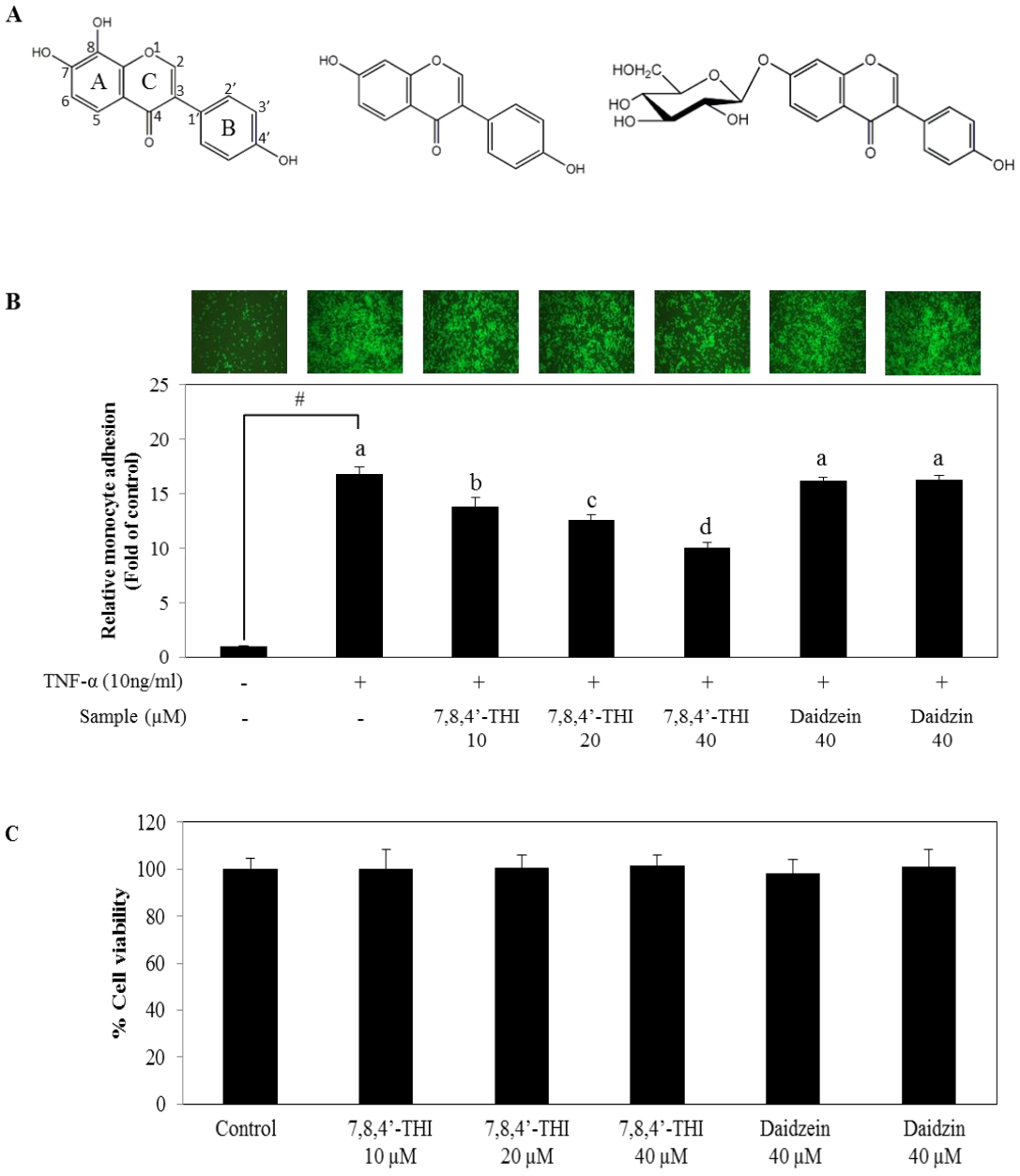

Figure 4.2. Comparison of monocyte-endothelial adhesion inhibitory effect between 7,8,4'-THI and its precursors daidzein and daidzin. (A) Structure of 7,8,4'-THI, daidzein, and daidzin (Left to right). (B) Representative images of calcein AM-labeled THP-1 in different treatment groups. Quantification of adhered THP-1 cells was described in the Materials and Methods. (C) Cell cytotoxicity was evaluated by MTT assay. Data are expressed as means ± SD (n = 4). Different letters denote significant differences ($P < 0.05$). Values bearing the same letters are not significantly different from one another.

7,8,4'-THI suppresses VCAM-1 and MCP-1 expression in TNF-α-induced HUVECs. Prevention effect of 7,8,4'-THI on TNF-α stimulated expression of VCAM-1 and MCP-1 in HUVECs was evaluated in order to examine proinflammatory cytokine stimulated mechanism involved in monocyte-endothelial adhesion. Western blot analysis showed that pretreatment with 7,8,4'-THI downregulated protein expression levels of TNF-α stimulated VCAM-1 in a dose dependent manner (Figure 4.3A). ELISA results showed that TNF-α stimulated MCP-1 protein secretion into the medium was reduced by pretreatment with 7,8,4'-THI (Figure 4.3B). In addition, RT-qPCR results showed that pretreatment with 7,8,4'-THI attenuated mRNA expression levels of TNF-α-stimulated VCAM-1 (Figure 4.3C) and MCP-1 (Figure 4.3D) in a dose-dependent manner.

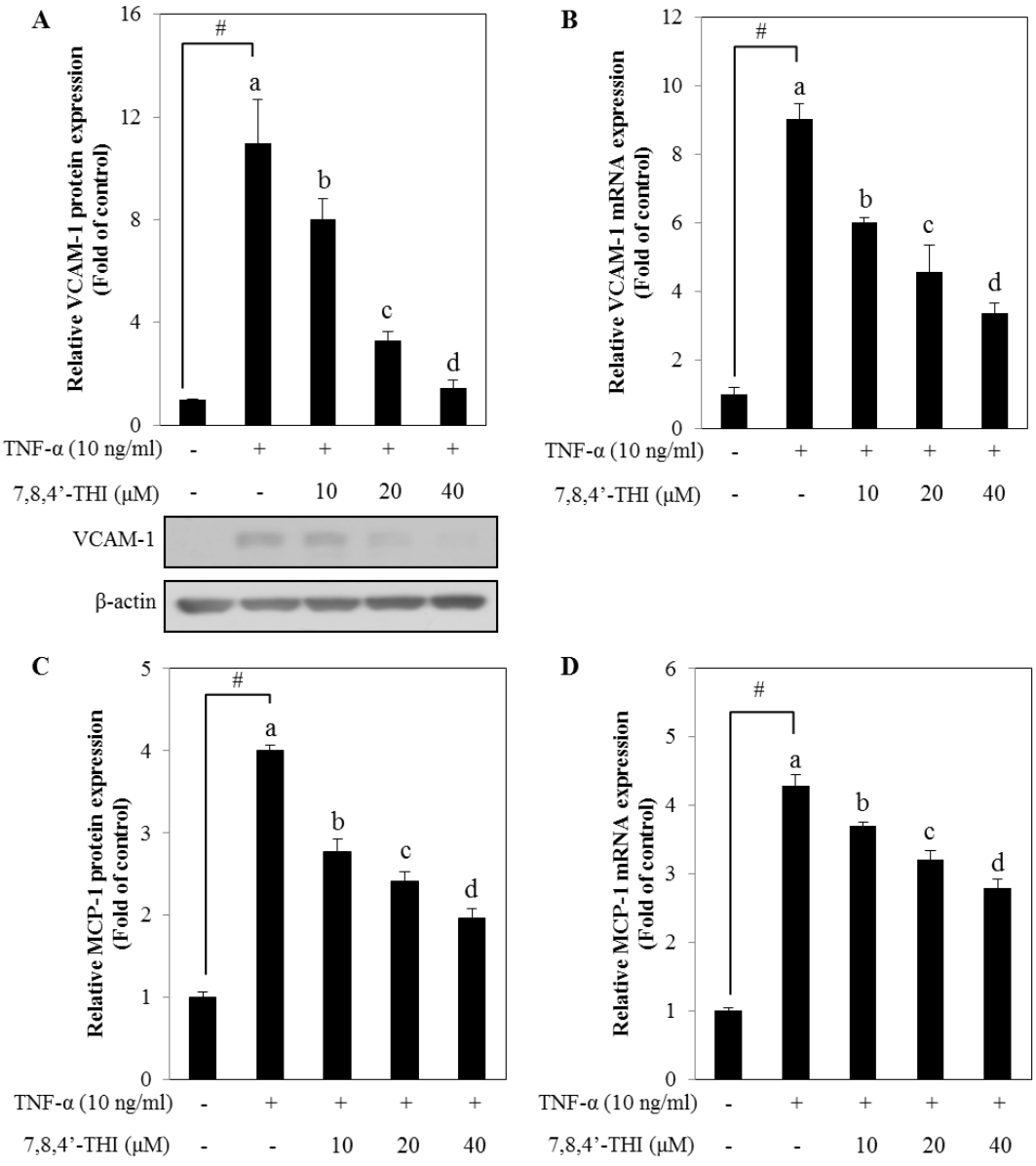

Figure 4.3. Effect of 7,8,4'-THI on VCAM-1 and MCP-1 expression levels in TNF-α-stimulated HUVECs. (A) Western blot analysis showing the effect of 7,8,4'-THI on TNF-α-stimulated VCAM-1 protein expression. (B) RT-qPCR showing the effect of 7,8,4'-THI on TNF-α-stimulated VCAM-1 mRNA expression. (C) ELISA analysis showing the effect of 7,8,4'-THI on TNF-α-stimulated MCP-1 protein expression. (D) RT-qPCR showing the effect of 7,8,4'-THI on TNF-α-stimulated MCP-1 mRNA expression. All the data is expressed as means ± SD of three or four independent experimental replications. Different letters denote significant differences ($P < 0.05$). Values bearing the same letters are not significantly different from one another.

7,8,4'-THI regulates NF-κB signaling in TNF-α-induced HUVECs.

Prevention effect of 7,8,4'-THI on TNF-α stimulated phosphorylation of IKK and IκBα in HUVECs was investigated to understand proinflammatory cytokine stimulated expression of VCAM-1 and MCP-1 as well as NF-κB signaling pathway. 7,8,4'-THI downregulated TNF-α stimulated phosphorylation of IKK (Figure 4.4A) and its downstream signaling protein IκBα (Figure 4.4B). Reduction of basal expression of IκBα caused by TNF-α stimulation was prevented by 7,8,4'-THI pretreatment as its concentration was increased (Figure 4.4C).

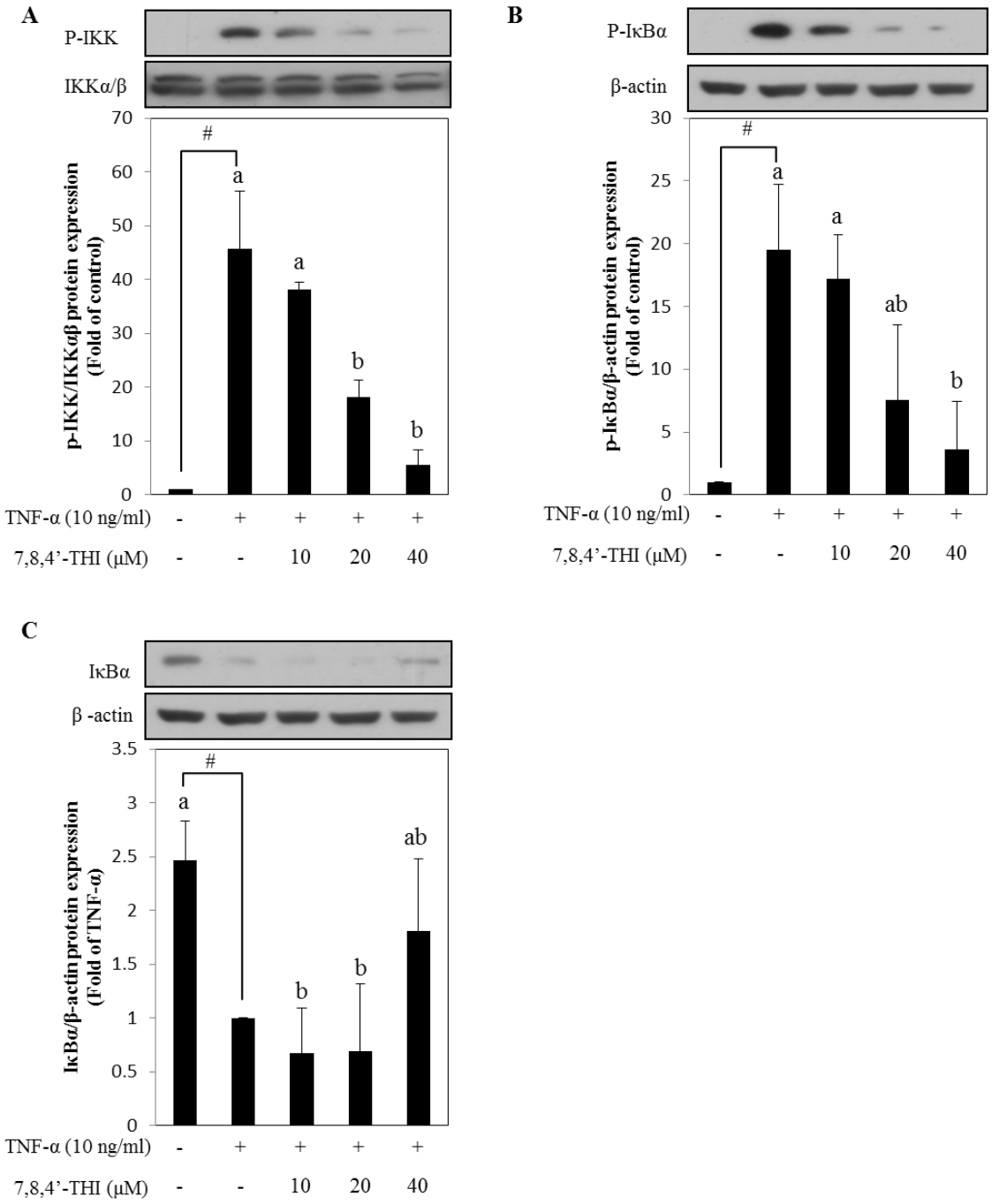

Figure 4.4. Effect of 7,8,4'-THI on NF-κB signaling in TNF-α-stimulated HUVECs. Western blot analyses show the effect of 7,8,4'-THI on TNF-α-stimulated (A) phosphorylated and total protein expression levels of IKK, (B) phosphorylated IκBα and (C) total protein expression levels of IκBα. All the data is expressed as means ± SD of three independent experimental replications. Different letters denote significant differences ($P < 0.05$). Values bearing the same letters are not significantly different from one another.

Discussion

A meta-analysis conducted by Zhan and Ho (2005) has found that intake of regular soy protein (*Glycine max*) together with a higher dose of isoflavone (80-185 mg/day) has higher impact on risk factors of CVDs compared to soy protein intake without isoflavones or lower doses of isoflavones (20-80 mg/day). However, native isoflavones are subjected to several metabolic biotransformation, leading to phase I and II metabolites (Barnes et al., 2011). Metabolites of isoflavones might have additive or synergistic effect on the potential preventive bioactivity of soy protein for CVDs. In the present study, rats were administered with YKE extract at an average of 12 mg of isoflavones per kg BW corresponding to human intake of about 110 mg isoflavones per 60 kg BW according to body surface area normalization (FDA, 2005). Following this acute supplementation, 7,8,4'-THI was found as the major circulating metabolite of isoflavone.

It has been previously reported that isoflavone aglycones are predominantly hydroxylated at ortho positions to original hydroxyl groups (Kulling, Honig, Simat & Metzler, 2000). It has been further found that 5,7,3'4'-tetrahidroxyisoflavone, 7,3',4'-THI, 6,7,4'-THI, and 7,8,4'-THI are four major hydroxylated metabolites of isoflavones found in human urine (Heinonen, Hoikkala, Wahala & Adlercreutz, 2003). Additionally, pharmacokinetic study of daidzein in humans has confirmed that these hydroxylated metabolites of daidzein are present in both urine and plasma at nano-molar concentrations after the intake of a single dose of daidzein (Rufer, Bub, Moseneder, Winterhalter, Sturtz & Kulling, 2008). 5,7,3'4'-tetrahidroxyisoflavone has been identified as the major hydroxylated metabolite of genistein (Kulling et al.,

2000). These metabolites are derived from both gut microbiota (Heinonen, Wahala, Liukkonen, Aura, Poutanen & Adlercreutz, 2004) and cytochrome P450 (Kulling, Honig & Metzler, 2001) specifically CYP1A2 P450 isozyme (Peng, Wang, Li, Abd El-Aty, Chen & Zhou, 2003). Inter-individual variability in gut microbiota and cytochrome P450 (Chen, Zhang, Huang & Cai, 2016; Poolsup, Po & Knight, 2000) can affect individual responses in producing different profiles of hydroxylated metabolites of isoflavones. In the present study, among the four previously observed hydroxylated metabolites, only 7,8,4'-THI (8-hydroxydaidzein) was identified in rat plasma. The highest average of 7,8,4'-THI was measured at 5.1 μM in rat plasma within 30–60 min after intake of an average of 3.77 mg daidzin derivatives/kg BW. To the best of our knowledge, this is the first *in vivo* study that reports the bioavailability of hydroxylated metabolites of isoflavones at micro-molar concentrations in plasma after intake of soy isoflavone mixture.

The preventive bioactivity of 7,8,4'-THI on CVDs was then investigated using monocyte-endothelial adhesion assay. It has been reported that soybean-derived compounds have protective effect on overall atherosclerosis mechanism (Nagarajan, 2010). It has been previously reported that soybean extract, soy isoflavone mixture, and genistein alone can inhibit monocyte-endothelial cell adhesion (Andrade, Sa & Toloi, 2012; Jia et al., 2013; Nagarajan, Stewart & Badger, 2006). However, hydroxylated metabolites of isoflavone including 7,8,4'-THI have not been studied for their atherosclerosis prevention activity. In the present study, we found that 7,8,4'-THI exerted stronger inhibitory effect on monocyte-endothelial adhesion compared to its precursor daidzein or daidzin. It was further demonstrated that 7,8,4'-THI could

attenuate protein and mRNA expression levels of atherosclerosis biomarkers such as VCAM-1 and MCP-1 stimulated by TNF-α. Additionally, 7,8,4'-THI suppressed the phosphorylation of IKK and IκBα in NF-κB signaling pathway. Therefore, 7,8,4'-THI appears to have significant impact for prevention of atherosclerosis.

Conclusion

A few previous studies have found that 7,8,4'-THI has tyrosinase and melanogenesis inhibitory activities (Tai, Lin, Wu & Chang, 2009), anti-pruritic effects (Kim et al., 2014) and antioxidant activity against lipid peroxidation (Esaki, Onozaki, Morimitsu, Kawakishi & Osawa, 1998). However, this study is the first one that reports its anti-atherosclerotic effect. We demonstrated that 7,8,4'-THI rather than its native forms of isoflavones might be the one highly bioavailable in the circulating system and exerting CVD preventive bioactivity after soybean intake. However, the potential CVDs prevention effect of 7,8,4'-THI needs to be confirmed *in vivo*. In addition, target kinase of 7,8,4'-THI needs to be identified in order to elucidate its mechanisms involved in atherosclerosis prevention.

Acknowledgements

The authors thank the Rural Development Administration, Republic of Korea, for providing a small black soybean cultivar (Registration number: 01-0003-2013-3). This work was carried out with the support of "Cooperative Research Program for Agriculture Science and Technology Development (Project No. PJ01133403)" Rural Development Administration, Republic of Korea. Also this work was supported by the

Korea Institute of Planning and Evaluation for Technology in Food, Agriculture, Forestry and Fisheries (IPET) through Agri-Bio industry Technology Development Program, funded by Ministry of Agriculture, Food and Rural Affairs (MAFRA) (514004), Republic of Korea.

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CHAPTER 5

CONCLUSIONS

Soybean (*Glycine max*) has been recognized as an effective functional food that may prevent cardiovascular diseases (CVDs). Of the several soybean-derived bioactive components known, the claim that soy protein reduces the risk of cardiovascular heart disease (CHD) has been approved by the FDA. A meta-analysis has demonstrated that isoflavones can elicit additive or synergistic effects towards the CHD preventive effect of soy protein. In addition to soy protein and isoflavones, soybean (especially cultivars with black seed coats) contains additional phenolic compounds including proanthocyanidins and anthocyanins, which have also shown promising associations with CVD risk reduction. Our research has demonstrated that Yak-Kong (YK), a small black soybean cultivar with a green embryo, is a highly potent anti-atherosclerotic agent. This was determined by comparing its antioxidant activities with that of common yellow (YS) and black soybean (BS) cultivars, analyzing the representative phenolic groups in the seed coat and embryo, and investigating the preventive effects of the metabolites on monocyte-endothelial adhesion.

In the first study, we compared the total phenolic content, as well as flavonoid and anthocyanin content present in YK with that found in common YS and BS using colorimetric assays. Additionally, the antioxidant activity in the YK seed coat and embryo were separately compared with YS and BS, as antioxidant activity has been recognized as an important property that may counteract several risk factors of CVD and atherosclerosis. We identified a number of other representative phenolics in each

part of YK using UHPLC-MSMS, and determined that both the YK seed coat and embryo contain higher antioxidant activity and total phenolic content than YS and BS. In addition, comprehensive phenolic analysis results showed that YK is an excellent source of proanthocyanidins and isoflavones, which have previously been strongly associated with reductions in CVD risk factors. In particular, YK seed coat contains a broad phenolics profile that includes A-type procyanidin dimers, anthocyanins, protocatechuic acid and phloridzin, which have shown anti-atherosclerotic bioactivity *in vivo*.

The majority of previous studies on the biological properties of functional foods and their phenolic compounds have been conducted using their native forms. Although their potential preventive effects against several diseases have been demonstrated from *in vitro* cell models to *in vivo* clinical studies, the bioavailability of such compounds is often overlooked. Bioavailability is a critical factor for the development of functional foods, as it accounts for the actual concentrations that end up circulating in the human body and reaching the target organs and tissues. We sought to focus on the atherosclerosis preventive effects of metabolites derived from YK phenolics using a monocyte-endothelial cell adhesion assay which mimics the conditions of the first phase of atherosclerosis.

It has been previously reported that proanthocyanidins are subjected to microbial catabolism, which is strongly influenced by an individual's gut microbiota profile. We therefore investigated major proanthocyanidin microbial metabolites that have been previously reported, and selected potential candidates that are likely to be bioavailable after ingestion of YK seed coat. Of the previously reported derivatives

including procyanidin A1, A2, B1 and B2, (epi)catechins, 5-(3',4'-dihydroxyphenyl)-γ-valerolactone (DHPV), 3-(3,4-dihydroxyphenyl)propionic acid and 2-(3,4-dihydroxyphenyl)acetic acid, we determined that DHPV elicits the most potent preventive effect on monocyte-endothelial adhesion.

In the third study, the CVD preventive effect of isoflavone-rich YK embryo was investigated. Among the previously reported isoflavone metabolites, hydroxylated isoflavones have shown more potent antioxidant activity compared to their precursors. We then analyzed several major hydroxylated isoflavones and their precursors in the plasma of rats acutely supplemented with YK embryo extract. A daidzein metabolite, 7,8,4'-trihydroxyisoflavone (7,8,4'-THI), was identified as being at the highest concentrations (average 5.1 μM) in rat plasma within 30–60 min after intake of an average of 3.77 mg daidzin derivative/kg per body weight of rat. Compared to its precursors daidzein and daidzin, 7,8,4'-THI showed a more potent preventive effect on TNF-α-stimulated monocyte-endothelial adhesion.

The mechanism responsible for the preventive bioactivity of DHPV and 7,8,4'-THI on monocyte-endothelial adhesion was revealed to involve the downregulation of vascular cell adhesion molecule-1, monocyte chemotactic protein-1 and the phosphorylation of I Kappa B Kinase in the NF-κB signaling pathway, all of which are associated with atherosclerosis.

Taken together, our studies demonstrate that YK is an excellent source of soy protein, proanthocyanidins and isoflavones, and both seed coat and embryo together may contribute toward additional cardioprotective bioactivity. Notably, the major metabolites DHPV and 7,8,4'-THI have more favorable bioavailability profiles and

are therefore more likely to exert actual anti-atherosclerotic effects.