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Effect of fermentation with *Monascus pilosus* on the antioxidant activities and phenolic acid contents of adzuki bean (*Vigna angularis*)

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ABSTRACT

Objective: To enhance physiological activities of adzuki bean (*Vigna angularis*) via fermentation with *Monascus pilosus* (*M. pilosus*).

Methods: The adzuki bean fermentation conditions with *M. pilosus* were optimized, and the effect of *Monascus*-fermentation on the antioxidant capacity and phenolic acid contents of adzuki bean was investigated.

Results: Optimal fermentation conditions were determined by the production of monacolin K. The highest monacolin K production was observed in 5% inoculum sized on day 15 in fermentation. Free and bound phenolic acids were isolated from native and fermented adzuki bean. A 1.9-fold decrease was observed in bound *p*-coumaric acid content, whereas the contents of bound ferulic and sinapic acids were increased by 28- and 1.7-fold, respectively. However, the contents of free phenolic acids such as *p*-coumaric, ferulic, and sinapic acids were increased by 2.6-, 5.2-, and 7.2-fold, respectively. The fermentation of adzuki bean by *M. pilosus* enhanced the activities of DPPH$^{\bullet}$ radical scavenging, ferrous ion-chelating, nitric oxide scavenging, and ferric antioxidant reducing activities 2.2-, 1.7-, 1.2-, and 1.8-fold, respectively.

Conclusions: Results from our study suggest that the contents of *p*-coumaric, ferulic, and sinapic acids in adzuki bean were highly increased by fermentation with *M. pilosus*, resulting in enhanced various antioxidant activities.

1. Introduction

The oxidation induced by reactive oxygen species (ROS) can result in cell membrane disintegration, membrane protein damage and DNA mutation, which can further initiate or propagate the development of many diseases, such as cancer, liver injury, and cardiovascular disorders[1]. Although there are defense mechanisms in the body to arrest the damaging properties of ROS, continuous exposure to pollutants may lead to the accumulation of large amounts of free radicals in the body, and cause irreversible oxidative damage[2]. Dietary compounds (*i.e.* vitamin C, vitamin E, phenolic compounds, and carotenoids), which are present in fruits, grains, vegetables, and legumes, reduce oxidative damages with its radical scavenging activities. Previous studies have reported beneficial effects of dietary antioxidant compounds on the oxidative stress-induced diseases[3,4].

Vigna angularis (*V. angularis*), also known as adzuki bean or small red beans, belongs to the plant family Fabaceae and has been widely cultivated in South Korea, China, Taiwan, and Japan. In the Orient, adzuki bean is a popular ingredient to make confections with its sweet flavor[5]. Adzuki bean is a rich source of phenolic compounds, including anthocyanin and other catechins,

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carbohydrates, proteins, and vitamins[6,7].

The fungi of the genera *Monascus*, have been used for food fermentation especially to make red yeast rice in China for centuries, and the *Monascus* fermentation is performed mainly by *Monascus pilosus* (*M. pilosus*), *Monascus purpureus*, and *Monascus anka*. Recent clinical observations revealed that red yeast rice has the ability to lower blood-lipid levels and cholesterol concentrations[8,9]. *Monascus* species were reported to produce diverse secondary metabolites with biological functions, including a group of pigments, anti-hypercholesterolemic agent (monacolin K), hypotensive agent (γ-aminobutyric acid), antioxidant compounds including dimerumic acid and antibacterial compounds[10]. In recent studies, other food materials (*i.e.* soybean and dioscorea) have also been fermented with *Monascus*, and the level of monacolin K and the antioxidant capacities were highly increased[11,12]. However, despite reports on the health benefits of adzuki bean[13-16], the effects of fermentation by *Monascus* on adzuki bean have not been studied yet.

Here, we report for the first time the increase of physiologically active substances in adzuki bean via fermentation with *M. pilosus* KCCM 60084 and the optimization of adzuki bean fermentation conditions. To investigate antioxidant properties of *Monascus*-fermented adzuki bean (MFAB), 1,1-diphenyl-2-picrylhydrazyl freeradical ($DPPH^{\bullet}$) scavenging assay and 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical scavenging assay, ferrousion-chelating assay, nitro oxide scavenging, and reducing power assay were conducted. The contents of monacolin K and bound phenolic acids in the MFAB were determined by high performance liquid chromatography (HPLC), and the changes of total polyphenols and total flavonoids were also measured.

2. Materials and methods

2.1. Sample material and preparation of cultures

Adzuki bean, which is harvested in the Republic of Korea in 2013, was used for this study. *M. pilosus* KCCM 60084 was obtained from the Korean Culture Center of Microorganisms (KCCM) and used as inoculum. To prepare stock cultures, *M. pilosus* KCCM 60084 was grown for 7 days on potato dextrose agar (Difco Laboratories, Detroit, MI) at 25 °C, and the spores were collected and washed in sterile saline solution and diluted to obtain a spore suspension of 1×10^7 spores/mL.

2.2. Solid state fermentations

Adzuki bean was washed with tap water, and then soaked in distilled water for 16 h. After removing water, adzuki bean was ground slightly to disrupt the seed coat. Each 100 g of adzuki bean was put into a 1 L flask and sealed with cotton stuff, and then autoclaved. After being cooled, the substrate was inoculated with spore suspension (1×10^7 spores/mL) in a 1%, 2%, 5%, and 10% (v/w). The inoculated substrate was fermented at 25 °C for 15 d.

2.3. Sample extraction

The MFAB was dried at 60 °C for 18 h. Each 1 g of ground adzuki bean and ground MFAB was extracted with 10 mL of 70% ethanol at room temperature with occasional shaking for 24 h. The extract was centrifuged at 4000 r/min and 25 °C for 15 min, and the supernatant was filtered through polytetrafluoroethylene filter (0.45 μm).

2.4. Proximate composition analysis of MFAB

Proximate composition of MFAB and non-fermented adzuki bean (NFAB) were determined according to the methods of AOAC[17]. The parameters determined include: moisture, crude protein, crude fiber, lipids, and ash content.

2.5. Determination of monacolin K contents

Pure monacolin K was obtained from Sigma (St. Louis, MO, USA) and dissolved in methanol to prepare the standard curve. Monacolin K was determined according to the methods of Friedrich and others with slight modifications[18]. Analysis was performed using a Hypersil ODS column (Agilent technologies 250 mm×4.0 mm inner diameter: 5 μm) connected to binary HPLC pump (Waters 1525) set at a flow rate of 1.0 mL per min. The photodiode array detector was set to 238 nm. The mobile phase consisted of water (pH adjusted to 2.5 with H_3PO_4)/acetonitrile/isopropanol (55:35:10, v:v:v) (solution A) and 100% acetonitrile (solution B), running at a ratio of 50:50 (v:v) for 20 min. Twenty microliters of extract was directly injected into the HPLC system and analyzed. The content of monacolin K was expressed as μg of monacolin K per g of dry weight (dw).

2.6. Determination of total phenolic content

The total phenolic content of the extract was determined using the Folin-Ciocalteu reagent. The extract (1 mL) was mixed with 1 mL of 20% Folin-Ciocalteau reagent and 1 mL of 10% sodium carbonate solution, and allowed to stand at room temperature for 1 h. The absorbance of the solution was measure at 570 nm by using a microplate reader (Tecan, infinite pro2000). The total phenolic contents were expressed as gallic acid equivalents (GAE) per mL of the extract[19].

2.7. Determination of total flavonoid content

Total flavonoid content of extract was determined using a modified method of Park and others[20]. The extract (1 mL) was added to a test tube containing 0.1 mL of 10% aluminum nitrate, 0.1 mL of 1 mol/L potassium acetate, and 4.3 mL of ethanol and the mixture was allowed to react for 30 min. The absorbance was measured at 415 nm by using a microplate reader (Tecan, infinite pro2000). The total flavonoid contents were expressed as quercetin equivalents (QE) per mL of the extract.

2.8. Determination of bound and free phenolic acid contents

Non-fermented and MFAB samples were analyzed for bound phenolic acids. The bound and free phenolic acids were extracted from 1.0 g of NFAB and MFAB according to the previous report[21]. Phenolic acids (caffeic acid, *p*-coumaric acid, ferulic acid, and sinapic acid) contents of the extract was analyzed using HPLC according to the previous report[22]. A reverse-phase column (YMC-Pack ODS-A. 250 mm×4.6 mm. 5 μm particle size; YMC, Japan) was used as a stationary phase. The injection volume was 20 μL. Photo diode array detector detection was performed between 210 and 325 nm. Standard compounds for analysis were obtained from Sigma (St. Louis, MO).

2.9. Determination of antioxidant activities

2.9.1. Ferric reducing antioxidant power

Ferric reducing antioxidant power of the extract was determined according to the modified method of Oyaizu[23]. A 2.5 mL of extract (0.25, 0.5, 1.0, and 2.0 mg/mL) was mixed with 2.5 mL of 200 mM sodium phosphate buffer (pH 6.6) and 2.5 mL of 1% potassium ferricyanide, and the mixture was incubated at 50 °C for 20 min. Then 2.5 mL of 10% trichloroacetic acid (w/v) was added, and the mixture was centrifuged at 4 000 r/min for 10 min. The 5 mL of upper layer was taken, and mixed with 5 mL of deionized water and 1 mL of 0.1% ferric chloride. Then the absorbance was measured at 700 nm using a microplate reader (Tecan, infinite pro2000). Higher absorbance values of the mixtures indicate higher reducing power.

2.9.2. $ABTS^{\bullet+}$ radical scavenging activity

$ABTS^{\bullet+}$ radical scavenging activity of the extracts was determined according to the modified method of Zhishen and others[24]. The $ABTS^{\bullet+}$ was produced by reacting 7 mmol/L ABTS stock solution with 2.45 mmol/L potassium persulfate and allowing the mixture to stand for 16 h at room temperature in the dark. The $ABTS^{\bullet+}$ solution was diluted with water to obtain an absorbance of 0.70±0.02 at 734 nm. A 10 μL of the different concentrations of extract (0.25, 0.5, 1.0, and 2.0 mg/mL)was added to 1 mL of a diluted $ABTS^{\bullet+}$ solution, and the mixture was incubated at room temperature for 30 min in the dark. The absorbance was measured at 734 nm using a microplate reader (Tecan, infinite pro2000). $ABTS^{\bullet+}$ radical scavenging capacity was calculated using the following formula: $ABTS^{\bullet+}$ radical scavenging activity (%)=$[(A_0 - A_s)/A_0)]\times100$, where A_0 is the absorbance of control (blank) and A_s is the absorbance of the sample extract.

2.9.3. Ferrous (Fe^{2+}) ion chelating activity

Ferrous ion chelating activity of the extracts was measured according to the modified method of Gülçin and others[25]. Hundred microliters of 2 mmol/L $FeCl_2$ was added to 1 mL of the different concentrations of extract (0.25, 0.5, 1.0, and 2.0 mg/mL) extract and mixed well. The reaction was initiated with 0.2 mL of 5 mmol/L ferrozine solution and incubated at room temperature for 10 min. The absorbance of the solution was measured at 562 nm. The results were expressed as the percentage inhibition of the ferrozine-Fe^{2+} complex formation and it was calculated using the same formula used to determine $ABTS^{\bullet+}$ scavenging activity.

2.9.4. $DPPH^{\bullet}$ radical scavenging activity

$DPPH^{\bullet}$ radical scavenging assay was conducted according to the method of Diaz and others[26]. A 50 μL volume of the different concentrations of extract (0.25, 0.5, 1.0, and 2.0 mg/mL) was added to 200 μL of 100 mmol/L DPPH solution and mixed well, and then allowed to react at room temperature in the dark for 30 min. The absorbance was measured at 517 nm using a microplate reader (Tecan, infinite pro2000). $DPPH^{\bullet}$ scavenging activity was determined using the same equation used to calculate $ABTS^{\bullet+}$ scavenging activity.

2.9.5. Nitric oxide (NO) scavenging activity

The scavenging activity of the extracts on NO was measured according to the method of Marcocci and others[27]. A 50 μL volume of the different (0.25, 0.5, 1.0 and 2.0 mg/mL) was mixed with 1 mL of sodium nitroprusside (5 mmol/L) and the mixture was incubated at 25 °C for 3 h. After the incubation, 50 μL of Griess reagent (1% sulphanilamide, 2% phosphoric acid, and 0.1% of napthylene diaminedihydrochloride) was added to the sample mixture. The absorbance of the sample mixture was measured at 540 nm and referred to the absorbance of standard solutions of sodium nitrite salt treated in the same way with Griess reagent. The NO scavenging activity was determined using the same equation to calculate $ABTS^{\bullet+}$ scavenging activity.

2.10. Statistical analysis

All data are presented as mean±SD and analyzed by Sigmaplot (version 12.5). One-way analysis of variance was performed by ANOVA procedures. Significant differences between means were determined by student's *t*-test. A *P*-value of less 0.01 was considered as significant.

3. Results

3.1. Optimization of adzuki bean fermentation with M. pilosus

In order to identify the most effective inoculum size for the fermentation of adzuki bean, the fermentation was performed with four different inoculum size (1%, 2%, 5%, and 10% v/w) for 15 d, and the monacolin K contents was determined. The production of monacolin K was analyzed from day 6 of fermentation (Figure 1). On day 6, the 10% inocula showed the highest monacolin K

production (223.43 μg/g dw, 250.22 μg/g dw, 305.04 μg/g dw, and 457.01 μg/g dw at 1%, 2%, 5%, and 10% inoculum density, respectively). However, at the end of fermentation (day 15), the highest monacolin K production was observed in the 5% inocula (1 200.42 μg/g dw, 1 577.03 μg/g dw, 1 650.00 μg/g dw, 1 500.33 μg/g dw at 1%, 2%, 5%, and 10% inoculum density, respectively).

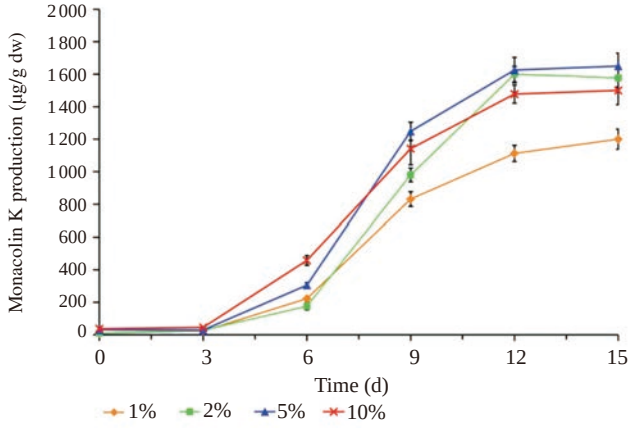

Figure 1. Monacolin K production by *M. pilosus* during fermentation of adzuki bean with different inoculum size.
Results are mean±SD of triplicate independent experiments (P<0.01; One-way ANOVA and student's *t*-test).

3.2. Determination of the proximate composition of adzuki bean before and after fermentation

The proximate compositions of adzuki bean that was fermented with *M. pilosus* for 15 d is shown in Table 1. The content of carbohydrate of MFAB had decreased 16% and the content of crude protein was increased 26% compared to the NFAB. The content of crude lipid and crude fiber had increased 5.5% and 78%, respectively, compared with NFAB. The ash content was not changed significantly (P<0.01).

Table 1
Proximate composition of adzuki bean and its fermented product (g/100g).

Constituents	NFAB	MFAB
Carbohydrate	65.00±5.20^{a}	54.50±5.24^{b}
Crude protein	23.31±1.31^{c}	29.34±5.20^{d}
Crude lipid	1.83±0.30^{e}	1.93±0.21^{e}
Crude fiber	5.63±0.33^{f}	9.92±0.20^{g}
Ash	4.30±0.21^{h}	4.33±0.20^{h}

Values are the mean±SD of triplicate independent experiments; *: Different letters in the same row mean significant differences (P<0.01); **: The same letter in the same row indicates not significantly different (P<0.01).

3.3. Effect of fermentation by M. pilosus on the contents of total phenolics and total flavonoids

The amount of total phenolics of NFAB and MFAB was (595.23±4.80) μg GAE/mL extractand (911.02±31.74) μg GAE/mL extract, respectively (Figures 2a and 2b). The amount of total flavonoids of NFAB and MFAB was 1.32 mg QE/mL extract and 1.80 mg QE/mL extract, respectively.

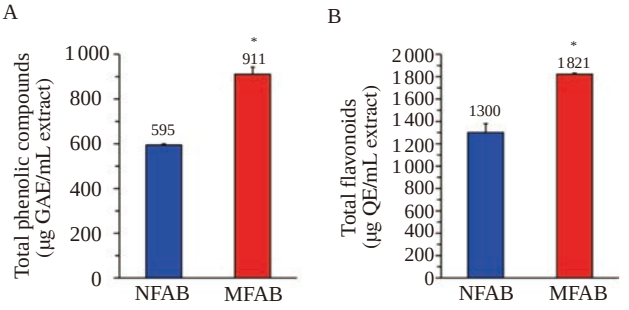

Figure 2. Total phenolic contents and total flavonoid contents of MFAB and NFAB at different concentrations (0.25-2.0 mg/mL). A: Total phenolic contents of NFAB and MFAB; B: Total flavonoid content of NFAB and MFAB.
Data are expressed as mean±SD of triplicate independent experiments (*P< 0.01, One-way ANOVA and student's *t*-test).

3.4. Changes in phenolic acid content after fermentation by M. pilosus

The results of the phenolic acid content analysis in the adzuki bean extracts are presented in Table 2. *p*-coumaric acid was found to be the major bound and free phenolic acid in the NFAB and caffeic acid was not detected both in NFAB and MFAB. After fermentation, bound form of *p*-coumaric acid was significantly decreased (P<0.01) by 1.9-fold (from 0.41 to 0.22 μg/mg extract), and the contents of bound form of ferulic and sinapic acids was significantly increased (P<0.01) by 28- (from 0.12 to 3.36 μg/mg extract) and 1.7-fold (from 0.06 to 0.10 μg/mg extract), respectively. The contents of free form of *p*-coumaric, ferulic, and sinapic acids was significantly increased (P<0.01) by 2.6- (from 0.81 to 2.10 μg/mg extract), 5.2- (from 0.53 to 2.80 μg/mg extract), and 7.2-fold (from 0.05 to 0.36 μg/mg extract), respectively, by *Monascus*-fermentation.

Table 2
Determination of bound and free phenolic acid content of non-fermented and MFAB extract (μg/mg extract).

Sample	*p*-coumaric acid		Ferulic acid		Sinapic acid	
	Bound	Free	Bound	Free	Bound	Free
NFAB	0.41±0.01^{a}	0.81±0.01^{c}	0.12±0.00^{e}	0.53±0.00^{g}	0.06±0.00^{i}	0.05±0.00^{k}
MFAB	0.22±0.00^{b}	2.10±0.01^{d}	3.36±0.00^{f}	2.78±0.05^{h}	0.10±0.00^{j}	0.36±0.04^{l}

Values are the mean±SD of triplicate independent experiments; *: Different letters in the same column mean significant differences (P<0.01).

3.5. DPPH• radical scavenging activity

DPPH is stable organic radical and the DPPH• radical scavenging capacity of biological reagents can be expressed as its magnitude of antioxidant activity[28]. Antiradical activities of MFAB and NFAB were determined by using DPPH• and the results are presented as percent inhibition of DPPH activity (Figure 3A). The DPPH• radical scavenging activity was significantly increased in dose dependent manner, and 2.0 mg/mL of MFAB extract showed 2.2-fold increase in (P<0.01) DPPH• radical scavenging activity compared with the same concentration of NFAB extract. The IC_{50} value of MFAB for DPPH• radicals was found to be 0.6 mg/mL.

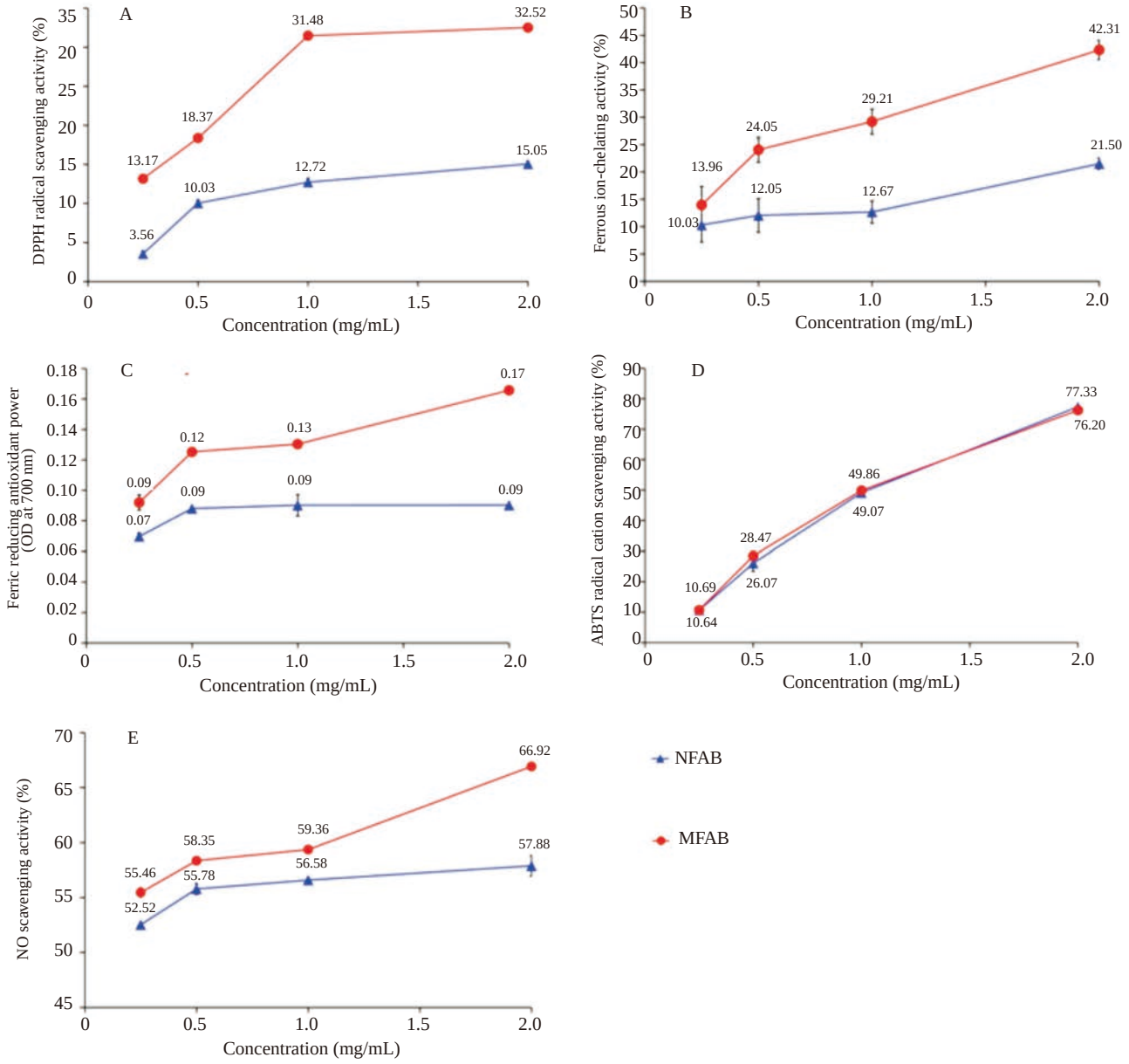

Figure 3. Antioxidant activities of MFAB and NFAB at different concentrations (0.25-2.0 mg/mL). A: DPPH$^{\bullet}$ radical scavenging activity; B: Ferrous ion chelating activity; C: Ferric reducing antioxidant power; D: ABTS$^{\bullet+}$ radical scavenging activity; E: NO scavenging activity.
Data are expressed as mean±SD of triplicate independent experiments (P<0.01; One-way ANOVA and student's t-test).

3.6. Ferrous (Fe^{2+}) ion chelating activity

The transition metal ions can allow the formation of many radicals with its ability to move single electrons, and the previous reports proposed that the transition metal ions contribute to the production of ROS that causes oxidative damage and ultimately neurodegenerative disorders[29]. The most effective strategy to reduce ROS generation is chelation of the metal ions. To determine the capacity of metal ion-chelating ability of MFAB, ferrous ion-chelating assay was performed (Figure 3B). The ion-chelating capacity was significantly increased with increasing concentrations of extract, and 2.0 mg/mL of MFAB extract displayed 1.7-fold increase (P<0.01) in ion-chelating activity compared with the same concentrations of NFAB extract.The IC_{50} value of MFAB for ion-chelating activity was found to be 3 mg/mL.

3.7. Ferric (Fe^{3+}) reducing antioxidant power

The electron-donating ability is one of antioxidative mechanism of antioxidant reagents and the antioxidant capacity of reducing reagents can be measured by the conversion of the Fe^{3+}/ferricyanide complex to the ferrous form[30,31]. The reducing capacity of MFAB was determined by potassium ferricyanide reduction assay. The ferric antioxidant reducing activities of NFAB and MFAB were increased in dose dependent manner (Figure 3C) and that of 2.0 mg/mL MFAB was 1.8-fold higher (P<0.01) compared with 2.0 mg/mL NFAB.

3.8. ABTS$^{\bullet+}$ radical scavenging activity

ABTS$^{\bullet+}$radical scavenging assay is frequently used to measure the

antioxidant capacities of foods[32]. $ABTS^{\bullet+}$, which is the stable cation radical, is generated by oxidation of ABTS with potassium persulfate and is reduced by hydrogen-donation antioxidants[33]. Figure 3D presents that the $ABTS^{\bullet+}$scavenging ability of adzuki bean was not changed significantly (P<0.01) after fermentation with *M. pilosus*.

3.9. NO scavenging activity

NO has been implicated in diverse physiological processes such as regulation of cell growth, differentiation, and apoptosis, and modulation of blood pressure[34]. However, abnormally high level of NO could cause various diseases such as sepsis and hepatic failure[35]. The previous studies have shown that natural antioxidants effectively decrease the level of NO, and polyphenols from dietary fruits, vegetables, and herbs generally have the potent NO scavenging activities[36,37]. Figure 3E shows that NO scavenging activities of NFAB and MFAB are increasing in dose dependent manner, and MFAB displays 1.2-fold increased (P<0.01) NO scavenging activity at 2.0 mg/mL compared with NFAB. The IC_{50} value of MFAB for NO scavenging activity was found to be 0.07 mg/mL.

4. Discussion

The purpose of this study was to enhance the physiological activities of adzuki bean (*V. angularis*) via fermentation with *M. pilosus*. Because adzuki bean contains rich amount of phenolic compounds and other bioactive compounds[6,7], it shows various physiological functions, such as anticancer, antioxidant, anti-obesity[13-16]. The edible fungi *Monascus* have been used to ferment rice, soybean, adlay, and dioscorea, and the previous studies reported that the fermentation of food materials by *Monascus* increases the contents of physiological active compounds, such as phenolic acids, γ-aminobutyric acid, free amino acids, isoflavone aglycones[38]. Moreover, because the genus of *Monascus* produce monacolin K, which has anti-hypercholesterolemic acitivity, the function of cholesterol-lowering can be added to the final product via the fermentation using *Monascus*.

To produce the MFAB, it was necessary to optimize inoculum size and incubation time for the fermentation. The fermentation of adzuki bean was conducted with four different (1%, 2%, 5%, and 10% v/w) inoculum size for 15 d, and the content of monacolin K was determined as a standard compound in fermented adzuki bean. The highest monacolin K content was observed in the 5% inocula (1500.33 μg/g dw) on day 15. This result demonstrated that a 5% inoculum size led to the highest production of monacolin K in the adzuki bean fermentation. According to these results, following adzuki bean fermentation with *M. pilosus* was performed with the 5% inoculum size during 15 days. Additionally, monacolin K has been reported that it can inhibit cholesterol biosynthesis and lower cholesterol level in blood with its inhibition activity on 3-hydroxyl-3-mehtylglutaryl coenzyme A reductase, which is the rate-limiting enzyme in cholesterol biosynthesis[10]. So, anti-obesity and antihyperlipidemic effect of adzuki bean can be expected via fermentation with *M. pilosus*.

Polyphenolic compounds of plants have a variety of biological effects such as antioxidant activity[39]. Among the phenolics, phenolic acids and flavonoids are known to the major antioxidant compound[40], and it has been reported that adzuki bean contains significant quantities of phenolic compounds and flavonoids[41]. In order to investigate the effect of fermentation with *Monascus*, the contents of total phenolics and total flavonoids in MFAB was determined. The amount of total phenolics of MFAB was significantlyincreased 1.5-fold (P<0.01) compared to NFAB [(911.02±31.74) μg GAE/mL extract of MFAB and (595.23±4.80) μg GAE/mL extract of NFAB], and the amount of total flavonoids of MFAB was also significantly increased 1.4-fold (P<0.01) compared to NFAB (1.80 mg QE/mL extract of MFAB and 1.32 mg QE/mL extract of NFAB).

The most abundant phyenylpropanoids in plant tissue are hydroxycinnamic acids (caffeic, *p*-coumaric, ferulic and sinapic) and they are present in free or bound forms; however, these phenolic acids are rarely found in the free form[42]. To identify the effect of *Monascus*-fermentation on the contents of phenolic acids in adzuki bean, bound and free form of these four phenolic acids were determined before and after fermentation. The analysis results of the contents of phenolic acids in the adzuki bean extracts revealed that the *Monascus*-fermentation increases the contents of bound form of ferulic acid and sinapic acids, and free form of *p*-coumaric, ferulic and sinapic acids.

The antioxidant activity of food constituents have to be determined with various methods, because they work on different targets with different reaction mechanisms[28,43]. In this study, the antioxidant capacity of MFAB was evaluated via $DPPH^{\bullet}$ scavenging, $ABTS^{\bullet+}$ scavenging, ferrous ion-chelating, NO scavenging, and reducing power assays. The results from various antioxidant test demonstrated that the adzuki bean fermentation by *M. pilosus* significantly increased the DPPH radical scavenging, ferrous ion-chelating, and NO scavenging activities.

The experimental results demonstrated that the fermentation of adzuki bean by *M. pilosus* increases the contents of total phenolic acids and total flavonoids, and enhances various antioxidant capacities. According to the previous reports[44,45], the enhanced antioxidant capacities of MFAB seems due to the increased total phenolics and total flavonoid contents. A previous study revealed that the metal ion-chelating activity is more affected by polysaccharides, proteins, or peptides than polyphenols[46]. The proximate composition analysis of MFAB demonstrated total protein contents was significantly increased 1.3-fold (P<0.01) compared with that of NFAB, and this might be the reason for the increased ferrous ion-chelating activity. The analysis of phenolic acid contents in MFAB and NFAB demonstrated that the contents of free *p*-coumaric, ferulic, and sinapic acids was markedly increased after fermentation by *M. pilosus*, and in the case of bound *p*-coumaric acid a decreasing was observed; however, the contents of bound ferulic and sinapic acids

was increased after fermentation. *p*-coumaric acid has antioxidant capacities that decrease the risk of stomach cancer by reducing the formation of carcinogenic nitrosamines[47,48]. Recent study has also identified that *p*-coumaric acid suppressed tumor growth with its antiangiogenic effect[49]. Sinapic acid is one of the most common hydroxycinnamic acids and it has been proposed as a potent antioxidant[50]. In recent years many researches have revealed that sinapic acid has anti-inflammatory, anticancer, and anti-anxiety activities[51-53]. Ferulic acid shows a wide range of therapeutic effects in the treatment of cancer, lung and cardiovascular diseases, antimicrobial and anti-inflammatory activities with its strong free radical scavenging capacities[54]. The increasing in *p*-coumaric, ferulic, and sinapic acid contents during *Monascus*-fermentation may enhances various biological activities of adzuki bean.

Fermentation in adzuki bean (*V. angularis*) with *M. pilosus* caused significant increases in crude protein and crude fiber contents, and total phenolics and total flavonoids contents, and DPPH free radical scavenging activity, and ferrous ion-chelating activity, ferrous antioxidant reducing power, and NO scavenging activity. Moreover, it was demonstrated that MFAB contains a large amount of monacolin K, ferulic, sinapic, and *p*-coumaric acids. Results obtained from present study suggest that MFAB will be helpful for the health problems associated to the oxidative stress and high blood cholesterol level.

Conflict of interest statement

We declare that we have no conflict of interest.

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References

[1] Liao K, Yin M. Individual and combined antioxidant effects of seven phenolic agents in human erythrocyte membrane ghosts and phosphatidylcholine liposome systems: importance of the partition coefficient. *J Agric Food Chem* 2000; **48**(6): 2266-2270.

[2] Tseng TH, Kao ES, Chu CY, Chou FP, Lin Wu HW, Wang CJ. Protective effects of dried flower extracts of Hibiscus sabdariffa L. against oxidative stress in rat primary hepatocytes. *Food Chem Toxicol* 1997; **35**(12): 1159-1164.

[3] Halliwell B, Gutteridge J. *Free radicals in biology and medicine.* 2nd ed. Oxford: Claredon Press; 1989.

[4] Gutteridge JM. Free radicals in disease processes: a compilation of cause and consequence. *Free Radic Res Commun* 1993; **19**: 141-158.

[5] Yoshida H, Tomiyama Y, Yoshida N, Shibata K, Mizushina Y. Regiospecific profiles of fatty acids in triacylglycerols and phospholipids from Adzuki beans (*Vigna angularis*). *Nutrients* 2010; **2**(1): 49-59.

[6] Yoshida K, Sato Y, Okuno R, Kameda K, Isobe M, Kondo T. Structural analysis and measurement of anthocyanin from colored seed coats of *Vigna*, *Phaseolus*, and *Glycine* Lugumes. *Biosci Biotechnol Biochem* 1996; **60**(4): 589-593.

[7] Ariga T, Hamano M. Radical scavenging action and its mode in procyanidins B-1, and B-3 from azuki beans to peroxyl radicals. *Agric Biol Chem* 1990; **54**(10): 2499-2504.

[8] Cheng MJ, Wu MD, Su YS, Chen IS, Yuan GF. Anti-inflammatory compounds from *Monascus pilosus*-fermented rice. *Phytochem Letters* 2012; **5**(1): 63-67.

[9] Ma J, Li Y, Ye Q, Li J, Hua Y, Ju D, et al. Constituents of red yeast rice, a traditional Chinese food and medicine. *J Agric Food Chem* 2000; **48**(11): 5220-5225.

[10] Wang JJ, Lee CL, Pan TM. Modified mutation method for screening low citrinin-producing strains of *Monascus purpureus* on rice culture. *J Agric Food Chem* 2004; **52**(23): 6977-6982.

[11] Pyo YH, Seong KS. Hypolipidemic effects of *Monascus*-fermented soybean extracts in rats fed a high-fat and -cholesterol diet. *J Agric Food Chem* 2009; **57**(18): 8617-8622.

[12] Chiang SS, Chang SP, Pan TM. Osteoprotective effect of *Monascus*-fermented dioscorea in ovariectomized rat model of postmenopausal osteoporosis. *J Agric Food Chem* 2011; **59**(17): 9150-9157.

[13] Kitano-Okada T, Ito A, Koide A, Nakamura Y, Han KH, Shimada K, et al. Anti-obesity role of adzuki bean extract containing polyphenols: *in vivo* and *in vitro* effects. *J Sci Food Agric* 2012; **92**(13): 2644-2651.

[14] Durak A, Baraniak B, Jakubczyk A, Swieca M. Biologically active peptides obtained by enzymatic hydrolysis of Adzuki bean seeds. *Food Chem* 2013; **141**(3): 2177-2183.

[15] Mukai Y, Sato S. Polyphenol-containing azuki bean (*Vigna angularis*) seed coats attenuate vascular oxidative stress and inflammation in spontaneously hypertensive rats. *J Nutr Biochem* 2011; **22**(1): 16-21.

[16] Nakaya K, Nabata Y, Ichiyanagi T, An WW. Stimulation of dendritic cell maturation and induction of apoptosis in leukemia cells by a heat-stable extract from azuki bean (*Vigna angularis*), a promising immunopotentiating food and dietary supplement for cancer prevention. *Asian Pac J Cancer Prev* 2012; **13**(2): 607-611.

[17] AOAC. *Official methods of analysis of the Association of Official Analytical Chemists*. 15th ed. Washington, DC: Association of Official Analytical Chemists; 1990.

[18] Friedrich J, Zuzek M, Bencina M, Cimerman A, Strancar A, Radez I. High-performance liquid chromatographic analysis of mevinolin as mevinolinic acid in fermentation broths. *J Chromatography A* 1995; **704**(2): 363-367.

[19] Guha G, Rajkumar V, Ashock Kumar R, Mathew L. Aqueous extract of *Phyllanthusamarus inhibits* chromium(VI)-induced toxicity in MDA-MB-435S cells. *Food Chem Toxicol* 2010; **48**(1): 396-401.

[20] Park YK, Koo MH, Ikegaki M, Contado JI. Comparison of the flavonoid aglycone contents of *Apis mellifera* propolis from various regions of Brazil. *Arq Biol Technol* 1997; **40**(1): 97-106.

[21] Hole AS, Grimmer S, Naterstad K, Jensen MR, Paur I, Johansen SG, et al. Activation and inhibition of nuclear factor kappa B activity by cereal extracts: role of dietary phenolic acids. *J Agric Food Chem* 2009; **57**(20): 9481-9488.

[22] Filipiak-Szok A, Piasta A, Kurzawa M, Szłyk E. Determination of flavonols and phenolic acids in *Puearia lobata* (Kudzu) root by HPLC-PDA method. *Curr Issues Pharm Med Sci* 2012; **25**(3): 278-281.

[23] Oyaizu M. Studies on product of browning reaction: antioxidative activities of products of browning reaction prepared from glucoseamine. *Jpn J Nutr* 1986; **44**(6): 307-315.

[24] Jia ZS, Tang MC, Wu JM. The determination of flavonoid contents in mulberry and their scavenging effects on superoxide radicals. *Food Chem* 1999; **64**(4): 555-559.

[25] Gülçin I, Elias R, Gepdiremen A, Boyer L. Antioxidant activity of lignans from fringe tree (*Chionanthus virginicus* L.). *Eur Food Res Technol* 2006; **223**(6): 759-767.

[26] Diaz P, Jeong SC, Lee S, Khoo C, Koyyalamudi SR. Antioxidant and anti-inflammatory activities of selected medicinal plants and fungi containing phenolic and flavonoid compounds. *Chin Med* 2012; **7**: 26.

[27] Marcocci L, Maguire JJ, Droy-Lefaix MT, Packer L. The nitric oxide-scavenging properties of *Ginkgo biloba* extract EGb 761. *Biochem Biophys Res Commun* 1994; **201**(2): 748-755.

[28] Ou B, Huang D, Hampsch-Woodill M, Flanagan JA, Deemer EK. Analysis of antioxidant activities of common vegetables employing oxygen radical absorbance capacity (ORAC) and ferric reducing antioxidant power (FRAP) assays: a comparative study. *J Agric Food Chem* 2002; **50**(11): 3122-3128.

[29] Aparadh VT, Naik VV, Karadge BA. Antioxidative properties (TPC, DPPH, FRAP, metal chelating ability, reducing power and TAC) within some cleome species. *Ann Bot* 2012; **2**: 49-56.

[30] Yen GC, Chen HY. Antioxidant activity of various tea extracts in relation to their antimutagenicity. *J Agric Food Chem* 1995; **43**(1): 27-32.

[31] Yildirim A, Mavi A, Oktay A, Kara AA, Algur ÖF, Bilaloglu V. Comparison of antioxidant and antimicrobial activities of tilia (*Tilia argentea* Desf Ex DC), sage (*Salvia triloba* l.), and black tea (*Camellia sinensis*) extracts. *J Agric Food Chem* 2000; **48**(10): 5030-5034.

[32] Huang D, Ou B, Prior RL. The chemistry behind antioxidant capacity assays. *J Agric Food Chem* 2005; **53**(6): 1841-1856.

[33] Re R, Pellegrini N, Progeggente A, Pannala A, Yang M, Reice-Evans C. Antioxidant activity applying an improved ABTS radical cation decolorization assay. *Free Rdical Biol Med* 1999; **26**(9-10): 1231-1237.

[34] Lloyd-Jones DM, Bloch KD. The vascular biology of nitric oxide and its role in atherogenesis. *Annu Rev Med* 1996; **47**: 365-375.

[35] Shah V, Lyford G, Gores G, Farrugia G. Nitric oxide in gastrointestinal health and disease. *Gastroenterology* 2004; **126**(3): 903-913.

[36] Uttara B, Singh AV, Zamboni P, Mahajan RT. Oxidative stress and neurodegenerative diseases: a review of upstream and downstream antioxidant therapeutic options. *Curr Neuropharmacol* 2009; **7**(1): 65-74.

[37] Perron NR, Brumaghim JL. A review of the antioxidant mechanisms of polyphenol compounds related to iron binding. *Cell Biochem Biophys* 2009; **53**(2): 75-100.

[38] Srianta I, Ristiarini S, Nugeahani I, Sen SK, Zhang BB, Xu GR, et al. Recent research and development of *Monascus* fermentation products. *Int Food Res J* 2014; **21**(1): 1-12.

[39] Kähkönen MP, Hopia AI, Vuorela HJ, Rauha JP, Pihlaja K, Kujala TS, et al. Antioxidant activity of plant extracts containing phenolic compounds. *J Agric Food Chem* 1999; **47**: 3954-3962.

[40] Wojdyło A, Oszmia ski J, Czemerys R. Antioxidant activity and phenolic compounds in 32 selected herbs. *Food Chem* 2007; **105**(3): 940-949.

[41] Lin PY, Lai HM. Bioactive compounds in legumes and their germinated products. *J Agric Food Chem* 2006; **54**(11): 3807-3814.

[42] Manach C, Scalbert A, Morand C, Rémésy C, Jiménez L. Polyphenols: food sources and bioavailability. *Am J Clin Nutr* 2004; **79**(5): 727-747.

[43] Cao G, Sofic E, Prior RL. Antioxidant and prooxidant behavior of flavonoids: structure-activity relationships. *Free Radic Biol Med* 1997; **22**(5): 749-760.

[44] Oboh HA, Omoregie IP. Total phenolic and antioxidant capacity of some Nigerian beverages. *Nig J Basic Appl Sci* 2011; **19**(1): 68-75.

[45] Farhan H, Rammal H, Hijazi A, Hamad H, Daher A, Reda M, et al. *In vitro* antioxidant activity of ethanolic and aquous extracts from crude *Malva parviflora* L. grown in Lebanon. *Asian J Pharm Clin Res* 2012; **5**(Suppl 3): 234-238.

[46] Wang T, Jónsdóttir R, Ólafsdóttir G. Total phenolic compounds, radical scavenging and metal chelation of extracts from Icelandic seaweeds. *Food Chem* 2009; **116**(1): 240-248.

[47] Ferguson LR, Zhu ST, Harris PJ. Antioxidant and antigenotoxic effects of plant cell wall hydroxycinnamic acids in cultured HT-29 cells. *Mol Nutr Food Res* 2005; **49**(6): 585-593.

[48] Zhang LY, Cosma G, Gardner H, Shi X, Castranova V, Vallyathan V. Effect of antioxidant protection by *p*-coumaric acid on low-density lipoprotein cholesterol oxidation. *Am J Physiol Cell Physiol* 2000; **279**(4): C954-960.

[49] Kong CS, Jeong CH, Choi JS, Kim KJ, Jeong JW. Antiangiogenic effects of *p*-coumaric acid in human endothelial cells. *Phytother Res* 2013; **27**(3): 317-323.

[50] Niciforovic N, Abramovic H. Sinapic acid and its derivatives: natural sources and bioactivity. *Compr Rev Food Sci Food Saf* 2014; **13**(1): 34-51.

[51] Yun KJ, Koh DJ, Kim SH, Park SJ, Ryu JH, Kim DG, et al. Anti-inflammatory effects of sinapic acid through the suppression of inducible nitric oxide synthase, cyclooxygase-2, and proinflammatory cytokines expressions via nuclear factor-kappaB inactivation. *J Agric Food Chem* 2008; **56**(21): 10265–10272.

[52] Hudson EA, Dinh PA, Kokubun T, Simmonds MS, Gescher A. Characterization of potentially chemopreventive phenols in extracts of brown rice that inhibit the growth of human breast and colon cancer cells. *Cancer Epidemiol Biomarkers Prev* 2000; **9**(11): 1163–1170.

[53] Yoon BH, Jung JW, Lee JJ, Cho YW, Jang CG, Jin C, et al. Anxiolytic-like effects of sinapic acid in mice. *Life Sci* 2007; **81**(3): 234–240.

[54] Paiva LB, Goldbeck R, Santos WD, Squina FM. Ferulic acid and derivatives: molecules with potential application in the pharmaceutical field. *Braz J Pharm Sci* 2013; **49**(3): 395-411.