Food Chemistry 112 (2009) 659–663

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

In vitro antioxidant activity of anthocyanins of black soybean seed coat in human low density lipoprotein (LDL)

Ignasius Radix Astadi [a,*], Mary Astuti [b], Umar Santoso [b], Prihati Sih Nugraheni [c]

[a] Department of Food Technology, Widya Mandala Surabaya Catholic University, Jl Dinoyo 42–44, Surabaya, Indonesia
[b] Department of Food and Agricultural Product Technology, Gadjah Mada University, Jl Socio Justitia, Yogyakarta, Indonesia
[c] Department of Fisheries, Gadjah Mada University, Jl Flora A-4, Yogyakarta, Indonesia

ARTICLE INFO

Article history:
Received 5 February 2008
Received in revised form 13 June 2008
Accepted 16 June 2008

Keywords:
Black soybean
Phenolic
Anthocyanin
DPPH
LDL
TBARS

ABSTRACT

The objectives of this study were to determine the phenolic and anthocyanin contents in black soybean *Mallika* and *Cikuray* variety seed coat extract and to examine antioxidant activity of extract against DPPH radical and LDL oxidation. Black soybean seed coat of *Mallika* (M) and *Cikuray* (C) was extracted using methanol-1%HCl. The phenolic and anthocyanin contents were determined with Folin–Ciocalteu and pH differential methods, respectively. Individual anthocyanidins were identified with HPLCdiode array detector, and antioxidant activity was examined, using DPPH and TBARS assay with LDL as the oxidation substrate. BHT and rutin were used as antioxidant references. The phenolic content in M and C were 8.15 ± 0.23 and 6.46 ± 0.11 g GAE/100 g, respectively. The anthocyanin contents were 11.36 ± 0.12 and 1.45 ± 0.13 g/100 g, respectively. Cyanidin, delphinidin, and pelargonidin were found as individual anthocyanidins. The optimum DPPH radical scavenging capacity (%) of M and C were 92.78% and 91.50%, respectively, BHT and rutin were 77.0% and 91.94%, respectively. The optimum inhibition of TBARS formation from M and C were 37.10 and 30.37 nmol MDA equivalents/g LDL protein, respectively, and rutin were 30.10 nmol MDA equivalents/g LDL protein, respectively. These results suggest that black soybean seed coat has high levels of phenolic and anthocyanin, and also demonstrated considerable antioxidant activity of black soybean seed coat.

1. Introduction

Black soybean has long been consumed in Indonesia. It was originally grown in Southeast Asia (Shurtleff & Aoyagi, 1979). According to Rhumpius report in 1750 (Mary-Astuti., 1996), black soybean has been planted by farmer in Java and Bali island and used for tempeh production. It has also been used as holy food in Indonesian tradition. In Japan and Korea, black soybean has been known as medicinal food, it has antioxidant compound especially anthocyanins in their seed coat (Inagaki, Morimura, Shigematsu, Kida, & Akutagawa, 2005; Kim, Yun, Chung, & Park, 2004; Shinomiya, Tokunaga, Shigemoto, & Kamei, 2005).

Many studies have demonstrated the antioxidant activities and health benefits of various beans (Aparicio-Fernandez, Yousef, Loarca-Pina, de Mejia, & Lila, 2005; Daaz-Batalla, Widholm, Fahey, Tostado, & Paredes-Lopez, 2006; Heimler, Vignolini, Dini, & Romani, 2005; Lee & Shibamoto, 2000; Madhujith, Amarowicz, & Shahidi, 2004; Madhujith & Shahidi, 2005). In addition, work on bean with different seed coat colour has been carried out by Choung, Choi, An, Chu, and Cho (2003) which reported on anthocyanin profile of Korean cultivated red and black kidney beans (*Phaseolus vulgaris* L.). Another work by Ranilla, Genovese, and Lajolo (2006) reported that the seed coat colour pattern and the type of bean cultivar showed an important influence on the variability of phenolic profiles and levels. However, little has been reported about the health functional properties of anthocyanins from black soybean. The objectives of this study were to determine the anthocyanins and phenolic contents in Indonesian black soybean *Mallika* and *Cikuray* variety seed coat extract and to examine antioxidant activity of extract against free radical DPPH and LDL oxidation.

2. Materials and methods

2.1. Materials

The samples of black soybean *Mallika* variety were obtained from farmers in Bantul district, Yogyakarta Special Province, Indonesia and *Cikuray* variety were obtained from Malang district, East Java Province, Indonesia.

* Corresponding author. Tel.: +62 31 5678478x110.
E-mail address: radix_astadi@yahoo.com (I.R. Astadi).

0308-8146/$ - see front matter © 2008 Elsevier Ltd. All rights reserved.
doi:10.1016/j.foodchem.2008.06.034

2.2. Chemical

Methanol, hexane, formic acid, acetonitrile, HCl, Folin–Ciocalteu's reagent, $CuSO_4$, thiobarbituric acid (TBA), and trichloroacetic acid (TCA) were purchased from Merck (Darmstad, Germany), and 2,2-diphenyl-1-picrylhydrazyl (DPPH), rutin, butylated hydroxytoluene (BHT), and gallic acid (GA) were purchased from Sigma Chemical Co. (St. Lois, MO). Anthocyanin standards (cyanidin, delphinidin, and pelargonidin) were purchased from Extrasynthese, Genay Cedex, France.

2.3. Sample preparation of extract

Black soybean seed coat was peeled from the grain manually, and then blended using a food processor (Model 2L-350W, Phillips, Indonesia) and passed trough 30 mesh sieve. The fat from seed coat powder was removed with hexane (1:5 w/v, 1 h × 3) in an Erlenmeyer. Erlenmeyer was shaken using a shaker (Model OS 752, Optima Inc., Japan) at 125 rpm at 27 °C. The resulting slurry was filtered trough a Whatman filter paper and the residue was air dried. The dried defatted powder was stored in vacuum packaged at −20 °C prior to analysis.

2.4. Preparation of crude extracts of black soybean seed coat

A quantity of 5 g (db) defatted powder was placed in Erlenmeyer and 50 ml of methanol-1% HCl was added, the Erlenmeyer was placed in a shaker and shaken at 125 rpm for 1 h, at room temperature. Then extracts were macerated at 4 °C for 24 h. After that, extracts were placed in a tube and centrifuged at 4000*g*, 4 °C for 10 min. After centrifugation the supernatant was filtered trough a Whatman no 42 paper and the volume was measured. The extraction was repeated three times and supernatants were combined.

2.5. Analysis of phenolic compounds

The phenolic content in the black soybean seed coat extracts was determined according to the Folin–Ciocalteu procedure (Singleton & Rossi, 1965) with a slight modification. In brief, 0.1 ml extract was placed in a tube, and 0.5 ml Folin–Ciocalteu reagent was added, mixed, and allowed to stand for 8 min. Then 4.5 ml of 2% sodium carbonate (Na_2CO_3) solution were added, mixed and placed in a dark room for 1 h at room temperature. Absorbance of the resulting blue complex was then measured at 765 nm using a Shimadzu UV-1601 spectrophotometer (Shimadzu, Kyoto, Japan). Methanol was used as the blank and gallic acid used as a standard. The used of gallic acid based on its stability and purity (Singleton & Rossi, 1965; Waterhouse, 2002). The results were expressed as g gallic acid equivalents (GAE)/100 g fresh weight of black soybean seed coat powder. Data were reported as means ± SD for three replications.

2.6. Quantification of anthocyanins

Anthocyanins quantification was performed by the pH differential method of Giusti and Wrolstad (Awika, Rooney, & Waniska, 2004) with a slight modification. Samples were diluted 1:170 in pH 1.0 and pH 4.5 buffers. Then the diluted samples were measured at 513 and 700 nm in a Shimadzu UV-1601 spectrophotometer. Absorbance readings were converted to total g of anthocyanins per 100 g fresh weight of black soybean seed coat powder using the molar extinction coefficient of 26,900, a molecular weight of 445 and an absorbance of (A) = $[(A_{513}-A_{700})$ pH 1.0$-(A_{513}-A_{700})$ pH 4.5$)]$. Data were reported as means ± SD for three replications.

2.7. HPLC identification of individual anthocyanidins

High Performance Liquid Chromatography (Shimadzu, Kyoto, Japan) coupled with a photodiode array (PDA) detector (SPD-M20A, Shimadzu, Kyoto, Japan) performed in 510–550 nm were used for identification of individual anthocyanidins (Nakajima, Tanaka, Seo, Yamazaki, & Saito, 2004). Briefly, 0.5 g black soybean seed coat powder was placed in 50 ml polyethylene centrifuge tube and 20 ml of 2 M HCl in methanol were added. The centrifuge tube was sealed and placed in 90 °C waterbath for 50 min. Then the mixture was centrifuged (1500*g*) and filtered trough a 0.2 μm nylon membrane. The supernatant (0.20 μl) was injected into a C-18 column. HPLC conditions were as follows: solvent A, 10% formic acid; solvent B, 100% acetonitrile; linear gradient, initial percentage of A (96–85%) and B (4–15%) from 0 to 8 min; from 8–23 min, isocratic elution A (85%) and B (15%); from 23–24 min, linear gradient, A(85–20%) and B (15–80%); from 24–27 min, isocratic elution A (20%) and B (80%); from 27–28 min, linear gradient, A (20–96%) and B (80–20%); column temperature 35 °C, flow rate, 0.8 ml/min. Cyanidin, delphinidin, and pelargonidin standards (Extrasynthese, Genay Cedex, France) were dissolved in 1.2 M HCl in methanol in 0.1–0.3 μg/μl concentration to obtain standard curves.

2.8. Determination of DPPH radical scavenging activity

Experiments were carried out according to Kordali et al. (2005) with a slight modification. Briefly, 0.5 mM DPPH solution in methanol was prepared, and then 0.5 ml of DPPH solution was mixed with 0.1 ml of 50, 100, and 150 ppm anthocyanins equivalents of the extract and vortexed thoroughly. Then, 4 ml of methanol was added to the solution and allowed to stand for 60 min in the dark room. The absorbance was measured at 516 nm using a Shimadzu UV-1601 spectrophotometer (Shimadzu, Kyoto, Japan). Decreasing the absorbance of the DPPH solution indicates an increase in DPPH radical scavenging activity. This activity is given as percent DPPH radical scavenging, which is calculated with the equation. % DPPH radical scavenging = [(control absorbance−sample absorbance)/control absorbance] × 100%. Control contained 0.5 ml of DPPH solution and 4 ml of methanol. Rutin and BHT were used as positive controls at 150 ppm concentration. Data were reported as means ± SD for three replications.

2.9. Inhibition of LDL oxidation

2.9.1. LDL isolation

The isolation of LDL was performed according to Katsube et al. (2005a,b). Plasma was obtained from healthy human volunteers after 12 h of fasting and dispersed into a tube containing ethylene diamine tetraacetic acid (EDTA) and plasma was immediately separated by centrifugation (3000*g*, 10 min). The plasma was then transferred to centrifuge tubes. Solid NaBr was added to adjust the density of plasma to 1.220 g/ml. Then 1.006 g/ml NaBr solution was added and centrifuged at 236,000*g*, 4 °C, for 45 min (Hitachi SCP 85H; 80T rotor) ultracentrifuge. After separation of the very low density lipoprotein (VLDL) fraction on the top and the next lower fraction, then 1.063 g/ml NaBr solution was added to the remained fraction and centrifuged at 236,000*g*, 4 °C for 45 min. The LDL fraction on the top was then collected and stored at −20 °C until used. The protein contents of the fraction were determined with Biuret method (Castro, Neto, & Gomes, 2006).

2.9.2. LDL oxidation

Protein level of LDL was adjusted to 8 g protein/l with phosphate buffer saline (PBS) pH 7.4. Then 0.4 ml of LDL fraction was

incubated with 70 μl 20, 40, 60 ppm anthocyanins equivalents of extract for 5 min. Then, 33.3 μl of 50 μM $CuSO_4$ was added to induce the LDL fraction and incubated at 37 °C for 24 h. After incubation, 33.3 μl EDTA (final concentration 27 mM) were added to prevent any further oxidation.

2.9.3. Determination of thiobarbituric acid reactive substances (TBARS)

The extent of LDL oxidation was determined by measuring the TBARS formation. TBARS assay was performed according to the procedures of Katsube et al. (2005a,b) with a slight modification. Briefly, 2 ml of 0.67% of thiobarbituric acid (TBA) and 15% of trichloroacetic acid (TCA) in 0.1 M HCl were added to the incubated LDL, and then the mixed reagents reacted at 95 °C for 1 h and cooled in ice for 5 min. Then the samples were centrifuged at 3000*g* for 15 min.

The formation of TBARS was measured at 532 nm and the results were expressed in nmol MDA equivalents/g LDL protein. A solution of 1,1,3,3-tetramethoxypropane (TMP) was used as standard and data were reported as means ± SD for five replications.

2.10. Statistical methods

A completely randomised design was used for the experiment. The data were analysed with SPSS version 12.0 using one way ANOVA followed by least significant different (LSD) test at the 95% confident level ($p < 0.05$).

3. Results and discussion

3.1. Phenolics and anthocyanins content

The phenolics and anthocyanins are usually extracted from plant materials with an acidified organic solvent, most commonly methanol-1% HCl. This solvent system destroys the cell membranes, simultaneously dissolves the phenolics and anthocyanins and stabilises them.

The content of phenolics and anthocyanins in black soybean seed coat *Mallika* and *Cikuray* variety is given in Table 1. The content of phenolics determined by the Folin–Ciocalteu method was higher in *Cikuray* variety (8.15 ± 0.23) than *Mallika* (6.46 ± 0.11). The differences of phenolic contents indicate that the type of cultivar showed an important influence on the variability of phenolic levels. In support of this observation, Ranilla et al. (2006) reported differences in phenolic content of seed coat and cotyledon of twenty five Brazilian and three Peruvian bean cultivars.

The Folin–Ciocalteu method is simple and can be useful in characterising and standardising botanical samples, for example sorghum (Awika et al., 2004), common beans *Phaseolus vulgaris* L. (Heimler et al., 2005), wheat (Liyana-Pathirana & Shahidi, 2006), soybean (Takahashi et al., 2005), and white and black sesame seed (Shahidi, Liyana-Pathirana, & Wall, 2006). However it suffers from a number of interfering substances (particularly sugars, aromatic amines, sulphur dioxide, ascorbic acid and other enediols and reductones, organic acids, and Fe(II).

Table 1
Phenolics and anthocyanins content of black soybean seed coat

Variety	Phenolics content*	Anthocyanins content**
Mallika	$6.46^{a} \pm 0.11$	$1.36^{c} \pm 0.12$
Cikuray	$8.15^{b} \pm 0.23$	$1.45^{c} \pm 0.13$

Mean values with the same letters are not significantly different at $p < 0.05$.
* Data expressed as g of gallic acid equivalents per 100 g of fresh weight.
** Data expressed as g of anthocyanin equivalents per 100 g of fresh weight.

Anthocyanin pigments undergo reversible structural transformations with a change in pH manifested by strikingly different absorbance spectra). The coloured oxonium form of anthocyanin predominates at pH 1.0 and the colourless hemiketal form at pH 4.5. The pH-differential method is based on this reactions, it measures the absorbance at two different pH values, and relies on the structural transformations of the anthocyanin chromophore as a function of pH (Giusti & Wrolstad, 2001). This method is simple, therefore it had been used by many scientist to determine anthocyanin content in various samples, for example sorghum (Awika et al., 2004), black bean (Salinas-Moreno, Rojas-Herrera, Sosa-Montes, & Pérez-Herrera, 2005), and purple wheat (Hoseinian, Li, & Beta, 2008). In this research, *Cikuray* variety (1.45 ± 0.13) was higher than *Mallika* (1.36 ± 0.12) but not significantly different. Recently, increased attention has focused on the possible health effects of naturally occurring phenolics and anthocyanins. There is much interest in replacing synthetic antioxidants with natural alternatives due to safety concerns (Shahidi et al., 2006). Antioxidant phytochemicals may also play an important role in human health via scavenging reactive oxygen and nitrogen species (Bravo, 1998).

3.2. Individual anthocyanidins

We used HPLC coupled with diode array detector to identify individual anthocyanin of black soybean seed coat *Mallika* variety. The identification of individual anthocyanidins in black soybean seed coat is given in Fig. 1.

Fig. 1 shows the individual anthocyanidins in *Mallika* variety as delphinidin, cyanidin, and pelargonidin detected in chromatograms compared with standard curves.

3.3. DPPH radical scavenging capacity

The antioxidant activity of black soybean seed coat extract *Mallika* and *Cikuray* variety was evaluated by the DPPH radical scavenging capacity. Fig. 2 shows the percent of DPPH radicals scavenging capacity with rutin and BHT as reference. The experimental data of the variety reveal that all of these extracts at various levels are likely to have the effect of scavenging free radicals. From Fig. 1 we observe that a dose–response relationship is found in the DPPH radical scavenging capacity; the activity increased as the concentration increased for each extract. Among the three levels used in the experiment, 150 ppm *Malika* variety was the strongest one with 92.78%, then 150 ppm rutin as standard with 91.94%, and 150 ppm *Cikuray* variety with 91.50%, which is not significantly different ($p < 0.05$) from rutin, otherwise 150 ppm BHT only found to be 77.00%. From the results, percent DPPH scavenging capacity of black soybean extract was higher than the reference, possibly due to the phenolics and anthocyanins content of extract which could act as a hydrogen donor antioxidant. In this experiment, only 50, 100, and 150 ppm anthocyanin equivalents were used to determine the optimum levels of extract because at 200 ppm concentration and higher, a higher absorbance value was read spectrophotometrically caused as by the red colour of the extract that could not turn to a pale yellow colour. Moreover, anthocyanins content in black soybean seed coat has colour interference with DPPH radical which could lead to underestimation of antioxidant activity (Awika et al., 2004). Thus the same levels of anthocyanins in black soybean seed coat do not necessarily correspond to the same DPPH scavenging capacity. DPPH has been widely used to test the ability of compounds or plant extracts to act as free radical scavengers, for example sorghum. (Awika, Rooney, Wu, Prior, & Zevallos, 2003), pea beans (Madhujith et al., 2004), common bean (*Phaseolus vulgaris*) (Rocha-Guzman et al., 2006), and barley (Madhujith & Shahidi, 2006).

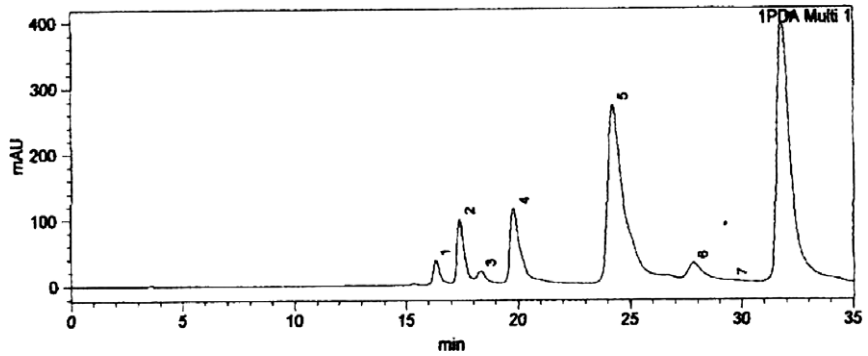

Fig. 1. HPLC chromatogram and detected peak number on diode array detector *Mallika* extract. Peak (2) delphinidin, (4) cyanidin, (6) pelargonidin.

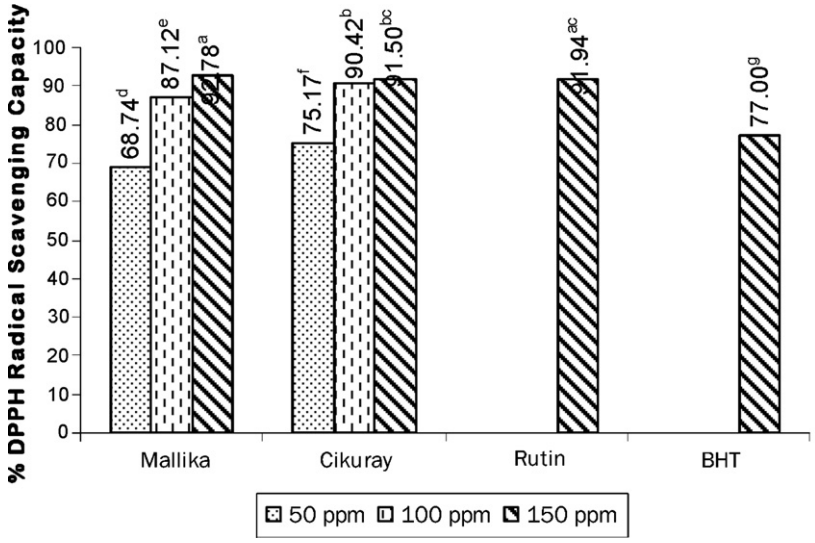

Fig. 2. Percent DPPH radical scavenging capacity of black soybean seed coat extract∗. ∗Mean values with the same letters are not significantly different at $p < 0.05$.

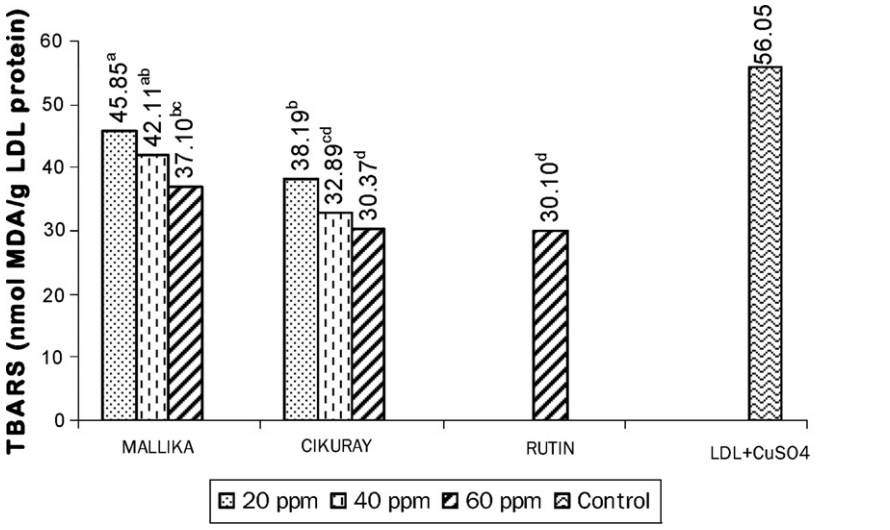

Fig. 3. Inhibition of Cu^{2+} induced human LDL oxidation in vitro by black soybean seed coat extract∗. ∗Mean values with the same letters are not significantly different at $p < 0.05$.

3.4. Inhibition of LDL oxidation in vitro

The low density lipoprotein (LDL) oxidation is one of the most studied free radical-mediated processes occurring in the body, because it is believed to play a crucial role in the formation and progression of early atherosclerotic lesions linked to cardiovascular diseases (Steinberg, 1997). In its native form, LDL does not form atherosclerotic plaques. However oxidised LDL is responsible for

the pathogenesis of atherosclerosis that may lead to the build up of plaque in the arteries. Thus, the consumption of dietary antioxidants is beneficial in preventing cardiovascular diseases, especially atherosclerosis. In this study, the antioxidant protection of LDL was evaluated as the inhibition of TBARS formation.

The antioxidant activity of black soybean seed coat extracts were investigated individually at three different levels of anthocyanins against inhibition of Cu^{2+}-induced oxidation of human LDL in vitro by monitoring TBARS formation. Our results showed that all these extracts at various levels were effective in inhibition of LDL oxidation. From Fig. 3, a dose–response relationship was also found in the inhibition of TBARS formation; the activity increased as the concentration increased for each extract and shown by lower formation of TBARS. Among the three levels used in the experiment, 60 ppm of *Cikuray* variety was the strongest one with 30.37 nmol MDA equivalents/g LDL protein, which is not significantly different ($p < 0.05$) from rutin as reference with 30.10 nmol MDA equivalents/g LDL protein, and then 60 ppm *Mallika* variety with 37.10 nmol MDA equivalents/g LDL protein. From the result, extract could inhibit production of TBARS formation in three given concentration. The protection of LDL by phenolics and anthocyanins in a copper-induced oxidation system could be due to both metal-chelating and radical scavenging capacity. However, the mechanism by which the extracts inhibit LDL oxidation in vitro remains unclear. Dorman, Peltoketo, Hiltunen, and Tikkanen (2003) suggested possible explanations for the protecting effects of phenolics and anthocanins on LDL: (i) scavenging of various radical species in the aqueous phase, (ii) interaction with peroxyl radicals at the LDL surface, (iii) partitioning into the LDL particle and terminating chain-reactions of lipid peroxidation by scavenging lipid radicals, and (iv) regenerating endogenous α-tocopherol back to its active antioxidative form.

4. Conclusions

Black soybean seed coat *Mallika* and *Cikuray* variety were found to have high levels of phenolics and anthocyanins. The antioxidant activity of *Mallika* extract was higher than *Cikuray*. Black soybean seed coat extract significantly scavenged DPPH radical and could inhibit LDL oxidation by reduced formation of TBARS.

References

Aparicio-Fernandez, X., Yousef, G. G., Loarca-Pina, G., de Mejia, E., & Lila, M. A. (2005). Characterization of polyphenolics in the seed coat of black jamapa bean (*Phaseolus* vulgaris L.). *Journal of Agricultural and Food Chemistry, 53*, 4615–4622.

Awika, J. M., Rooney, L. W., Wu, X. L., Prior, R. L., & Zevallos, L. C. (2003). Screening methods to measure antioxidant activity of sorghum (sorghum bicolor) and sorghum products. *Journal of Agricultural and Food Chemistry, 51*, 6657–6662.

Awika, J. M., Rooney, L. W., & Waniska, R. D. (2004). Anthocyanins from black sorghum and their antioxidant properties. *Food Chemistry, 90*, 293–301.

Bravo, L. (1998). Polyphenols: Chemistry, dietary sources, metabolism and nutritional significance. *Nutrition Reviews, 56*, 317–333.

Castro, S. H., Neto, H., & Gomes, M. B. (2006). Platelet-activating factor acetylhydrolase (PAF-AH) activity in patients with type 1 diabetes mellitus. *Brazilian Cardiology, 88*, 156–161.

Choung, M. G., Choi, B. R., An, Y. N., Chu, Y. H., & Cho, Y. S. (2003). Anthocyanin profile of Korean cultivated kidney bean (*Phaseolus* vulgaris L.). *Journal of Agricultural and Food Chemistry, 51*, 7040–7043.

Daaz-Batalla, L., Widholm, J. M., Fahey, G. C., Tostado, E., & Paredes-Lopez, O. (2006). Chemical components with health implications in wild and cultivated Mexican common bean seeds (*Phaseolus* vulgaris L.). *Journal of Agricultural and Food Chemistry, 54*, 2045–2052.

Dorman, H. J. D., Peltoketo, A., Hiltunen, L., & Tikkanen, M. J. (2003). Characterisation of the antioxidant properties of de-odourised aqueous extracts from selected Lamiaceae herbs. *Food Chemistry, 83*, 255–262.

Heimler, D., Vignolini, P., Dini, M. G., & Romani, A. (2005). Rapid tests to assess the antioxidant activity of *Phaseolus vulgaris L. dry beans*. *Journal of Agricultural and Food Chemistry, 53*, 3053–3056.

Hoseinian, F. S., Li, W., & Beta, T. (2008). Measurement of anthocyanins and other phytochemicals in purple wheat. *Food Chemistry, 109*, 916–924.

Giusti, M., & Wrolstad, R. (2001). *Characterization and Measurement of Anthocyanins by UV-Visible Spectroscopy*. John Wiley & Sons, Inc.. All rights reserved.

Inagaki, S., Morimura, S., Shigematsu, T., Kida, K., & Akutagawa, H. (2005). Apoptosis induction by vinegar produced from boiled extract of black soybeans in human monoblastic leukemia U937 cells: Difference in sensitivity to cell toxicity compared to normal lymphocytes. *Food Science and Technology Research, 11*, 311–317.

Katsube, T., Iwamaka, N., Kawano, Y., Yamazaki, Y., Shiwaku, K., & Yamane, Y. (2005a). Antioxidant flavonol glycosides in mulberry (*Morus alba* L.) leaves isolated based on LDL antioxidant activity. *Food Chemistry, 97*, 25–31.

Katsube, T., Watanabe, M., Yamasaki, M., Kitajima, K., Yamane, Y., & Shiwaku, K. (2005b). Dietary antioxidant for prevention of cardiovascular diseases. *Journal of Rural Medicine, 1*, 4–14.

Kim, Y.H., Yun, H.T., Chung, W.K., Park, K.Y. (2004). Investigation of biological effect in black colored soybean. In Proceedings of the 4th international crop science congress. Brisbane, Australia.

Kordali, S., Kotan, R., Mavi, A., Cakir, A., Ala, A., & Yildirim, A. (2005). Determination of the chemical composition and antioxidant activity of the essential oil of *Artemisia dracunculus* and of the antifungal and antibacterial activities of Turkish *Artemisia absinthium, A. dracunculus, Artemisia santonicum, and Artemisia spicigera* essential oils. *Journal of Agricultural and Food Chemistry, 53*, 9452–9458.

Lee, K. G., & Shibamoto, T. (2000). Antioxidant properties of aroma compounds isolated from soybeans and Mung beans. *Journal of Agricultural and Food Chemistry, 48*, 4290–4293.

Liyana-Pathirana, C. M., & Shahidi, F. (2006). Importance of insoluble-bound phenolics to antioxidant properties of wheat. *Journal of Agricultural and Food Chemistry, 54*, 1256–1264.

Madhujith, T., Amarowicz, R., & Shahidi, F. (2004). Phenolic antioxidants in beans and their effects on inhibition of radical-induced DNA damage. *Journal of American Oil Chemists' Society, 81*, 691–696.

Madhujith, T., & Shahidi, F. (2005). Antioxidant potential of pea beans (*Phaseolus* vulgaris L.). *Journal of Food Science, 70*, S85–S90.

Madhujith, T., & Shahidi, F. (2006). Optimization of the extraction of antioxidative constituents of six barley cultivars and their antioxidant properties. *Journal of Agricultural and Food Chemistry, 54*, 8048–8057.

Mary-Astuti. (1996). Sejarah Perkembangan Tempe, dalam Bunga Rampai Tempe Indonesia, Yayasan Tempe Indonesia. Jakarta (in Bahasa Indonesia).

Nakajima, J., Tanaka, I., Seo, S., Yamazaki, M., & Saito, K. (2004). LC/PDA/ESI-MS profiling and radical scavenging activity of anthocyanins in various berries. *Journal of Biomedicine and Biotechnology, 5*, 241–247.

Ranilla, L. G., Genovese, M. I., & Lajolo, F. M. (2006). Polyphenols and antioxidant capacity of seed coat and cotyledon from Brazilian and Peruvian bean cultivars (*Phaseolus vulgaris* L.). *Journal of Agricultural and Food Chemistry, 55*, 90–98.

Rocha-Guzman, N. E., Herzog, A., Gonzalez-Laredo, R. F., Ibarra-Perez, F. J., Zambrano-Galvan, G., & Gallegos-Infante, J. A. (2006). Antioxidant and antimutagenic activity of phenolic compounds in three different colour groups of common bean cultivars (*Phaseolus vulgaris*). *Food Chemistry, 103*, 521–527.

Salinas-Moreno, Y., Rojas-Herrera, L., Sosa-Montes, E., & Pérez-Herrera, P. (2005). Anthocyanin composition in black bean (*Phaseolus vulgaris* L.) varieties grown in México. *Agrociencia, 39*, 385–394.

Shahidi, F., Liyana-Pathirana, C. M., & Wall, D. (2006). Antioxidant activity of white and black sesame seeds and their hull fractions. *Food Chemistry, 99*, 478–483.

Shinomiya, K., Tokunaga, S., Shigemoto, Y., & Kamei, C. (2005). Effects of seed coat extract of black soybeans on radial maze performance in rats. *Clinical and Experimental Pharmacology and Physiology, 32*, 757–760.

Shurtleff, W., & Aoyagi, A. (1979). *The Book of Tempeh*. New York, USA: Harper & Row Publisher Inc.

Singleton, V. L., & Rossi, J. A. (1965). Colorimetry of total phenolics with phosphomolybdic–phosphotungstic acid reagents. *American Journal of Enology and Viticulture, 16*, 144–158.

Steinberg, D. (1997). Low density lipoprotein oxidation and its pathobiological significance. *Journal of Biological Chemistry, 272*, 20963–20966.

Takahashi, R., Ohmori, R., Kiyose, C., Momiyama, Y., Ohsuzu, F., & Kondo, K. (2005). Antioxidant activities of black and yellow soybeans against low density lipoprotein oxidation. *Journal of Agricultural and Food Chemistry, 53*, 4578–4582.

Waterhouse, A. (2002). Folin Ciocalteu micro method for total phenol in wine. Department of viticulture & enology. University of California, Davis.