Food Anal. Methods (2012) 5:1354–1361
DOI 10.1007/s12161-012-9377-7

Analytical Methods for Enzyme and DPPH Radical Scavenging Activities of Natural Pigments from Some Plants

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Received: 6 January 2012 / Accepted: 9 February 2012 / Published online: 4 March 2012

Abstract The enzyme activities in different fractions of *Dioscorea japonica* Thunb. and 1,1-diphenyl-2-picryl hydrazyl radical scavenging activity in 15 natural plant pigments from black rice, purple sweet potato, yellow bitter melon, yellow paprika, red cabbage, yellow gardenia, blue gardenia, Chinese foxglove, mulberry leave, onion peel, grape peel, mulberry, red beet, gromwell, and cactus were determined. The antioxidant activity in the cosmetic composition of mulberry leaves, grape peel, mulberry, and red cabbage pigments was relatively high in comparison with all other studied plants. Enzyme activities in investigated plants were evaluated as superoxide dismutase (SOD), catalase (CAT), and ascorbate peroxidase (APX). The cosmetic composition of mulberry leaf pigment had the highest SOD enzyme activity of 67.1% while onion peel pigment showed the lowest SOD enzyme activity of 15.3%. The activity of CAT and APX from cosmetic composition of natural plant pigments has also been investigated. Both CAT and APX showed higher values in the cactus, mulberry, and red cabbage cosmetic compositions in comparison with other plant pigments. The cosmetic composition in EtOAc extract of *D. japonica* Thunb. had the highest SOD enzyme activity while the BuOH and EtOH extracts were comparatively low. CAT and APX activities showed significantly high values in EtOH and EtOAc extracts. The antioxidant enzyme activities of *D. japonica* Thunb. differ significantly in different plant pigments during their extraction. In conclusion, we showed that the plant pigments and *D. japonica* Thunb. had the potent biological activities. Therefore, these plant resources having anti-aging components could be good materials for development of source of natural cosmetics.

Keywords *Dioscorea japonica* Thunb. · Plant pigment · Cosmetic composition · Antioxidant enzyme · DPPH radical scavenging activity

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Introduction

Natural pigments have been used from the beginning of human history. Difficulties in productivity, stability and storage of natural pigments have been developed the synthetic ones. However, in the recent years, with improved quality of life and the growing interest of nature-friendly environment for living, the desire to produce natural materials is getting stronger. In response to this trend in recent cosmetic resources, textile dyeing, and functional healthy supplements for health and safety, the use of natural pigments is gradually expanding. The most promising sources of natural anti-tumor drugs are plants. Some natural plant pigments such as β-carotene, lycopene, and others have high antioxidant activity (Chang et al. 2011). Pigments, which are less widespread in the plant kingdom or of less importance for plant pigmentation, are of commercial significance, suggesting that natural plant pigments have biologically active properties (Bovy 2005). The research on the

use of natural pigments is carried out actively, but the available kinds of natural pigments in their functionality, safety, and reliability were not supported by empirical research, therefore systematic variety research in these fields is needed. Features of natural pigments compared with synthetic colors have been known to have low impact on the environment, significantly low or almost harmless for human toxicity and antibacterial, anticancer, and anti-inflammatory efficacy (Kim and Cho 2008). In addition, discoloration or fading color becomes a natural, graceful, even delicate color, producing stable richness as well as various colors depending on the nature of the material (Park et al. 2010).

Yam has been classified as one of the important staples in the diets of many tropical countries because of the carbohydrate composition (Chou et al. 2006). In general, yam is a major dietary source in certain African countries, and some yams are also used as medicines in oriental countries to prevent diarrhea and diabetes (Hsu et al. 2002, 2003), thus yams are considered to be helpful for human health. *Dioscorea japonica* Thunb., especially, of Korean native yam cultivar is considerably consumed in Korea due to its nutritional values and certain specific functionalities. Bhandari and Kawabata (2004) found Nepal wild yam tubers to have significant antioxidant activities, as evaluated by DPPH (1,1-diphenyl-2-picryl hydrazyl) free radical scavenging, ferrous ion chelating, reducing power, and total antioxidant activity. The consumption of fresh yam tubers for health benefit was thus recommended. Diosgenin, a steroid sapogenin of yam, has been utilized to manufacture steroid hormones such as cortisone, estrogen, and progesterone (Araghiniknam et al. 1996). Diosgenin is transformed in the human intestine into serum dehydroepiandrosterone which is associated with reduced lipid peroxidation, lowered serum triglycerides and LDL, and elevated HDL (Araghiniknam et al. 1996; McAnuff et al. 2002). Recent research has also found that dioscorin might be beneficial in controlling high blood pressure and for scavenging DPPH and hydroxyl free radicals (Hsu et al. 2002; Iwu et al. 1999). In addition, yam tuber mucilage was found to exhibit antioxidative ability (Hou et al. 2002). Freeze-drying of various yam species (Tai-Nung no. 2; Ta-Shan; Ming-Chien) was found to yield higher antioxidative ability than hot-air drying and drum-drying (Hsu et al. 2003). Many researchers have shown that yam extracts showed antioxidative activity (Chan et al. 2004; Farombi et al. 2000). Farombi et al. (2000) demonstrated that brown yam flour contained natural antioxidants and might mitigate damage and diseases caused by oxidative components. Therefore, in this experiment, *D. japonica* Thunb. was used for the development of the possibility of anti-aging cosmetic products due to these antioxidant characteristics. In this study, the application of natural pigment materials which are widespread in plants was studied: purple sweet potato, red cabbage, grape peel, mulberry, black rice, and cactus as anthocyanin pigments; red beet, as betalain pigment; onion peel, as a kind of quercetin pigment with yellow color; yellow bitter melon and yellow paprika, as carotenoid pigment system; and mulberry leaf, as a major component of the green pigment chlorophyll. In general, paprika is included as the pigment components such as a capsanthine, β-cryptoxanthine, and zeaxanthine ingredients; and yellow gardenia is included as the crocin pigment component belonging to the carotenoid (Yoon and Kim 1999). The pigment of gromwell is known to be the shikonin, acetylshikonin, isobutyrylshikonin, and prpionylshikonin components induced by naphthoquinone (Ju et al. 2010). Bluish and reddish purple-colored anthocyanin pigments, as functional materials with excellent antioxidant availability, are known to be of superior efficacy in anticancer, anti-aging, and anti-inflammatory, and betalain pigments extracted from amaranth (Amaranthaceae) plants have also been reported to have a powerful antioxidant activity (Cai et al. 2003). The extraction procedure is important. The effect of commonly used techniques and solvents in the antioxidant activities of pink-flesh guava fruit were studied (Musa et al. 2011; Zhao et al. 2012). Natural pigments used in this study were also suspected to have relatively high antioxidant and antibacterial effects and were selected because of relatively easy possibility to obtain them in large quantities for actual application of the product.

Materials and Methods

Experimental Materials

In this experiment, 15 kinds of plant materials (black rice, purple sweet potato, yellow bitter melon, yellow paprika, red cabbage, yellow gardenia, blue gardenia, Chinese foxglove, mulberry leave, onion peel, grape peel, mulberry, red beet, gromwell, and cactus) of natural plant pigments and wild yam (*D. japonica* Thunb.) were used. These plants were chosen because of the possibility to obtain natural pigments and various physiological functionalities. Liquid nitrogen was used for prevention of oxidation of the polyphenols. Each sample was freeze-dried and then ground. Each pigment powder was stored at −20 °C for further experiments.

DPPH Assay

One hundred microliters of various concentrations (100, 250, 500, 1,000, and 2,500 mgL^{-1}) of pigment compositions of investigated plants were added to 900 μL of 100% methanol containing 100 μM DPPH, and the reaction mixture was shaken vigorously. After storage at room temperature for 30 min in darkness, the absorbance of DPPH was determined by spectrophotometer at 517 nm. The DPPH radical-scavenging activity was calculated according to the

following equation: scavenging effect of DPPH radical (%)=[(*A*−*B*)/*A*]×100, where *A* is the absorbance at 517 nm without pigment compositions and *B* is the change in absorbance at 517 nm with pigment compositions incubation (Brand-Williams et al. 1995).

Enzyme Assays

Protein Extraction

Sample solution of 100 μL was dissolved in 900 μL of 100 mM potassium phosphate buffer (pH 7.5) containing 2 mM ethylenediaminetetraacetic acid, 1% (*w/v*) polyvinypyrrolidone, and 1 mM phenylmethylsulfonyl fluoride. The suspension was centrifuged at 15,000×*g* for 20 min at 4 °C. Protein concentration was determined by the method of Bradford (1976) with bovine serum albumin as standard.

SOD Activity Assay

The superoxide dismutase (SOD) activity was measured using SOD assay Kit-WST purchased from Sigma-Aldrich. This assay is based on the colorimetric assay for the measurement of total antioxidant capacity of crude aqueous fractions. The 60 μL of sample solution (sample and blank2) or double-distilled water (blank1 and blank3) was mixed with 600 μL of WST working solution. For Blank2 and Blank3, 60 μL of dilution buffer was added. Then, 60 μL of enzyme working solution was added to each sample and blank1. The plate was incubated at 37 °C for 20 min, and the OD was determined at 450 nm using a spectrophotometer. SOD activity (inhibition rate percent) was calculated using the following equation: SOD activity=$\{[(A_{\text{blank 1}}-A_{\text{blank3}})-(A_{\text{sample}}-A_{\text{blank 2}})]/(A_{\text{blank 1}}-A_{\text{blank 3}})\}\times 100$.

CAT Activity Assay

Catalase (CAT) activity was assayed by the method of Mishra et al. (1993). The reaction mixture contained 50 mM potassium phosphate buffer (pH 7.0), 11 mM H_2O_2, and the crude enzyme extract. The reaction was initiated by addition of H_2O_2 to the mixture, and enzyme activity was determined by monitoring the decline in absorbance at 240 nm (ε= 36 M^{-1} cm^{-1}), because of H_2O_2 consumption.

APX Activity Assay

Ascorbate peroxidase (APX) activity was determined by monitoring the decline of absorbance at 290 nm as ascorbate (ε= 2.8 mM^{-1} cm^{-1}) was oxidized, by the method of Chen and Asada (1989). The reaction mixture contained 100 mM potassium phosphate buffer (pH 7.5), 0.5 mM ascorbate, and 0.2 mM H_2O_2. POX activity was determined specifically with guaiacol at 470 nm (ε=26.6 M^{-1} cm^{-1}), following the method of Egley et al. (1983). The reaction mixture contained 40 mM potassium phosphate buffer (pH 6.9), 1.5 mM guaiacol, and 6.5 mM H_2O_2 in 1 ml with crude enzyme extract.

Table 1 DPPH radical scavenging activities of cosmetic composition having the natural plant pigments and *D. japonica* extract

Cosmetic composition	DPPH radical scavenging activity, % of control				
	Concentration (mg/L)				
	100	250	500	1,000	2,500
Black rice	9.9±0.42c	11.3±0.42e	15.2±0.59g	20.4±0.94fg	34.2±1.29f
Purple sweet potato	9.6±0.33cde	9.3±0.26i	12.3±0.61ij	15.7±0.11jk	27.7±0.31g
Mature bitter melon	9.4±0.57cde	12.1±0.15d	17.9±0.52e	25.8±0.77e	48.7±1.05d
Paprika	9.5±0.26cde	10.7±0.24f	13.4±0.35h	16.5±0.50jk	37.4±0.77e
Red cabbage	11.7±0.24a	15.2±0.26b	21.7±0.48c	34.1±0.74d	67.1±1.57b
Yellow gardenia	9.2±0.26def	10.2±0.37fgh	11.7±0.15jk	15.4±0.48k	24.1±0.83h
Blue gardenia	9.1±0.26ef	9.9±0.35h	11.0±0.35k	15.1±1.40k	20.8±0.59i
Chinese foxglove	9.6±0.31cde	10.1±0.26gh	12.3±0.31ij	17.2±1.88ij	25.4±0.85h
Mulberry leaves	10.6±0.28b	18.8±0.50a	27.6±0.70a	46.9±1.51a	75.0±0.03a
Onion peel	9.9±0.20c	12.7±0.09c	16.1±0.37f	21.5±0.59f	37.5±0.77e
Grape peel	11.6±0.44a	15.5±0.26b	23.7±0.81b	37.8±1.27c	63.6±1.40c
Mulberry	8.8±0.22fg	9.3±0.24i	19.9±0.28d	41.1±0.28b	67.8±0.46b
Red beet	9.8±0.15c	11.3±0.46e	13.6±0.90h	18.9±0.46gh	34.9±0.96f
Gromwell	9.7±0.20cd	10.5±0.37fg	13.2±0.17hi	16.4±0.42jk	27.8±0.48g
Cactus	8.3±0.07g	9.0±0.22i	12.8±0.17hi	18.7±0.20hi	28.1±0.52g

Data represent the mean values± SE of three independent experiments. Means with the same letter in a column are not significantly different at $p<0.05$ level by Duncan's multiple range test

Table 2 APX activities of cosmetic composition having the natural plant pigments and various partition extracts in *Dioscorea japonica*

Cosmetic composition	APX activity (μmol ascorbate oxidized/min/mg protein)			
	Pigment composition	Pigment composition+EtOH extract	Pigment composition+BuOH extract	Pigment composition+EtOAc extract
Black rice	554.7±45.79g	845.2±51.96f	2,635.9±104.18bcd	3,233.0±81.82g
Purple sweet potato	875.3±39.85f	839.6±26.41f	1,560.6±132.17fg	2,001.2±48.73i
Mature bitter melon	863.6±65.53f	782.1±61.30f	2,654.1±146.23bcd	4,635.7±94.75d
Paprika	1,191.6±68.23de	1,232.9±107.56e	2,468.9±87.73cde	5,062.3±127.05c
Red cabbage	1,715.7±143.34c	2,433.1±55.36b	1,938.0±44.46defg	4,375.3±189.09e
Yellow gardenia	458.0±22.71gh	945.4±79.35f	1,125.2±145.38gh	1,451.6±49.28j
Blue gardenia	379.7±78.35h	508.4±50.78g	399.3±31.58h	901.7±89.34k
Chinese foxglove	1,146.0±68.02de	1,299.4±37.36e	2,590.8±42.75bcd	5,161.2±107.29c
Mulberry leaves	1,287.3±62.87d	279.2±52.14h	1,541.8±49.24fg	2,052.3±106.94i
Onion peel	714.6±86.08f	2,260.5±206.44b	2,824.4±170.20bc	3,433.0±90.50g
Grape peel	1,662.8±125.33c	1,598.5±129.32d	5,266.4±122.70a	5,839.5±114.68b
Mulberry	2,183.5±105.66b	1,255.8±40.40e	2,209.4±85.52cdef	3,413.0±137.25g
Red beet	1,052.3±75.29e	2,344.4±157.37b	3,381.0±179.01b	3,654.6±181.50f
Gromwell	1,290.9±146.20d	1,992.2±57.38c	1,732.4±69.73efg	2,565.1±131.51h
Cactus	2,633.3±143.48a	2,738.0±252.38a	4,819.9±136.74a	6,262.2±145.62a

Data represent the mean values±SE of three independent experiments. Means with the same letter in a column are not significantly different at $p<0.05$ level by Duncan's multiple range test

Data Analysis

The statistical analysis was performed using the procedures of the Statistical Analysis System. ANOVA procedure followed by Duncan test was used to determine the significant difference ($p<0.05$) between treatment means.

Results and Discussion

DPPH Radical Scavenging Activities

The measurement results of free radical scavenging activity are shown in Table 1 of the investigated samples. The investigation of the antioxidant activity of natural substances is based on the measuring of the electron donor capacity of DPPH with the ability to inhibit the oxidation by donating electrons in free radicals causing this lipid peroxidation. Active oxygen caused by in vivo metabolism removed by the body's antioxidant system, but excessive free radicals induced stress, causing the lipid peroxidation by combining with unsaturated fatty acids in the cell membrane, and brought intracellular structural and functional damage. Looking at the results, the antioxidant capacity of the cosmetic composition, containing natural pigments, showed relatively high scavenging activity in red cabbage, paprika, and grape peel and especially in onion peel and mulberry pigment but in a relatively low concentration. Overall, the

Fig. 1 The cosmetic composition having natural plant pigments and extracts of *D. japonica* thumb. *A*: Red cabbage, *B*: gromwell, *C*: mulberry, *D*: cactus, *E*: purple sweet potato, *F*: black rice, *G*: grape peel, *H*: red beet, *I*: blue gardenia, *J*: yellow gardenia, *K*: Chinese foxglove, *L*: paprika, *M*: mulberry leaves, *N*: mature bitter melon, *O*: onion peel

DPPH radical scavenging activity in most of the natural pigments mixed compositions showed that the increase was proportional to the concentration.

Cells are oxidized and damaged by the free radical, depending on the growth of cells. It has been reported that phenolic compounds have antioxidant capacity to inhibit the oxidation by donating electrons to the free radical due to strong reduction (Sanchez et al. 2007; Saija et al. 1998). The content of phenolic compounds increases the radical scavenging activity, which also is reported to be increased (Boo et al. 2011; Oki et al. 2002). The natural pigments are also a sort of phenolic compound, therefore the antioxidant capacity is assumed to be excellent. Kim et al. (2010) reported that the DPPH radical scavenging activity of anthocyanin pigment separated from soybean showed a similar degree to the high antioxidant capacity of α-tocopherol, especially soybean cultivar containing a lot of anthocyanin pigment, which is reported to be high in general DPPH radical scavenging activity than other cultivars. The physicochemical and antioxidative properties of red and black rice varieties from Thailand, China, and Sri Lanka can be explained by the deposits of anthocyanins in different layers (Sompong et al. 2011). Anthocyanins isolated from the purple-fleshed sweet potato attenuate the proliferation of hepatic stellate cells. The pigments from the purple sweet potato were more stable than the pigments of strawberries (Choi et al. 2011). Most of the bioactive compounds in mulberry leaves were extracted as flavonoid rutin (Kim et al. 2007). Chen et al. (2008) revealed that all yam species displayed a different degree of DPPH scavenging effects at different pHs. The results indicated better DPPH scavenging effect at acidic environments for all yam species. Many researchers have shown specific functionalities of yams, and some yams behaved differently in their antioxidative ability.

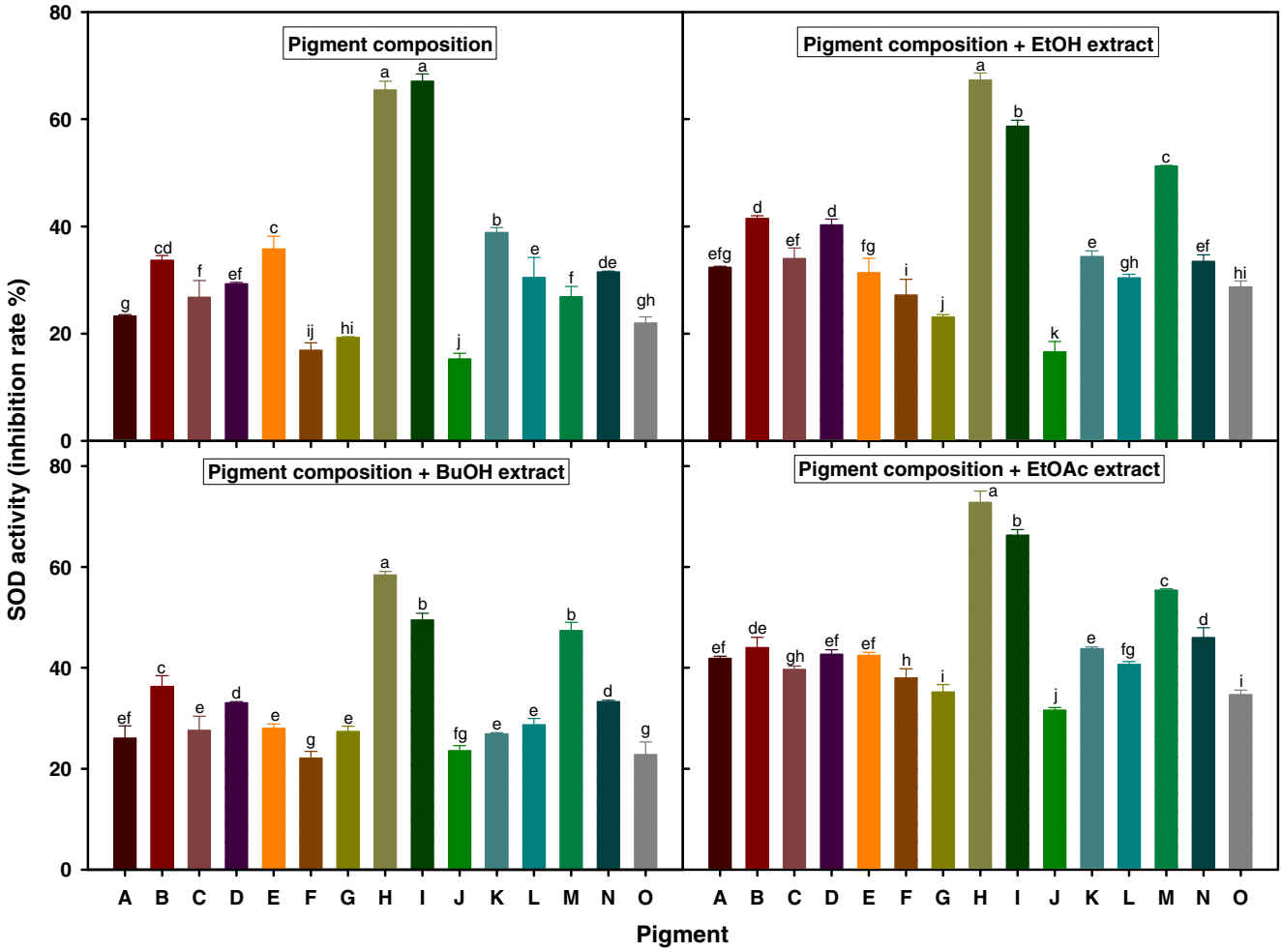

Fig. 2 SOD activities of cosmetic compositions having the natural plant pigments and various partition extracts in *D. japonica*. Means with the same *letter* in a *column* are not significantly different at $p < 0.05$ level by Duncan's multiple range test. The *bars* represent the standard error. *A*: black rice, *B*: purple sweet potato, *C*: mature bitter melon, *D*: paprika, *E*: red cabbage, *F*: yellow gardenia, *G*: blue gardenia, *H*: Chinese foxglove, *I*: mulberry leaves, *J*: onion peel, *K*: grape peel, *L*: mulberry, *M*: red beet, *N*: gromwell, *O*: cactus

Yam species with good antioxidative ability should gain more attention from farmers and consumers in terms of profitability and food nutrition. The high antioxidant activity of yams enhanced the potential interest in these under-exploited wild tubers for improving the efficacy of different products as nutraceutical and pharmacological agents. The consumption of these wild yams may play a role in preventing human diseases in which free radicals are involved, such as cancer, cardiovascular disease, and aging. However, further investigations on individual phenolic compounds, their in vivo antioxidant activity, and the different antioxidant mechanisms are warranted (Bhandari and Kawabata 2004).

In our study, the DPPH radical scavenging activity appears to be high and concentration-dependent. Our results are similar to others, showing that, if the content of phenolic compounds is high, then the electron donor capacity is also high (Matkowski and Piotrowska 2006; Rhim and Choi 2010).

Antioxidant Enzyme Activities

The comparative results of the antioxidant enzyme activity in the 15 natural plant pigments are shown in Table 2 and Figs. 1–3. The cosmetic composition of mulberry leaves pigments had the highest SOD enzyme activity of 67.1% while onion peel pigment showed the lowest SOD enzyme activity of 15.3%. The activity of CAT and APX from cosmetic composition of natural plant pigments has also been investigated. Both CAT and APX showed higher values in the cactus, mulberry, and red cabbage pigment cosmetic compositions in comparison with other plant pigments. The cosmetic composition in EtOAc extract of *D. japonica* Thunb. had the highest SOD enzyme activity while the BuOH and EtOH extracts were comparatively low. CAT and APX activities showed significantly high values in EtOH and EtOAc extracts. The antioxidant enzyme activities of *D. japonica* Thunb. were significantly different in different plant pigments extracts. Our results of solvent

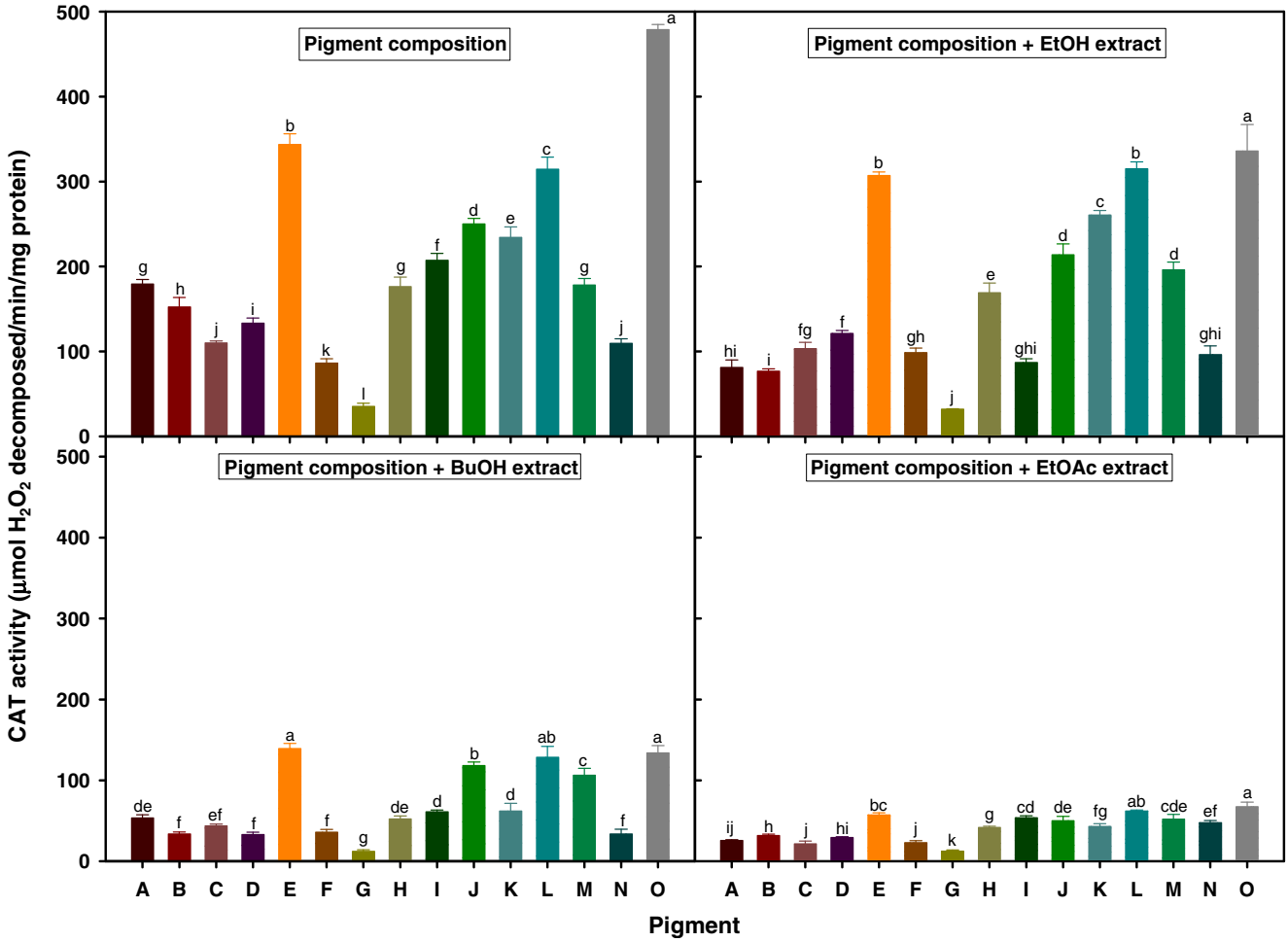

Fig. 3 CAT activities of cosmetic compositions having the natural plant pigments and various partition extracts in *D. japonica*. Means with the same *letter* in a *column* are not significantly different at $p < 0.05$ level by Duncan's multiple range test. The *bars* represent the standard error. *A*: black rice, *B*: purple sweet potato, *C*: mature bitter melon, *D*: paprika, *E*: red cabbage, *F*: yellow gardenia, *G*: blue gardenia, *H*: Chinese foxglove, *I*: mulberry leaves, *J*: onion peel, *K*: grape peel, *L*: mulberry, *M*: red beet, *N*: gromwell, *O*: cactus

extractions can be compared with the data of Musa et al. (2011) where, for the extraction of the bioactive compounds, methanol, ethanol, and acetone were used at three different concentrations (50%, 70%, and 100%) and with 100% distilled water, and the antioxidant activity of the fruits was evaluated using DPPH. Enhanced extraction yields were obtained from solvent containing higher water concentrations, and 50% acetone is a recommended solvent for extracting antioxidants compounds from pink-flesh guava fruit. Zhao et al. (2012) used acetonitrile for extraction of dyes in chili powder and paste compounds and were purified using freezing lipid filtration. Our results can be discussed with another report (Singh et al. 2009), where the five extracts/fractions of red onion peel were studied for their total content of free radical scavenging activity, assayed by DPPH radical in the terms of anti-radical power, and the value for the ethyl acetate (EA) fraction was about 75.3%. EA fraction had markedly higher antioxidant capacity than butylated hydroxytoluene in preventive or scavenging capacities against $FeCl_3$-induced lipid peroxidation, hydroxyl (site-specific and non-site-specific), superoxide anion, and nitric oxide radicals. The large amount of polyphenols contained in EA fraction may cause its strong antioxidant and antimutagenic properties. This information shows that EA fraction of red onion peel can be used as natural antioxidant in nutraceutical preparations.

The SOD is one of the enzymes, in vivo, to catalyze the reaction that converts the harmful reduced oxygen formed in cell due to rancidity into hydrogen peroxide; is generated in most aerobic or anaerobic biological organisms; is switched to water and oxygen by the CAT and APX, and loses then its toxicity. Typically, the APX plays the most important scavenger role in the cytoplasm and chloroplasts of plants, and ascorbic acid is used as a reduction substrate (Wheeler et al. 1998). The CAT is also an antioxidant enzyme that protects cells by dispatching of in vivo harmful oxygen and is a typical enzyme that acts to decompose and scavenge the H_2O_2 together with APX. The antioxidant enzymes, indicating a high activity to remove harmful free radicals, have the effect of prevention and inhibition of various diseases and aging, and in the natural plant pigments, we can also expect to see these benefits for the next variety of natural foods and cosmetics where the need to apply pigments may also be required. Therefore, the results of this experiment are believed to be meaningful. That is, with this study, in a variety of natural plant pigments, we can expect to take advantage of their higher value as cosmetics and natural dyeing materials, as they showed higher antioxidant enzyme activity. Therefore, in the future, developing the natural cosmetics, healthy functional food, and natural antioxidants can be possible.

Acknowledgments This research was supported by Bio-industry Technology Development Program of IPET, Ministry for Food, Agriculture, Forestry, and Fisheries, Republic of Korea.

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