Industrial Crops and Products 40 (2012) 129–135

Contents lists available at SciVerse ScienceDirect

Industrial Crops and Products

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

Extraction and characterization of some natural plant pigments

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ARTICLE INFO

Article history:
Received 6 January 2012
Received in revised form 24 February 2012
Accepted 27 February 2012

Keywords:
Natural plant pigments
Antioxidant activity
Phenolics
Flavonoids
Antimicrobial activity

ABSTRACT

Antioxidant activities, total polyphenols and flavonoids, and antimicrobial effects in some plant pigments were determined in order to use these natural materials for cosmetics. The DPPH (1,1-diphenyl-2-picryl hydrazyl radical scavenging activity, % of control at maximum plants extract concentrations of 2500 mg/L) in the thirteen natural plant pigments (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 and red beet) ranged from 88.9% for red cabbage to 18.0% for blue gardenia. The highest total polyphenol content (404.2 μg/ml) was measured in the onion peel pigment, and the lowest was in Chinese foxglove pigment (11.4 μg/ml). The red cabbage had the highest total flavonoid amount which was 95.5 μg/ml. The antimicrobial activities of the natural plant pigments were evaluated using the agar diffusion method. Most of the natural pigments for *Bacillus subtilis*, *Micrococcus luteus*, *Escherichia coli*, and *Vibrio parahaemolyticus* showed the clear zone formation of growth inhibition. Purple sweet potato, mulberry, mulberry leaf, grape peel, and blue gardenia showed high antimicrobial activities. These findings suggest that the pigments derived from natural plants had high biological activities, and exhibited different properties depending on each kind of pigments. Therefore these plant resources, having active functional components, can be used as excellent materials for natural cosmetics and food supplements.

1. Introduction

Currently pigments of various kinds and forms have been used as additives or supplements in the food industry, cosmetics, pharmaceuticals, livestock feed and other applications (Boo et al., 2011; Katsube et al., 2003; Lazze et al., 2004). However, because of the problems of the synthetic pigments that cause toxicity and carcinogenicity in the human body, the use of them is gradually has decreased. Therefore interest in natural pigments, that can replace synthetic ones, which caused many side effects, is increasing (Puupponen-Pimiä et al., 2005; Wang and Mazza, 2002). Recently in response to this trend, tend to use natural pigments as adding natural materials in the natural dyeing, healthy functional foods, cosmetic products for human health and safety have been gradually expanded (Bener et al., 2010; Jensen et al., 2011; Venkatasubramanian et al., 2011). Kim et al. (2011) investigated eight different varieties of Korean sweet potatoes in order to develop new healthy foods. In general terms, the colors are organic or inorganic compounds that can absorb the wavelength range between 350 and 750 nm of visible light (Humphrey, 2004; Singh et al., 2005). The color for the consumer is an important factor for the selection of the final product, which is important for the pigments in current food industry (Boo et al., 2012; Wrolstad, 2004). Plant pigments include a variety of different kinds of components, including anthocyanins, carotenoids, betalains and chlorophylls (Gandia-Herrero et al., 2010; Jensen et al., 2011). Plant pigments can be mainly classified into the fat-soluble pigments that exist in plastid of plant protoplasm (protoplasts) and water-soluble ones which are dissolved in the cell sap (Humphrey, 2004; Kong et al., 2003; Wrolstad, 2004; Zhoh et al., 2010). The fat-soluble pigments are chlorophylls and carotenoids, and water-soluble pigments include anthoxanthins (common flavonoids) and anthocyanins. Anthocyanins can appear from red to blue colors, according to pH. They occur in all tissues of higher plants providing color in leaves, plant stem, roots, flowers, and fruits (Burdulis et al., 2009; Jensen et al., 2011). Anthocyanins are involved in a wide range of biological activities (Kong et al., 2003) that affect positively the health properties. Anthocyanins decrease the risk of cancer (Lazze et al., 2004; Martin et al., 2003; Katsube et al., 2003; Kim et al., 2012), reduce inflammatory insult (Yuodim et al.,

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doi:10.1016/j.indcrop.2012.02.042

2002) and modulate immune response (Wang and Mazza, 2002). Carotenoids are red, orange, or yellow tetraterpenoids. The most familiar carotenoids are carotene (orange pigment), lutein (yellow pigment), and lycopene (red pigment). Carotenoids act as antioxidants and promote healthy eyesight in humans. Chlorophyll is the primary pigment that gives green color in plants. Betalains are red or yellow pigments and like anthocyanins they are water-soluble, but unlike anthocyanins they are indole-derived compounds synthesized from tyrosine. Betalains are responsible for the deep red color of beets, and are used commercially as food-coloring agents. Puupponen-Pimiä et al. (2005) reported that antimicrobial berry compounds, especially dietary flavonoids, may have important applications in the future as natural antimicrobial agents for the food industry as well as for the field of medicine. Other reports confirm the properties of pigments (Ferdes et al., 2009; Park and Lee, 2011; Kostic et al., 2010). Therefore in this study in plant natural pigments that are suitable for this experiment, and can be used as natural pigments, the polyphenol and flavonoid contents, their antioxidant activities and antimicrobial activities for each of the pigment were investigated. A comparative analysis of the polyphenol content and the antimicrobial activities of each color category will provide in the future the basis data for the potential possibility of developing functional health foods and natural antimicrobial materials.

2. Materials and methods

2.1. Experimental materials

As plant materials in this study were used thirteen kinds (black rice (*Oryza sativa* L.), purple sweet potato (*Ipomoea batatas* L.), mature bitter melon (*Momordica charantia* L.), yellow paprika (*Capsicum annuum* L.), red cabbage (*Brassica oleracea* L.), yellow gardenia (*Gardenia jasminoides* Ellis), blue gardenia (*Gardenia jasminoides* Ellis); Chinese foxglove (*Rehmannia glutinosa* Liboschitz); mulberry leaves (*Morus alba* L.); onion peel (*Allium cepa* L.), grape peel (*Vitis vinifera* L.); mulberry (*Morus alba* L.); and red beet (*Beta vulgaris* L.)) of natural plant pigments. Liquid nitrogen was used for prevention of oxidation of the polyphenols and flavonoids. Each sample was freeze-dried and then grinded. Each pigment powder was stored at −20 °C for further experiments. Extracts of all the samples were dissolved in distilled water only without any organic solvent for safety to apply to the health food and cosmetic products. These plants were chosen because of the possibility to obtain natural pigments and various physiological functionalities.

2.2. Determination of total phenolic content

The concentration of total phenolics was measured using the Folin–Ciocalteu assay (Singleton et al., 1999). Briefly, 3 mL of Nanopure water, 1.0 mL of sample, and 1.0 mL of Folin–Ciocalteu reagent were added to a 25 mL volumetric flask. The contents were mixed and allowed to stand for 5 min at room temperature. Then 1.0 mL of a sodium carbonate saturated solution was added, and followed by the addition of Nanopure water filled to volume. Solutions were mixed and allowed to stand at room temperature for 2 h. Sample aliquots were filtered through a Whatman 0.45 m poly (tetrafluoroethylene) filter prior to the determination of total phenolics concentration using a UV spectrophotometer monitoring 640 nm. Total phenolics content was standardized against ferulic acid and expressed as μg/ml of ferulic acid equivalents (FAE). The linearity range for this assay was determined as 0.5–5.0 mg/L FAE (R^2 = 0.9990), giving an absorbance range of 0.050–0.555 AU.

2.3. Determination of total flavonoid content

The total flavonoid content was determined according to Zhishen et al. (1999). In brief, about 0.1 g of the dried and ground each sample was extracted with 75% methanol for overnight at room temperature. The sample was mixed with a reagent containing aluminum chloride and sodium nitrite, and a pink-colored flavonoid-aluminum complex was formed in alkaline medium. A solution corresponding to 30 μl of sodium nitrite (10%), 60 μl of aluminum chloride hexahydrate (20%), 200 μl of NaOH (1 M) and 400 μl of water was added to 100 μl of each sample. The absorbance readings at 510 nm were started 5 min after the addition of the sample, and were performed every 20 s for 1 min. A reagent blank containing methanol instead of sample was used. The total flavonoid content was determined using a standard curve of naringin.

2.4. DPPH assay

100 μl of various concentrations (100, 250, 500, 1000 and 2500 mg/L) of pigment compositions of the 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 on DPPH radical $(\%) = [(A - B)/A] \times 100$, 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).

2.5. Antimicrobial screening test

2.5.1. Strains and media

For the purpose of antimicrobial evaluation, 2 g of positive bacteria, and 4 g of negative bacteria were employed. These microorganisms were purchased from the Korean Collection for Type Culture (KCTC, Daejeon, Korea) and cultured in nutrient agar.

2.5.2. Agar diffusion method

The antimicrobial activities of the natural plant pigments were evaluated using the agar diffusion method. Inocula of approximately 10^7 CFU were inoculated onto the surface of pre-dried agar. Sterile 8-mm filter paper discs were placed on the plates and impregnated with 30 μl of sample extract. After allowing 1 h at room temperature for the extracts to facilitate diffusion across the surface, the plates were incubated at 37 °C for 24 h for the bacteria. However, *Salmonella typhymurium* was incubated at 30 °C for 24 h. The antimicrobial activity was measured as the size of the clear zone of growth inhibition. The kanamycin was used as the control.

2.6. 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.

3. Results and discussion

3.1. Total phenolics and flavonoids contents

Polyphenol substances that are widely present in plants are known to play an important role for antioxidant effects and defense action in the plant or the human body, and phenolic compounds are

Fig. 1. Powders of natural plant pigments used in this experiment.

generally have different physico-chemical properties and physiological functions depending on their structure (Bener et al., 2010; Borchardt et al., 2009; Chon et al., 2009; Piluzza and Bullitta, 2011). These phenolic substances give a special color in plants, and act as substrates in oxidation–reduction reactions. The ability of phenolics to protect the plant itself is important in preventing against microbial attack. Phenolic compounds are part of the products of secondary metabolism distributed widely in the plant kingdom, having carious structures and molecular weights. Phenolic hydroxyl radicals couple with giant molecules such as protein, and have functions of physiological activation such as antioxidant effect. In this study, in the selected plant natural pigments that are suitable for this purpose (Table 1, Fig. 1) the contents of total polyphenol and flavonoid compounds were determined (Fig. 2). Looking at the results of the total phenolic compounds measured in 13 species of selected plant natural pigments, the polyphenol content was the highest in onion peel, and showed relatively high levels also in red cabbage and mulberry. The flavonoid content was the highest in red cabbage, and followed by yellow gardenia, mulberry leaves, and mulberry. Our results about the phenolic and flavonoid contents can be compared with others (Jensen et al., 2011), who showed that the stability of the Davidson's plum (*Davidsonia pruriens*, F. Muell.), extract towards heat treatment at 95 °C was higher than that of commercial mulberry colorant, but inferior to colorants derived from red cabbage and purple sweet potato. An addition of a variety of phenolic acids significantly increased color intensity indicating the formation of copigmentation complexes. Commercial chlorogenic acid as well as extract from a native Australian herb rich in chlorogenic acid, Tasmania pepper leaf (*Tasmannia lanceolata*, R. Br.), was both tested in model soft drink solutions subjected to light irradiation and heat treatment. The

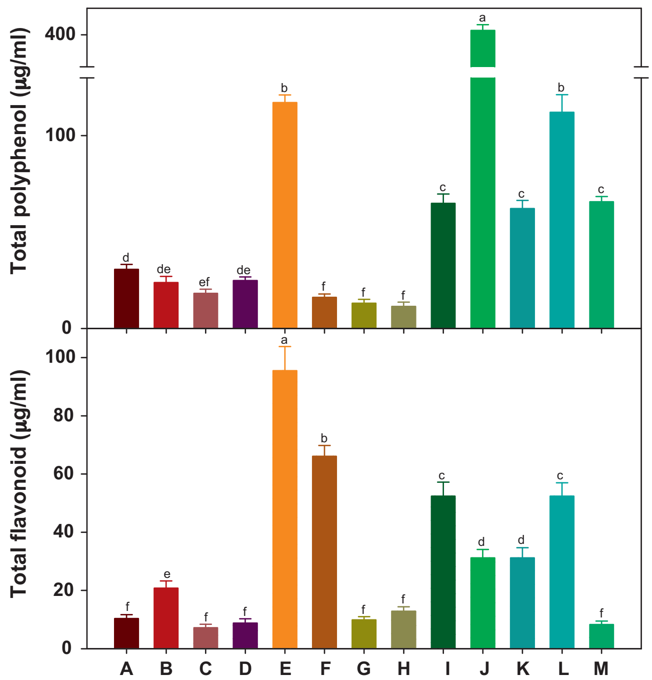

Fig. 2. Total polyphenol and flavonoid contents of natural plant pigments. *Data represent the mean values ± SE of three independent experiments. Means with the same letters in column are not significantly different at $p < 0.05$ level by Duncan's multiple range test. The bars represent the standard 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 leave, J: onion peel, K: grape peel, L: mulberry, and M: red beet.

Table 1
Selected natural plant pigments.

Material source	Class of coloring matter
Red and purple group	
Purple sweet potato	Anthocyanins
Red beet	Betalines
Red cabbage	Anthocyanins
grape peel	Anthocyanins
Yellow group	
Mature bitter melon	Carotenoids (including xanthophylls)
Paprika	Carotenoids (including xanthophylls)
Yellow gardenia	Carotenoids (including xanthophylls)
Chinese foxglove	Carotenoids (including xanthophylls)
onion peel	Carotenoids (including xanthophylls)
Black group	
Black rice	Anthocyanins
Mulberry	Anthocyanins
Blue group	
Blue gardenia	Iridoids, flavonoids
Green group	
Mulberry leave	Porphyrins (chlorophylls)

addition of the copigment resulted in a lasting increase in color intensity. Davidson's plum extract can successfully be utilized as a source of natural food color. The conclusion of this report that utilization of Davidson's plum fruit as a source of natural color will allow the industry to increase the range of natural pigments and will create new opportunities for the emerging native food industry corresponds to our obtained results.

3.2. DPPH radical scavenging activities

The antioxidant capacity (Fig. 3) of natural pigments showed relatively high in onion peel, red cabbage, mulberry, paprika, and grape peel. Onion peel and mulberry pigment in a relatively low concentration showed high scavenging activity (Boo et al., 2011). In our study, the DPPH radical scavenging activity appears to be concentration-dependent. A similar result to other studies showed that the higher the content of phenolic compounds the electron donor capacity is also higher (Joung et al., 2007; Lee et al., 2005; Rhim and Choi, 2010). Our results on red cabbage can be compared with Jacob et al. (2011), where using different extraction procedures, the antioxidant properties of green and red cabbage extracts were evaluated. The results demonstrate that the total antioxidant capacity of green and red cabbages correlated well with the total phenolics and total flavonoids content present in the extracts. The 2,2′-azino-bis (3-ethylbenzo-thiazoline-6-sulphonic acid) (ABTS) radical scavenging capacity of red cabbage was much higher than that of green cabbage extract. There is a correlation between the polyphenols, antioxidant and enzyme activities in the studied plants. Our results were as well in accordance with Debnath et al. (2011), where the water and ethanol extracts were investigated in Gardenia *jasminoides Ellis* (GJE). The IC_{50} values for

Table 2
List of strains and cultivation conditions used for screening of antimicrobial activity test.

Strains	Cultivation condition
Gram positive bacteria	
Bacillus subtilis (KCCM 11316)	37 °C, trypticase soy agar
Micrococcus luteus (KCCM 11326)	37 °C, nutrient agar
Gram negative bacteria	
Escherichia coli (KCCM 11234)	37 °C, trypticase soy agar
Salmonella typhymurium (KCCM 40253)	30 °C, MEDIUM 211
Vibrio parahaemolyticus (KCCM 11965)	37 °C, nutrient agar with 3% NaCl
Proteus mirabilis (KCCM 40888)	37 °C, nutrient agar

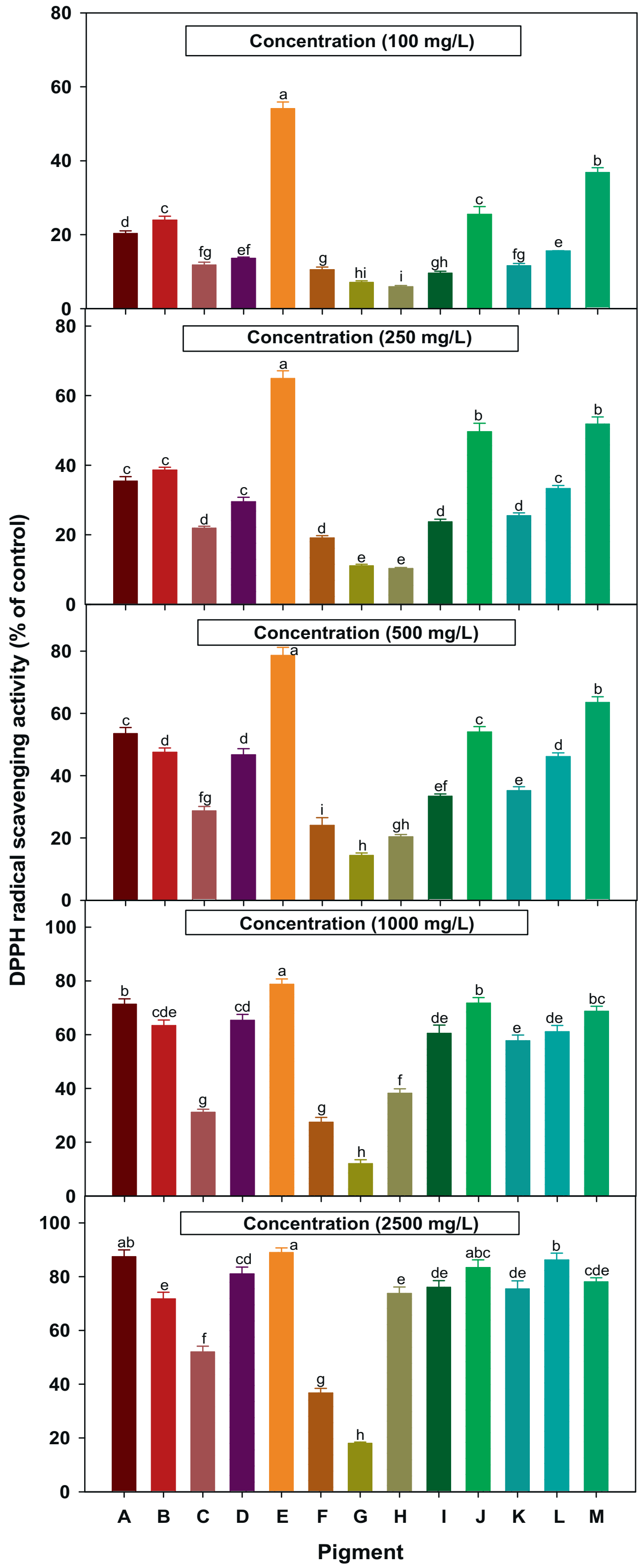

Fig. 3. DPPH radical scavenging activities according to each kind of the natural plant pigment. Means with the same letter in column are not significantly different at $p < 0.05$ level by Duncan's multiple range test. The bars represent the standard error. A: black rice, B: purple sweet potato, C: mature 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, and M: red beet.

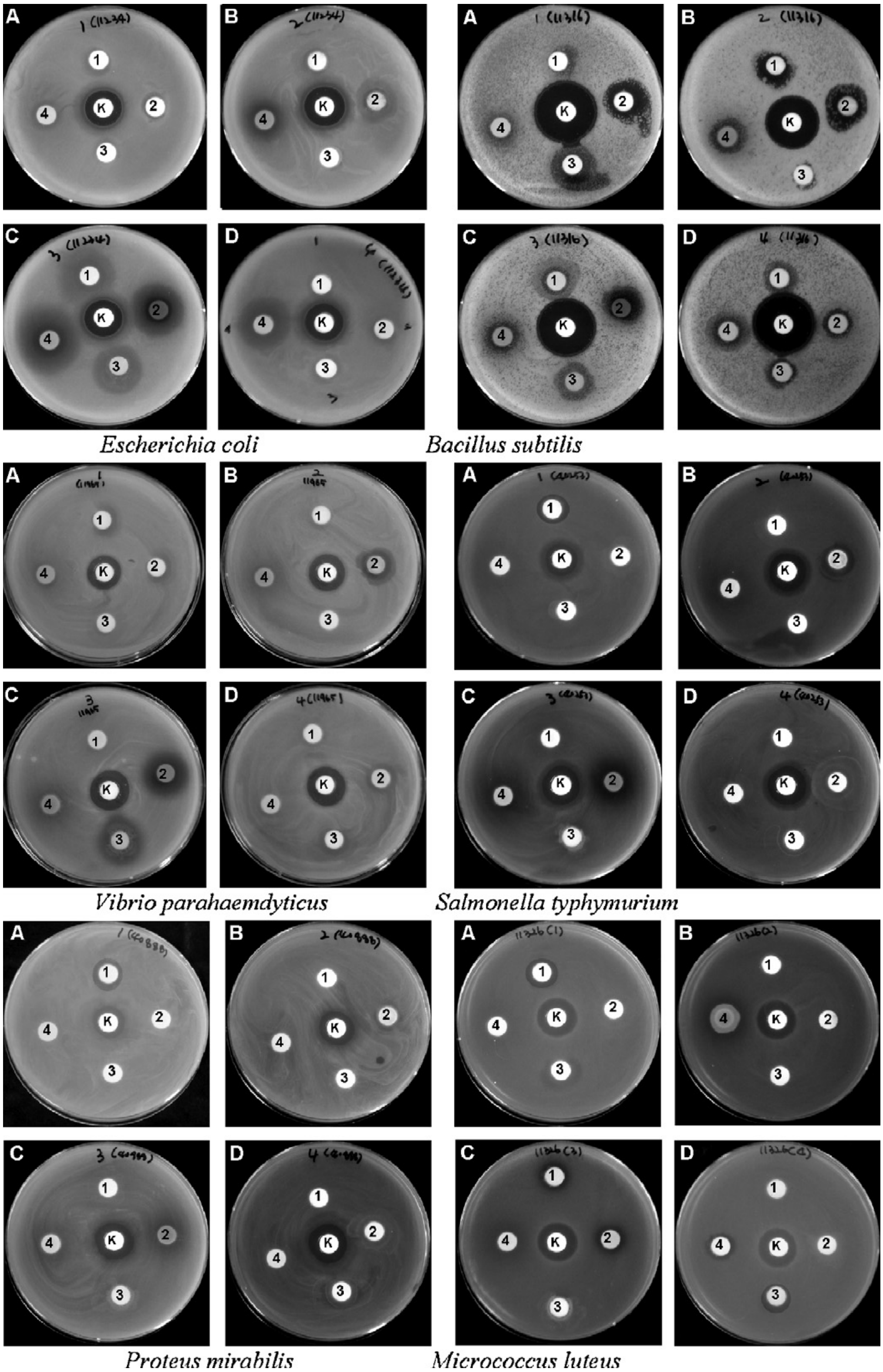

Fig. 4. Inhibition activity of the natural plant pigments against the microorganisms in paper disc diffusion assay. Kanamycin (center) was taken as the standards for antibacterial activity. A—1: mature bitter melon (yellow); 2: paprika; 3: Chinese foxglove; 4: yellow gardenia, B—1: immature bitter melon (green); 2: mulberry leave; 3: onion peel; 4: blue gardenia, C—1: purple sweet potato; 2: mulberry; 3: grape peel; 4: red cabbage, D—1: red beet; 2: black rice; 3: mature bitter melon (yellow); 4: purple sweet potato (in a clockwise direction).

DPPH, ABTS, hydroxyl and superoxide radical-scavenging activities were 0.14, 0.21, 1.08 and 1.43 mg/ml for the water-based extract, and 0.36, 0.39, 1.56 and 1.99 mg/ml for the ethanol-based extract, respectively. The extracts also showed strong reducing power, nitrite-scavenging activity, inhibition of linoleic acid oxidation, superoxide dismutase-like (SOD-like) and catalase activities. The water extract had a higher antioxidant activity than the ethanol extract. In addition, the antioxidant activities were highly

Table 3
Antimicrobial activity of the natural plant pigments.

Plant pigment	Inhibition zone size, mm					
	Escherichia coli	*Bacillus subtilis*	*Vibrio parahaemolyticus*	*Salmonella typhymurium*	*Proteus mirabilis*	*Micrococcus luteus*
Black rice	++	+++	+++	++	++	+
Purple sweet potato	++++	++++	+	–	–	++
Mature bitter melon	++	++	++	++	++	++
Immature bitter melon	++	++++	+	–	–	+++
Paprika	++	++++	++	+	–	++
Red cabbage	++++	+++	++	+++	–	+
Yellow gardenia	+++	++	++	–	–	+++
Blue gardenia	++++	++++	++	–	++	+++
Chinese foxglove	+	++++	+	–	++	+++
Mulberry leave	++++	++++	+++	+++	++	++
Onion peel	+	+	–	–	–	+
Grape peel	++++	++++	++++	++	+++	+
Mulberry	++++	++++	++++	+++	+++	++
Red beet	++	++++	++	–	–	+

++++: larger than 10 mm; +++: 5–10 mm; ++: 2–5 mm; +: smaller than 2 mm; –: not detected.

correlated with the observed phenolic and flavonoid contents. Therefore, our study as well as Debnath et al. (2011), strongly suggests that extracts derived from this fruit and other pigments could be an excellent source of antioxidants as dietary supplements. It was shown in our very recent publication (Boo et al., 2012) that the cosmetic composition of mulberry leaves pigments had the highest superoxide dismutase (SOD), enzyme activity of 67.1%, while onion peel pigment showed the lowest SOD enzyme activity of 15.3%. The activity of CAT (catalase) and APX (ascorbate peroxidase) of cosmetic composition of natural plant pigments showed higher activity in mulberry, and red cabbage pigments cosmetic composition in comparison with other plant pigments.

3.3. Antimicrobial activity

The comparative analysis results of the antimicrobial activity of selected microbes (*Escherichia coli*, *Bacillus subtilis*, *Vibrio parahaemolyticus*, *Salmonella typhymurium*, *Proteus mirabilis*, *Micrococcus luteus*) using agar diffusion test in the thirteen natural plant pigments are shown in Tables 2 and 3 and Fig. 4. *B. subtilis, M. luteus* of gram positive bacteria and *E. coli*, *V. parahaemolyticus*, of gram negative bacteria showed the clear zone formation of growth inhibition. Antimicrobial activities of *P. mirabilis* and *S. typhymurium* were relatively low compared to other strains. Most of the natural pigments except onion peel in the case of *E. coli* and *B. subtilis* showed high antimicrobial activity. The antimicrobial activities in *V. parahaemolyticus* and *P. mirabilis* were the highest in the grape peel and mulberry. The *S. typhymurium* showed high activity in red cabbage, mulberry leave, mulberry and the *M. luteus* was high in gardenia and Chinese foxglove. Especially, the mulberry and grape peel showed the antimicrobial activity in most of the strains. However, the antimicrobial activity is thought to look different depending on the strains of antibacterial type and the kinds of natural plant pigments. It is apparent that the selected plant pigments are bactericidal in nature and not bacteriostatic, based on the results of the inhibition obtained from the clear zone. Currently a wide range of natural substances known to have antimicrobial activity, but few studies related to the antimicrobial efficacy and the application of the natural plant pigment has been done, some are not made up. Kim et al. (2011) reported that Korean purple-fleshed sweet potatoes (SPs) contained the highest amounts of anthocyanins and the total phenolic compounds (2.43–3.35 mg/g, and 454.13–638.79%, respectively) compared to white/cream-fleshed dry-type SPs. The antioxidant activities of the purple-fleshed SPs were higher than those of other SPs. Our results in connection with Korean sweet potato correspond with Kim et al. (2012) where it was found that the anthocyanins of the Korean purple-fleshed sweet potato variety "Shinzami" were composed of mono- or di-acylated forms of p-hydroxybenzoic acid, caffeic acid and ferulic acid with the basic structure of cyanidin 3-sophoroside-5-glucoside or peonidin 3-sophoroside-5-glucoside. After steaming, the total anthocyanin content was reduced by nearly a half, while roasting only slightly reduced the total anthocyanin content. This is very important for the functional foods. Also, Jeon et al. (2005) reported that the colored potato extract was indicated with the high antimicrobial activity against *B. subtilis* and *E. coli*. The natural pigments of this study as well as in many other studies (Jeon et al., 2005; Kim and Rhyu, 2008; Zhoh et al., 2010) showed a relatively high antimicrobial activity. These results are suggested to have high value avaluable natural materials for the future development of colored cosmetics. It is obvious that antimicrobial properties are closely related to the pigment component, especially in the presence of functional groups.

Acknowledgements

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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