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Bioactivities of Citrus (*Citrus unshiu*) Peel Extracts Subjected to Different Extraction Conditions, Storage Temperatures, and Irradiation

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Abstract

Effects of extraction conditions, gamma-irradiation and storage conditions on bioactivities of Citrus (*Citrus unshiu*) peel extract were investigated. The Hunter color L*- and a*-values of the extract increased but b*-value decreased with an increase in absorbed irradiation dose. DPPH radical scavenging, tyrosinase inhibition and nitrite scavenging activities were not affected by irradiation but reduced by increased storage time. Nitrite scavenging activity of the extract was the highest at pH 1.2 followed by pH 4.2 and 6.0 and not changed by storage. Results indicated that there is potential for using citrus peel byproducts as a bioactive ingredient, and that gamma irradiation brightens the color of the extract without adversely altering its biological activity.

Key words: citrus peel extract, bioactivity, irradiation, storage

INTRODUCTION

Certain food components provide health benefits and play roles in disease risk prevention. The beneficial components are referred to as phytochemicals, functional or bioactive components (1). Antioxidant activity is one of the major features of bioactive components. Many of the biological properties such as anti-mutagenicity, anti-carcinogenicity and anti-aging originate from this property (2). Oxidative changes in food are responsible for the development of off flavors by the formation of compounds that result in a decrease in sensory and nutritional qualities. Antioxidants are added to food to prevent these changes. Most antioxidants currently employed are synthetic (BHA, BHT) and studies have shown that these are sometimes toxic (3). There is consumer preference for natural foods and food ingredients that are believed to be safer, healthier and less hazardous than their synthetic counterparts (4). There is need to develop antioxidants which are natural i.e. originate from food and produced in bulk using a process which uses minimum chemicals and has a high antioxidative activity (5).

The peels of fruits and vegetables are known to be a source of good antioxidants. The majority (96%) of citrus fruits in major citrus producing countries (Brazil and the United States) are used to produce juice. Since the juice yield is about half of the fruit weight, a very large amount of citrus peel is generated as a byproduct of the juice industry (6). Citrus peel is known to be rich in polyphenolic compounds, which are in turn known to have numerous biological activities such as antioxidant (6), anticancer (7) and anti-microbial (8). Citrus peel extract is potentially of interest to the food industry since it retards oxidative changes in food and thereby improves the quality and nutritional value of food. Recent studies in our laboratory on green tea leaf extract have shown that irradiation treatment can improve the color characteristics without adversely affecting the physiological properties (9). In the present study we examine the physiological activity of citrus peel extract as possible source of natural bioactive compound for the food industry.

MATERIALS AND METHODS

Sample preparation

Frozen citrus peel was finely ground and extracted with 70% ethanol using two methods. The first method sample was an extraction with 70% ethanol (sample to solvent ratio 1:10) at room temperature (20°C) for 72 hours with several agitations. The second method sample was an extraction with 70% ethanol (sample to solvent ratio 1:10) in a Soxhlet apparatus at 85°C for 3 hours. The extracts were filtered using Whatman filter paper

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(No. 4). Both groups of filtrates were sub-divided into four irradiation groups (0, 5, 10 and 20 kGy). The filtrates were stored at 4°C in screw-capped bottles (2 L capacity).

Irradiation

The samples were irradiated in a Co^{60} irradiator (point source. AECL, IR-79, MDS Nordion, Ottawa, Canada) with 0, 5, 10 and 20 kGy absorbed doses as described by Jo et al. (9). After irradiation, samples were centrifuged (4,000 rpm for 10 minutes at 4°C) in a refrigerated centrifuge (Union 5KR, Hanil, Korea). Samples were stored in screw-capped tubes (50 mL) at 4°C and room temperature, respectively.

The °Brix of all the citrus peel extract was measured using a refractometer (AST Japan). All samples were measured in triplicate.

Color measurement

A Color Difference Meter (spectrophotometer CM-3500d, Minolta Co., Ltd., Osaka, Japan) was used for color analyses. The instrument was calibrated with standard black and white tiles before measurement. Samples (9 mL) were put into a glass cell (CM A-98, 10 mm in width) and measured. All the measurements were made in triplicate using a large size aperture. The Hunter color L* (lightness), a* (redness) and b* (yellowness) values were reported for 0 and 20 kGy-irradiated samples through a computerized system using Spectra Magic software (Version 2.11, Minolta Cyberchrom Inc., Osaka, Japan). A numerical total color difference (ΔE) was calculated as follows;

$$(\Delta E) = [(\Delta L)^2 + (\Delta a)^2 + (\Delta b)^2]^{1/2}$$

Total phenolic content

The total polyphenols were estimated colorimetrically using the Folin-Ciocalteu method (10). The diluted sample aliquot (500 μL) was added to the Folin-Ciocalteu reagent (500 μL). Sodium carbonate solution (10%) was added and vortexed. The absorbance was measured using a spectrophotometer (UV 1600 PC, Shimadzu, Tokyo, Japan) at 700 nm after incubation for 1 hour at room temperature. Quantification was done based on a standard curve generated with gallic acid.

1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging assay

The effect of citrus peel extract on the DPPH radical was estimated according to the method of Blois (11). Citrus peel extract (5 μL) was added to 495 μL of 70% ethanol and 500 μL of methanolic DPPH solution (0.2 mM). The mixture was vortexed and left to stand at room temperature for 30 minutes. A tube containing 500 μL of 70% ethanol and 500 μL of methanolic DPPH solution (0.2 mM) served as the control. The absorbance of the solution was measured spectrophotometrically at 517 nm (UV 1600 PC, Shimadzu, Tokyo, Japan). The percentage of DPPH reduction by citrus peel extract was compared with the control.

Measurement of tyrosinase inhibition

Tyrosinase inhibition capacity of the citrus peel extracts was estimated according to the method of Masamoto et al. (12). A citrus peel extract (0.2 mL) was added to the reaction mixture containing 10 mM L-3, 4-dihydroxyphenyl-alanine (L-DOPA, Sigma Chemical Co., Ltd) solution, 1/15 M potassium phosphate buffer (pH 6.5) and mushroom tyrosinase (100 unit/mL, Sigma Chemical Co., Ltd.). The reaction mixture was incubated at 37°C for 20 min. The amount of dopachrome produced in the reaction mixture was spectrophotometrically (UV 1600 PC, Shimadzu, Tokyo, Japan) determined at 475 nm. The tyrosinase inhibition effect was calculated as follows;

$$\text{Inhibition } (\%) = \{1 - (S_{(Abs)} - B_{(Abs)})\} / C_{(Abs)} \times 100$$

Where $S_{(Abs)}$, $B_{(Abs)}$, and $C_{(Abs)}$ were the absorbance of the sample, the solution with 0.1 mL of distilled water instead of the enzyme solution, and the solution with 0.2 mL of distilled water instead of the citrus peel extracts.

Measurement of nitrite scavenging

Nitrite scavenging effect was determined by the method of Gray and Dugan (13) with some modification. A sample (1 mL) was added into a nitrite solution (1 ppm) (1 mL) made up to a 10 mL solution with the pH sets at 1.2 using 0.1 N HCl or at 4.2 and 6.0 using a 0.2 M citric acid buffer. The reaction mixture was incubated at 37°C in a water bath for 1 hr. Then 1 mL of the sample with 5 mL of 2% acetic acid and 0.4 mL of Griess reagent (1:1 solution of 1% sulfanilic acid in 30% acetic acid and 1% of naphthylamine in 30% acetic acid) in tubes were vortexed and kept at room temperature for 15 min. The amount of residual nitrite in the reaction mixture was measured spectrophotometrically (UV 1600 PC, Shimadzu, Tokyo, Japan) at 520 nm. The calculation of the nitrite scavenging effect was as follows;

$$\text{Nitrite scavenging effect } (\%) = \{1 - (S_{(Abs)} - B_{(Abs)})\} / C_{(Abs)} \times 100$$

Where $S_{(Abs)}$, $B_{(Abs)}$, and $C_{(Abs)}$ were the absorbance of the reaction mixture containing sample extracts, the sample extracts (Sample blank without Griess Reagent), and the reaction mixture containing distilled water (Standard).

Statistical analysis

One way Analysis of Variance was performed using SAS (SAS Institute, Cary, NC USA) software (14) and the Duncan's multiple range tests were used to compare the differences among mean values. Mean values with pooled standard errors of the mean (SEM) were reported, and the significance was defined at $p<0.05$.

RESULTS AND DISCUSSION

Brix values

The mean Brix values of all the citrus peel extract samples were 21°. There was no effect from the extraction method employed, irradiation treatment and storage period.

Color changes

The color values of differently treated citrus peel extracts stored at 4°C and 20°C are shown in Table 1. Initial analyses suggest that the Hunter color L*-, a*- and b*-values of non-irradiated citrus peel extract varied with extraction procedures. The samples extracted by heating had lower L*-value but but higher a*- and b*-values when compared to those of the room temperature extracted samples. The immediate effect of irradiation treatment was a dose dependent increase in L*- and a-values of the samples extracted by both procedures (data not shown). The L*-value in the case of the room temperature extracted samples was increased from 98.40 in the non-irradiated control to 101.11 by 20 kGy-irradiation. The Hunter color a*-value, which represents the redness of the sample, was also increased from 10.91 in the non-irradiated control to -5.43 by 20 kGy-irradiation. However, b*-value (yellowness) decreased with irradiation dose. The overall color difference from the original was calculated as ΔE, which also differed significantly with radiation ($p<0.05$). Destruction of color components such as chlorophyll by gamma irradiation has also been reported in other studies (15). Similarly, Jo et al. (9) reported reduction of brown color in green tea leaf extract by gamma irradiation treatment. There

Table 1. Hunter color value of irradiated citrus peel extract (70% ethanol) during storage

Color parameter	Extraction method[1)]	Storage (day)	4°C[2)]		20°C	
			0 kGy	20 kGy	0 kGy	20 kGy
L*	RT	0	98.40^{bx}	101.11^{ax}	98.40^{bx}	101.11^{ax}
		30	98.19^{y}	98.21^{y}	97.07^{by}	97.29^{ay}
		SEM[3)]	0.021	0.009	0.019	0.012
	Heat	0	94.89^{by}	100.53^{ax}	94.89^{by}	100.53^{ax}
		30	95.26^{bx}	98.49^{ay}	95.74^{bx}	95.85^{ay}
		SEM[3)]	0.018	0.020	0.019	0.008
a*	RT	0	-10.91^{by}	-5.43^{ay}	-10.91^{by}	-5.43^{ay}
		30	-9.95^{bx}	-3.31^{ax}	-7.12^{bx}	-4.89^{ax}
		SEM[3)]	0.009	0.007	0.018	0.009
	Heat	0	-6.78^{bx}	-4.60^{ay}	-6.78^{bx}	-4.60^{ay}
		30	-7.48^{by}	-3.85^{ax}	-6.77^{bx}	-1.97^{ax}
		SEM[3)]	0.009	0.006	0.012	0.005
b*	RT	0	51.09^{ax}	16.01^{by}	51.09^{ax}	16.01^{by}
		30	48.21^{ay}	23.18^{bx}	44.17^{ay}	29.60^{bx}
		SEM[3)]	0.056	0.013	0.057	0.011
	Heat	0	56.52^{ax}	15.77^{by}	56.52^{ax}	15.77^{by}
		30	54.71^{ay}	21.71^{bx}	51.39^{ay}	28.33^{bx}
		SEM[3)]	0.014	0.012	0.015	0.010
ΔE	RT	0	111.41^{ax}	102.15^{bx}	111.41^{ax}	102.15^{by}
		30	109.84^{ay}	100.96^{by}	106.89^{ay}	102.40^{bx}
		SEM[3)]	0.042	0.009	0.041	0.011
	Heat	0	110.66^{ax}	101.87^{bx}	110.66^{ax}	101.87^{bx}
		30	110.11^{ay}	100.93^{by}	108.87^{ay}	99.96^{by}
		SEM[3)]	0.021	0.019	0.023	0.008

[a,b]Different letters within the same row differ significantly ($p<0.05$).
[x,y]Different letters within the same column with the same treatment differ significantly.
[1)]RT: Extracted at room temperature (20°C) for 72 hours with several agitation.
Heat: Extracted in Soxhlet apparatus at 85°C for 3 hours.
[2)]Storage temperature after extraction.
[3)]Pooled standard errors of the mean (n = 6).

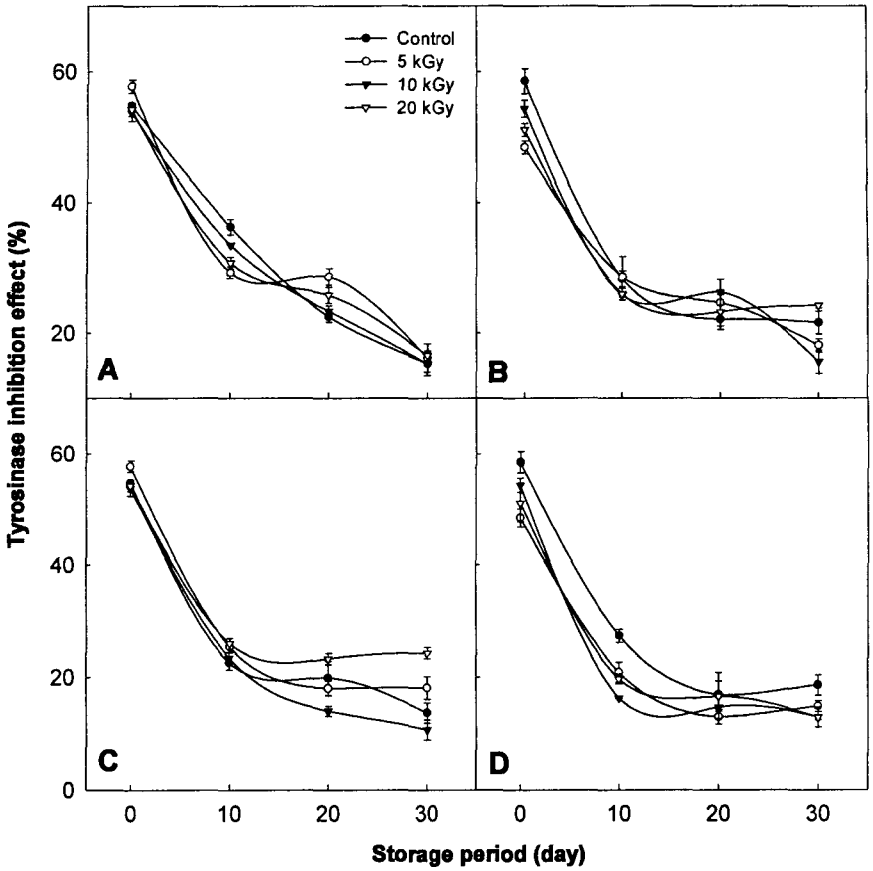

Fig. 1. DPPH free radical scavenging activities of citrus peel extracts with different storage temperatures. A, extracted at room temperature (20°C) for 72 hours and stored at 4°C; B, extracted in Soxhlet apparatus at 85°C for 3 hours and stored at 4°C; C, extracted at room temperature (20°C) for 72 hours and stored at 20°C; D, extracted in Soxhlet apparatus at 85°C for 3 hours and stored at 4°C.

was inclination for the color of the citrus peel extract to darken during storage, irrespective of irradiation treatment and storage temperatures. It was observed that the effects of storage temperatures on b*-value were reduced, whereas L*- and a*-values increased with storage time. Only data from days 0 and 30 are shown in the Tables. However, data from days 10 and 20 had similar trends (data not shown). Tendency of color change in gamma irradiated green tea leaf extract during storage has also been reported (9).

Phenolic content

Phenolic content of samples extracted at room temperature was 3.7±0.30 mg%, whereas that of the samples extracted by heat treatment was 4.2±0.40 mg%. It was observed that phenolic content was not affected by irradiation treatment and storage time at both temperatures.

DPPH free radical scavenging activity

Determination of DPPH free radical scavenging activity is a fast method to determine antioxidant activity. In this method anti-radical power of an antioxidant is measured as the decrease in the absorbance of DPPH. As a result, the color changes from purple to yellow. It is most widely employed to characterize antioxidant activity of plant material (16). Effects of the extraction procedure, gamma-irradiation, and storage on DPPH free radical scavenging activity of citrus peel extracts are shown in Fig. 1. It can be seen that initially the extracts prepared by both procedures had scavenging activity of about 33%. The radical scavenging activity decreased significantly during storage, regardless of irradiation treatment. A similar tendency to lose anti-radical activity during storage has been reported for green tea leaf extract (9). However, in the case of irradiated fermented foods, it has reported that radical scavenging activity was not altered by storage (17). Antioxidant properties of foods can remain unaffected, increased or decreased upon processing and storage, which could be attributed to the formation of new compounds which have either more potent antioxidant or prooxidant activities (18).

Tyrosinase inhibition effect

Synthesis of melanin pigment in human skin plays an important role in the protection against skin photocar-

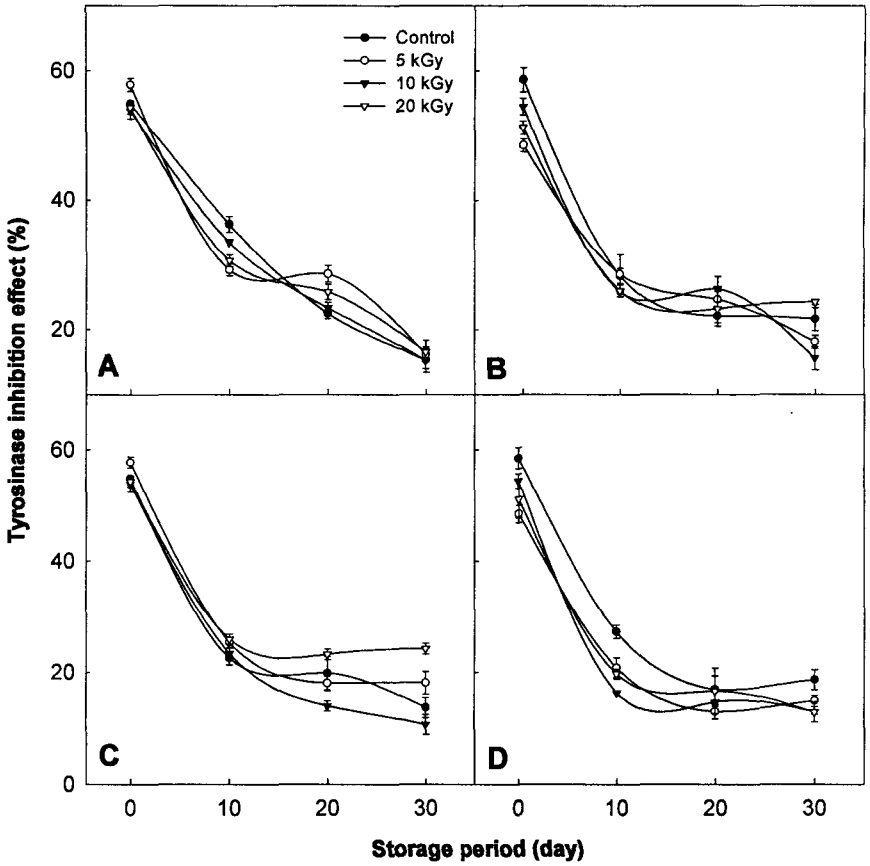

Fig. 2. Tyrosinase inhibition effect of citrus peel extracts stored at different temperatures. A, extracted at room temperature (20°C) for 72 hours and stored at 4°C; B, extracted in Soxhlet apparatus at 85°C for 3 hours and stored at 4°C; C, extracted at room temperature (20°C) for 72 hours and stored at 20°C; D, extracted in Soxhlet apparatus at 85°C for 3 hours and stored at 4°C.

cinogensis. Although it is a major defense mechanism against ultraviolet light damage to the skin, increased and redistributed melanin darkens skin color which is often considered a serious aesthetic problem (19). Tyrosinase is mainly responsible for melanin biosynthesis (melanogenesis) in animals and enzymatic browning (melanosis) in plants. Green and black teas are reported to have high tyrosinase inhibition activity (20). The tryrosinase inhibition effect of citrus peel extract was investigated for possible application as a bioactive natural skin whitening-agent. The initial analyses of citrus peel extract showed about 55% tyrosinase inhibition in both the non-irradiated and irradiated samples (Fig. 2). There was time-dependent reduction in the activity at both storage temperatures. Similar findings were observed in previous studies where no difference in tyrosinase inhibition effect between non-irradiated and irradiated extracts were reported in green tea leaf (9), licorice root or stolon (19), and soybean-based fermented food (15). On the other hand, in the case of *Lonicera japonica* extract, gamma irradiation treatment increased the tyrosinase inhibition effect (20). The tyrosinase inhibition effect was reduced significantly by increased storage time. The result suggests that the active compound was unstable.

Nitrite scavenging effect

Carcinogenic nitrosamines are formed as a result of the reaction between nitrite and secondary or tertiary amines in protein rich foods, medicines and residual pesticides. When nitrite is present in various foods and the food is consumed, the formation of nitrosamine is expected in human stomach, which is highly acidic (21). Therefore, effective nitrite scavenging under acidic conditions is very important in inhibiting the formation of carcinogenic nitrosamines. Both non-irradiated and irradiated citrus peel extracts showed 80 to 85% nitrite scavenging effect (Fig. 3) at pH 1.2. However, at pH 4.2 and 6.0, these values were reduced to 35% and 20%, respectively. The nitrite scavenging effect of the sample stored at 20°C was similar trend to that of the sample stored at 4°C (Fig. 3). Phenolic compounds are reported to have high nitrite scavenging effects (22). It has been reported that the phenolic compounds have higher nitrite scavenging effects in low environmental pH conditions (23). The differences between the non-irradiated and irradiated samples are not shown from a statistical point

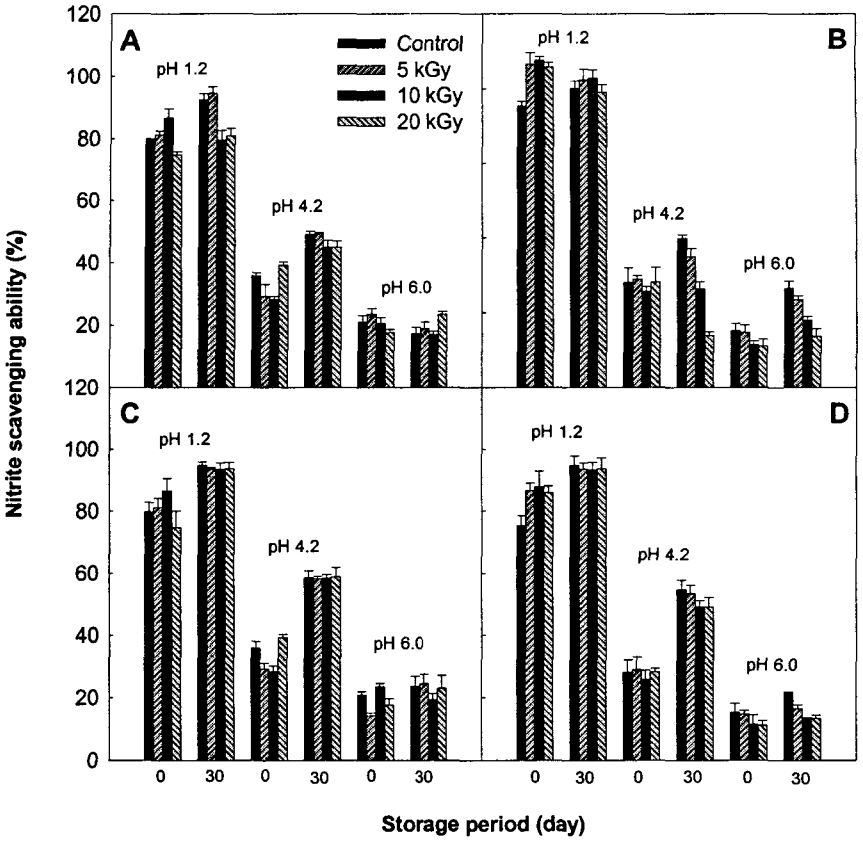

Fig. 3. Nitrite scavenging activities of citrus peel extracts stored at different temperatures. A, extracted at room temperature (20°C) for 72 hours and stored at 4°C; B, extracted in Soxhlet apparatus at 85°C for 3 hours and stored at 4°C; C, extracted at room temperature (20°C) for 72 hours and stored at 20°C; D, extracted in Soxhlet apparatus at 85°C for 3 hours and stored at 4°C.

of view.

It can be concluded that extract of citrus peel by-products can be used as a natural source of bioactive compounds in the cosmetic and food industry with merits that include: waste management of citrus processing industry byproducts in a manner that is cost-effective and environmentally friendly. However, reduction of bioactivities of citrus peel extract during storage should be considered. Irradiation treatment can be utilized to lighten the color without adversely influencing the biological activities of citrus peel extracts.

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