J. Appl. Biol. Chem. **51**(1), 7-12 (2008)

Article

Citrus Peel Wastes as Functional Materials for Cosmeceuticals

Sang-Suk Kim[1], Jung-A Lee[2], Ji-Young Kim[2], Nam Ho Lee[1], and Chang-Gu Hyun[2,*]

[1]*Department of Chemistry, Cheju National University, Jeju 690-756, Korea*
[2]*Laboratory of Bioresources, Jeju Biodiversity Research Institute (JBRI), Jeju Hi-Tech Industry Development Institute (HiDI), Jeju 697-943, Korea*

Received September 21, 2007; Accepted December 6, 2007

The suitability of CPWs, by-products of the juice industry, was investigated as a source for the production of cosmeceuticals. Four kinds of CPWs, CW, CWE, CWER, and CWEA, were examined for their antioxidant potentials in terms of DPPH radical-scavenging ability for anti-wrinkle applications, inhibition of tyrosinase or melanin production for whitening products, and anti-inflammatory effects to treat various skin diseases such as atopic dermatitis and acne as well as for anti-bacterial activity against acne-inducing pathogens. Of the four extracts, CWER was the most potent tyrosinase inhibitor (IC_{50} value: 109 μg/mL), and CWEA (IC_{50}: 167 μg/mL) showed good antioxidative effects. CWE and CWEA samples had dose-dependent inhibitory effects on the melanin production. The cytotoxic effects of the four CPWs were determined by colorimetric MTT assays using human keratinocyte HaCaT cells. Most extracts exhibited low cytotoxicity at 100 μg/mL. These results suggest CPWs are attractive candidates for topical applications on the human skin.

Key words : *acid lysis, acne, citrus peel waste, DPPH, interleukin-8, melanin, tumor necrosis factor α*

Food and beverage processing industries create large quantities of by-products, which are difficult to dispose of due to their high biological oxygen demand. These plant material wastes sometimes contain high levels of biological compounds that can have an adverse environmental impact. Positive impacts of industrial wastes on human health include inhibition of oxidation of low-density lipoprotein thereby reducing the risk of heart disease. Anti-inflammatory, antioxidant and anticarcinogenic properties of the biological compounds have also been reported [Kang *et al*., 2006].

Citrus is an economically important fruit of Jeju Island. Because of its special flavor, the fruit is processed into juice as an ingredient for sauces and salad dressings. After the juice extraction, the fruit pulps are mostly dumped as waste at large expense. This waste, which amounts to several tons per day in a medium-sized processing plant, causes many economical and environmental problems due to its fermentability [Tripodo *et al*., 2004]. Furthermore, because of the high water content (about 86%) of the waste, drying with common industrial drying devices is difficult, and the high organic matter content stands in the way of an easy disposal. In Korea, about 40,000 tons of citrus peel by-products are produced from 150,000 tons of citrus peels yearly during the citrus juice processing. Therefore, it is worthwhile to determine how to make use of the *Citrus unshiu* waste [Kang *et al*., 2006; Kato-Noguchi and Tanaka, 2004; Patel-Predd, 2006].

Citrus peel, called 'Jin-Pi', is used in the traditional medicine for the treatment of severe dermatitis, atopic dermatitis, for the recovery of fatigue, and as a digestant [Choi *et al*., 2007]. However, there is little information on the biological potential of the juice-extracted waste of the citrus fruit. The present study focused on whether the CPWs have antibacterial and anti-inflammatory effects against the acne-inducing bacteria, induce low cytotoxicity in THP-1 and HaCaT cell lines, and inhibit the melanin production in the melan-a cells.

*Corresponding author
Phone: +82-64-720-2811; Fax: +82-64-720-2801
E-mail: cghyun@jejuhidi.or.kr

Abbreviations: CPW, citrus peel waste; CW, ethanol extracts; CWE, ethyl acetate extracts; CWER, acid-lysate of CW at room temperature; CWEA, acid-lysate of CW by autoclave; DMSO, dimethylsulfoxide; EDTA, ethylenediamine tetraacetic acid; ELISA, enzyme-linked immunosorbent assay; IL-8, interleukin-8; MIC, minimal inhibitory concentration; PBS, phosphate-buffered saline; PMA, phorbol myristate acetate; TNF-α, tumor necrosis factor α.

Materials and Methods

Plant material and extraction. The fruit waste of *C. unshiu* after juice extraction (peel and attached membranes) was obtained from a local food processing company (Jeju Provincial Development Co., Jeju, Korea), and stored frozen (−20°C) until use. These materials were extracted with 20 volumes of 100% aqueous ethanol for 3 days. The extract was then filtered through the filter paper (No. 2, Toyo, Tokyo, Japan), and the filtrate was evaporated to dryness at 40°C *in vacuo* (CW). A solution of the CW extract (6 g) in 600 mL of 6% HCl was stored at room temperature for 6 h (CWER) or at 120°C for 15 min in an autoclave (CWEA). These reaction mixtures were then extracted with EtOAc. As a control, the CW extract was also extracted with EtOAc (extract CWE).

Antibacterial tests. Two Gram-positive bacterial species, *Propionibacterium acnes* ATCC 6919 and *Staphylococcus epidermidis* KCTC 3958, which are involved in the formation of acne, were selected as test microorganisms according to their pathological capacities. *P. acnes* ATCC 6919 was cultured at 37°C for 48 h in a GAM broth (Nissui, Tokyo, Japan) under anaerobic conditions, and *S. epidermidis* KCTC3958 was cultured at 37°C for 24 h in the *Corynebacterium* medium before the assay. MIC was estimated by the broth dilution method. The MIC was taken as the lowest concentration that prevented visible bacterial growth after 24 h incubation at 37°C.

DPPH radical-scavenging assay. The reaction was carried out in 100% ethanol containing 0.1 mM DPPH and various concentrations of CPWs. The scavenging effect against DPPH radical was assessed at room temperature for 10 min. The change in absorbance was measured at 517 nm in a 96-well reader. The percent scavenging effect on DPPH radical was calculated as follows:

$$\text{Scavenging effect on DPPH radical (\%)} = [(A-B)/A] \times 100$$

where A is the absorbance at 517 nm without CPWs and B is the change in absorbance at 517 nm following the incubation with CPWs.

Tyrosinase inhibition assay. Tyrosinase inhibition of each CPW sample was assayed by measuring its effect on the tyrosinase activity in a 96-well reader (Power Wave, Bio-Tek Inc., Winooski,VT). The reaction was carried out in 100 mM sodium phosphate buffer (pH 6.7) containing 1 mM L-tyrosine and 80 unit/mL mushroom tyrosinase at 37°C. The reaction mixture was pre-incubated for 10 min before adding the substrate. The change in the absorbance at 475 nm was measured. The percent inhibition of tyrosinase was calculated as follows:

$$\text{Inhibition (\%)} = [(A-B)/A] \times 100$$

where A is absorbance at 475 nm without CPWs and B is the change in absorbance at 475 nm following the incubation with CPWs.

Melan-a cell culture. Melan-a melanocytes were a kind gift from Dr. D. C. Bennett (St. George's Hospital, London, UK). Melan-a cells are non-tumorigenic melanocyte cell lines derived from C57BL mice and are more similar to the primary mouse melanocytes than the tumorigenic B16 melanoma cell. Melan-a cells were grown in a humidified incubator at 37°C in an atmosphere of 10% CO_2. The cells were routinely passaged in RPMI 1640 supplemented with 5% heat-inactivated fetal bovine serum, 100 mM β-mercaptoethanol, 50 U/mL penicillin, 50 mg/mL streptomycin, 2 mM L-glutamine, and 200 nM PMA [Chang *et al*., 2007]. The cells were seeded at a density of 1.5×10^5 cells/well in 6-well plates, and the drug treatment was started 24 h after the seeding. After 3 days, the cells were harvested using 0.5 mL of 0.25% trypsin/EDTA. The melanin content was determined in triplicate for each treatment.

Melanin content assay. After the treatment and the extraction as described above [Virador *et al*. 1999], the cell extracts were centrifuged for 5 min at 15,000 rpm. The resulting pellets were lysed with 200 mL of 1 N NaOH and transferred to 96-well plates in triplicates. The relative melanin content was determined by measuring the absorbance at 405 nm in a PowerWave X340 ELISA reader (Power Wave, Bio-Tek Inc.).

Measurement of cell proliferation. The MTT assay was used to measure the cell proliferation. After treating the cells as described above [Virador *et al.,* 1999], 100 mL aliquots of the harvested cells were plated, and allowed to attach and grow overnight at 37°C. After the media were discarded, 100 mL of 0.5 mg/mL MTT was added to each well, and the cells were incubated at 37°C for 4 h. Subsequently, the plate was centrifuged for 10 min at 3,000 rpm. After the supernatants were discarded, 200 mL isopropanol was added to the plate and incubated at room temperature for 4 h. The formazan precipitates were quantitated based on the absorbance measured at 570 nm in an ELISA reader (Power Wave, Bio-Tek Inc.).

Measurement of cytokine production. Human monocytic THP-1 cells (1×10^6) in the serum-free medium were incubated for 48 h with 100 μg/mL *P. acnes*, alone or in combination with CPWs at the indicated concentrations. The culture supernatants were then harvested. The concentrations of interleukin-8 (IL-8) and TNF-α in the culture supernatants were measured using the ELISA kit (BioSource, Camarillo, CA).

Cytotoxicity assay of citrus peel wastes. The HaCaT

and THP-1 cells were cultured in the Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (Gibco, Calsbad, CA) and penicillin-streptomycin at 37°C in a humidified 95% air/5% CO_2 atmosphere. The cells were seeded on 24-well plates, and the drug treatment began 24 h after the seeding. The general viability of the cultured cells was determined by the MTT assay, in which MTT is reduced to formazan. After the normal fibroblast cells were incubated with various concentrations of CPW for 24 h at 37°C in a 5% CO_2 atmosphere, MTT (1 mg/mL in PBS) was added to each well in 1/10 volume of the medium. The cells were incubated at 37°C for 3 h, and DMSO was added to dissolve the formazan crystals. The absorbance was then measured at 570 nm with a spectrophotometer (Power Wave, Bio-Tek Inc.).

Statistical evaluation. Means±SEM were calculated, and the statistical analyses of the results were performed using Student's t-test for independent samples. Values of $P<0.05$ were considered significant.

Results and Discussion

Because tyrosinase catalyzes the melanin synthesis, inhibitors of tyrosinase or melanin production are important for cosmetic skin-whitening purpose. Oxidative stress contributes to skin aging and can adversely affect the skin health; thus, the antioxidants active in the skin cells may promote the skin health [Wang *et al*., 2006]. Therefore, the CPWs that might be effective for skin-whitening and promotion of skin health were examined, and their antibacterial and anti-inflammatory effects were evaluated.

Antibacterial effects of citrus peel wastes. The disk diffusion method was used to elucidate the antibacterial activities of CPWs against the acne-inducing pathogens. Erythromycin was employed as a positive control. CWEA was found to have a moderate antibacterial activity against *P. acnes,* but not against *S. epidermidis*. The antibacterial activities of CPWs were further evaluated by determining the MIC, which is the lowest concentration that yields no bacterial growth. The MICs of CPWs were determined by a two-fold serial dilution. Among the CPWs, only CWEA inhibited the growth of *P. acnes* at 1 mg/mL (data not shown).

Scavenging effects of citrus peel wastes on DPPH radicals. Several studies have reported on the association of aging with the formation of free radicals [Kim *et al*., 2007]. Oxidative stresses can be generated in the connective tissues and the skin cells by photodamage and inflammatory processes. The skin, when exposed to UV or visible light, will produce free radicals, which can then induce skin damages such as phototoxicity and aging [Leu *et al*., 2006]. DPPH is a purple, stable radical that is used to screen the free radical-scavenging ability of compounds and the antioxidant activity of the plant extracts [Huang *et al*., 2005; Zhu *et al*., 2004]. Antioxidants react with DPPHs and convert them into the colorless α-α-diphenyl-β-picryl hydrazine. The amount of DPPH reduced can be quantified by measuring the decrease in absorbance at 517 nm. Four kinds of the citrus peel wastes obtained by ethanol extraction or acid-catalyzed lysates were screened for their possible antioxidant activities using the DPPH free radical-scavenging systems.

Table 1. IC_{50} values of CPWs against DPPH free radicals and Inhibitory effect of CPWs on mushroom tyrosinase

Samples	DPPH (IC_{50}; μg/mL)	Tyrosinase inhibition (IC_{50}; μg/mL)
CW	489	311.61
CWE	279	138.7
CWEA	167	146.2
CWER	489	109

Dose-response curves for the DPPH radical-scavenging activity were observed in all CPWs. The IC_{50} values were calculated and are presented in Table 1. The most active extract was CWEA (167 μg/mL), followed by CWE (279 μg/mL). CW and CWER exhibited the weakest antioxidant activities in this test system with IC_{50} value of 489 μg/mL. These results indicate that CPWs contain constituents with strong proton-donating abilities [Sawai *et al*., 2005].

Effects of citrus peel wastes on melanin production, cell proliferation, and tyrosinase enzymes. Melanin production in the human skin is an important defense mechanism against UV and a major determinant of the skin color. Despite the protective role of melanins, abnormal hyperpigmentation including freckles, chloasma, and other forms of melanin hyperpigmentation can sometimes cause serious aesthetic problems. Therefore, potent active agents have been sought for cosmetic use to control hyperpigmentation [Costin and Hearing, 2007]. Many chemicals such as hydroquinone, arbutin, kojic acid, and ascorbic acid are known for their melanogenic-inhibitory functions. Although these chemicals are often used for skin-lightening and cosmetic formulations, they also have some disadvantages such as poor safety and limited effectiveness [Chang *et al*., 2007].

To investigate the effects of CPWs on the melanin production, the melan-a cells were treated with various concentrations of CPWs for 4 d. CWE and CWEA showed remarkable dose-dependent depigmenting effects (Fig. 1). At 10 μg/mL, CWE and CWEA decreased the melanin content by almost 55 and 35%, respectively. In the selection of a skin-lightening compound for cosmetic

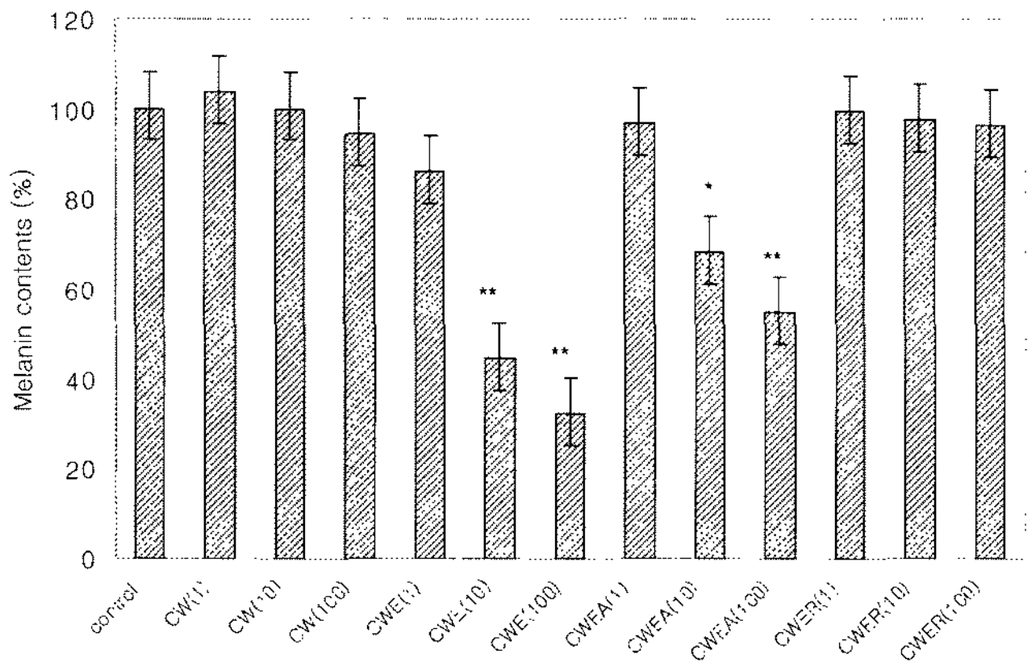

Fig. 1. CWE and CWEA decreased the pigmentation of melan-a cells. Melan-a cells were treated with CPWs (1 to 100 μg/mL) for 4 d and then harvested. Harvested cells were pelleted, and their melanin contents were assayed as described in Materials and Methods. The melanin contents are expressed as percent of the control. CWE and CWEA showed remarkable dose-dependent depigmentation effects. At 10 μg/mL, CWE and CWEA decreased the melanin content by almost 55 and 35%, respectively.

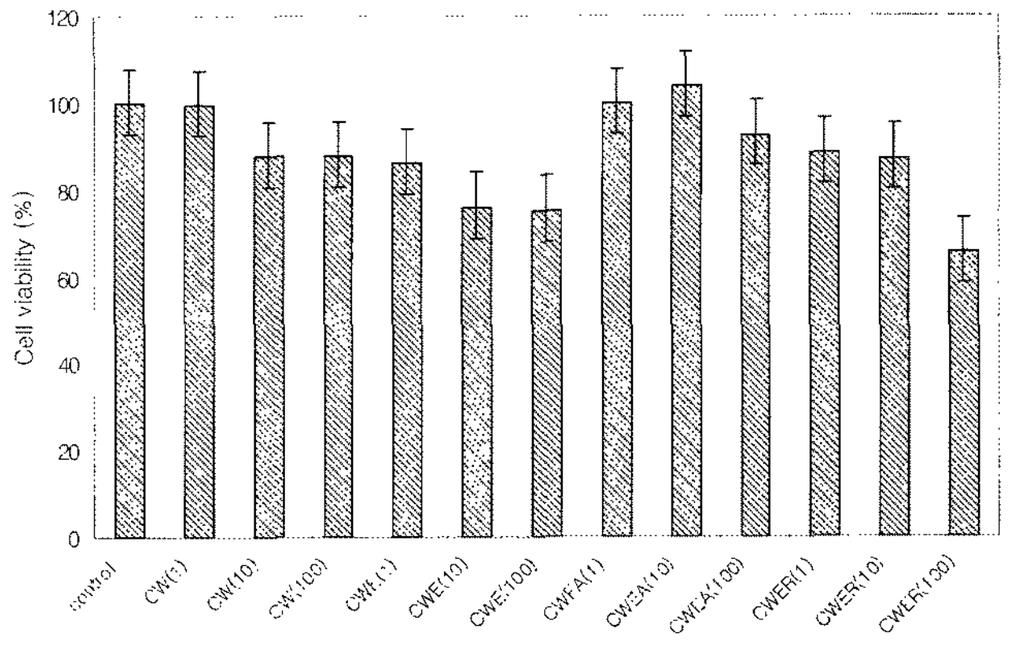

Fig. 2. CWE and CWEA did not inhibit melan-a cell proliferation. Melan-a cells were treated with CPWs (1 to 100 μg/mL) for 4 d, and then harvested. The MTT assay was performed as described in Materials and Methods. The formazan precipitates were quantitated by measuring the absorbance at 562 nm. CWE and CWEA showed little inhibitory effects on the cell proliferation at the tested concentrations, suggesting that the inhibitory effects of CWE and CWEA on the melanin production do not cause cytotoxicity.

formulation, one of the most important characteristics to consider is that the compound has a minimal effect on the melanocyte cell proliferation. Thus, proliferation of the cells treated with CPWs for 4 d was evaluated using the MTT assay. CWE and CWEA showed little inhibitory effects on the cell proliferation at the tested concentrations, suggesting that the inhibitory effects of CWE and CWEA on the melanin production do not contribute to cytotoxicity (Fig. 2).

Melanin is a heteropolymer of the indole compounds that is produced inside the melanosomes by the action of the tyrosinase on the tyrosine precursor material in the melanocytes. Although other factors such as metal ions and the TRP-1 and TRP-2 enzymes have also recently been shown to contribute to the melanin production, because tyrosinase plays a critical regulatory role in the melanin biosynthesis [Solano *et al.*, 2006], the tyrosinase-inhibitory activities of CPWs were estimated. Table 1 summarizes the results of the assessment of mushroom tyrosinase inhibition by CPWs, with the inhibition expressed as IC_{50} values. Of the four CPWs, CWER was the strongest inhibitor of tyrosinase with an IC_{50} value of 109 μg/mL.

Effects of citrus peel wastes on inflammation. The main pro-inflammatory mediators induced by bacteria and their cell components are cytokines, primarily TNF-α and interleukin-8 [Park *et al.*, 2004]. Therefore, to investigate the anti-inflammatory effects of CPWs, the concentrations of interleukin-8 and TNF-α in the THP-1 cells were measured by ELISA. The results showed CPWs only inhibited the release of TNF-α. CW and CWEA slightly reduced the *P. acnes*-induced production of TNF-α in the THP-1 cells (Fig. 3A). However, because it is possible that the reduction of the proinflammatory cytokines was induced by the cytotoxic effects of CW, MTT assays were performed on the THP-1 cells. Almost 100% cell viabilities of THP-1 cells treated with CW and CWEA below 100 and 10 μg/mL, respectively, were observed (Fig. 4A). Although other extracts such as CWEA and CWE showed anti-inflammation effects at 100 μg/mL, they also had severe cytotoxicity on the THP-1 cells.

Cytotoxicity of citrus peel wastes on human keratinocytes HaCat cells. The cytotoxic effects of CPWs were also examined in the HaCaT cells, a human keratinocyte cell line (Fig. 4B), because CPWs could have cytotoxic effects on the human skin cells when applied as therapeutic agents [Park *et al.*, 2006]. If so, they would not be suitable therapeutic agents. The cell viability was almost 100% with CPWs at concentrations below 10 μg/mL, whereas at 100 μg/mL, the viability of the HaCaT cells was over 80% (Fig 4B). These data suggest that CPWs have low cytotoxicity against the mammalian cell lines. Interestingly, different patterns of cytotoxicity were observed between THP-1 (Fig. 4A) and HaCaT (Fig. 4B) cells, which could be attributed to the differences in the cell types such as the growth pattern and the origin of the cell line. That is, the THP-1 cell line shows an anchorage-independent growth pattern, and its origin is the human monocyte [Park *et al.*, 2004].

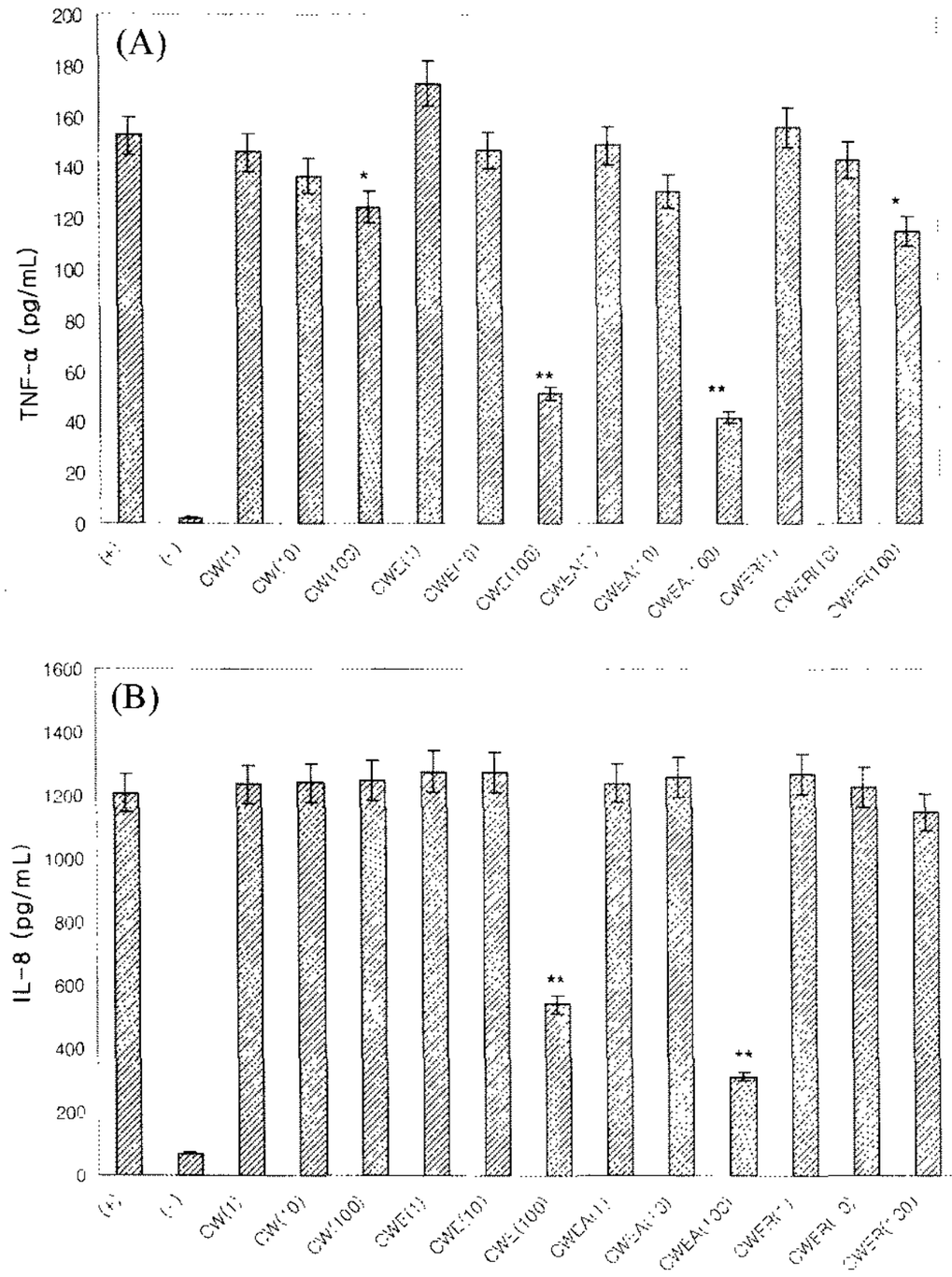

Fig. 3. CPWs inhibit *P. acnes*-induced secretion of the proinflammatory cytokine, TNF-α (A), but not of IL-8 (B). THP-1 cells were stimulated with or without the heat-killed *P. acnes* and the supernatants were harvested after 48 h for IL-8 or TNF-α measurement. CPWs only inhibited the TNF-α release. CW and CWEA slightly reduced the *P. acnes*-induced production of TNF-α.

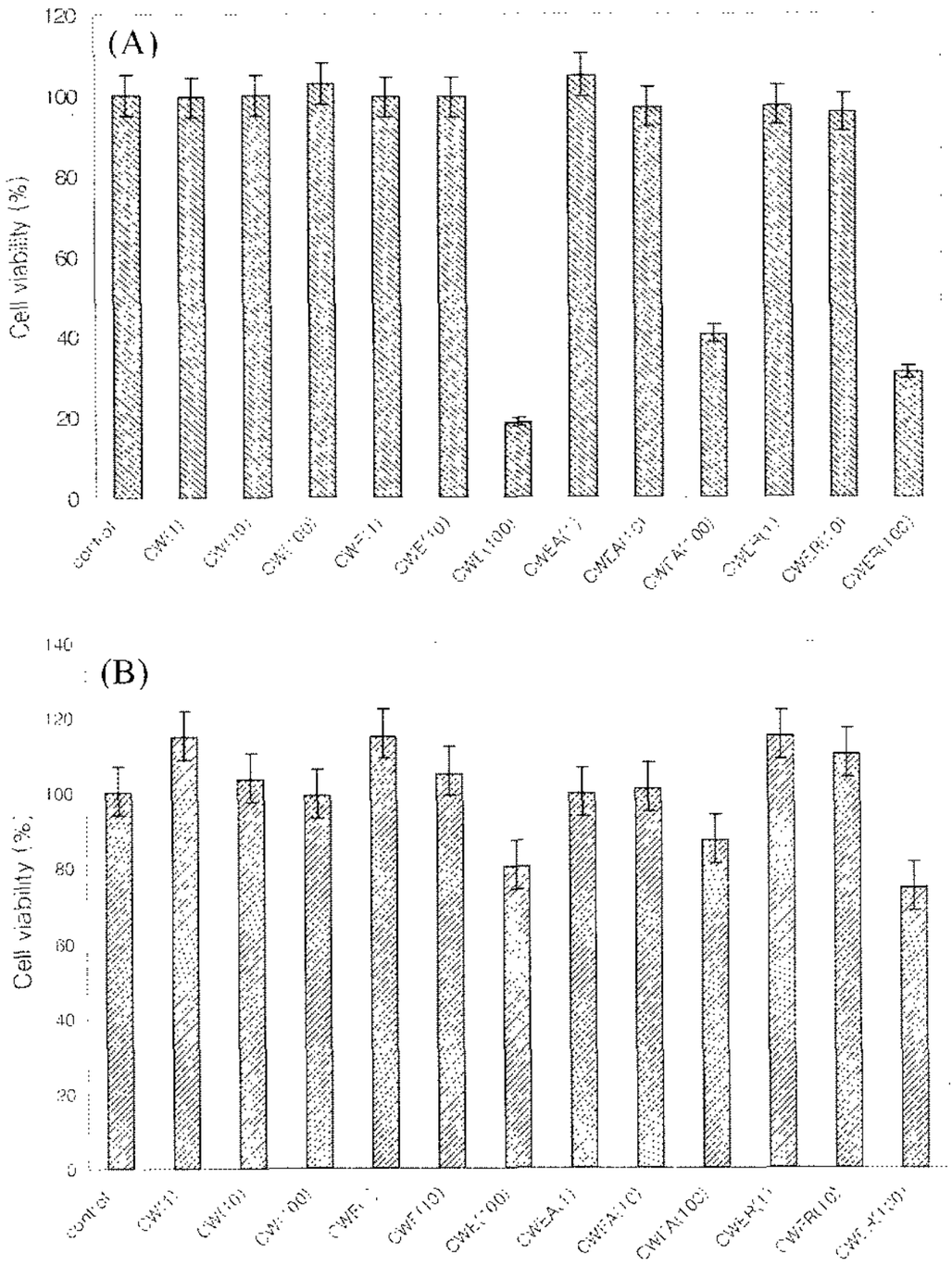

Fig. 4. Cytotoxicity of CPWs against THP-1 (A) and HaCaT cells (B). THP-1 or HaCaT cells were cultured for 24 h in the medium with or without the test agents. Cellular cytotoxicity was determined according to the rapid colorimetric MTT assay and expressed as the mean± S.E.M. Cell viability of the HaCaT cells was almost 100% with CPWs at concentration below 10 μg/mL, whereas over 80% at 100 μg/mL.

The suitability of CPWs as a source for cosmeceuticals was investigated. Because most polyketide compounds such as flavonoids and stilbenes are extracted from the EtOAc fraction, four kinds of EtOAc extracts were prepared. Of the four extracts, acid-lysates (aglycone complex) such as CWER and CWEA showed potent tyrosinase inhibition or good antioxidative effects. Furthermore, CWE and CWEA samples had dose-dependent inhibitory effects on the melanin production. In order to apply CPWs to the human skin, the cytotoxic effects of the four CPWs were determined by MTT assays using the human keratinocyte HaCaT cells. Most extracts exhibited low cytotoxicity at 100 μg/mL. In addition, CW and CWEA extracts slightly reduced the heat-killed *P. acnes*-induced secretion of TNF-alpha in the THP-1 cells. Results of this study indicate that some CPWs have the potential as antioxidant, anti-inflammatory, and whitening agents and may be useful in the cosmetic industries. Our results contribute to better the valorization of these wastes by the juice industry. Other biological tests to search for more activities of these CPWs may also be worthwhile.

Acknowledgments. This research was partially supported by the Regional Technology Innovation Program (RTI04-02-07) and the Development Program of the Regional Industry Technology (10027152) from the Ministry of Commerce, Industry, and Energy of the Korean Government.

References

Chang YH, Kim C, Jung M, Lim YH, Lee S, and Kang S (2007) Inhibition of melanogenesis by selina-4(14),7(11)-dien-8-one isolated from *Atractylodis Rhizoma Alba*. *Biol Pharm Bull* **30**, 719-723.

Choi SY, Hwang JH, Ko HC, Park JG, and Kim SJ (2007) Nobiletin from citrus fruit peel inhibits the DNA-binding activity of NF-kappaB and ROS production in LPS-activated RAW 264.7 cells. *J Ethnopharmacol* **113**, 149-

155.
Costin GE and Hearing VJ (2007) Human skin pigmentation: melanocytes modulate skin color in response to stress. *FASEB J* **21**, 976-994.
Huang D, Ou B, and Prior RL (2005) The chemistry behind antioxidant capacity assays. *J Agric Food Chem* **53**, 1841-1856.
Kang HJ, Chawla SP, Jo C, Kwon JH, and Byun MW (2006) Studies on the development of functional powder from citrus peel. *Bioresour Technol* **97**, 614-620.
Kato-Noguchi H and Tanaka Y (2004) Allelopathic potential of *Citrus junos* fruit waste from food processing industry. *Bioresour Technol* **94**, 211-214.
Kim SS, Hyun CG, Lee J, Lim JH, Kim JY, and Park D (2007) *In vitro* Screening of Jeju Medicinal Plants for Cosmeceutical Materials. *J Appl Biol Chem* **50**, 215-220.
Leu SJ, Lin YP, Lin RD, Wen CL, Cheng KT, Hsu FL, and Lee MH (2006) Phenolic constituents of *Malus doumeri* var. *formosana* in the field of skin care. *Biol Pharm Bull* **29**, 740-745.
Park LJ, Ju SM, Song HY, Lee JA, Yang MY, Kang YH, Kwon HJ, Kim TY, Choi SY, and Park J (2006) The enhanced monocyte adhesiveness after UVB exposure requires ROS and NF-êB signaling in human keratinocyte. *J Biochem Mol Biol* **39**, 618-625.
Park J, Lee J, Jung E, Park Y, Kim K, Park B, Jung K, Park E, Kim J, and Park D (2004) *In vitro* antibacterial and anti-inflammatory effects of honokiol and magnolol against *Propionibacterium* sp. *Eur J Pharmacol* **496**, 189-195.
Patel-Predd P (2006) Fueling the future with citrus waste. *Environ Sci Technol* **40**, 5170-5171.
Sawai Y, Moon JH, Sakata K, and Watanabe N (2005) Effects of structure on radical-scavenging abilities and antioxidative activities of tea polyphenols: NMR analytical approach using 1,1-diphenyl-2-picrylhydrazyl radicals. *J Agric Food Chem* **53**, 3598-3604.
Solano F, Briganti S, Picardo M, and Ghanem G (2006) Hypopigmenting agents: an updated review on biological, chemical and clinical aspects. *Pigment Cell Res* **19**, 550-571.
Tripodo MM, Lanuzza F, Micali G, Coppolino R, and Nucita F (2004) Citrus waste recovery: a new environmentally friendly procedure to obtain animal feed. *Bioresour Technol* **91**, 111-115.
Virador VM, Kobayashi N, Matsunaga J, and Hearing VJ (1999) A standardized protocol for assessing regulators of pigmentation. *Anal Biochem* **270**, 207-219.
Wang KH, Lin RD, Hsu FL, Huang YH, Chang HC, Huang CY, and Lee MH (2006) Cosmetic applications of selected traditional Chinese herbal medicines. *J Ethnopharmacol* **106**, 353-359
Zhu YZ, Huang SH, Tan BK, Sun J, Whiteman M, and Zhu YC (2004) Antioxidants in Chinese herbal medicines: a biochemical perspective. *Nat Prod Rep* **21**, 478-489.