Journal of Bioscience and Bioengineering
VOL. 127 No. 1, 59–65, 2019

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Comparison of biofunctional activity of *Asparagus cochinchinensis* (Lour.) Merr. Extract before and after fermentation with *Aspergillus oryzae*

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Received 4 April 2018; accepted 20 June 2018
Available online 8 August 2018

Asparagus cochinchinensis **root (ACR) is used in traditional Chinese medicine. In this study, ACR was first extracted with 25% ethyl acetate (EA) and then fermented by *Aspergillus oryzae* to enhance its antioxidant activity and evaluate its potential antityrosinase activity. The physiological activity and cytotoxicity of *A. oryzae*-fermented ACR extract, along with its antityrosinase activity and effects on melanogenic factor levels in human epidermal melanocytes (HEMs), were analyzed and compared with those of the unfermented extract. The results showed that the physiological activity of the fermented extract in vitro or in cells was significantly higher than that of the unfermented extract. The IC_{50} values for 2,2-diphenyl-1-picrylhydrazine radical scavenging activity, reducing power, and antityrosinase activity in vitro for the fermented extract were 250.6 ± 32.5, 25.7 ± 3.5, and 50.6 ± 3.1 mg/L, respectively. The fermented extract favored cellular antityrosinase activity with low melanin production in human melanoma cells compared with the unfermented extract. The inhibitory mechanism of melanin synthesis by unfermented extract was independent of the tested melanogenesis-related proteins. However, the inhibitory mechanism of the fermented extract was possibly caused by synergistic inhibition of these proteins. Thus, *A. oryzae*-fermented ACR extract may be used for developing new health food or cosmetic ingredients.**

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[**Key words:** Antioxidant; Cytotoxicity; Fermentation; Melanogenesis; Tyrosinase]

The pursuit of having lighter skin is a global trend (1). In particular, Asian women prefer light skin, resulting in a great demand to develop safer and more effective skin-whitening agents (2). Factors considered in determining the human skin color and melanogenesis is the most crucial (3). It is a multistage process and can be regulated at the subcellular level, where the expression of various melanogenic enzymes, melanin transport, and melanosome release occur (4,5). Melanogenic enzymes include tyrosinase and tyrosinase-related proteins 1 (TRP-1) and 2 (TRP-2) (6). Among melanogenic enzymes, the expression of tyrosinase is the key factor affecting melanin formation. Tyrosinase is widely distributed in microorganisms, plants, and animals—including humans (7). Tyrosinase is a copper-containing monooxygenase and a polyphenol oxidase, found inside melanosomes (8). It is involved in the first two steps of melanin biosynthesis: it converts L-tyrosine or 3,4-dihydroxyphenylalanine eventually to melanin, for protecting skin from ultraviolet radiation (9,10). Overproduction of melanin pigment, particularly on the face, is undesirable for some people (11).

Chinese herbs are essential remedies used in oriental integrative medicine over hundreds of years for preventing and treating various diseases (12,13). Their low toxicity and various functions generate increasing interest (14). Chinese herbs are essential sources of various natural antioxidants. Some Chinese herbs possess higher antioxidant activity than do fruits and vegetables (15). Therefore, recent studies on the antioxidant activity of Chinese herbs have focused on the natural potential antioxidants (16). To increase the antioxidant or other biological activities of Chinese herbs, specific extraction processes can be applied (15). Various solvents, including acetone, ethanol, ethyl acetate, methanol, and water, have been used for the extraction of bioactive compounds (17). Although these herb extracts may have high biological activity, certain herb extracts are potentially cytotoxic to normal cells (18).

Fermentation may increase the physiological and biochemical activities of substrates by modifying their original molecules; therefore, fermented products may be more multifunctional than unfermented products (19,20). Successful fermentation often depends on strain selection. Appropriate microorganisms used for fermentation can not only metabolize herbs or herbal extracts but also increase their physiological activity and decrease their cytotoxicity (21). Some lactic acid bacteria, including *Lactobacillus* spp. and *Bifidobacterium* spp., and fungi, including *Aspergillus* spp., have been considered for this (20, 22–24). If fermented products have high physiological activity with low cytotoxicity, further purification of bioactive components is not required, because the benefits

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https://doi.org/10.1016/j.jbiosc.2018.06.015

of fermented products may stem from the synergistic effects of phytochemicals (23).

Asparagus cochinchinensis root (ACR), herbal medicine with high physiological activity, has been used for treatment of airway inflammatory disorders, lung disease, immune system diseases, and signs of aging (25,26). Moreover, solvent extracts of ACR have moderate antibacterial activity, antioxidant activity, anti-inflammatory effects, and anticancer effects (27,28). However, few studies have clarified the antimelanogenic effects of ACR extracts. In our previous studies, *Bifidobacterium bifidum* fermentation moderately improved the antioxidant activity of various solvent ACR extracts (17). Moreover, the melanogenic inhibitory effect of ACR extracts was evaluated on murine B16F10 cells (17). Nevertheless, the physiological activity of ACR should be improved by extracting with appropriate solvents and then fermenting with appropriate strains to meet the commercial demand; furthermore, its melanogenic inhibition in human melanocyte cells should be evaluated.

In this study, the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity, reducing power, tyrosinase inhibition, total phenolic contents, and phenolic composition of unfermented and fermented ACR extracts were analyzed and compared. The melanogenic inhibitory effects on various melanoma cell lines, and antimelanogenic mechanisms of the unfermented and fermented ACR extracts were firstly examined. To the best of our knowledge, this study is the first report to illustrate the difference between the antioxidant and tyrosinase inhibitory activities of ACR extracts before and after fermentation with *Aspergillus oryzae*. Moreover, the feasibility of fermented ACR extracts as safe antioxidants and more effective skin-whitening ingredients was confirmed.

MATERIALS AND METHODS

Chinese herbs, fungus, cells, and reagents *A. cochinchinensis* (Lour.) Merr. was purchased from a vendor on Dihua Street, Taipei City, Taiwan. *A. oryzae* (BCRC 32288), normal human skin fibroblast cell line CCD-966SK (ATCC CRL-1881), and human malignant melanoma cell line A375.S2 (ATCC CRL-1872) were obtained from the Bioresource Collection and Research Center (Hsinchu, Taiwan). HaCaT human keratinocytes were kindly provided from Professor GH Wang (Xiamen Medical College, China). HEMs (Cascade cat. C-102-5C, Cascade Biologics, Inc., Portland, OR, USA) obtained from neonatal foreskin were propagated in Medium 254 (cat. M-254-500) supplemented with human melanocyte growth supplement (cat. S-002-5, Cascade Biologics). Mushroom tyrosinase (350 U/mL), 2,2-diphenyl-1-picrylhydrazyl (DPPH), and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Cell culture reagents were purchased from Thermo Fisher Scientific (Waltham, MA, USA). MITF, Tyrosinase, TRP-1, TRP-2, and β-actin antibodies were purchased from Santa Cruz Biotechnology (Dallas, TX, USA). Other chemicals used in our experiment were analytical grade and obtained from Sigma–Aldrich.

Extraction and fermentation of A. cochinchinensis The dried root of *A. cochinchinensis* was ground into powder, and 100 g of 0.3-mm powder was extracted using 300 mL 25% EA at 40 °C for 2 h. The extraction procedure was conducted three times. The final extracts were filtered and concentrated using a rotary vacuum evaporator at 50 °C. The residue was then freeze-dried and refrigerated until further use.

A. oryzae was cultured in potato dextrose broth and incubated at 28 °C for 5 d. For fermentation, 200 mL of sterile broth containing 2 g of freeze-dried ACR extract was inoculated with 1 mL of *A. oryzae* spore suspension (2×10^8 spores/mL). These mixtures were then incubated at 28 °C under aerobic conditions. After 5 d of fermentation, the fermented solution was centrifuged at $8000 \times g$ for 10 min, and the supernatant was collected, filtered (0.45-μm filter), and concentrated in the rotary vacuum evaporator at 50 °C. The residue (*A. oryzae*-fermented ACR extract) was freeze-dried and stored at −20 °C.

Measurement of DPPH radical scavenging activity The scavenging activities of the DPPH radical of the unfermented and fermented ACR extracts were estimated according to a previous method (14). Different concentrations (100–1000 mg/L) of unfermented or fermented extracts (40 μL) in DMSO were added to 1 mM DPPH solution (80 μL) in methanol and mixed for 30 min in the dark. The absorbance of the mixture was measured spectrophotometrically at 517 nm. BHT was used as a positive control. The DPPH radical scavenging activity was calculated as:

$$\text{DPPH radical scavenging activity (\%)} = \left[1 - \left(\frac{\text{Abs sample}}{\text{Abs control}}\right)\right] \times 100 \quad (1)$$

Measurement of reducing power The reducing power of unfermented and fermented ACR extracts was determined through the method described by Jayanthi and Lalitha (29). The extracts (1 mL) at different concentrations were mixed with phosphate buffer solution (PBS, 1 mL, pH 6.6) and 1% potassium ferricyanide (1 mL). The mixture was kept at 50 °C for 20 min and then 1 mL of 10% trichloroacetic acid was added. Subsequently, the solution was centrifuged at $3000 \times g$ for 10 min. A mixture was created of 0.5 mL of the supernatant, 0.5 mL of distilled water, and 0.2 mL of 0.1% $FeCl_3$. After a 10-min reaction, the absorbance of the reaction solution was measured at 700 nm. BHA, ascorbic acid, and α-tocopherol were used as positive controls.

Measurement of tyrosinase inhibition and melanin content in HEMs The inhibition of mushroom tyrosinase activity was measured through a method modified from that described by Zheng et al. (30). The reaction mixture containing L-tyrosine (0.03%, 100 μL) dissolved in PBS, with or without ACR extract at different concentrations (50–1000 mg/L, 20 μL), was added to a 96-well microplate. Finally, 20 μL of mushroom tyrosinase solution was added, and the plate was incubated at 25 °C for 20 min in the dark. After incubation, absorbance was measured at 490 nm using a microplate in an Epoch ELISA reader (Bio-Tek Instruments, Winooski, VT, USA). α-arbutin and kojic acid were used as positive controls. The inhibition of tyrosinase activity by the tested sample was calculated as follows:

$$\text{Tyrosinase inhibition (\%)} = \frac{[(A-B)-(C-D)]}{(A-B)} \times 100\% \quad (2)$$

where A is absorbance at 490 nm without the extracts; B is absorbance at 490 nm without the extracts and enzyme; C is absorbance at 490 nm with the extracts and enzyme; and D is absorbance at 490 nm without the enzyme.

Tyrosinase inhibition in HEMs was analyzed according to the method of Lee et al. (31), with minor modifications. The HEMs (10^6 cells/mL) were incubated in a CO_2 incubator at 37 °C with 5% CO_2 for 24 h. Cells were grown in 24-well plates and treated with the ACR extracts at different concentrations. After 24-h treatment, the HEMs were collected, washed three times with phosphate-buffered saline, and lysed with a lysis solution including 0.1 M PBS, 1% Triton X-100, and protease inhibitors. The cells were sonicated at 4 °C and centrifuged at $10{,}000 \times g$ for 10 min. The lysates were added to 96-well plates, containing 2.5 mM L-DOPA in 0.1 M PBS in each well, and then incubated at 37 °C for 1 h. After incubation, absorbance was measured at 475 nm using a microplate in an Epoch ELISA reader (Bio-Tek Instruments).

Melanin contents in HEMs and A375.S2 were measured according to the method of Lin et al. (32), with few modifications. Melanoma cells (5×10^6 cells/wel1) were treated with various concentrations of the ACR extracts for 24 h. After washing with PBS, the cells were harvested through trypsinization. The cell pellets were treated with 1 N NaOH containing 10% DMSO at 80 °C for 1 h to dissolve melanin. The absorbance of the solutions was measured at 450 nm using an ELISA reader. The melanin content was measured by comparison with the synthetic melanin standard.

Analysis of phenolic compounds Total phenolic content in the unfermented and fermented ACR extracts was measured according to the method of Wang et al. (20), who used gallic acid as the standard. The extracts were mixed with 1 mL of the Folin–Ciocalteu reagent and 1 mL of 20% Na_2CO_3 solution and shaken. After 60 min of incubation, the absorbance of the mixture was measured at 725 nm using an UV–vis spectrophotometer (Shimadzu, Kyoto, Japan). Results were expressed as the gallic acid equivalent (mg-GAE/g-dried extract).

To determine the primary chemical compositions and concentrations of the fermented extracts, these extracts were first dissolved in 95% ethanol, filtered through a 0.45-μm nylon membrane filter, and injected into a high-performance liquid chromatography (HPLC) system (Hitachi, Tokyo, Japan). The HPLC analysis was followed the method used by Wang et al. (17). A 5-μm, 4.6 × 250-mm Econosil column was used, and column temperature was maintained at 20 °C. The acetonitrile and acidified water containing 3% formic acid as the mobile phase were introduced at a flow rate of 1-mL/min. Chromatograms were acquired at three wavelengths, namely 280, 330, and 350 nm, according to the absorption maxima of analyzed compounds. Each compound was identified by its retention time and by spiking with standards under the same conditions. The identities of constituents were confirmed with a photodiode array detector by comparison with the UV spectrum of standards in wavelengths of 200–450 nm.

Cytotoxicity assay Cytotoxicity effects of the unfermented or fermented ACR extracts on cell line CCD-966SK, HaCaT, HEMs, and A375.S2 were evaluated using the MTT assay (5). The CCD-966SK and HEMs were cultivated in minimum essential medium supplemented with 10% fetal bovine serum and Medium 254 supplemented with human melanocyte growth supplement, respectively. HaCaT cells were cultivated in Dulbecco's modified Eagle's medium supplemented with

10% fetal bovine serum and antibiotics (100 U/mL penicillin, and 100 μg/mL streptomycin). A375.S2 cells were cultivated in minimum essential medium, supplemented with 10% fetal bovine serum and antibiotics (100 U/mL penicillin, 100 μg/mL streptomycin). After 24 h of incubation, the cells (5×10^6 cells/wel1) were washed in fresh medium (control) and treated with the culture medium or ACR extracts for 24, 48, and 72 h at different concentrations. These cells were then rewashed and incubated with 0.02% MTT solution at 37 °C for 4 h. Afterward, the MTT solution was removed and 0.1 mL DMSO was added to solubilize the formazan crystals. The absorbance was measured at 570 nm using microplate in an Epoch ELISA reader (Bio-Tek Instruments). The viability of the cells was calculated using the following formula:

$$\text{Cell viability (\%)} = \left(\frac{\text{Abs sample}}{\text{Abs control}}\right) \times 100\% \quad (3)$$

Western blotting HEMs were treated with the unfermented and fermented ACR extracts. After 24 h, cells were washed with 1 × PBS, lysed in an optimal amount of protein extraction solution on ice for 30 min, and centrifuged at 12,000 × *g* for 5 min; supernatant protein was quantified using Bradford assay reagent. Proteins (50 μg) were resolved by SDS-polyacrylamide gel electrophoresis and electrophoretically transferred to a polyvinylidenefluoride membrane at 20 V for 1 h. The membrane was blocked in 1% BSA solution for 1 h. After a brief wash using the PBST buffer, the membrane was incubated with anti-MITF (1:2000), antityrosinase (1:5000), anti-TRP-1 (1:2000), anti-TRP-2 (1:2000), and β-actin antibodies (1:2000) overnight at 4 °C. The membranes were then washed with PBST buffer and incubated antimouse HRP-conjugated secondary antibody (1:10,000) for 2 h at room temperature. Subsequently, the proteins were visualized using an enhanced chemiluminescence detection system (Santa Cruz Biotechnology). β-Actin was used as the internal control. The protein bands were detected and scanned using a MultiGel-21 gel image system (Topbio Co., New Taipei City, Taiwan).

Statistical analysis All tests were performed in triplicate. The experimental data were expressed as means ± SEM. Statistical analysis was performed with one-way ANOVA followed by Tukey's *post hoc* test for multiple comparisons tests. Mean differences were considered significant when $P < 0.05$. The IC_{50} values were calculated by using Origin software.

RESULTS AND DISCUSSION

Antioxidant activity through DPPH scavenging activity Chinese herbs are sources of various phytochemicals, many of which possess vital antioxidant activity (33). *A. cochinchinensis* possesses antibacterial, anticancer, and antioxidant activity (34,35). *Aspergillus* spp., *Monascus* spp., *Bifidobacterium* spp., and *Lactobacillus* spp. were recently inoculated into substrates to increase antioxidant activity of the products (20,22,36). The DPPH scavenging activities of the unfermented and fermented ACR extracts are shown in Fig. 1. The DPPH scavenging activities of the fermented extracts at 100–1000 mg/L were much higher than those of the unfermented extracts. The IC_{50} values for the unfermented and fermented extracts were 586.9 ± 86.1 and 250.6 ± 32.5 mg/L, respectively. Moreover, the DPPH radical scavenging activity of fermented extracts was significantly higher than that of 1105.3 ± 285.6 mg/L BHT, the positive control. The IC_{50} values of water ACR extract, *B. bifidum*-fermented 50% EA extract, *B. bifidum*-fermented 100% EA extract, and *Weissella cibaria*-fermented butanol extract (BEW) are 1800, 330, 400, and 31.62 mg/L, respectively (17,26,35). Extraction solvents and microbial strains influence the antioxidant activity of ACR by producing new ingredients or increasing active compound concentrations. Although the DPPH scavenging activity of BEW was superior to that of *A. oryzae*-fermented 25% EA extract (this study), the antityrosinase activity of BEW remains unknown.

Reducing power assay Reducing power may be an indicator of potential antioxidant properties (37). The antioxidant activity of a tested sample could interrupt free radical processes by donating a hydrogen atom (38). The sample concentration providing 0.5 of absorbance is called IC_{50}, and a low IC_{50} value indicates high antioxidant activity. Fig. 2 shows the effects of different concentrations on the reducing power of the unfermented and fermented extracts. A sample with higher absorbance always indicates a sample with higher reducing power. The reducing power of the unfermented and fermented extracts increased with the tested concentrations. The IC_{50} values of reducing power for unfermented and fermented extracts were 59.0 ± 6.2 and 25.7 ± 3.5 mg/L, respectively. The reducing power of fermented ACR extracts was higher than that of 35.4 ± 2.6 mg/L BHA, 76.8 ± 6.2 mg/L ascorbic acid, and 53.2 ± 3.7 mg/L α-tocopherol, the positive control groups. Furthermore, it was significantly superior to that of the products (IC_{50} = 980 ± 70 to 3890 ± 70 mg/L) obtained by extracting ACR with various solvents and then fermenting with *B. bifidum* (17). Thus, fermentation was not a panacea; appropriate microbial strains are necessary to obtain high physiological activity. According to the results, the fermented ACR extract obtained using 25% EA before fermentation with *A. oryzae* was a potential antioxidant.

Analysis of antityrosinase activity and phenolic composition and contents The effects of different concentrations on mushroom tyrosinase activity of the unfermented and fermented ACR extracts are depicted in Fig. 3. Antityrosinase activity of fermented

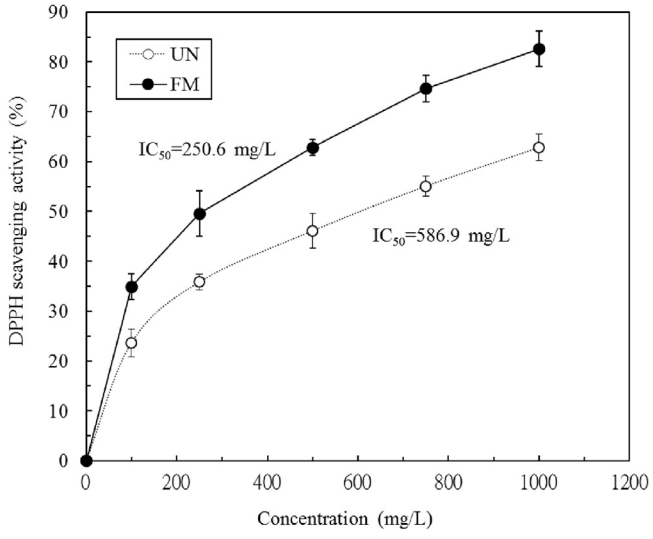

FIG. 1. Effects of different concentrations on the DPPH radical scavenging activity of unfermented (UN) and fermented (FM) ACR extracts. ACR was first extracted using 25% EA and fermented by *A. oryzae* for 5 d.

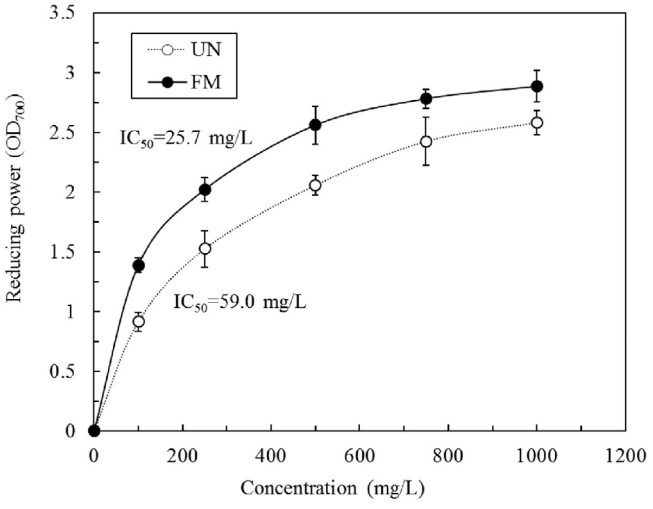

FIG. 2. Effects of different concentrations on the reducing powder of unfermented (UN) and fermented (FM) ACR extracts. ACR was first extracted using 25% EA and fermented by *A. oryzae* for 5 d.

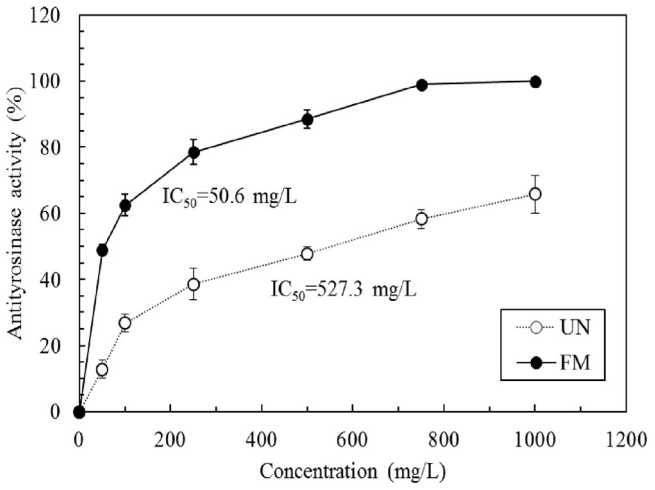

FIG. 3. Effects of different concentrations on mushroom tyrosinase activity inhibition of unfermented (UN) and fermented (FM) ACR extracts. ACR was first extracted using 25% EA and fermented by *A. oryzae* for 5 d.

extracts was significantly higher than that of unfermented extracts. The highest antityrosinase activity of fermented extracts was found at 750 mg/L with 99.0% ± 0.2%, much higher than that of the unfermented extracts (65.8% ± 5.6%). The IC_{50} values of the antityrosinase activity for unfermented and fermented extracts were 527.3 ± 23.6 and 50.6 ± 3.1 mg/L, respectively. By contrast, the IC_{50} values of the antityrosinase activity for the positive controls kojic acid and α-arbutin were 18.6 ± 0.5 and 261.4 ± 12.8 mg/L, respectively.

To understand possible reasons for the increase in the physiological activity of the *A. oryzae*-fermented ACR extracts, the phenolic composition and contents of the ACR extracts were analyzed. Results indicated the total phenolic contents of the unfermented and fermented extracts were 71.6 ± 1.8 and 186.3 ± 4.2 mg-GAE/g-dried extract, respectively. The phenolic content in fermented extract was 2.6 times that of the unfermented extract. The increases in the total phenolic content of the ACR extracts following fermentation are consistent with findings for soybeans (39). Thus, ACR extract fermentation favored phenolic compound formation, which through creation of new ingredients or increasing active compound concentrations significantly enhanced its physiological activity (antioxidant and antityrosinase activity). The previously reported phenolic contents of aqueous extract of ACR (26), *B. bifidum*-fermented 50% ethanol ACR extract (17), and BEW (35) were 0.459, 154.6 and 2.0 mg-GAE/g-dried extract, respectively. By contrast, our fermented extracts exhibited considerably higher phenolic content. According to HPLC analysis (Fig. 4), the fermented extract contained 15 phenolic compounds at least, and the primary phenolic compounds were rosmarinic acid (1020 ± 15.6 μg/g-dried extract), apigenin (805 ± 9.6 μg/g-dried extract), caffeic acid (670 ± 5.7 μg/g-dried extract), and benzoic acid (260 ± 1.3 μg/g-dried extract), which together accounted for 90% of the phenolic compounds in the product. These four phenolic compounds can be antioxidants or potential skincare ingredients (40–43). Thus, the high total phenolic concentrations of major phenolic compounds in the fermented ACR extracts increased its antioxidant and antityrosinase activities.

Two possible reasons of the increase in antioxidant and antityrosinase activities after fermentation were speculated: ACR extract was degraded and produced new ingredients or increased active compound concentrations (hypothesis 1); the ACR extract acted an inducer to improve the *A. oryzae* produced the antioxidant and anti-tyrosinase activity, or the more activities were only from *A. oryzae* (hypothesis 2). According to the results of the pre-test, potato dextrose broth (PDB) and the *A. oryzae*-fermented PDB did not exhibit antioxidant and antityrosinase activities. *A. oryzae* was difficult to survive in ACR extract solution alone. Based on analysis of phenolic composition and contents in the unfermented and fermented ACR extract, hypothesis 1 was suggested. Besides, an additional experiment was conducted by adding different ACR extracts in *A. oryzae*-fermented broth to clarify hypothesis 2. Results indicated the increase in the antioxidant and antityrosinase activities of broth with the increasing ACR extracts was not observed. Thus, we think fermentation increase the physiological and biochemical activities of substrates by modifying their original molecules.

Assessment of cell viability MTT assay is a common colorimetric assay to measure the cytotoxicity of potential toxic compounds potentially affecting cell viability and growth (44). For user safety, the cytotoxic effects of ACR extracts on cells should be assessed. The possible cytotoxic effects of the unfermented and fermented ACR extracts were evaluated at different concentrations (100–1000 mg/L) in four cell lines, the CCD-966SK, HaCaT, HEMs, and A375.S2, for 72 h. Fig. 5 indicates the cell viability exceeded 95.6% ± 1.2% and 94.5% ± 0.8% for human skin cells (Fig. 5A) and human melanoma cells (Fig. 5B), respectively; cytotoxicity was nonsignificantly different compared with the control ($P < 0.05$). A nonsignificant dose-dependent trend was observed on the tested cells. The IC_{50} values for DPPH radical scavenging activity, reducing power, and antityrosinase activity of the unfermented and fermented extracts were 59.0 ± 6.2–586.9 ± 86.1 and 25.7 ± 3.5–250.6 ± 32.5 mg/L, respectively. At these concentrations, ACR extracts would either not or mildly inhibit cell viability, even if 100% physiological activity is achieved. Thus, the inhibitory activity of the ACR extracts is independent of the cytotoxic effect on HEMs, and the fermented ACR extracts are safe for industrial application.

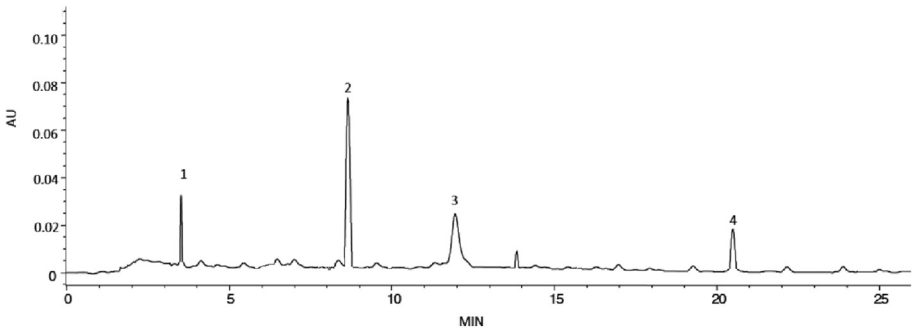

FIG. 4. HPLC chromatogram of the free phenolic compounds from fermented ACR extracts. Peak identification: peak 1, caffeic acid; peak 2, rosmarinic acid; peak 3, apigenin; peak 4, benzoic acid.

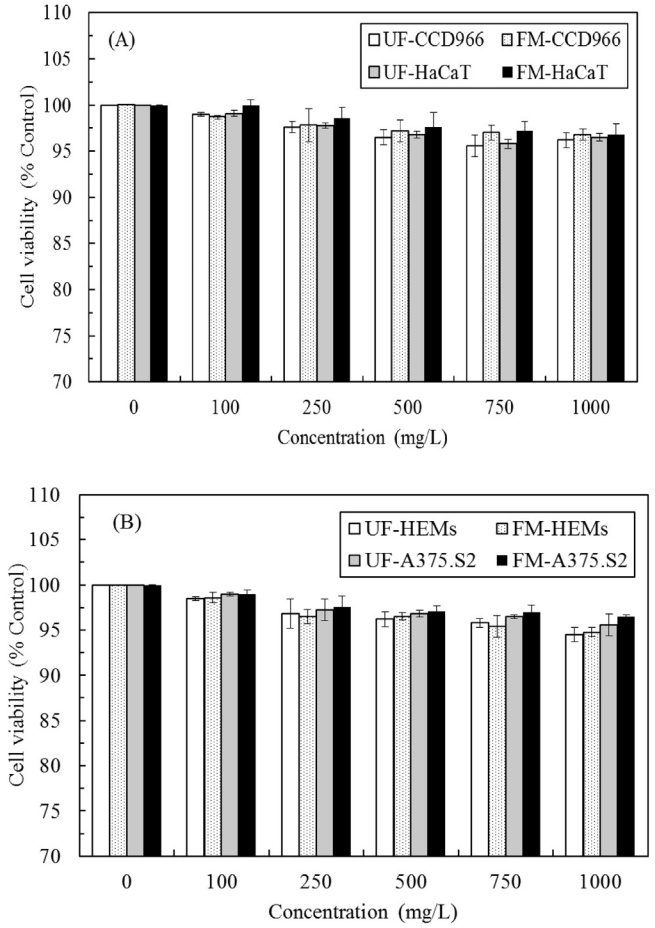

FIG. 5. Effects of unfermented (UN) and fermented (FM) ACR extracts on the viability of (A) human normal cells and (B) human melanoma cells. Cells were treated with various concentrations of ACR extracts for 72 h, and analyzed using the MTT assay. Data are expressed as the means standard deviations of 3 independent experiments.

Antityrosinase activity and melanin content in HEMs and A375.S2 The third section detailed the in vitro mushroom tyrosinase inhibitory effects of the unfermented and fermented ACR extracts. A study used B16F10 melanoma cells to evaluate cellular tyrosinase inhibition of active compounds (17). However, cellular tyrosinase inhibitory should be evaluated in human melanocytes to approximate the effects on human body better than the in vitro mushroom test (17,45). Fig. 6A shows the antityrosinase activity in HEMs and A375.S2 of the unfermented and fermented ACR extracts with different concentrations. Cellular antityrosinase activity increased with the concentration of extracts. Antityrosinase activity was 23.6% ± 2.0%–28.4% ± 1.3% and 48.6% ± 1.8%–55.8% ± 2.3% for the unfermented and fermented extracts, respectively, at the 1000-mg/L concentration. At this concentration, the ACR extracts were not cytotoxic to the cell (see the previous section at 1000 mg/L). However, the cellular antityrosinase activity in HEMs of the fermented extract (IC_{50} = 717.3 ± 32.1 mg/L) was significantly lower than its mushroom antityrosinase activity (IC_{50} = 50.6 ± 3.1 mg/L). Therefore, the high mushroom antityrosinase activity was not reflected in melanocytes.

Fig. 6B shows the melanin content in HEMs and A375.S2 after exposure to unfermented and fermented ACR extracts at different concentrations. The inhibition of melanin production in HEMs and A375.S2 was dose-dependent. The extent of inhibition of melanin production was proportional to the antityrosinase activity of unfermented extract; however, the extent of inhibition of melanin

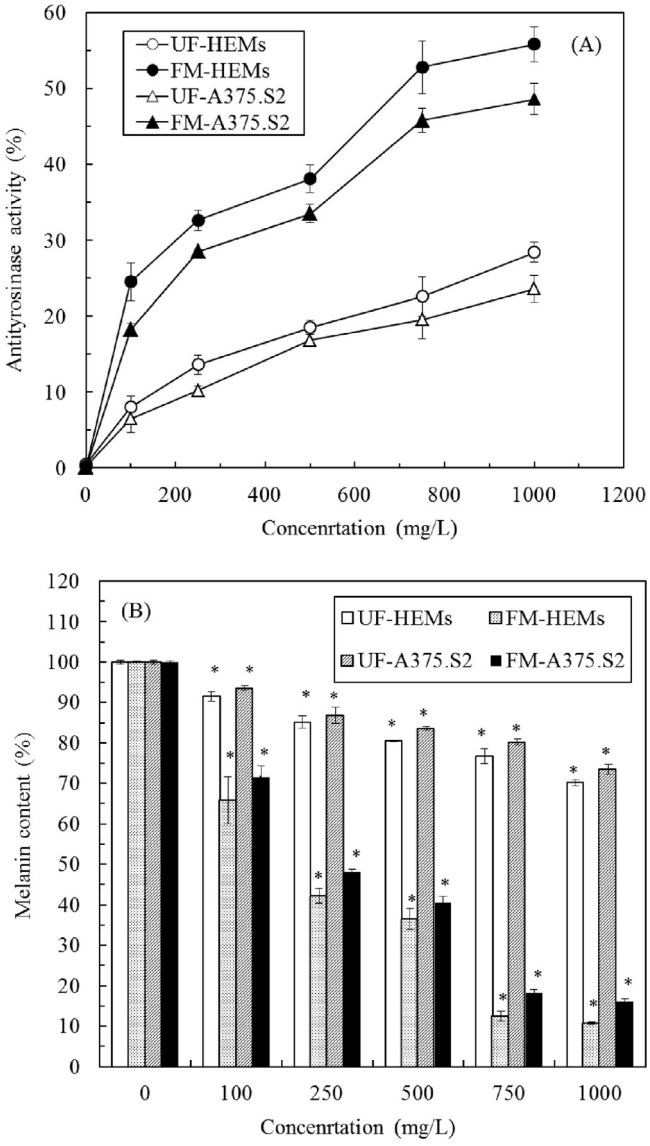

FIG. 6. Effects of unfermented (UN) and fermented (FM) ACR extracts on (A) antityrosinase activity in human melanoma cells and (B) melanin content of human melanoma cells. Cells were treated with various concentrations of ACR extracts for 24 h. Data are expressed as the means standard deviations of 3 independent experiments (*$P < 0.05$ vs. blank control).

production was much higher than the antityrosinase activity of fermented ACR extract by calculating the theoretical melanin content, according to the results in Fig. 6A. The inhibition in HEMs of the *A. oryzae*-fermented ACR extracts at 100, 250, 500, 750, and 1000 mg/L increasingly reduced melanin content to 65.8% ± 5.1%, 42.3% ± 1.8%, 36.5% ± 2.2%, 12.5% ± 1.0%, and 10.8% ± 0.3%, respectively. Similar inhibition tendency was also observed when A375.S2 melanoma cells were tested. It suggests that the inhibitory mechanism of unfermented extract is directly related to tyrosinase or tyrosinase-related protein. By contrast, the inhibitory mechanism of the fermented extract is more complicated and may involve mRNA, tyrosinase, or related proteins (32). To the best of our knowledge, this study is the first to evaluate the antityrosinase activities of unfermented and fermented ACR extracts in human melanoma cells.

Effect of ACR extracts on melanogenesis-related protein expression in HEMs Tyrosinase is crucial for melanin synthesis. TRP-1 and TRP-2 are two related enzymes involved in the

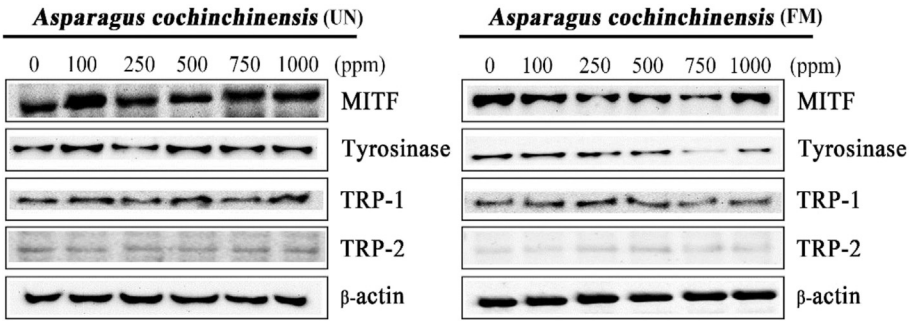

FIG. 7. Effects of unfermented (UM) and fermented (FM) ACR extracts on the protein levels of melanogenic factors in HEMs cells. ACR was first extracted using 25% EA and fermented by *A. oryzae* for 5 d.

melanogenesis pathway (11). MITF acts as a transcription factor for tyrosinase (10). To understand the melanin synthesis inhibition mechanism of unfermented and fermented ACR extracts, tyrosinase, TRP-1, TRP-2, and MITF levels were assayed though Western blotting. A study used murine melanoma cell line B16F10 to evaluate the antimelanogenic effect of active compounds because they are relatively easy to culture (46). A recent study also has focused on HEMs (32,45).

Because ACR extracts inhibited the melanin formation in HEMs was slightly superior to that in A375.S2 (Fig. 6B), the effects of ACR extracts on the protein levels of melanogenic factors in HEMs were evaluated. Fig. 7 shows MITF, tyrosinase, and TRP-1 levels were nonsignificantly affected by unfermented extracts; however, fermented ACR extracts in the range of 100–750 mg/L reduced MITF, tyrosinase, and TRP-1 levels in a dose-dependent manner. The difference in TRP-2 expression was nonsignificant. Although melanin production was related to the antityrosinase activity of the unfermented extract (Fig. 6), the same phenomenon was not observed in the protein levels of melanogenic factors in HEMs. Thus, the inhibitory mechanism of melanin synthesis by unfermented extracts may be attributable to regulation of other signaling pathways (e.g., MAPK/ERK or SAPK/JNK pathway) (47). Moreover, the inhibitory mechanism of fermented extracts in concentrations from 100 to 750 mg/L may be caused by MITF degradation, tyrosinase and TRP-1 downregulation, and melanin production inhibition. An additional detailed study on the inhibitory mechanism will be conducted in future.

The effects of unfermented and fermented ACR extracts on in vitro biofunctional activity, cytotoxicity, and melanogenic protein expression in HEMs were compared for the first time. The *A. oryzae*-fermented ACR extracts improved various physiological activities and total phenol content of the ACR extracts. This is attributable to the biosynthesis of new active compounds or an increase in concentration of original active compounds during fermentation. The fermented extract exhibited relatively higher reducing power than do some well-known antioxidants. In addition, it exhibited relatively higher antityrosinase activity than did α-arbutin. The mechanism of inhibition of melanin synthesis by the unfermented extract is attributable to regulation of other signaling pathways, not melanogenic enzymes. By contrast, the underlying mechanism of the fermented extract is the synergistic inhibition of the expression of MITF, tyrosinase, and TRP-1. Few reports have shown that ACR extracts can be applied to reduce melanogenesis. These findings suggest that fermented ACR extracts can be used as natural antioxidants and in skin-whitening cosmetics.

ACKNOWLEDGMENTS

The work was partially supported by Grant NSC 100–2632-B-157-001-MY3 from the Ministry of Science and Technology.

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