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# Evaluation of tyrosinase inhibitory and antioxidant activities of *Angelica dahurica* root extracts for four different probiotic bacteria fermentations

Guey-Horng Wang,[1,‡] Chih-Yu Chen,[2,‡] Teh-Hua Tsai,[3] Ching-Kuo Chen,[3] Chiu-Yu Cheng,[4] Yi-Hsin Huang,[4] Min-Chi Hsieh,[4] and Ying-Chien Chung[4,*]

*Fujian Provincial Key Laboratory of Biological Engineering on Traditional Herbs and Research Center of Natural Cosmeceuticals Engineering, Xiamen Medical College, No. 1999, Guankou Road, Xiamen, Fujian 361023, China,[1] Department of Tourism and Leisure, Hsing Wu University, No. 101, Sec. 1, Fenliao Rd., New Taipei City 24452, Taiwan,[2] Department of Chemical Engineering and Biotechnology, National Taipei University of Technology, No. 1, Sec. 3, Zhongxiao E. Rd., Taipei City 10608, Taiwan,[3] and Department of Biological Science and Technology, China University of Science and Technology, No. 245, Sec. 3, Academia Rd., Taipei City 11581, Taiwan[4]*

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***Angelica dahurica* root (ADR), which shows strong antioxidant activity, is used in Chinese medicine. This study evaluated the tyrosinase inhibitory and antioxidant activities of ADR extracts fermented by four different probiotic bacteria: *Bifidobacterium bifidum*, *Bifidobacterium lactis*, *Lactobacillus acidophilus*, and *Lactobacillus brevis*. The ADR was first extracted using distilled water, 70% ethanol, and ethyl acetate, and then fermented by probiotic bacteria. The physiological characteristics of these fermented extracts, namely the antityrosinase activity, antioxidant activity, phenolic composition, and phenolic content, were evaluated and compared with those of unfermented extracts. Results showed that the water extracts after fermentation by probiotic bacteria exhibited the most favorable physiological characteristics. Among the extracts fermented by these probiotic bacteria, *L. acidophilus*-fermented ADR extract showed the most favorable physiological characteristics. The optimal $IC_{50}$ values for antityrosinase activity, DPPH radical scavenging activity, and reducing power for *L. acidophilus*-fermented ADR extract were 0.07 ± 0.03, 0.12 ± 0.01, and 0.68 ± 0.06 mg/mL, respectively. Furthermore, the physiological activities of fermented extracts were considerably higher than those of unfermented extracts. The tyrosinase inhibition and melanin content of B16F10 melanoma cells, and cytotoxicity effects of the fermented ADR extracts on B16F10 cells were also evaluated. We found that the *L. acidophilus*-fermented ADR extract at 1.5 mg/mL showed significant cellular antityrosinase activity with low melanin production in B16F10 cells and was noncytotoxic to B16F10 cells. Among all probiotic bacteria, water-extracted ADR fermented by *L. acidophilus* for 48 h was found to be the best skincare agent or antioxidant agent.***



[**Key words:** B16F10 cell; Fermentation; Melanin; Antioxidant activity; Tyrosinase inhibition]

The distribution of pigments throughout an organism's body results in the different colors of different organisms. Pigmentation in the human body is regulated by genetic and environmental factors that vary the amount and distribution of the melanin (1). Melanin serves an important role in protecting human skin against harmful ultraviolet radiation, but pigmentation disorders can cause abnormal accumulations of melanin (2). Melanogenesis, the creation of melanin, can be controlled in several ways, including through the inhibition of tyrosinase gene expression and inactivation of the related enzymes (3). The use of tyrosinase inhibitors has become more prominent in medication and in cosmetics to prevent hyperpigmentation by inhibiting the function of tyrosinase. Natural products with tyrosinase-inhibiting activity have become potential sources of skin whiteners (4). Antioxidants are good inhibitors of tyrosinase activity and melanin production. Actually, certain antioxidants have been applied as melanogenesis inhibitory agents (5). For example, Carthamus yellow-the major component of the yellow pigments of *Carthamus tinctorius* L., showed the anti-oxidation and anti-melanogenesis activities (4).

Fermentation can increase the physiological and biochemical activities of biological substrates by modifying their original molecules (6). That is, fermentation can be used to produce new compounds as novel pharmaceutical and cosmeceutical agents. Fermentation with various species of microorganisms has the potential to decrease or eliminate an herbal extract's cytotoxicity (7,8). Also, many fermented natural products have proven their usefulness in promoting gastrointestinal health and skincare (9).

Chinese herbs are rich sources of bioactive chemicals without harmful side effects (10,11). There is an increased interest in finding natural tyrosinase inhibitors from herbs and applying them as skin care products. Baizhi, the dried root of *Angelica dahurica*, is an important herbal medicine showing strong antioxidant activity that has been used as an antipyretic and analgesic for cold, headaches, coryza, hypertension, and toothaches (12,13). Additionally, *A. dahurica* has shown a strong melanogenic inhibitory effect on B16F10 cells (14). Zheng et al. (15) has further shown the anticancer effects of the active compounds extracted from *A. dahurica root* (ADR).

* Corresponding author. Tel.: +886 2 26086593; fax: +886 2 26086523.
*E-mail address:* ycchung@cc.cust.edu.tw (Y.-C. Chung).
‡ The first two authors contributed equally to this work.

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In our previous studies, it was shown that fermentation with *Bifidobacterium bifidum* significantly improved the tyrosinase inhibitory and antioxidant activities of walnut, Moutan Cortex Radicis, and asparagus root extracts, demonstrating the ability to produce different bioactive compounds by different metabolic pathways through fermentation using various bacterial strains. In this study, the tyrosinase inhibitory and antioxidant activities of the ADR extracts fermented by four different probiotic bacteria were evaluated. The probiotic bacteria were the *Lactobacillus* and *Bifidobacterium* genera. These probiotic bacteria are some of the most important taxa involved in food microbiology and human nutrition as a result of their role in food fermentation, production, and preservation (16). In this study, the reducing powers, phenolic compositions, and phenolic contents of the fermented extracts were also analyzed. Furthermore, the cytotoxic effects and cellular tyrosinase inhibition of the fermented extracts on B16F10 melanoma cells were examined. To the best of our knowledge, this study is the first to evaluate the antioxidant and tyrosinase inhibitory activities of ADR extracts fermented by probiotics.

## MATERIAL AND METHODS

**Chinese herbs, the probiotic bacteria, tested cells, and tyrosinase** The ADR (Fisch. ex Hoffm.) Benth. et Hook. f. was purchased from a vendor on Dihua Street, Taipei City, Taiwan. The four strains of probiotic bacteria, *B. bifidum* (American Type Culture Collection (ATCC) number: 29521), *Bifidobacterium lactis* (ATCC number: 25741), *Lactobacillus acidophilus* (ATCC number: 4356), *Lactobacillus brevis* (ATCC number: 8287), murine melanoma cell line B16F10 (ATCC number: CRL-6475), and normal human skin fibroblast cell line CCD-966SK (ATCC number: CRL-1881) were purchased from the Bioresource Collection and Research Center (Hsinchu, Taiwan). Mushroom tyrosinase was purchased from Sigma Chemical Co. (St. Louis, USA).

**Extraction and fermentation of *A. dahurica*** The dried root of *A. dahurica* (0.5 kg) was extracted using three solvents: distilled water, 70% ethanol, and ethyl acetate. First, the solvent containing 0.3 mm ADR powder was sonicated at 40°C for 2 h. Then, the extracts were filtered and concentrated in a rotary vacuum evaporator at 50°C. The residue was freeze-dried and then refrigerated until further use. The probiotic bacteria used in our research were cultured according to our previous reports (17). The ingredients of the culture medium were as follows: tryptone 15 g/L, meat extract 2.5 g/L, yeast extract 7.5 g/L, $K_2HPO_4$ 4.5 g/L, cysteine HCl 0.05 g/L, lactose 2.5 g/L, glucose 7.5 g/L, and Tween 80 mL/L. The pH of the broth was adjusted to 6.5 ± 0.1 by 0.1 N HCl or NaOH and incubations were conducted under anaerobic conditions at 37°C. For fermentation, the pH of the solution (200 mL) containing the herb extracts (0.2 g) was adjusted to 6.5 ± 0.1 before 1 mL of the individual probiotic culture was inoculated (with *B. bifidum*, *B. lactis*, *L. acidophilus*, and *L. brevis*). The initial number of cells of the inoculation was 2.2 ± 0.4 × $10^7$ cfu/mL. These mixtures were incubated under anaerobic conditions and incubation temperature was controlled at 37°C using a water bath. The optimal fermentation periods for various herb extracts were evaluated by their antityrosinase activities.

**Analysis of crude extracts** After fermentation, the solution was centrifuged at 8000 ×*g* for 25 min, and the supernatant was collected, filtered, and concentrated in the rotary vacuum evaporator at 50°C. The residues were freeze-dried and stored under refrigeration. To analyze their chemical compositions, the fermented and unfermented extracts were first dissolved in 70% ethanol, transferred to vials, and filtered through a 0.45-μm nylon membrane filter before injection into a high-performance liquid chromatography (HPLC) system (Hitachi, Japan).

**Analysis of antityrosinase activity** The antityrosinase activities of the fermented and unfermented ADR extracts were analyzed using the method reported by Zheng et al. (18). First, the herb extracts were dissolved in a dimethyl sulfoxide (DMSO) solution (1 g/L) and diluted to different concentrations further using DMSO. Second, a 30-μL mixture was removed and mixed with 970 μL sodium phosphate buffer (0.05 mM) and subsequently added to 1 mL of 100 mg/L l-tyrosine and 1 mL of mushroom tyrosinase solution (350 units/mL). All 3 mL of this reaction solution was homogeneously mixed and the absorbance was measured at 490 nm using a UV−vis spectrophotometer (Shimizu, Japan). The absorbance of the solution was measured at 490 nm again after 20 min of incubation. The concentration, at which half the original tyrosinase activity was inhibited ($IC_{50}$), was calculated for fermented and unfermented extracts. Antityrosinase activity of the ADR extracts is expressed as a percentage of tyrosinase inhibition using the following formula:

$$\text{Tyrosinase inhibition}(\%) = \frac{[(A-B)-(C-D)]}{(A-B)} \times 100 \quad (1)$$

where *A* is the absorbance at 490 nm without the herb extracts (control), *B* is the absorbance at 490 nm without the herb extracts and enzyme (blank), *C* is the absorbance at 490 nm with the herb extracts and enzyme (experimental group), and *D* is the absorbance at 490 nm without the enzyme (blank of *C*).

Antityrosinase activity in B16F10 murine melanoma cells was analyzed using the method reported by Peng et al. (2). The B16F10 cells were cultured in culture flasks in a $CO_2$ incubator in an atmosphere of 5% $CO_2$ at 37°C, and supplemented with 10% fetal bovine serum (Sigma, USA). Cells were grown in 24-well plates and treated with the fermented ADR extracts at different concentrations for 48 h. The B16F10 cells ($10^6$ cells/mL) were collected through trypsinization and washed 3 times with ice-cold phosphate-buffered saline (PBS). A lysis buffer included 0.1 M sodium phosphate buffer (pH 6.8) containing 0.1% Triton-X and protease inhibitors. The cells were sonicated at 4°C and centrifuged at 15,000 rpm for 30 min. The reaction mixtures containing the ADR extracts, 2 mg/mL l-DOPA, and a 0.1 M sodium phosphate buffer (pH 7.0) were mixed in a 96-well plate and cultivated at 37°C for 2 h. The activity of mammalian tyrosinase used in the studies was diluted to 350 units per milliliter, which was the same as that of the mushroom tyrosinase. Absorbance was analyzed at 450 nm using an ELISA plate reader.

**Analysis of antioxidant activity** The scavenging activity of the 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical was used as an index of the antioxidant activity levels of the ADR extracts and was estimated according to Chen et al. (19). The DPPH solution at 100 μM was prepared in pure ethanol (97%). ADR extracts at different concentrations (1 mL) were individually added to ethanol (1 mL) and the DPPH solution (500 μL). The absorbance of this mixture was read at 517 nm using a UV−vis spectrophotometer versus a blank without the ADR extracts after 1-h incubation at 25°C in the dark. The scavenging activities of the DPPH radical of the fermented and unfermented extracts were calculated in the following way:

$$\text{DPPH scavenging activity}(\%) = \left(\frac{A_0 - A}{A_0}\right) \times 100 \quad (2)$$

where $A_0$ is the absorbance of the blank (without extract) and *A* is the absorbance of the test compound. The $IC_{50}$ of the DPPH radical by the fermented or unfermented extract was evaluated at 50% scavenging activity.

**Analysis of reducing power** The ferric reducing power method was applied using the method described by Fejes et al. (20). Various concentrations of the ADR extracts (1 mL) were mixed with 2.5 mL of 0.2 M phosphate buffer and 2.5 mL of 1% potassium ferricyanide. The mixture was incubated at 50°C for 20 min and then 2.5 mL of 10% trichloroacetic acid was added. After that, the reaction solution was centrifuged at 4000 ×*g* for 20 min and the supernatant of the reaction solution was collected. A 2.5 mL sample of the upper layer was mixed with 0.5 mL of 0.1% ferric chloride and 2.5 mL of deionized water. The absorbance of the solution was measured at 700 nm using a UV−vis spectrophotometer after 10 min of mixing. The concentrations of ADR extracts providing an absorbance of 0.5 (i.e., $IC_{50}$) were calculated from the graph of absorbance at 700 nm versus the concentrations of ADR extracts in the solution.

**Analysis of phenolic compounds** Total phenolic contents in the unfermented and fermented extracts were estimated as gallic acid equivalents essentially according to the description of Zheng and Wang with minor modifications (21). ADR extracts were first mixed with 1 mL of a Folin−Ciocalteu phenol reagent (Sigma) and 1 mL of a $Na_2CO_3$ solution (20%), and then the mixture was shaken for 10 min. Absorbance was measured at 725 nm using a UV−vis spectrophotometer. The results were expressed as the gallic acid equivalent (mg-GAE/g-dried extract).

To determine total phenolic content in the unfermented and fermented extracts, their phenolic compositions were also analyzed using an HPLC method modified from Li et al. (22). A 5-μm and 4.6 × 250-mm Econosil column was used and the flow rate and injection volume of tested samples were 0.5 mL/min and 40 μL, respectively. The separation was performed isocratically with a mobile phase consisting of 0.1% (v/v) water acetic acid and acetonitrile (4:1). The detection wavelength was set at 330 nm. Individual phenolic compounds were collected and identified by comparing their retention times against those of the standard samples.

**The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay** The cytotoxicity of the ADR extracts fermented by *L. acidophilus*, and *L. brevis* on B16F10 cells were assessed through the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method. The cell viability assay was modified from that in the studies described by Wodnicka et al. (23). After 12-h incubation, the cells (3 × $10^6$ cells/wel1) were washed in fresh medium and treated with the culture medium or different concentrations of fermented ADR extracts. After 72-h incubation, the B16F10 cells and the CCD-966SK cells were rewashed and incubated in a 5% $CO_2$ incubator with 1 g/L MTT solution (0.05 mL) at 37°C for 4 h in the dark. Afterward, the solution was carefully discarded and 0.1 mL DMSO was added. After a 10-min reaction, the absorbance was measured at 540 nm using a microplate reader (Plate Chameleon V, Hidex, Finland). The number of viable cells was counted using a hemocytometer and the number of viable cells after each treatment was expressed as a percentage of the control.

**Cellular melanin content** Melanin content in the B16F10 cell was measured according to the method of Hosoi et al. (24) with slight modifications. B16F10 melanoma cells (2 × $10^6$ cells/well) were seeded in 6-well culture plates and

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