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Tyrosinase inhibitory and antioxidant activities of three *Bifidobacterium bifidum*-fermented herb extracts

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ABSTRACT

Chinese herb extracts can potentially be used for developing new cosmetic or health food ingredients. This study evaluates the tyrosinase inhibitory and antioxidant activities of *Bifidobacterium bifidum*-fermented extracts of 3 Chinese herbs: walnut, Moutan Cortex Radicis (MCR), and asparagus root. First, the herbs were extracted using distilled water, 95% ethanol, 50% ethanol, 100% ethyl acetate, and 50% ethyl acetate. These extracts were further fermented using *B. bifidum* for different fermentation periods, and were evaluated for their tyrosinase inhibition activities, phenolic composition and content, and antioxidant activities. To understand user safety and the preliminary tyrosinase inhibitory mechanism of these extracts, we evaluated their cytotoxicity by analyzing the viability of normal skin fibroblast cells, CCD-966SK, and murine melanoma cells, B16F10. The 50% ethanol extracts of all 3 herbs exhibited the highest tyrosinase inhibition activities, phenolic content, and antioxidant activities. Furthermore, the physiological activities of fermented extracts were considerably higher than those of nonfermented extracts. The optimal IC_{50} values for tyrosinase inhibition for fermented walnut, MCR, and asparagus root extracts were 420, 380, and 260 μg/mL, respectively. Even at 300–900 μg/mL, all 3 fermented extracts examined were noncytotoxic to both CCD-966SK and B16F10 cells. Among all of the fermented herb extracts, asparagus root extract obtained using 50% ethanol before fermentation with *B. bifidum* for 24 h was found to be the best skin-whitening agent with the highest antioxidant potential.

1. Introduction

Melanin is essential for protecting human skin against radiation. However, its undesired accumulation in the basal layer of the epidermis leads to melanogenesis or skin pigmentation (Hearing, 2011). Melanogenesis can be controlled by inhibiting the activity of tyrosinase, a key enzyme involved in melanin biosynthesis in plants and animals (Gillbro and Olsson, 2011). The use of tyrosinase inhibitors is probably the most noninvasive strategy for melanogenesis control: tyrosinase inhibitors specifically target melanogenetic cells and do not lead to side effects compared with other melanogenesis inhibitors (Chang, 2012; Peng et al., 2013). Antioxidants are good inhibitors of tyrosinase activity and melanin production (Cha et al., 2012). Moreover, certain antioxidants, such as vitamin C, *N*-acetylcysteine, and Trolox, have been applied as melanogenesis inhibitory agents (Kim et al., 2014).

Chinese herbs are essential remedies used in oriental integrative medicine (Chen et al., 2011). These herbs have several medicinal functions such as melanogenesis inhibition and antioxidation, and exhibit low cytotoxicity and environmentally friendly properties compared with synthetic chemicals (Lee et al., 1997; Maltaş et al., 2010; Zengin et al., 2014, 2015a; Zengin, 2016). The well-known Chinese herbs walnut, Moutan Cortex Radicis (MCR), and asparagus root have numerous medical properties (Reiter et al., 2005; He et al., 2014; Samad et al., 2014). Walnut contains unsaturated fatty acids (e.g., linoleic and α-linolenic acids), proteins, and antioxidants (Salcedo et al., 2010). Certain byproducts derived from the walnut tree have several applications in the cosmetic and pharmaceutical industries (Stampar et al., 2006). The major extracts of MCR can inhibit tyrosinase activity (Bu et al., 2008). Asparagus is a plant found near seashores, and its tuber is used as a traditional herbal medicine. The asparagus root extract has antioxidant

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properties (Samad et al., 2014). These herb extracts have strong antioxidant and tyrosinase inhibitory activities because they contain various phenolic compounds. However, certain herb extracts are potentially cytotoxic to normal cells (Cha et al., 2012).

Fermentation is used to break down or convert certain common substrate components into compatible components. Fermentation can typically increase physiological and biochemical activities of biological substrates by modifying their naturally occurring molecules (Choi et al., 2008), and can potentially be used to develop novel pharmaceutical and cosmeceutical agents (Cha et al., 2012). Fermented herbs have beneficial health effects (Bose et al., 2012). The cytotoxicity of certain herb extracts can potentially be reduced or eliminated after treatment with various microbial species (Cha et al., 2012). Some probiotics, for example *B. bifidum*, may have the potential of producing new ingredients or reducing the cytotoxicity of herb extracts by fermentation (Goulet, 2015; Hussain et al., 2016).

This study evaluates whether the fermented extracts of 3 herbs—walnut, MCR, and asparagus root—can inhibit tyrosinase activity. Next, the antioxidant activities and reducing powers of the fermented extracts are determined. Afterward, the tyrosinase inhibitory and antioxidant activities of the fermented and nonfermented Chinese herb extracts are compared. The phenolic composition and content of the 3 fermented extracts are also determined. Finally, the cytotoxic effects of the fermented extracts on normal CCD-966SK cells and B16F10 melanoma cells are examined. To the best of our knowledge, this study is the first to evaluate the antioxidant and tyrosinase inhibitory activities of *B. bifidum*-fermented herb extracts.

2. Material and methods

2.1. Chinese herbs, the probiotic bacterium, tested cells and tyrosinase

Walnut (*Prunus persica* (L.) Batsch), MCR (*Paeonia suffruticosa* Andr.), and asparagus root (*Asparagus cochinchinensis* (Loureiro) Merrill.) were purchased from Dihua Street, Taipei City, Taiwan. The probiotic bacterium *B. bifidum* (American Type Culture Collection (ATCC) number: 29521), 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 Bioresource Collection and Research Center (Hsinchu, Taiwan). Mushroom tyrosinase was purchased from Sigma Chemical Co (St. Louis, USA).

2.2. Analysis of crude extract with or without fermentation

A 0.1–0.3-mm fine powder of walnut, MCR, and asparagus root (0.2 kg each) was extracted individually using 5 solvents: distilled water, 95% ethanol, 50% ethanol, 100% ethyl acetate, and 50% ethyl acetate. The extraction process was performed with sonication at 40 °C for 2 h. The extracts were collected, filtered, and concentrated in a rotary vacuum evaporator at 50 °C. The residual extracts were freeze-dried and then refrigerated until further use.

Bifidobacterium bifidum was cultured in broth containing: 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 growth medium was adjusted to 6.5 and incubations were carried out under anaerobic conditions at 37 °C (Goulas et al., 2007). For fermentation, the pH of the solution (200 mL) containing the herb extracts (2 g) was first adjusted to 6.2–6.5 before 1 mL of the *B. bifidum* culture (initial cell number: 2×10^7 cfu/mL) was inoculated. The mixtures were incubated at 37 °C under anaerobic conditions in a thermostatically controlled water bath. The fermentation periods were 0, 12, 24, 36, and 48 h. After fermentation, the solution was centrifuged at 8000*g* for 20 min, and the supernatant was collected, filtered, and concentrated in the rotary vacuum evaporator at 50 °C. The residual extracts were freeze-dried and stored under refrigeration. The optimal fermentation period for the various herbs was set at 24 h based on their physiological activities. To determine their chemical composition, the extracts were dissolved in 95% ethanol, transferred to a vial, and filtered through a 0.45-μm nylon membrane filter before injection into a high-performance liquid chromatography (HPLC) system.

2.3. Analysis of phenolic compounds

The total phenolic content in the herb extracts was measured using a modified method adopted from Zheng and Wang (2001) by using gallic acid as the standard. The herb extracts were added to test the cuvettes, and then 1 mL of a Folin–Ciocalteu reagent and 1 mL of a Na_2CO_3 solution were added, and the mixture was appropriately shaken. The optical density (OD) of the solution was measured at 725 nm using an UV–vis spectrophotometer (Shimizu, Japan) after 60 min of incubation. The total phenolic content was expressed as the gallic acid equivalent (mg-GAE/g-dried extract).

2.4. HPLC analysis

The fermented herb extracts were analyzed by using an HPLC method modified from that used by Zheng and Wang (2001). It was performed using a 5-μm, 4.6 × 250-mm Econosil column at a 1-mL/min flow rate and a 30-μL injection volume. The mobile phase was acetonitrile (A) and acidified water containing 3% formic acid (B). The gradient was as follows: 0 min, 5% A; 5 min, 10% A; 30 min, 20% A; 40 min, 40% A; 50 min, 60% A; 60 min, and 100% A; and was held for 15 min before it returned to the initial conditions. The detection wavelengths were set at 280, 330, and 350 nm. Scanning was performed between 200 and 450 nm, and data were collected using a photodiode array. Individual phenolic compounds were identified by comparing their retention times against those of the standard samples.

2.5. Measurement of tyrosinase inhibition

The tyrosinase inhibitory activities of the herb extracts were determined using the spectrophotometric method described by Zheng et al. (2012). The herb extracts were first dissolved in DMSO at a concentration of 1 g/l, and then diluted to different concentrations using DMSO. Subsequently, a 30-μl mixture was diluted with a 0.05-mM sodium phosphate buffer (970 μL) in the tubes, followed by the addition of 100 mg/l L-tyrosine (1 mL), and finally, 350 units per milliliter of a mushroom tyrosinase solution (1 mL); 3 mL of this reaction solution was mixed by vortexing, and the initial absorbance was measured at 490 nm. After incubation for 20 min at 25 °C, the final absorbance of the reaction solution was measured at 490 nm. The tyrosinase inhibitory effect of the test samples was generally evaluated at a concentration of 500 μg/mL, unless stated otherwise. The concentration at which half the original tyrosinase activity was inhibited (IC_{50}) was calculated if required. The inhibitory effects of the test samples were expressed as percentage of tyrosinase inhibition, as follows:

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

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