Journal of Bioscience and Bioengineering
VOL. 127 No. 6, 738–743, 2019

www.elsevier.com/locate/jbiosc

Anti-melanization effects and inhibitory kinetics of tyrosinase of bird's nest fern (*Asplenium australasicum*) frond extracts on melanoma and human skin

Wen-Wen Zeng and Lih-Shiuh Lai*

Department of Food Science and Biotechnology, National Chung Hsing University, 145 Xingda Rd, Taichung 40227, Taiwan

Received 25 June 2018; accepted 9 November 2018
Available online 11 January 2019

Some bioactive properties of *p*-coumaric acid and fucose-rich polysaccharide in skin health have been studied, including melanogenesis inhibition of the phenolic acid and growth inhibitory effects of the polysaccharide on melanoma. The dermatological benefits of bird's nest fern extracts (BNFE), containing both substantial fucose-rich polysaccharide and *p*-coumaric acid, like promoting collagen production and growth of fibroblast cell and further improving the elasticity and dryness of human skins have been demonstrated in our previous study. Besides, the anti-melanization effects of various BNFE on B16-F10 melanoma and human skin were first studied here. The promising extracts revealed that the main phenolic acid, *p*-coumaric acid, in BNFE resulted in suppression against tyrosinase activity from melanogenesis. The inhibitory kinetics on the diphenolase activity indicated that AE40 was a noncompetitive inhibitor of mushroom tyrosinase. On the other hand, the fucose-rich mucilage of BNFE showed pronouncedly suppressing effect on B16-F10 melanoma viability. Clinical trial was performed by recruiting 46 female volunteers and the results indicated that the lotions with 1% of BNFE was non-irritant and reduced effectively the pigmentation on human skin after 7–14 days of continuous application. It was suggested that the fucose-rich mucilage and *p*-coumaric acid in BNFE may have potential for nutricosmetics and phytotherapy applications as a natural hypopigmenting agent.

© 2018, The Society for Biotechnology, Japan. All rights reserved.

[**Keywords:** Fern; *p*-Coumaric acid; Fucose-rich mucilage; Melanization; Melanoma; Tyrosinase]

The edible fronds of *Asplenium australasicum* (J. Sm.) Hook, also called bird's nest fern, is a popular fern vegetable in Taiwan. Its safety assessment had been demonstrated insignificant effect on hematology and serum biochemistry of rats after a 28-days feeding trial with water-extracted liquid of bird's nest fern (15 g/kg body weight/day) (1). It was reported that the aqueous ethanol extracts of *A. australasicum* fronds have some dermatological benefits such as enhancing collagen production and growth of fibroblast (NIH-3T3) and further improving the elasticity and dryness of human skins in an emulsion model (2). The extracts of *A. australasicum* fronds also demonstrated anti-oxidant activities and the main phenolic compounds in those extracts was reported as *p*-coumaric acid (*p*-CA) (3), which has been identified as a potent inhibitor against melanin biosynthesis in both cultured human melanocyte and human skin (4). Cutaneous hyperpigmentation, one of the common maladies characterized by lentigo, senile lentigines, pigmented acne scars and melasma, is stimulated by some factors such as exposure to ultraviolet (UV) radiation and chronic inflammation, resulting from the overacting melanogenesis (5,6). Human melanogenesis is regulated by a critical enzyme called tyrosinase, a copper-containing multifunctional oxidase widely found in nature, which catalyses the two initial rate-limiting reactions in the biosynthetic pathway of melanin in melanocytes: the hydroxylation of tyrosine to 3,4-dihydroxyphenylalanine (DOPA) by monophenolase activity and the oxidation of DOPA to dopaquinone by diphenolase activity (7,8). Thus, many skin whitener in cosmetics and medication have tyrosinase inhibitory activity, and natural products with this activity have become potential sources to prevent skin hyperpigmentation (9). Usually, antioxidants are good inhibitors of tyrosinase activity and melanin production (10). The aim of this study is to investigate the relation between the melanogenesis inhibitory activities and the potential bioactive compounds in bird's nest fern extracts (BNFE) for the applications of fern vegetable in functional foods and nutricosmetics by employing edible resources.

MATERIALS AND METHODS

Materials The edible fronds of bird's nest fern (*A. australasicum*), harvested in September 2011 were purchased from a contracted farmer in Hualien, Taiwan, which showed consistent features with voucher specimen (No. 248383) identified by Assoc. Prof. Chen-Meng Kuo (Institute of Ecology and Evolutionary Biology, National Taiwan University, Taiwan) and deposited at Herbarium of National Taiwan University, Taiwan. The bird's nest fern fronds were freeze-dried and then ground, sieved (40 meshes) for further analysis.

The chemicals used in this study including *p*-CA, mushroom tyrosinase, 3,4-dihydroxy-phenylalanine (L-DOPA), sodium hydroxide and alpha-melanocyte stimulating hormone (α-MSH) were purchased from Sigma–Aldrich (St. Louis, MO, USA); stearic acid, glycerol, calcium chloride, sodium hydroxide, diethyl ether and ethyl acetate were purchased from Union Chemical Works Ltd. (Hsinchu, Taiwan); span 80 and tween 20 were purchased from Choneye Pure Chemicals (Taipei, Taiwan); jojoba oil, capric triglyceride, complex preservatives were purchased from Jian Jia Chemical Co., Ltd. (Taichung, Taiwan); fetal bovine serum (FBS), penicillin/streptomycin, Dulbecco's modified Eagle medium (DMEM), Dulbecco's

* Corresponding author. Tel.: +886 4 22853758; fax: +886 4 22876211.
E-mail address: lslai@dragon.nchu.edu.tw (L.-S. Lai).

1389-1723/$ – see front matter © 2018, The Society for Biotechnology, Japan. All rights reserved.
https://doi.org/10.1016/j.jbiosc.2018.11.005

phosphate-buffered saline (DPBS, pH 7.0–7.3), and trypsin–EDTA (0.25%) were purchased from Gibco, Life Technologies (Grand Island, NY, USA). Other chemicals, including ethanol (95%) (Jam-Mao Corporation, Taichung, Taiwan), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Molecular Probes, Labeling & Detection Technologies, Eugene, OR, USA) and dimethyl sulfoxide (DMSO, Riedel-de Haën, Honeywell International Inc., Seelze, Germany), were all in analytical grade.

Preparation of bird's nest fern extracts and mucilage The freeze-dried powders (15 g) of bird's nest fern were suspended individually in 750 ml of deionized water, 25% and 40% aqueous ethanol. The ultrasonic extraction was performed at 80 Hz, 100% power (330 W), 40°C for 30 min by using an ultrasonicator (Elmasonic P 120H, Elma Schmidbauer GmbH, Singen, Germany). The extract was then centrifuged (6000 ×*g*, 4°C, 15 min) and filtered through a Whatman filter paper with a mean pore size of 11 μm. The filtrate was concentrated by using a rotary evaporator at 40°C and the concentrate was lyophilized (Eyela FDU-506, Eyela, Tokyo, Japan) to obtain various extracts. BNFEs prepared with water, 25% and 40% ethanol aqueous solution are denoted as WE, AE25 and AE40, respectively, hereafter.

The mucilage in the water extract was further isolated by adding three volumes of 95% (v/v) ethanol to the filtrates (the one extracted with water as descried above) and kept at 4°C overnight in order to precipitate the mucilaginous polymers. After centrifugation (6000 ×*g*, 20 min), the precipitate was then lyophilized to obtain the water-extracted mucilage which is denoted as WM hereafter.

Inhibitory effect and kinetic analysis on mushroom tyrosinase activity Tyrosinase activity assay for oxidation of L-DOPA was performed according to the method of Lu et al. (11) with slight modification. One hundred microliters of BNFE (10 mg/ml in water) or *p*-CA (100 μg/ml in DMSO) was mixed with 100 μl of phosphate buffer solution (25 mM, pH 6.8) and 75 μl of L-DOPA solution (7 mM). Mushroom tyrosinase solution (25 μl, 200 units/ml) was added to the mixture, which was then incubated for 10 min at room temperature. The absorbance of the mixture at a wavelength of 475 nm was measured immediately. The inhibition of dopachrome formation was calculated as the inhibition percentage according to the following equation:

$$\text{Inhibition (\%)} = \frac{A_C - (A_s - A_O)}{A_C} \times 100 \quad (1)$$

where A_s is the absorbance in the presence of sample, A_C is the absorbance in the absence of sample (i.e., sample was replaced with an equal volume of water or DMSO) and A_O is the absorbance in the absence of enzyme (i.e., tyrosinase was replaced with an equal volume of PBS buffer).

For the kinetic study, the velocity (V) of the enzymatic reaction in the presence of AE40 (15 mg/ml) were calculated by using the extinction coefficient of dopachrome formation (3.4 mM^{-1} cm^{-1}) which were carried out in 0.1 M PBS buffer (pH 7.0) at 25°C monitoring at 475 nm (12). The Michaelis constant (K_m) and the maximal velocity (V_{max}) of the tyrosinase activity were determined from the double reciprocal form of Michaelis–Menten plot (13,14) at various concentrations of L-DOPA.

Resistant against ultraviolet light To understand the resistant of BNFE against ultraviolet light, the absorbance spectrum of those extracts (200 μg/ml) was measured by a wavelength scanning from 200 to 800 nm. The area of absorbance between 280 and 400 nm was divided into two parts, including 320–400 nm (UVA) and 280–320 nm (UVB), and integrated separately.

The effect of BNFE and WM on the viability of melanoma B16-F10 The murine skin melanoma B16-F10 (BCRC 60031) were purchased from the Bioresource Collection and Research Center (BCRC) of Food Industry Research and Development Institute (FIRDI, Hsinchu, Taiwan). The B16-F10 cells were cultured in DMEM media with 10% FBS and then maintained in a humidified incubator (Forma Direct Heat CO_2 incubator, Bioway corporation, Taipei, Taiwan) with 5% CO_2 at 37°C. For the cell viability assays, B16-F10 cells were seeded in a 24-well plate at a density of 5000 cells/well and incubated for 24 h. Then, the culture medium was replaced with the media containing BNFE, WM or *p*-CA (as sample, A_s) by dissolving the sample powder in the media with 10% FBS. Then, these test media were filtered through a 0.2-μm-filter and diluted to the appropriate concentrations for an additional incubation time of 24 and 48 h. Cells treated with media plus 10% FBS only is used as the control (A_C). Cell viability of B16-F10 was determined via an MTT assay (15) and the resulting formazan was detected at 540 nm. Blank (A_B) was carried out by a test of culture media without cell seeding. The cytotoxic effect of each treatment was expressed as a percentage of cell viability relative to the untreated control cells, calculated by $(A_s - A_B)/(A_C - A_B) \times 100$.

Melanin production and tyrosinase activity of B16-F10 cell This assay was performed according to the methods of Park and Lee (16) with slight modifications. B16-F10 cells were seeded in a 24-well plate at a density of 5×10^4 cells/well with or without the 100 nM α-MSH then incubated at 37°C in 5% CO_2/air for 3 h. The cells were then incubated in medium with or without test sample for an additional 48 h. One part of the cells were washed with DPBS buffer, dissolved in 100 μl of 1 N NaOH and incubated at 800 rpm at 60°C for 1 h to solubilize the melanin. Melanin production was quantified by measuring the absorbance at 405 nm and was calculated as (sample *O.D.*/control *O.D.*) × 100.

For murine tyrosinase activity, after incubating for an additional 48 h, another part of the cells were washed with DPBS buffer and lysed in 150 μl of 50 mM PBS (pH 6.8) containing 1% Triton X-100 and 0.1 mM phenylmethylsulfonyl fluoride and then frozen at −80°C for 30 min. After thawing (30 min), cellular extracts were clarified by centrifugation at 12,000 ×*g* for 30 min at 4°C. One hundred and twenty five microliters of supernatant and 20 μl of L-DOPA (2 mg/ml) were placed in a 96-well plate, and the absorbance of dopachrome was read at 490 nm every 10 min for 80 min at 37°C using an ELISA plate reader (Infinite M200 Pro, Tecan, Zürich, Switzerland).

Ethical statement Forty six female volunteers with healthy skin were examined in the present study. Before the clinical experiment, each subject was informed about the details of this study and signed a letter of consent, which was approved by the Institutional Review Board of National Cheng-Kung University Hospital, Taiwan. All procedures performed in this study involving human participants were in accordance with the ethical standards of the institutional or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Study protocol of human clinical experiment Model emulsions with 1.0% WE, AE and control were prepared for the human experiment according to the method of Zeng and Lai (2). Forty six healthy female subjects in the age range of 18–40 years old volunteered to undergo an allergic test via smearing the tested formula emulsions at their inner skin of the forearms (5 × 5 cm^2) for 5 h. The subjects without allergic reaction to tested emulsions were divided into two groups (n = 23/group) for emulsion-WE/control and emulsion-AE40/control. The formula emulsions with 1.0% WE or 1.0% AE40 was used on a constant arm skin for each subject while the control emulsion (vehicle) was used on the other arm. The tested emulsions were applied on subjects' forearm twice daily by self-control for four weeks according to the approach of Chang et al. (17). After cleaning their forearm with water and detergent, subjects were asked to stay in the room where the skin properties measurement would be performed prior to the skin properties measurements for at least 30 min for full skin adapt to the temperature and humidity of the room.

Melanin pigmentation of human skin The melanin pigment of skin was measured with a Multi Skin Test Center MC 1000 (Courage-Khazaka Electronic GmbH, Cologne, Germany) connected with a melanin measurement probe with a foam ring for avoiding the interference of ambient light. The melanin content in a range of 0–99, based on the absorption/reflection principle, was calculated through the quantity of light with defined wavelength (660 and 880 nm, that correspond to pigments absorption (18)) absorbed by the tested skin. The inhibition of melaninization was determined by taking the measured value of before-using (0 day) minus that of after-using for each subject every week during the testing period.

Statistical analysis All statistical analyses were performed by using IBM SPSS software version 19 (SPSS Inc., Chicago, IL, USA). Student's *t*-test was used for comparisons between control and treatments, and the intergroup of sample analysis was performed by using Duncan's multiple-comparison test. Significant differences between means were determined under a confidence level of 95% ($p < 0.05$).

RESULTS AND DISCUSSION

Inhibiting effect on tyrosinase activity and phenolics content in BNFE The extraction yield for WE, AE25 and AE40 was about 26.8, 29.4 and 28.1 g per 100 g dried frond of bird's nest fern, respectively. The main phenolic acid of BNFE was reported to be *p*-CA in our previous study (2). As shown in Table 1, there was a correlation between *p*-CA content in BNFE and inhibitory effect on mushroom tyrosinase activity, which was in the order of AE40 > AE25 > WE. The inhibitory effect of pure *p*-CA against mushroom tyrosinase was 29.2% and 95.7% for 2 μg/ml and 100 μg/ml, respectively. Some literature have been reported that *p*-CA is a strong inhibitor of cellular melanogenesis and the cream containing *p*-CA can reduce UV-induced erythema formation and subsequent pigmentation in human skin (4,19). The main physiological stimulus for human melanogenesis and cutaneous photoageing is ultraviolet radiation (20). For protection against ultraviolet radiation, quite obvious absorbance on UV light (200–400 nm) but no absorbance on visible light (400–800 nm) was found for all BNFE solution as shown in Fig. 1. The faint-colored extract solutions from bird's nest fern seemed to show a chemical protection from UVA and UVB, especially for UVB range (Table 1). That might be attributed to the aromatic groups of phenolics (e.g., *p*-CA, which showed a maximum

TABLE 1. Potential bioactive components, tyrosinase inhibitory activity and resistance against ultraviolet radiation of bird's nest fern extracts (BNFE).

	Carbohydrate content[a,b] (GE %)	*p*-Coumaric acid content[b] (mg/g)	Inhibition on tyrosinase[c] (%)	UVB[d] (A × nm)	UVA[d] (A × nm)	UVB+A[d] (A × nm)
WE	24.7 ± 0.3a	3.3 ± 0.0c	53.21 ± 0.46c	14.7 ± 0.1c	11.1 ± 0.1c	25.9 ± 0.2c
AE25	19.8 ± 0.2b	4.2 ± 0.2b	62.93 ± 1.41b	16.3 ± 0.3b	12.0 ± 0.1b	28.3 ± 0.2b
AE40	14.0 ± 0.5c	7.5 ± 0.0a	71.40 ± 1.17a	23.9 ± 1.2a	17.6 ± 0.6a	41.5 ± 1.7a

a–c, Means with different letters within the same column differ significantly ($p < 0.05$). Each data was expressed as the mean of three replications ± standard deviation.
[a] Total carbohydrate content was expressed as glucose equivalent (GE).
[b] Data taken from Zeng and Lai (2).
[c] The inhibition on tyrosinase was measured in presence of 10 mg/ml BNFE.
[d] The absorbance of UVA (320–400 nm) and UVB (280–320 nm) were measured in presence of 200 μg/ml BFNE. The integration area from absorbance vs. wavelength plot (A × nm) were calculated for the wavelength range specified.

absorbance at 295 nm) in BNFE, since the one with higher phenolic content usually showed greater spectral absorbance for the whole UV range.

Inhibition kinetics of tyrosinase activity by BNFE The inhibitory kinetics of AE40 on the diphenolase activity was investigated using Lineweaver–Burk plots as presented in Fig. 2A. The Lineweaver–Burk double-reciprocal plots indicated that AE40 was a noncompetitive inhibitor of mushroom tyrosinase due to its decreasing effect on the apparent value of V_{max} and no changing on that of K_m (Fig. 2). To further justify the noncompetitive-type inhibition mechanism, Lineweaver–Burk plots was rewritten as:

$$\frac{1}{V} = \frac{K_{mapp}}{V_{maxapp}}\frac{1}{[s]} + \frac{1}{V_{maxapp}} \quad (2)$$

$$\text{Slope} = \frac{K_{mapp}}{V_{maxapp}} = \frac{K_m}{V_{max}} \times \frac{[I]}{K_I} + \frac{K_m}{V_{max}} \quad (3)$$

$$\text{y-intercept} = \frac{1}{V_{maxapp}} = \frac{1}{V_{max}} \times \frac{[I]}{K_{I2}} + \frac{1}{V_{max}} \quad (4)$$

where K_{mapp} and V_{maxapp} are the apparent K_m and apparent V_{max}, respectively. The inhibition constant (K_I and K_{I2}) of an inhibitor was then obtained from the secondary plot of Lineweaver–Burk plots, i.e., a linear regression plot for various inhibitor concentration (5, 10, 13, 15, 20 mg/ml AE40) [*I*] versus the slope and the *y*-intercept, respectively (21,22). The inhibitory constants K_I (about 14.6 mg/ml) and K_{I2} (about 14.2 mg/ml) obtained from Fig. 2B, for the binding affinity of AE40 to free tyrosinase and AE40 to tyrosinase–dopa complex, respectively, were found to be essentially equal. This result confirmed the noncompetitive-type inhibition mechanism of AE40 on tyrosinase. It was reported that the inhibition by *p*-CA was noncompetitive against fungal tyrosinase (*ortho*-diphenol oxidases) as well, but was competitive against apple tyrosinase (regardless of substrate) (23).

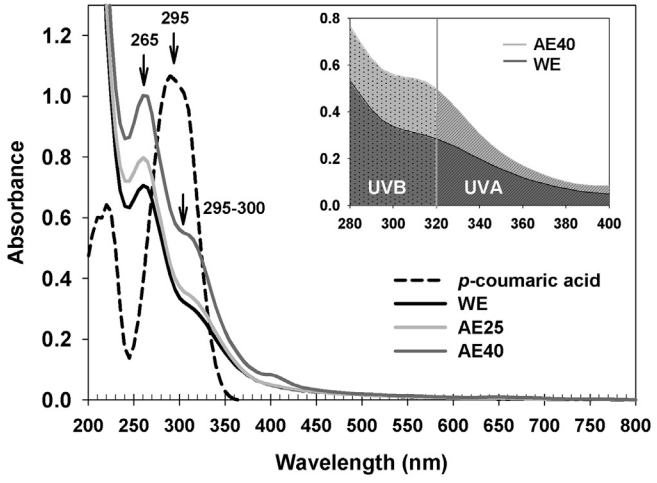


FIG. 1. The absorbance of bird's nest fern extracts (200 μg/ml) and *p*-coumaric acid (10 μg/ml) in the wavelength range of 200–800 nm.

Proliferative depressing effects of BNFE and WM on B16-F10 melanoma After two-day treatment with BNFE, the viability of B16-F10 melanoma generally decreased in both a dose-dependent and time-dependent way, in which the viability got lower than that of one-day treatment at the same concentration level, and usually decreased significantly with increasing BNFE concentration (Table 2). The suppressing effect on B16-F10 melanoma growth was in the order of WE > AE25 > AE40 treatment, which was probably related to the polysaccharide content in BNFE (Table 1). The viability of B16-F10 was decreased significantly with increasing WM concentration (10–100 μg/ml) and treatment time, which was essentially consistent with that of WE treatment (Table 2). On the other hand, we have tested the toxicity of BNFE to normal skin cell in our previous study (2), all BNFE not only show no cytotoxicity to fibroblast NIH-3T3 at the addition level (50–400 μg/ml) but also showed enhanced cell proliferation. WM, principally consisting of polysaccharide isolated from bird's nest fern, has been reported to be mainly composed of 24% galactose, 23% fucose, 13% xylose, 12% arabinose, and 10% glucuronic acid (2). The studies about fucose-rich sulfated polysaccharides from seaweeds have also pointed out many biological activities such as anti-proliferation effect on skin melanoma B16-F10 cells via induction of apoptosis through activation of caspase-3 (24). It was found that BNFE with more mucilage usually led to the intense growth suppression on B16-F10 melanoma. Furthermore, the viability of B16-F10 cell also decreased by WM treatment but under a lower concentration than WE. However, the proliferative depressing effects of WM on B16-F10 melanoma were about 18% and 27% depressing ratio at 50 and 100 μg/ml, respectively, which was not declined as fast as that via caspase apoptosis pathway and as WE at the comparable concentration (100 and 400 μg/ml). A kind of polyphenolics like epigallocatechin-gallate (EGCG), not only found in green tea but also in *Asplenium* sp. fern (25), was demonstrated to inhibit melanoma cell growth at physiological doses (0.1–1 μM) (26). In a study about the EGCG-mediated melanoma cell suppression, EGCG treatment was found to downregulate the inflammasome component NLRP1 and reduced caspase-1 activation, leading to inhibition of IL-1β secretion from melanoma cells and NF-κB activity, then decreased cell growth (26). Therefore, it was thought that the fucose-rich mucilage and a small amount of polyphenolics (e.g., EGCG) in BNFE may cooperatively cause the suppression effect on melanoma viability pronouncedly.

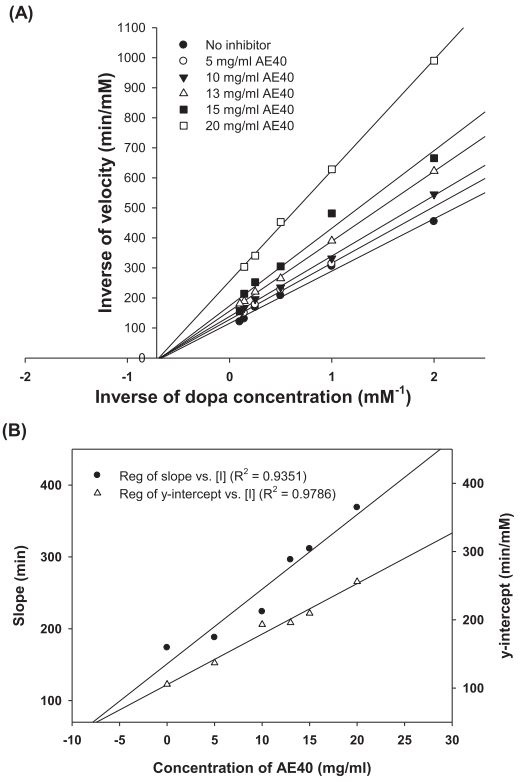
Inhibitory effect of BNFE on murine melanogenesis and tyrosinase activity of B16-F10 As shown in Fig. 3A, B16-F10 melanoma was stimulated to enhance melanin synthesis by adding α-MSH. As comparing to the control in the presence of α-MSH, the inhibitory effects of BNFE on melanogenesis were in the order of AE40 > AE25 > WE. It seems that BNFE with more

TABLE 2. Effect of bird's nest fern extracts (BNFE), water-extracted mucilage (WM) and *p*-coumaric acid (*p*-CA) on the viability of B16-F10 melanoma.

Sample	Days	Concentration of aqueous extracts (μg/ml)										
		0.5	1	2	5	10	25	50	100	200	300	400
WE	1	–	–	–	–	102.3 ± 5.9a	96.6 ± 2.5ab	92.1 ± 2.6bc	89.3 ± 3.4c	80.2 ± 4.7d	65.1 ± 1.7e	47.3 ± 4.7f
	2	–	–	–	–	99.3 ± 3.5a	93.1 ± 2.1b	85.9 ± 1.2c	85.0 ± 1.3c	75.5 ± 0.5d	65.2 ± 2.4e	41.4 ± 4.4f
AE25	1	–	–	–	–	102.5 ± 3.2a	100.0 ± 2.0a	92.2 ± 2.3b	90.9 ± 1.1b	85.7 ± 3.6c	76.3 ± 3.3d	56.1 ± 3.1e
	2	–	–	–	–	101.3 ± 1.7a	93.9 ± 4.0b	86.5 ± 1.8c	84.1 ± 0.8cd	79.1 ± 4.9d	68.1 ± 3.2e	47.7 ± 0.5f
AE40	1	–	–	–	–	99.7 ± 2.4a	94.4 ± 3.5ab	91.7 ± 0.8b	89.5 ± 7.3b	87.5 ± 4.0b	68.1 ± 6.5c	55.6 ± 1.7d
	2	–	–	–	–	98.4 ± 1.1a	96.6 ± 1.2ab	91.2 ± 2.8bc	88.3 ± 0.9c	86.7 ± 5.7c	73.7 ± 4.4d	54.6 ± 2.1e
WM	1	–	–	–	–	91.4 ± 4.2a	87.1 ± 2.5ab	82.1 ± 0.7bc	73.6 ± 3.7d	–	–	–
	2	–	–	–	–	86.8 ± 1.4a	82.2 ± 4.0ab	79.7 ± 2.1b	70.2 ± 1.1c	–	–	–
p-CA	1	99.2 ± 0.9b	100.5 ± 0.5ab	97.4 ± 3.5b	102 ± 2.0ab	105.4 ± 4.4a	–	–	–	–	–	–
	2	100.9 ± 3.4a	99.6 ± 2.1a	101 ± 1.4a	100 ± 1.5a	98.0 ± 3.1a	–	–	–	–	–	–

a–f, each data was expressed as the mean of three replications ± standard deviation. Means with different lowercase letters within the same row differ significantly ($p < 0.05$).

phenolic acid content like *p*-CA usually were more efficient for inhibiting melanin synthesis of murine B16-F10 cell. An et al. (27) have pointed that *p*-CA is a weaker inhibitor of mushroom tyrosinase but much stronger inhibitor of human or murine tyrosinase as comparison with kojic acid and arbutin. Besides, AE40 showed a comparable activity with kojic acid which has been shown to function as whitening agents and tyrosinase inhibitor (28). The anti-melanogenesis activity of BNFE may be related to the phenolic acids content in BNEE, hence *p*-CA and AE40 were further investigated for their effects on tyrosinase activity of B16-F10. As shown in Fig. 3B, the murine tyrosinase activity was significantly inhibited with increasing AE40 concentration. Similarly, *p*-CA also behaved a significant inhibition on murine tyrosinase under a lower concentration of 1–2 μg/ml which were also approximately equivalent to the *p*-CA content in 200 μg/ml of AE40 (Table 1). It implied that the repressing effect on cellular melanogenesis by AE40 treatment mainly result from the inhibiting effects of murine tyrosinase



	V_{max} (μM/min)	K_m (mM)	K_I (mg/ml)	K_{I2} (mg/ml)	Inhibition type
no inhibitor	8.96 ±0.46	1.51 ±0.01	-	-	-
AE40[1]	5.97 ±0.27 ***	1.48 ±0.00	14.56	14.18	Noncompetitive

***Means with *** differ significantly ($p < 0.001$) as compared with the control (no inhibitor). Each data was expressed as the mean of three replications ±SD.

FIG. 2. Lineweaver–Burk plots for inhibition of AE40 on mushroom tyrosinase catalyzing L-DOPA at 25°C, pH 6.8 (A). The secondary plot of Lineweaver–Burk plots for inhibition of AE40 on mushroom tyrosinase was used to determine the inhibition constants K_I (obtained from the plot of [AE40] vs. slope) and K_{I2} (obtained from the plot of [AE40] vs. *y*-intercept) (B).

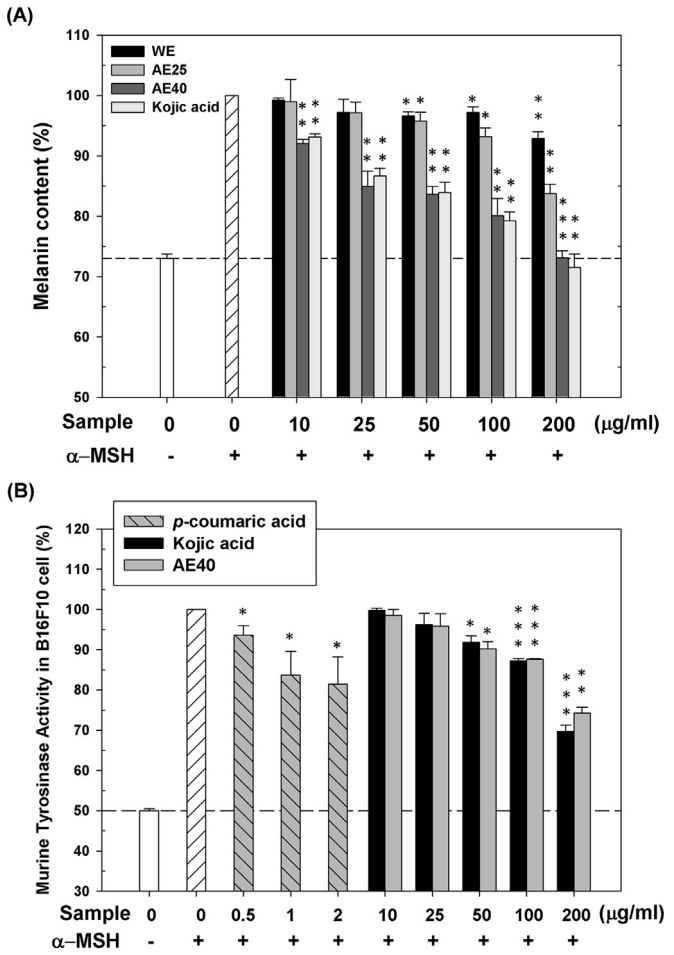


FIG. 3. α-MSH-induced melanin synthesis content (A) and tyrosinase activity (B) of melanoma B16-F10. Each data is expressed as the mean of three replications with standard deviation bar. $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$, means differ significantly.

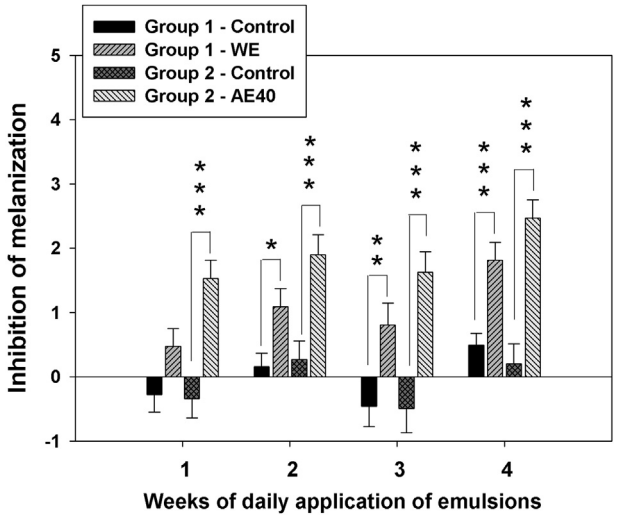


FIG. 4. Depigmentation effect on the forearms of female volunteers during 4-week period of daily application of emulsions with WE and AE40. Values are means with S.E. bar of 23 individuals. $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$, the mean is significantly greater than that of control at the same week (analyzed by independent-*t* test).

activity by the *p*-CA in BNFE and the slightly-decreased viability of B16-F10 melanoma by the mucilage in BNFE.

Whitening effect of BNFE on human skin during 28-day clinical trial Because cells are much weaker than human organs, we performed the cell test by using a lower concentration than that of human trial. For example, Lin et al. (29) completed *in vitro* test by using 176 μM biochanin A from *Trifolium pratense* on B16-F10 cells but *in vivo* test by applying a cream containing 2% biochanin A. In our previous study (2), we have also treated human skin by applying the same emulsion formula with 1% BNFE (WE and AE40 were used), and no allergic reaction was noticed in 46 participated volunteers. Besides, they showed a higher increase in skin hydration in each week and also showed a better improvement of dry and flaky shin than control for the same individual, and an increase in skin elasticity. Therefore, WE (with higher mucilage content) and AE40 (with higher *p*-CA) were formulated into a model skincare emulsion of 1% extracts and examined for a placebo-controlled clinical trial with intra-individual comparison of two formulations on the forearm. As comparing with control emulsion (vehicle), the skin melanization of female subjects was significantly decreased after applying the emulsions with 1% AE40 or 1% WE (Fig. 4). Moreover, the reduction in skin melanization seemed to have an increasing trend with applying time after applying emulsions, 1% WE and AE40. Besides, the good solubility in water of BNFE could prevent the hurt caused by the frequent use of topical irritants like alcohol which can remove skin surface lipids and induce xerotic itch (30). In conclusion, the formulation with 1% AE40 exhibited a pronounced inhibitory effect on melanization of human skin, probably resulted from the suppression of tyrosinase activity by a great amount of phenolics (including *p*-CA) in BNFE. Moreover, the spectral absorbance of BNFE implied that the AE40 has the potential benefits for chemical sunscreen in sun care products against UVB and UVA, but to a lesser extent. On the other hand, WE with significant amount of fucose-rich mucilage showed a suppressing activity on viability of skin cancer cell (B16-F10 melanoma). The various BNFE with different proportion of *p*-CA and fucose-rich mucilage have shown different bioactive properties on melanoma cell and human skin.

ACKNOWLEDGMENTS

This work was supported by Ministry of Science and Technology (MOST), Taiwan (grant number: NSC 101-2313-B-005-048-MY3) for the promotion of science. We appreciate sincerely the kind sponsorship of MOST without which this work would not have been possible.

References

1. **Tsai, M. C., Liao, J. W., Tseng, S. H., Chuan, C. H., Wu, S. S., Yang, S. H., Wang, S. C., and Hwang, J. S.:** Safety evaluation of processed agricultural foods, Plant Prot. Bull., **47**, 403–418 (2005).
2. **Zeng, W.-W. and Lai, L.-S.:** Multiple-physiological benefits of bird's nest fern (*Asplenium australasicum*) frond extract for dermatological applications, Nat. Prod. Res., **33**, 736–741 (2019).
3. **Chang, C. H.:** Effect of hot-air drying and extraction conditions on the anti-oxidant and functional properties of *Asplenium australasicum* (J. Sm.) Hook, Master thesis. National Chung Hsing University, Taichung (2012).
4. **Seo, Y. K., Kim, S. J., Boo, Y. C., Baek, J. H., Lee, S. H., and Koh, J. S.:** Effects of *p*-coumaric acid on erythema and pigmentation of human skin exposed to ultraviolet radiation, Clin. Exp. Dermatol., **36**, 260–266 (2010).
5. **Fu, B., Li, H., Wang, X., Lee, F. S., and Cui, S.:** Isolation and identification of flavonoids in licorice and a study of their inhibitory effects on tyrosinase, J. Agric. Food Chem., **53**, 7408–7414 (2005).
6. **Amen, Y., Arung, E. T., Afifi, M. S., Halim, A. F., Ashour, A., Fujimoto, R., Goto, T., and Shimizu, K.:** Melanogenesis inhibitors from *Coix lacryma-jobi* seeds in B16-F10 melanoma cells, Nat. Prod. Res., **31**, 2712–2718 (2017).

7. **Husni, A., Jeon, J. S., Um, B. H., Han, N. S., and Chung, D.:** Tyrosinase inhibition by water and ethanol extracts of a far eastern sea cucumber, *Stichopus japonicus*, J. Sci. Food Agric., **91**, 1541–1547 (2011).
8. **Ochiai, A., Tanaka, S., Imai, Y., Yoshida, H., Kanaoka, T., Tanaka, T., and Taniguchi, M.:** New tyrosinase inhibitory decapeptide: molecular insights into the role of tyrosine residues, J. Biosci. Bioeng., **121**, 607–613 (2016).
9. **Chen, Y.-S., Lee, S.-M., Lin, C.-C., Liu, C.-Y., Wu, M.-C., and Shi, W.-L.:** Kinetic study on the tyrosinase and melanin formation inhibitory activities of carthamus yellow isolated from *Carthamus tinctorius* L, J. Biosci. Bioeng., **115**, 242–245 (2013).
10. **Wang, G.-H., Chen, C.-Y., Tsai, T.-H., Chen, C.-K., Cheng, C.-Y., Huang, Y.-H., Hsieh, M.-C., and Chung, Y.-C.:** Evaluation of tyrosinase inhibitory and antioxidant activities of *Angelica dahurica* root extracts for four different probiotic bacteria fermentations, J. Biosci. Bioeng., **123**, 679–684 (2017).
11. **Lu, R., Liu, X., Gao, S., Zhang, W., Peng, F., Hu, F., Huang, B., Chen, L., Bao, G., and Li, C.:** New tyrosinase inhibitors from *Paecilomyces gunnii*, J. Agric. Food Chem., **62**, 11917–11923 (2014).
12. **Selinheimo, E., NiEidhin, D., Steffensen, C., Nielsen, J., Lomascolo, A., Halaouli, S., Record, E., O'Beirne, D., Buchert, J., and Kruus, K.:** Comparison of the characteristics of fungal and plant tyrosinases, J. Biotechnol., **130**, 471–480 (2007).
13. **Lineweaver, H. and Burk, D.:** The determination of enzyme dissociation constants, J. Am. Chem. Soc., **56**, 658–666 (1934).
14. **Johnson, K. A. and Goody, R. S.:** The original Michaelis constant: translation of the 1913 Michaelis–Menten paper, Biochemistry, **50**, 8264–8269 (2011).
15. **Kim, E.-K., Kwon, K.-B., Shin, B.-C., Seo, E.-A., Lee, Y.-R., Kim, J.-S., Park, J.-W., Park, B.-H., and Ryu, D.-G.:** Scopoletin induces apoptosis in human promyeloleukemic cells, accompanied by activations of nuclear factor κB and caspase-3, Life Sci., **77**, 824–836 (2005).
16. **Park, K. H. and Lee, M. W.:** Anti-oxidative, anti-inflammatory and whitening effects of phenolic compounds from Bambusae Caulis in Liquamen, Nat. Prod. Res., **26**, 1687–1691 (2012).
17. **Chang, M.-J., Huang, H.-C., Chang, H.-C., and Chang, T.-M.:** Cosmetic formulations containing *Lithospermum erythrorhizon* root extract show moisturizing effects on human skin, Arch. Dermatol. Res., **300**, 317–323 (2008).
18. **Soica, C., Oprean, C., Borcan, F., Danciu, C., Trandafirescu, C., Coricovac, D., Crăiniceanu, Z., Dehelean, C. A., and Munteanu, M.:** The synergistic biologic activity of oleanolic and ursolic acids in complex with hydroxypropyl-γ-cyclodextrin, Molecules, **19**, 4924–4940 (2014).
19. **Garcia-Jimenez, A., Munoz-Munoz, J. L., García-Molina, F., Teruel-Puche, J. A., and García-Cánovas, F.:** Spectrophotometric characterization of the action of tyrosinase on *p*-coumaric and caffeic acids: characteristics of *o*-caffeoquinone, J. Agric. Food Chem., **65**, 3378–3386 (2017).
20. **Aberdam, E., Roméro, C., and Ortonne, J.-P.:** Repeated UVB irradiations do not have the same potential to promote stimulation of melanogenesis in cultured normal human melanocytes, J. Cell Sci., **106**, 1015–1022 (1993).
21. **Hu, W.-J., Yan, L., Park, D., Jeong, H. O., Chung, H. Y., Yang, J.-M., Ye, Z. M., and Qian, G.-Y.:** Kinetic, structural and molecular docking studies on the inhibition of tyrosinase induced by arabinose, Int. J. Biol. Macromol., **50**, 694–700 (2012).
22. **Liao, W. C., Wu, W. H., Tsai, P.-C., Wang, H.-F., Liu, Y.-H., and Chan, C.-F.:** Kinetics of ergothioneine inhibition of mushroom tyrosinase, Appl. Biochem. Biotechnol., **166**, 259–267 (2012).
23. **Ferrar, P. H. and Walker, J. R.:** Inhibition of diphenol oxidases: a comparative study, J. Food Biochem., **20**, 15–30 (1996).
24. **Ale, M. T., Maruyama, H., Tamauchi, H., Mikkelsen, J. D., and Meyer, A. S.:** Fucose-containing sulfated polysaccharides from brown seaweeds inhibit proliferation of melanoma cells and induce apoptosis by activation of caspase-3 in vitro, Mar. Drugs, **9**, 2605–2621 (2011).
25. **Živković, S., Skorić, M., Šiler, B., Dmitrović, S., Filipović, B., Nikolić, T., and Mišić, D.:** Phytochemical characterization and antioxidant potential of rusty-back fern (*Asplenium ceterach* L.), Lekovite Sirovine, **37**, 15–20 (2017).
26. **Ellis, L. Z., Liu, W., Luo, Y., Okamoto, M., Qu, D., Dunn, J. H., and Fujita, M.:** Green tea polyphenol epigallocatechin-3-gallate suppresses melanoma growth by inhibiting inflammasome and IL-1β secretion, Biochem. Biophys. Res. Commun., **414**, 551–556 (2011).
27. **An, S. M., Koh, J. S., and Boo, Y. C.:** *p*-Coumaric acid not only inhibits human tyrosinase activity in vitro but also melanogenesis in cells exposed to UVB, Phytother Res., **24**, 1175–1180 (2010).
28. **Cha, S. H., Ko, S. C., Kim, D., and Jeon, Y. J.:** Screening of marine algae for potential tyrosinase inhibitor: those inhibitors reduced tyrosinase activity and melanin synthesis in zebrafish, J. Dermatol., **38**, 354–363 (2011).
29. **Lin, V. C., Ding, H.-Y., Tsai, P.-C., Wu, J.-Y., Lu, Y.-H., and Chang, T.-S.:** In vitro and in vivo melanogenesis inhibition by biochanin A from *Trifolium pratense*, Biosci. Biotechnol. Biochem., **75**, 914–918 (2011).
30. **Yosipovitch, G.:** Dry skin and impairment of barrier function associated with itch – new insights, Int. J. Cosmetic Sci., **26**, 1–7 (2004).