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Inhibitory effect of *Cinnamomum osmophloeum* Kanehira ethanol extracts on melanin synthesis via repression of tyrosinase expression

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Melanin contributes to skin color, and tyrosinase is the enzyme that catalyzes the initial steps of melanin formation. Therefore, tyrosinase inhibitors may contribute to the control of skin hyperpigmentation. The inhibition of tyrosinase activity by *Cinnamomum zeylanicum* extracts was previously reported. In this report, we test the hypothesis that *Cinnamomum osmophloeum* Kanehira, an endemic plant to Taiwan, contains compounds that inhibit tyrosinase activity, similar to *C. zeylanicum*. The cytotoxicity of three sources of *C. osmophloeum* Kanehira ethanol extracts was measured in B16-F10 cells using a methyl thiazolyl tetrazolium bromide (MTT) assay. At concentrations greater than 21.25 μg/mL, the ethanol extracts were toxic to the cells; therefore, 21.25 μg/mL was selected to test the tyrosinase activities. At this concentration, all three ethanol extracts decreased the melanin content by 50% in IBMX-induced B16-F10 cells. In addition to the melanin content, greater than 20% of the tyrosinase activity was inhibited by these ethanol extracts. The RT-PCR results showed that tyrosinase and transcription factor MITF mRNAs expression were down-regulated. Consistent with the mRNA results, greater than 40% of the human tyrosinase promoter activity was inhibited based on the reporter assay. Furthermore, our results demonstrate that the ethanol extracts protect cells from UV exposure. *C. osmophloeum* Kanehira neutralized the IBMX-induced increase in melanin content in B16-F10 cells by inhibiting tyrosinase gene expression at the level of transcription. Moreover, the ethanol extracts also partially inhibited UV-induced cell damage and prevented cell death. Taken together, we conclude that *C. osmophloeum* Kanehira is a potential skin-whitening and protective agent.

[**Key words:** B16-F10 melanoma cells; *Cinnamomum osmophloeum* Kanehira ethanol extract; MITF; Skin whitening; Tyrosinase; Tyrosinase promoter activity]

Melanin contributes to skin, hair and eye color in humans, and the melanin pigment plays an important role in the prevention of skin injury induced by sun radiation (1). However, overexposure to sun radiation, particularly UV radiation, can lead to hyperpigmentation, wrinkling, melasma, and skin cancer (2). Tyrosinase is the enzyme that catalyzes the initial steps in the formation of melanin. Therefore, tyrosinase is responsible for the coloring of the skin, hair and eyes in humans (3). Any natural product or chemical that represses tyrosinase activity and/or inhibits tyrosinase gene expression is a candidate whitening agent for the cosmetics industry. Plant extracts, such as those from *Acer barbinerve* (4), *Magnolia grandiflora* L. (5) *Paeonia suffruticosa* (6) and *Rhodiola rosea* (7), have been reported as candidates. However, some plant extracts have adverse effects. For example, the primary active compounds of the roots of *Panax ginseng* (8) and Radix *Polygoni multiflora* (9) increase the cellular melanin content and tyrosinase activity.

The expression of tyrosinase is tightly regulated and depends on the cooperation of two DNA elements of a gene, that is, a promoter, which is located before the transcription start site, and the locus control region, which is located at −15 kb upstream of the gene (10). Hormones and several other factors also affect tyrosinase gene expression. Tyrosinase gene expression is up-regulated by estrogen (11), which is a female sex hormone that binds to its receptor and increases cellular cyclic adenosine monophosphate (cAMP) signaling. The chemical 3-isobutyl-1-methylxanthine (IBMX) elicits cellular cAMP and increases cellular tyrosinase activity and melanin content, whereas H89, a PKA inhibitor, reduces intracellular cAMP levels and decreases the melanin content (12).

UV radiation from sunlight is a risk factor for skin damage and may accelerate skin aging (13). According to the wavelength, UV radiation has been divided into UVA (315–400 nm), UVB (280–315 nm) and short wavelength UVC (100–280 nm) radiation. Because the ozone in the stratospheric layer adsorbs UVB and UVC, 95% of the UV that reaches the earth is UVA (14,15). UV induces reactive oxygen species (ROS) formation in skin cells, and ROS interact with lipid-rich membranes, enzymes and cellular DNA, leading to oxidative stress and, consequently, cell death. Several plant extracts with antioxidant activities protect cells from this UV-induced damage (8,9,14,15).

Cinnamon, that is, *Cinnamomum zeylanicum*, is a Chinese traditional herb that is also widely used worldwide. *C. zeylanicum* increases the activity of antioxidant enzymes when fed to rats (16).

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The extract also exhibits antityrosinase activity and reduces the formation of insoluble melanin flakes from tyrosine (17). Moreover, the essential oil of another member of the cinnamon family, *Cinnamomum cassia,* inhibits α-MSH-induced melanin production (18). In addition to *Cinnamomum osmophloeum,* Kanehira is an endemic cinnamon plant of Taiwan, and its leaves are widely used as a flavor additive substitute for *C. zeylanicum* (17). In the preparation of the report of this material, we found that Lee et al. (19) reported that *C. osmophloeum* Kanehira extracts have properties of tyrosinase suppression, wound repair promotion, and antioxidant activity. In this study, three different sources of *C. osmophloeum* Kanehira, including two different chemical types (20) and one unidentified type, were used to evaluate the effects on tyrosinase activity and cell protection against UV stress. The results revealed that all three *C. osmophloeum* Kanehira ethanol extracts inhibited tyrosinase mRNA expression and tyrosinase activity and consequently reduced melanin accumulation in B16-F10 murine melanoma cells. The ethanol extracts also protected the cells against UV-induced cell damage. We conclude that *C. osmophloeum* Kanehira ethanol extract is a potential natural skin-whitening and protective agent.

MATERIALS AND METHODS

Reagents and cell lines Murine melanoma B16-F10 cells were purchased from the Bioresource Collection and Research Center (BCRC), BCRC 60031, Taiwan ROC. Dulbecco's modified Eagle's medium (DMEM), glutamine, penicillin/streptomycin solution (P/S), and fetal calf serum were purchased from Gibco. Two different *C. osmophloeum* Kanehira chemical types were used (20). G2 is a cinnamaldehyde and cinnamylacetate chemotype, and P3 is a mixed type. Both types were collected from different counties in Taiwan and were cultivated in the nursery of the Lienhuachih branch stations, Taiwan Forestry Research Institute, and an additional tested sample was an un-characterized product collected by the Taipei Farmer Association (TFA). The chemicals methyl gallate (MG), 3-isobutyl-1-methylxanthine (IBMX), melanin, 1-(4,5-dimethylthiazol-2-yl)-3,5-diphenylformazan, thiazolyl blue formazan (MTT), L-DOPA and ortho-nitrophenyl-β-galactoside (ONPG) were purchased from Sigma. The reporter vector pGL3-Basic was obtained from Promega. Linear polyethyleneimine (PEI, 25 KDa) was purchased from Polysciences.

***C. osmophloeum* Kanehira ethanol extracts preparation** The leaves were collected and stored at −80°C until use. The active ingredients in the leaves were extracted with ethanol as described by Wang et al. (21) with minor modifications. The frozen cinnamon leaves were combined with 10 times the volume of ethanol (v/w) and were extracted with a Waring Blender. The solution was incubated at room temperature for three days to extract the active ingredients. The extractions were performed three times and combined. The ethanol was removed using a vacuum evaporator, and the ethanol-free extracts were freeze-dried. The dried powder was weighed and dissolved in ethanol to obtain the desired experimental concentration and sterilized by passing through a 0.2-μm filter.

Tyrosinase reporter plasmid construction The human lung cancer CL 1-0 cell line was a kind gift from Dr. Meng-Feng Tsai, Da-Yeh University. The genomic DNA (gDNA) extraction and restriction enzyme *Bam* HI digestion were performed as described by Sambrook et al. (22). Digested gDNA (100 ng) was used as the template for nested-PCR to amplify either the tyrosinase promoter (GenBank HSU03039) or the 5′ upstream locus control region (GenBank AY180962). The two amplified DNA fragments were sequenced, and the confirmed sequences were cloned into the pGL3 basic vector in which the promoter sequence is upstream of the luciferase gene and the locus control sequence is downstream of luciferase gene. The construct was named the pGL3-tyrosinase reporter (pGL3-TR).

B16-F10 cell culture and cell transfection Murine melanoma B16-F10 cells were grown in DMEM medium supplemented with 10% heat-inactivated fetal bovine serum, 1% penicillin/streptomycin, and 2 mM glutamine and were cultured at 37°C in a humidified atmosphere of 5% CO_2. IBMX with or without the *C. osmophloeum* Kanehira extracts was added to the cells.

The pGL3-TR (1 μg) and 0.1 μg pCMV-LacZ plasmids were mixed and co-transfected into B16-F10 cells using the PEI method with a NP ratio of 20. The preparation of the PEI solution and the PEI transfection method were performed as described by Boussif et al. (23).

Determination of cytotoxicity against mouse skin melanoma B16-F10 cell lines Cell cytotoxicity was determined using an MTT assay as described by Chen et al. (24) with minor changes. Briefly, 2×10^3 cells/well B16-F10 cells were cultured in 96-well plates for 24 h, and 2× serial dilutions of *C. osmophloeum* Kanehira ethanol extracts were added to the culture wells. Control cells were treated with 1% ethanol. After additional 72 h incubation, the medium was aspirated, and 20 μL of MTT solution (5 mg/mL in PBS) was added to each well. The cells were incubated at 37°C for 4 h. Then, 200 μL DMSO and 25 μL Sorensen's glycine buffer (100 mM NaCl in 0.1 M glycine, pH 10.5) were added to dissolve the crystals in each well. The optical densities were determined at 540 nm. The maximum concentration that was not toxic to the cells (safety concentration) was used for the assays.

TABLE 1. Primer sequences for PCR.

Primer name (accession no.)	DNA sequence (5′→3′)
Human tyrosinase promoter[a] (U03039)	
ProF	ACACGTGTAGGCCAGAGGAG
ProR	CCTCACAAGGTCTGCAGGAA
NheProF2	GCTAGCCCAGAGGAGACAGTGGCCTA
XhoProR2	CTCGAGTCTGCAGGAACTGGCTAATTG
Human tyrosinase locus control region[a] (AY180962)	
LcrF	CAAGCTCAGTTCAGGCATCA
LcrR	CGCAAGTCAGTGGTTGAAAA
BamLcrF2	GGATCCGCATCATCTCTCTCTAGGAGG
SalLcrR2	GCTGACTTCTCTGGCTCAGGAGGCTA
Mouse MITF mRNA, tyrosinase mRNA and GAPDH mRNA[b] (NM_008601, NM_011661.4 and NM_001289726.1)	
mMITFF	GGAAATGCTAGAATACAGTCACTA
mMITFR	GTCGCCAGGCTGGTTTGGACA
mTyrF	TGGGGATGAGAACTTCACTG
mTyrR	ACGTAATAGTGGTCCCTCAGGT
GAPDHF	ACTCACGGCAAATTCAACGG
GAPDHR	GACTCCACGACATACTGAGC

[a] Nested PCR was used to amplify the human tyrosinase promoter and tyrosinase locus control region sequence. The ProF, ProR and LcrF LcrR primer sets were used to first PCR amplify, and then the amplicons were nested PCR-amplified with the NheProF2, XhoPro2, BamLcrF2, and SalLcrR2 primers.
[b] PCR primers sequences based on Kumagai et al. (26).

Melanin content assay The melanin content was determined according to the method of Yu and Kim (25). Briefly, the cells were seeded in 6-well plates at 1×10^5 cells/well and cultured for 24 h. The IBMX and extracts were then added. The cells were cultured for two days and collected by trypsinization. The collected cell pellets were dissolved in 1 mL 1 M NaOH at 60°C for 1 h, and the optical densities were determined at 405 nm and melanin was used as standard to calculate the cellular melanin.

Cellular tyrosinase activity assay The cellular tyrosinase activity was monitored as described previously (25) with minor modifications. B16-F10 cells were seeded in 6-well plates (4×10^5 cells/well) and allowed to adhere at 37°C for 24 h. Then, the *C. osmophloeum* Kanehira ethanol extracts were added to wells, while control cells were treated with 1% ethanol. After a 24-h incubation, the cells were washed with PBS and lysed in PBS (pH 7.5) containing 1% Triton X-100 and 0.1 mM PMSF. The lysates were centrifuged to pellet the cell debris, and the protein concentrations were determined with the Bradford reagent according to the manufacturer's instructions (BioRad). The protein (30 μg) was transferred into 96-well plates, and 100 μL of freshly prepared 2 mg/mL L-3,4-dihydroxyphenylalanine (L-DOPA) in phosphate-buffer solution was added to each lysate. Following 1-h incubation, the optical densities were determined at 450 nm. The tyrosinase activity was expressed as a percentage of the IBMX treated control.

MITF and tyrosinase gene expression For RNA isolation, the cells were cultured and treated with the *C. osmophloeum* Kanehira ethanol extracts as described. The cells were incubated for 2 days and then trypsinized and collected by centrifugation. After removing the media, 1 mL Trizol (Invitrogen) was added to lyse the cells, and the RNA was extracted as recommended by the manufacturer. The extracted RNAs were reverse-transcribed with MMLV reverse transcriptase (Invitrogen), and PCR was used to amplify the mouse microphthalmia-associated transcription factor (MITF; NM_008601), tyrosinase (NM_011661.4) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; NM_001289726.1) using the specific primers (26) listed in Table 1. The amplified fragments were electrophoresed, and the intensities of the bands were determined with the AlphaDigiDOc 1201 program. The housekeeping gene GAPDH was used as the loading control.

Luciferase and β-galactosidase assay The PGL3-TR and pCMV-LacZ transfected cells were cultured for 24 h to express the reporter genes, and the IBMX and *C. osmophloeum* Kanehira ethanol extracts were then added. After 48 h of culture, the media were removed, and the cells were washed twice with PBS. The cells were then lysed with 100 μL CCLR (Promega) for 10 min. The cell debris were removed by centrifugation, 20 μL of the supernatant was used for the luciferase assay (luciferase assay system, Promega) and 10 μL was used for the β-galactosidase assay (β-galactosidase enzyme assay system, Promega). The luciferase activities were normalized to the β-galactosidase levels to account for the transfection efficiency (27). The reporter assays were performed in triplicate in at least three independent experiments.