Characterization of Phenethyl Cinnamamide Compounds from Hemp Seed and Determination of Their Melanogenesis Inhibitory Activity

Jae Kwon Kim, Hee-Young Heo, SeonJu Park, Haheon Kim, Jeong Ju Oh, Eun-Hwa Sohn, Se-Hui Jung,* and Kooyeon Lee*

ABSTRACT: Hyperpigmentation is induced by the overactivation of tyrosinase, which is a rate-limiting enzyme in melanogenesis. The defatted extract of hemp (Cannabis sativa L.) seed is known to have inhibitory effects on melanogenesis; however, effective compounds in the extract have not been identified yet. In this study, three phenethyl cinnamamides present in hemp seed extract were prepared by purification and chemical synthesis and were assessed for their inhibitory effects on melanogenesis in B16F10 melanoma cells. A comparison of the anti-melanogenesis and anti-tyrosinase activity of hemp seed solvent fractions revealed that the ethyl acetate fraction possessed the greatest potential for suppressing melanogenesis in melanoma cells by decreasing tyrosinase activity. We tentatively identified 26 compounds in the ethyl acetate fraction by comparing spectroscopic data with the literature. Three phenethyl cinnamamides such as N-trans-caffeoyletyramine, N-trans-coumaroyltyramine, and N-trans-feruloyltyramine present abundantly in the ethyl acetate fraction were prepared and their anti-melanogenesis and anti-tyrosinase activities in melanoma cells were evaluated. We found that N-trans-caffeoyletyramine and N-trans-feruloyltyramine inhibited alpha melanocyte stimulating hormone (α-MSH)-induced melanogenesis without cytotoxicity, while N-trans-coumaroyltyramine inhibited melanogenesis with cytotoxicity. IC50 values of N-trans-caffeoyletyramine, N-trans-feruloyltyramine, and N-trans-coumaroyltyramine for inhibition of α-MSH-mediated tyrosinase activation were 0.8, 20.2, and 6.3 μM, respectively. Overall, N-trans-caffeoyletyramine possessed the strongest anti-melanogenesis activity among the three phenethyl cinnamamides evaluated. The inhibitory effect of N-trans-caffeoyletyramine was verified by determining the melanin content and tyrosinase activity in melanoma after treating the cells with synthetic compounds. Thus, N-trans-caffeoyletyramine isolated from hemp seed extract could be useful in cosmetics as a skin-whitening agent.

INTRODUCTION

Melanin is a type of natural pigment that gives color to the hair, skin, and eyes in humans and animals.1 It is produced by specialized melanocytes which are dendritic cells located at the junction between the epidermis and the dermis and plays an important role in protecting human skin from ultraviolet (UV) lights and free radicals.2 However, dysregulation in melanin synthesis results in hyper pigmentary disorders, including melasma, freckles, age spots, solar lentigo, and hyperpigmentation syndromes.3 Melanin is synthesized from tyrosine via a multistep enzymatic process involving a number of proteins. Tyrosinase, known as the rate-limiting enzyme, is critical for melanin synthesis.4 Thus, to develop therapies that can improve or prevent hyper pigmentary disorders, inhibition of tyrosinase activity is generally targeted.1,5 Till date, numerous synthetic...
chemical inhibitors against tyrosinase activity have been developed for the application in the medical and cosmetic industries. However, due to the limits of synthetic compounds in terms of cytotoxicity and their associated side effects, there is still a demand for novel chemicals with improved safety, stability, and efficacy.6

Bioactive compounds extracted from natural sources, including plants, bacteria, and fungi, for efficient skin-lightening ingredients in the cosmetic industry have emerged as attractive sources because they contain numerous biologically active molecules with promising anti-inflammatory and antioxidant properties.1,7 Due to legalization in various countries, non-drug varieties of *Cannabis sativa* L. have recently emerged as a promising source of important bioactive compounds for applications in functional foods, medicines, and psychoactive medicines.8,9 *C. sativa* is an annual plant from the Cannabaceae family originating from Central Asia that has been used in folk medicine and also as a source of textile fiber since ancient times.9 The hemp seed is recognized as a super food because it contains a large number of beneficial molecules such as essential amino acids, unsaturated fatty acids, dietary fiber, and vitamins belonging to B family, which are not produced in the human body.8 hemp seeds contain numerous biologically active molecules, including cannabinoids, terpenes, and phenolic compounds.8,9 Hemp seed has gained much attention due to its therapeutic potential, which is based on high antioxidant, anti-inflammatory, and antiaging properties with low psychoactive effects.8b Furthermore, hemp seed is considered as an appealing functional cosmetic material in the cosmetic industry for skin whitening and wrinkle treatment. However, compounds that are responsible for the skin whitening effect have not been identified yet.

In this study, we prepared crude hemp seed extracts and solvent fractions via serial fractionation to investigate on their inhibitory effects on melanin biosynthesis in B16F10 melanoma cells. Additionally, we identified 26 compounds in the ethyl acetate (EtOAc) fraction and further assessed the effects of
phenethyl cinnamamides isolated from the hemp seed EtOAc fraction on the inhibition of melanogenesis.

**RESULTS AND DISCUSSION**

**Comparative Analysis of Hemp Seed Solvent Fractions for ABTS Radical Scavenging Activity and Tyrosinase Activity.** The defatted ethanol (EtOH) extract of hemp seed was fractionated with various solvents including hexane (n-Hex), methylene chloride (CH2Cl2), EtOAc, and butanol (n-BuOH) to identify and characterize ingredients inhibiting melanogenesis. To evaluate the potential of hemp seed solvent fractions against melanogenesis, we performed comparative analysis of the four different hemp seed solvent fractions for the radical scavenging and anti-tyrosinase activities. Intracellular ROS generation is involved in cell signaling for melanogenesis in melanoma cells by regulation of tyrosinase activity. To evaluate the antioxidant activity of the four solvent fractions, we determined the 2,2’-azinobis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) radical scavenging activities of the fractions in the concentration range of 2–200 μg/mL. All test samples exhibited potent ABTS scavenging activities in a concentration-dependent manner (Figure 1A). In particular, EtOAc and n-Hex fractions completely scavenged ABTS radicals at a concentration of 50 μg/mL. The ABTS radical scavenging activity increased in the following order: EtOAc > n-Hex > n-BuOH and CH2Cl2. These findings are consistent with a previous report. Furthermore, the inhibitory effects of the four solvent fractions on tyrosinase activity increased in a concentration-dependent manner. For instance, the maximal inhibitory activities of EtOAc and n-Hex fractions were over 40%, while those of CH2Cl2 and n-BuOH fractions were below 40% (Figure 1B). The inhibitory activity increased in the following order: EtOAc > n-Hex > n-BuOH and CH2Cl2. Furthermore, IC50 values of n-Hex, CH2Cl2, EtOAc, and n-BuOH for tyrosinase inhibition were 99.3, 65.5, 24.5, and 157.7 μg/mL, respectively.

**Inhibitory Potential of Hemp Seed Solvent Fractions against Melanogenesis.** To assess the anti-melanogenesis activity of the four solvent fractions, we conducted anti-melanogenesis tests in melanoma cells. First, the effects of the hemp seed solvent fractions on melanoma cell proliferation were investigated by treating B16F10 cells with 20 μg/mL of each different fractions or arbutin in the presence of α-melanocyte stimulating hormone (α-MSH), a hormone known to promote melanogenesis through microphthalmia-associated transcription factor induction. The results showed that none of the fractions exhibited significant effect on melanoma cell proliferation (Figure 1C). We then investigated the inhibitory effect of the solvent fractions on melanogenesis stimulated by α-MSH in melanoma cells. Treatment with α-MSH induced an approximately 1.7-fold increase in the intracellular melamin content of B16F10 cells (Figure 1D). Findings showed that α-MSH-mediated melanogenesis was completely inhibited by the EtOAc fraction (p < 0.001) but partially inhibited by n-Hex (75%, p < 0.001), CH2Cl2 (33%, p < 0.05), and n-BuOH (50%, p < 0.01) fractions (Figure 1D). Furthermore, since tyrosinase plays a key role in melanogenesis, the inhibitory effects of the solvent fractions on tyrosinase activity mediated by α-MSH were also determined. The EtOAc (61%, p < 0.001), n-Hex (28%, p < 0.05), and n-BuOH (22%, p < 0.05) fractions reversed tyrosinase activation mediated by α-MSH, whereas CH2Cl2 fractions did not show significant inhibitory property, as shown in Figure 1E.

To identify functional ingredients in hemp seed extracts that could be responsible for the inhibition of melanogenesis in melanoma cells, we determined the total phenolic content in each solvent fraction. Results demonstrated that phenolic compounds were most abundant in the EtOAc fraction [192.3 ± 4.5 mg of the gallic acid equivalents (GAE)/g extract], followed by n-Hex (95.8 ± 2.4 mg of the GAE/g extract), n-BuOH (51.4 ± 3.5 mg of the GAE/g extract), and CH2Cl2 (44.5 ± 7.0 mg of the GAE/g extract), as shown in Figure 1F. These results indicated that the inhibition rate of each solvent fraction on melanogenesis followed the same order as that of total phenolic content (i.e., EtOAc > n-Hex > n-BuOH > CH2Cl2). Phenolic compounds are known to have antioxidant, anti-tyrosinase, and anti-inflammatory effects. Taken together, these results suggested that the bioactive ingredients inhibiting melanin synthesis were mainly found in the EtOAc fraction, one of which is phenolic compounds.

**Identification and Characterization of Bioactive Compounds of Hemp Seed Extracts.** In order to identify and characterize compounds inhibiting melanogenesis, we established an in-house library isolated from hemp seed extracts (up to 2020). Previous studies reported the presence of various compounds such as lignanamides and phenolic acids in hemp seed extracts. Herein, the coupled ultra-performance liquid chromatography to quadrupole time-of-flight mass spectrometry (UPLC-QTOF-MS/MS) tentatively identified 26 compounds in the hemp seed extract including phenolic compounds within 18 min based on their negative ion electrospray ionization (ESI) mass spectra (Figure 2). Compounds were identified based on the m/z values of the molecular ion [M – H]-, and the identifications were supported by comparing obtained mass spectra with mass spectra available in the MassLynx V4.1 Library (Waters Corporation, Milford, MA, USA). However, three major peaks (peaks 16, 20, and 22) could not be identified using the in-house library. Details on their observed masses and calculated molecular formula are given in Table 1. In accordance with previous investigations of chemical components of hemp seeds, compounds identified by MS/MS were predominantly phenylpropionamides, namely, phenolic amides and lignanamides.

To identify compounds inhibiting melanogenesis, out of the 26 identified compounds, we prepared three phenethyl cinnamamides such as N-trans-caffeoyltyramine, N-trans-coumaroyltyramine, and N-trans-feruloyltyramine which were expected to be associated with melanogenesis. N-trans-caffeoyltyramine was obtained from the hemp seed EtOAc fraction using prep-high-performance liquid chromatography, while N-trans-coumaroyltyramine and N-trans-feruloyltyramine were obtained by chemical synthesis. The three compounds were then identified by comparing their 1H and 13C NMR data.
Table 1. Tentative Identification of Compounds in the EtOAc Fraction of Hemp Seed Extracts

| no. | RT (min) | m/z [M-H]^- | mass error (ppm) | response | MS^2 (m/z) | tentative identification | references |
|-----|----------|-------------|-----------------|----------|------------|--------------------------|------------|
| 1   | 1.97     | 137.0245    | 0.8             | 57,489   | 4-hydroxybenzoic acid   | 14         |
| 2   | 2.12     | 153.0195    | 1.1             | 1389     | protocatechuic acid     | 14a        |
| 3   | 2.16     | 179.0352    | 0.9             | 4301     | cinnamic acid           | 14a, 25    |
| 4   | 2.33     | 167.0352    | 1.6             | 2135     | vanillic acid           | 14a        |
| 5   | 2.63     | 314.1038    | 1.4             | 12,851   | N-trans-cafeoyltyramine | 14, 26     |
| 6   | 2.86     | 121.0297    | 1.8             | 19,847   | benzoic acid            | 14b        |
| 7   | 3.22     | 163.0403    | 1.2             | 31,986   | coumaric acid           | 14a        |
| 8   | 3.59     | 298.1092    | 2.3             | 9048     | N-trans-coumaroyltyramine | 26        |
| 9   | 3.76     | 447.0940    | 1.6             | 1472     | kaempferol 3-O-beta-glucopyranoside | 27 |
| 10  | 3.76     | 314.1039    | 1.7             | 22,146   | N-trans-cafeoyltyramine | 14, 26     |
| 11  | 3.79     | 193.0508    | 0.9             | 21,838   | ferulic acid            | 14a        |
| 12  | 4.39     | 298.1093    | 2.3             | 10,576   | N-trans-cafeoyltyramine | 14         |
| 13  | 4.99     | 298.1093    | 2.6             | 1,117,935| N-trans-cafeoyltyramine | 14         |
| 14  | 5.55     | 187.0977    | 0.9             | 20,532   | azeleic acid            | 14a        |
| 15  | 5.84     | 137.0245    | 0.9             | 224,46   | salicylic acid          | 14a        |
| 16  | 6.10     | 811.4535    | -1.1            | 1,173    | C_{13}H_{22}O_{12}     | 25, 28     |
| 17  | 6.72     | 282.1133    | -0.9            | 95,485   | N-trans-coumaroyltyramine | 14, 26     |
| 18  | 6.88     | 593.1917    | -2.01           | 225,488  | cannabinol A            | 14, 26     |
| 19  | 7.75     | 593.2088    | -0.9            | 310,177  | cannabinol B            | 14, 26     |
| 20  | 7.47     | 899.5063    | -0.6            | 140,141  | C_{39}H_{80}O_{22}      | 26         |
| 21  | 7.66     | 312.1242    | 0.6             | 67,534   | N-trans-feruloyltyramine | 14, 26     |
| 22  | 8.29     | 943.5325    | -0.5            | 106,940  | C_{40}H_{82}O_{23}      | 26         |
| 23  | 8.96     | 285.0405    | 0.3             | 22,053   | luteolin                | 14b, 26    |
| 24  | 9.25     | 595.2088    | 0.4             | 5476     | cannabinol B isomer     | 14b, 26    |
| 25  | 9.85     | 609.2231    | -0.3            | 26,613   | N-trans-cafeoyltyramine | 26         |
| 26  | 10.32    | 609.2236    | -1.1            | 30,701   | cannabinol C            | 14, 26     |
| 27  | 11.38    | 609.2231    | -0.2            | 3630     | demethylcannabigerol    | 26         |
| 28  | 12.00    | 609.2234    | 0.9             | 4254     | cannabinol C isomer     | 14b, 26    |
| 29  | 13.90    | 269.0461    | 2.1             | 3645     | apigenin                | 28         |
| 30  | 14.51    | 623.2399    | 0             | 7437     | cannabinol D            | 14b, 26    |
| 31  | 15.41    | 595.2081    | -0.8            | 4351     | 3,3'-dimehtylcannabigerol | 26 |
| 32  | 16.30    | 623.2397    | -0.2            | 3096     | grossamide              | 26         |
| 33  | 17.48    | 641.2510    | 0.9             | 4778     | cannabinol E            | 26         |
| 34  | 17.69    | 490.1877    | 1.2             | 969      | grossamide K            | 26         |
| 35  | 24.76    | 595.2097    | 0.7             | 4354     | N-trans-cafeoyltyramine | 26         |

Inhibitory Potential of Phenethyl Cinnamamide Compounds Identified in Hemp Seed Extracts against Melanogenesis. To assess the inhibitory activity of the three phenethyl cinnamamide derivatives identified in hemp seed extracts, we conducted anti-melanogenesis tests in melanoma cells. First, the effects of the three compounds on melanoma cell proliferation were investigated by treating B16F10 cells with 50 μM of each compound in the presence of α-MSH. The results showed that N-trans-cafeoyltyramine and N-trans-feruloyltyramine exhibited no significant effect on melanoma cell proliferation.
proliferation, while N-trans-coumaryltaryamine inhibited melanoma proliferation by more than 50% of control (Figure 4A). Furthermore, we investigated the inhibitory effect of the three derivatives on α-MSH-induced melanin synthesis in B16F10 cells. Treatment with α-MSH induced approximately a 1.7-fold increase in the intracellular melanin content of B16F10 cells (Figure 4B). It is observed that compound 2 (N-trans-feruloyltaryamine) partially inhibited α-MSH-induced melanogenesis (45%, p < 0.05), whereas compounds 1 (N-trans-cafeoyltaryamine) and 3 (N-trans-coumaryltaryamine) completely reversed α-MSH-induced melanogenesis (Figure 4B, p < 0.001). We also investigated the effect of the three compounds on tyrosinase activity in B16F10 cells. Results showed that tyrosinase activity mediated by α-MSH was partially reversed by...
N-trans-caffeoyltyramine (68%, \( p < 0.001 \)) and N-trans-feruloyltyramine (36%, \( p < 0.05 \)). IC\(_{50}\) values of N-trans-caffeoyltyramine, N-trans-feruloyltyramine, and N-trans-coumaroyltyramine for inhibition of \( \alpha \)-MSH-mediated tyrosinase activation were 0.8, 20.2, and 6.3 \( \mu \)M, respectively (data not shown). Interestingly, N-trans-coumaroyltyramine showed the strongest inhibitory activity on melanogenesis at 50 \( \mu \)M concentration and demonstrated anti-proliferative activity on...
cancer cells, which is consistent with a previous report. Thus, N-trans-coumaroyltyramine could be more appropriate for the development of future anticancer agents rather than functional cosmetic materials. Furthermore, our results suggested that the three phenethyl cinnamamide derivatives with similar structures but different substitution groups on the same positions showed different biological activities on melanogenesis and cell proliferation. Among the three phenethyl cinnamamide derivatives under investigation, N-trans-cafeoyltyramine exhibited potent inhibitory activity against melanogenesis and lowest IC_{50} values for inhibition of α-MSH-mediated tyrosinase activation in melanoma cells.

To predict interaction between the three phenethyl cinnamamides and tyrosinase, a computational molecular docking study was performed using the Flare V4.0 program. The best poses for the three compounds, docked to tyrosinase, are depicted in Figure 5. The molecular docking study revealed that N-trans-cafeoyltyramine and N-trans-coumaroyltyramine established a π−π stacking with His 263 and H-bond with Glu 322, while N-trans-feruloyltyramine established a π−π stacking with Phe 264 and a series of H-bond with Met 280 and Thr 261. Furthermore, binding energies between tyrosinase and the three compounds, including N-trans-cafeoyltyramine, N-trans-feruloyltyramine, and N-trans-coumaroyltyramine, were −11.44, −10.35, and −10.64 kJ/mol, respectively. These results indicated that binding energy between tyrosinase and N-trans-cafeoyltyramine was higher than the other two molecules, which is consistent with the cell-based evaluation. In addition, we observed that the hydroxyl group at the meta position of the benzene ring helped the hydroxyl group at the para position of the benzene ring to interact with tyrosinase.

Veriﬁcation of the Anti-melanogenesis Effect of N-trans-cafeoyltyramine. To verify the inhibitory effect of N-trans-cafeoyltyramine against melanogenesis, we determined the inhibitory effect of the synthetic compound on melanin synthesis mediated by α-MSH. Treatment of melanoma cells with N-trans-cafeoyltyramine in the concentration range of 2−100 μM exhibited no signiﬁcant effect on melanoma cell proliferation (Figure 6A). The result showed that N-trans-cafeoyltyramine reversed α-MSH-mediated melanin synthesis in a concentration-dependent manner, with complete inhibition reported at 50 and 100 μM (Figure 6B). Furthermore, N-trans-cafeoyltyramine also reversed α-MSH-mediated tyrosinase activation in a concentration-dependent manner, with maximal inhibition at 50 and 100 μM (Figure 6C). These results indicated that N-trans-cafeoyltyramine inhibited α-MSH-mediated melanin synthesis through inhibiting tyrosinase activity.

MATERIALS AND METHODS

Materials. The hemp seed was purchased from “Nature and Life” company in Hongcheon in South Korea and dried under ambient conditions, before use. Reagents, such as ABTS, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide...
(MTT), α-MSH, Folin-Ciocalteu reagent, and mushroom tyrosinase enzyme, were obtained from Sigma-Aldrich (St. Louis, MO, USA). Potassium persulfate (K₂S₂O₈) and 3,4-dihydroxy-L-phenylalanine (L-DOPA) were purchased from Alfa Aesar (Haverhill, MA, USA).

**Extraction and Fractionation of Hemp Seeds.** Dried hemp seeds (960 g) were pulverized using a pulverizer (HMF-3250s, Hanil Electric, Korea), then defatted with petroleum ether (9.6 L) at room temperature (RT) for 2 h, as described previously. Defatted hemp seeds were added to 70% EtOH (9.6 L), and the mixture was sonicated three times for 15 min at 20% amplitude (Bandelin, HD 4200, Germany). The resulting extract was vacuum filtered using Advantec filter papers no. 1 and no. 2 (Toyo Roshi Kaisha, Ltd, Tokyo, Japan) and concentrated using a Hei-VAP Advantage rotary evaporator (Heidolph, Germany) to obtain 27 g of the EtOH crude extract. The crude extract was suspended in dH₂O (270 mL) and successively portioned with n-Hex, CH₂Cl₂, EtOAc, and n-BuOH to yield n-Hex (1.44 g, 5.3%), CH₂Cl₂ (4.50 g, 16.7%), EtOAc (0.85 g, 3.1%), and n-BuOH (1.01 g, 3.7%) fractions, as described previously. The crude extracts and fractions were freeze-dried and kept at −80 °C prior to use.

**Determination of ABTS Radical Scavenging Activity.** In order to evaluate antioxidative potential of hemp seed solvent fractions, the ABTS radical scavenging activity was assessed, according to the previously described method. To prepare fresh ABTS radical solution, 10 mL of 7 mM ABTS was mixed with 176 μL of 140 mM potassium peroxysulfate in dH₂O and reacted in the dark at RT for 16 h. The ABTS radical solution was then diluted with methanol to obtain an absorbance of approximately 0.7 at 732 nm. Aliquots of 100 μL of each fraction in the concentration range of 2–200 μg/mL were mixed with 100 μL of the diluted ABTS radical solution and incubated for 10 min in the dark at RT. The absorbance was then taken at 732 nm using a SpectraMax M5 Multi-Mode microplate reader (Molecular Devices, Sunnyvale, CA, USA). The percentage of ABTS radical scavenging activity was calculated as follows

\[
\text{ABTS radical scavenging activity (\%)} = 1 - \left( \frac{A_{\text{sample}}}{A_{\text{control}}} \right) \times 100
\]  

(1)

**In Vitro Tyrosinase Inhibition.** The inhibitory potential of hemp seed solvent fractions on tyrosinase activity was assessed by measuring the total amount of dopachrome generated during the catalytic enzyme reaction of tyrosinase. Briefly, 50 μL of each fraction with the concentration range of 1–100 μg/mL was added to mushroom tyrosinase solution (50 U/mL) prepared in 50 mM phosphate-buffered saline (PBS; 8.1 mM Na₂HPO₄, 1.2 mM KH₂PO₄, pH 6.8, 2.7 mM KCl, 138 mM NaCl) in a 96-well plate and incubated for 30 min at RT. Then, equal quantities of 1 mM L-DOPA were added to the reaction mixtures, followed by further incubation for an additional 10 min at 37 °C. The absorbance of the resulting solution was measured at 475 nm, using a SpectraMax M5 Multi-Mode microplate reader.

**Determination of the Total Phenolic Content.** The total phenolic content of each solvent fraction was measured via the Folin-Ciocalteu method adopted in the previous report with slight modifications. Briefly, diluted crude extracts or solvent fractions of hemp seeds (25 μL) were mixed with 100 μL of the diluted Folin-Ciocalteu reagent (1:4) and incubated for 5 min. Then, 75 μL of sodium carbonate solution (100 g/L) was added to the reaction mixture and further incubated for 2 h. The absorbance was measured at 765 nm using a microplate reader.

Gallic acid dilutions (10–200 mg/L) were used as standards for calibration. The total phenolic content was expressed as mg GAE per g of the extract or fraction.

**Cell Culture.** B16F10 murine melanoma cells were cultured in Dulbecco’s modified eagle medium (Gibco, Gaithersburg, USA) supplemented with 10% heat-inactivated fetal bovine serum (Gibco), 100 units/mL penicillin, and 100 μg/mL streptomycin (Gibco) at 37 °C in a humidified atmosphere of 5% carbon dioxide (CO₂).

**Cell Viability.** The viability of B16F10 cells was determined using an MTT assay, as previously described. Briefly, B16F10 cells were seeded in 24-well plates (1 × 10⁵ cells/well) and incubated for 24 h. The cells were treated with hemp seed solvent fractions or chemical compounds at concentrations of 20 μg/mL or 50 μM, respectively. After being incubated for 48 h, the cells were then incubated with MTT solution for 4 h, and the generated formazan crystals were dissolved in dimethyl sulfoxide (DMSO). 200 μL of the resulting solution was transferred to 96-well plates, and the absorbance was measured at 540 nm using a SpectraMax M5 Multi-Mode microplate reader.

**Melanin Content Determination.** The melanin content was determined, as previously described, with some modifications. The melanoma cells were seeded in a 6-well plate and cultured for 24 h. They were treated with hemp seed solvent fractions or chemical compounds at concentrations of 20 μg/mL or 50 μM, respectively, for a further 48 h in the presence of 100 nM α-MSH. After washing twice with chilled Dulbecco’s PBS supplemented with calcium chloride and magnesium chloride (D-PBS, Gibco), the resulting cells were collected via trypsinization. After centrifugation at 1000 rpm for 3 min, the cell pellet was dissolved in 150 μL of 1 N NaOH containing 10% DMSO for 1 h at 60 °C. The melanin content was determined by the absorbance at 405 nm, using a microplate reader.

**Determination of Cellular Tyrosinase Activity in Melanoma Cells.** Tyrosinase activity in B16F10 cells was examined based on the amount of dopachrome produced from the catalytic reaction of intracellular tyrosinase. Briefly, melanoma cells were cultured in a 6-well plate for 24 h, followed by treatment with different concentrations of hemp seed solvent fractions or chemical compounds for a further 48 h in the presence of 100 nM α-MSH. After washing twice with ice-cold D-PBS, the cells were lysed in 200 μL of radio-immunoprecipitation assay buffer (Sigma-Aldrich) containing protease and phosphatase inhibitors. The cell lysate from each well was collected after being centrifuged at 15,000g for 15 min. 100 μL of the supernatant was mixed with 100 μL of 1 mM L-DOPA in PBS (pH 6.8), followed by incubation for 30 min at 37 °C. The absorbance of dopachrome was measured at 475 nm using a microplate reader. Data were normalized with protein concentration determined by bicinchoninic acid assay.

**Characterization of Bioactive Compounds Using UPLC-QTOF-Mass Spectrometry/Mass Spectrometry.** The EtOAc fraction was analyzed using the Waters Acquity UPLC I-Class system (Waters Co., Milford, MA, USA) coupled with mass spectrometry, as previously described. The EtOAc fraction was dissolved in 70% methanol at a concentration of 100 ppm and filtered through a 0.2 μm PTFE syringe filter. The Waters Acquity UPLC BEH C18 column (150 mm × 2.1 mm, 1.7 μm) was used for chromatographic separation. The separation conditions were as follows: mobile phases: 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B), and the following gradient was used: 0–5 min, 10–20% B; 5–25 min, 20–22% B; 25–45 min, 22–25% B; 45–65 min, 25–30%
B1 and 65–85 min, 30% B. The injection volume was 2 μL. The mass spectrometry (MS) experiments were performed on a Waters Xevo G2 QTof mass spectrometer (Waters MS Technologies, Manchester, UK) equipped with an ESI interface. The MS/MS ion patterns were obtained using a collision energy ramp from 15 to 45 eV in the MSe mode. The ESI parameters were set as follows: in the negative ion mode in a continuum format, a capillary voltage of 2.5 kV, a cone voltage of 45 V, a source temperature of 120 °C, a desolvation temperature of 350 °C, a cone gas flow of 50 L/h, and a desolvation gas flow of 800 L/h. The ion acquisition rate was 0.25 s with resolution in excess of 20,000 fwhm, and the inter-scan delay time was 0.014 s. The mass range was from m/z 100 to 1500. The instrument was calibrated using a sodium formate solution as the calibration standard. Leucine encephalin (m/z 554.23165 in the negative mode) was used as a reference or lock mass at a concentration of 200 pg/μL and a flow rate of 5 μL/min and was sprayed into the MS instrument every 10 s to ensure accuracy and reproducibility. Data acquisition was controlled by MassLynx V4.1 software (Waters Corporation, Milford, USA), and identification of components was performed by UNIFI.

**Tentative Identification by UNIFI and Search of an In-House Library.** Compound identifications were achieved by UNIFI 1.8 (Waters, Milford, USA). The Traditional Chinese Medicine (TCM) library in UNIFI consisted of more than 7500 natural product compounds. The library was updated with compounds that were either isolated or analyzed from hemp seed (up to 200) by searching databases such as Dictionary of Natural Product (CRC, 2009), SciFinder Scholar of the American Chemical Society, and Natural Product Activity & Species Source Database. The database and structure files that were prepared using ChemBioDraw 15.0 were saved as a mol file individually into UNIFI. Key parameters within UNIFI were set as follows: peak detection time, 1–85 min; intensity threshold, 3000 and 300 counts for low-energy and high-energy acquisitions, respectively; and mass accuracy, ±5 ppm. Both the TCM library and the in-house library were retrieved in identification.

**Molecular Modeling.** The crystal structure of Agaricus bisporus mushroom tyrosinase (PDB dose: 2Y9X) for molecular modeling was obtained from Protein Data Bank (PDB). The enzyme was prepared using the protein wizard preparation workflow embedded in the Flare V4.0 program (Cresset Software Inc., England). Water and all the other molecules present in the pdb files were removed. Molecular docking was performed using the standard docking mode and the extra precision mode.

**Statistical Analysis.** All data in this study were expressed as the mean ± standard deviation (SD) from three independent experiments. Statistical analyses were performed using Graph-Pad Prism 8.0 (GraphPad Software Inc., La Jolla, CA, USA). The differences between the mean values of the control and the exposed groups were analyzed using one-way analysis of variance (ANOVA), followed by Dunnett’s post hoc test. The threshold for statistical significance for all analyses was P < 0.05 (two-tailed).

**CONCLUSIONS**

In this study, we demonstrated that the EtOAc fraction of hemp seed extracts scavenged ABTS radicals and exhibited potent inhibitory effects on melanin biosynthesis, indicating that bioactive compounds might be abundant in this fraction. We tentatively identified 26 compounds, including phenethyl cinnamamides, contained in the EtOAc fraction by comparison of spectroscopic data with those in the library. We prepared three phenethyl cinnamamides such as N-trans-cafeoyltyramine, N-trans-coumaroyltyramine, and N-trans-feruloyltyramine and determined their chemical structures. Comparative evaluation of the three compounds for their anti-melanogenesis activity in melanoma cells revealed that the purified N-trans-cafeoyltyramine exhibited the strongest potential for anti-melanogenesis activity. The inhibitory effect of N-trans-cafeoyltyramine was verified by determining the melanin content and tyrosinase activity in melanoma after treating the cells with synthetic compounds. It can be concluded that N-trans-cafeoyltyramine isolated from hemp seed extracts could be useful in cosmetics as a skin-whitening agent.

**AUTHOR INFORMATION**

**Corresponding Authors**

Se-Hui Jung — Department of Bio-Health Technology, College of Biomedical Science, Kangwon National University, Chuncheon 24341, Republic of Korea; Research Institute, K-medichem Co., Ltd., Chuncheon 24341, Republic of Korea; orcid.org/0000-0002-9323-2745; Phone: +82-33-255-6477; Email: sh0603@kangwon.ac.kr

Kooyeon Lee — Department of Bio-Health Technology, College of Biomedical Science, Kangwon National University, Chuncheon 24341, Republic of Korea; Research Institute, K-medichem Co., Ltd., Chuncheon 24341, Republic of Korea; orcid.org/0000-0003-2180-6209; Phone: +82-33-250-6477; Email: lky@kangwon.ac.kr

**Authors**

Jae Kwon Kim — Department of Bio-Health Technology, College of Biomedical Science, Kangwon National University, Chuncheon 24341, Republic of Korea

Hee-Young Heo — Department of Bio-Health Technology, College of Biomedical Science, Kangwon National University, Chuncheon 24341, Republic of Korea

SeonJu Park — Chuncheon Center, Korea Basic Science Institute (KBSI), Chuncheon 24341, Republic of Korea

Haheon Kim — Department of Bio-Health Technology, College of Biomedical Science, Kangwon National University, Chuncheon 24341, Republic of Korea

Jeong Ju Oh — Department of Bio-Health Technology, College of Biomedical Science, Kangwon National University, Chuncheon 24341, Republic of Korea

Eun-Hwa Sohn — Department of Herbal Medicine Resource, Institute of Bioscience and Biotechnology, Kangwon National University, Samcheok 25949, Republic of Korea

Complete contact information is available at: https://pubs.acs.org/10.1021/acsomega.1c04727

**Notes**

The authors declare no competing financial interest.

**ACKNOWLEDGMENTS**

This study was partially supported by grants from the Research and Development of Chuncheon Hemp (Chuncheon city, Korea), Presidential Committee for Balanced National Development (PCBND), and the Ministry of Land, Infrastructure and Transport (MOLIT) through the pilot project for regional development investment agreement (B0070128000223). HR-ESI-MS spectra were obtained from Korea Basic Science Institute (KBSI, Chuncheon center).

31953 https://doi.org/10.1021/acsomega.1c04727

ACS Omega 2021, 6, 31945–31954
DEDICATION
This paper is dedicated to Professor Phil Ho Lee (Kangwon National University) on the occasion of his 60th birthday.

REFERENCES
(1) Zolghadri, S.; Bahrami, A.; Hassan Khan, M. T.; Munoz-Munoz, J.; Garcia-Molina, F.; Garcia-Canovas, F.; Saboury, A. A. A comprehensive review on tyrosinase inhibitors. J. Enzym. Inhib. Med. Chem. 2019, 34, 279–309.

(2) Brown, D. A. Skin pigmentation enhancers. J. Photochem. Photobiol. B 2001, 63, 148–161.

(3) Chiang, H.-M.; Chien, Y.-C.; Wu, C.-H.; Kuo, Y.-H.; Wu, W.-C.; Pan, Y.-Y.; Su, Y.-H.; Wen, K.-C. Hydroalcoholic extract of Rhodiola rosea L. (Crassulaceae) and its hydrolylate inhibit melanogenesis in B16F0 cells by regulating the CREB/MITF/tyrosinase pathway. J. Investig. Dermatol. 2007, 127, 751–761.

(4) Ando, H.; Kondoh, H.; Ichihashi, M.; Hearing, V. J. Approaches to identify inhibitors of melanin biosynthesis via the quality control of tyrosinase. J. Invest. Dermatol. 2007, 127, 751–761.

(5) Montaz, S.; Lall, N.; Basson, A. Inhibitory activities of mushroom tyrosine and DOPA oxidation by plant extracts. South Afr. J. Bot. 2008, 74, 577–582.

(6) Lee, S. Y.; Baek, N.; Nam, T.-g. Natural, semisynthetic and synthetic tyrosinase inhibitors. J. Enzyme Inhib. Med. Chem. 2016, 31, 1–15.

(7) Cazaroli, L.; Zanatta, L.; Alberton, E.; Bonorino Figueiredo, M. S.; Folador, P.; Damazio, R.; Pizzolatti, M.; Barreto Silva, F. R. Flavonoids: prospective drug candidates. Mini Rev. Med. Chem. 2008, 8, 1429–1440.

(8) Kushnina, N. D. T.; Lamb, A. J. Recent advances in understanding the antibacterial properties of flavonoids. Int. J. Antimicrob. Agents 2011, 38, 99–107.

(9) Yan, X.; Tan, J.; dos Santos Passos, C.; Nurisso, A.; Simões-Pires, C. A.; Li, M.; Hou, H.; Fan, P. Characterization of Lignanamides from Hemp (Cannabis sativa L.) Seed and Their Antioxidant and Acetylcholinesterase Inhibitory Activities. J. Agric. Food Chem. 2015, 63, 10611–10619.

(10) Zhou, Y.; Wang, S.; Lou, H.; Fan, P. Chemical constituents of hemp (Cannabis sativa L.) seed with potential anti-neuroinflammatory activity. Phytochem. Lett. 2018, 23, 57–61.

(11) Andre, C. M.; Hausman, J.-F.; Guerriero, G. Cannabis sativa: The Plant of the Thousand and One Molecules. Front. Plant Sci. 2016, 7, 19.

(12) Smiriglio, A.; Galati, E. M.; Monforte, M. T.; Lanuzza, F.; D’Angelo, V.; Circosta, C. Polyphenolic Compounds and Antioxidant Activity of Cold-Pressed Seed Oil from Finola Cultivar of Cannabis sativa L. Phytother Res. 2016, 30, 1298–1307.

(13) Kim, Y. J.; Kang, K. S.; Yokozawa, T. The anti-melanogenic effect of pycnogenol by its anti-oxidative actions. Food Chem. Toxicol. 2008, 46, 2466–2471.

(14) Chou, T.-H.; Ding, H.-Y.; Hung, W. J.; Liang, C. H. Antioxidative characteristics and inhibition of alpha-melanocyte-stimulating hormone-stimulated melanogenesis of vanillin and vanillic acid from Origanum vulgare. Exp. Dermatol. 2010, 19, 742–750.

(15) Yang, J.; Kwon, Y. S.; Kwon, Y. S.; Lim, J. D.; Yu, C. Y.; Kim, M. J. Antioxidant and Anticancer Properties of the Extracts from Lepisorus thunbergianus (Kaulf.) Ching. Korean J. Medicinal Crop Sci. 2015, 23, 324–333.

(16) Shim, E.; Song, E.; Choi, K. S.; Choi, H.-J.; Hwang, J. Inhibitory effect of Gastrodia elata Blume extract on alpha-melanocyte stimulating hormone-induced melanogenesis in murine B16F10 melanoma. Natr. Res. Pract. 2017, 11, 173–179.

(17) Re, R.; Pellegrini, N.; Protegente, A.; Pannala, A.; Yang, M.; Rice-Evans, C. Antioxidant activity of rutin isolated from Magnolia officinalis L. (Magnoliaceae) leaves by using liquid chromatography–ion liquid chromatography electrospray ion trap mass spectrometry. J. Chromatogr. A 2015, 1430–1438.

(18) Jung, S.-H.; Kim, J.; Eum, J.; Cho, J. W.; Kim, H. H.; Lee, K. Velutin, an Aglycone Extracted from Korean Mistletoe, with Improved Inhibitory Activity against Melanin Biosynthesis. Molecules 2019, 24, 2549.

(19) Huang, H.-C.; Hsieh, W.-Y.; Niu, Y.-L.; Chang, T.-M. Inhibitory effects of adlay extract on melanin production and cellular oxygen stress in B16F10 melanoma cells. Int. J. Mol. Sci. 2016, 17, 212.

(20) Miao, S. H.; Mariadoss, A. V. A.; Wang, M. H. Metabolite Profiling of Methanolic Extract of Gardenia jasminoides withLC-MS/MS and GC-MS and Its Anti-Diabetic, and Anti-Oxidant Activities. Pharmaceuticals 2021, 14, 102.

(21) Abu-Reidah, I. M.; Ali-Shtayeh, M. S.; Jamous, R. M.; Arráez-Román, D.; Segura-Carretero, A. Comprehensive metabolite profiling of Arum palaestinum (Araceae) leaves by using liquid chromatography–tandem mass spectrometry. Food Res. Int. 2015, 70, 74–86.

(22) Nigro, E.; Crescine, G.; Formato, M.; Pecoraro, M. T.; Mallardo, M.; Piccolella, S.; Daniele, A.; Pacifico, S. Hempseed Lignanamides Rich-Fraction: Chemical Investigation and Cytotoxicity towards U-87 Globlastoma Cells. Molecules 2020, 25, 1049.

(23) Li, R.; Liu, S.-k.; Song, W.; Wang, Y.; Li, Y.-l.; Qiao, X.; Liang, H.; Ye, M. Chemical analysis of the Tibetan herbal medicine Carduus nutans an aqueous extract of Carduus nutans an aqueous extract. Analytical Methods 2014, 6, 7181–7189.

(24) Fabre, N.; Rustan, I.; de Hoffmann, E.; Quinet-Leclercq, J. Determination of flavones, flavonol, and flavanone aglycones by negative ion liquid chromatography electrospray ion trap mass spectrometry. J. Am. Soc. Mass Spectrom. 2001, 12, 707–715.