Original Research

Inhibitory activity of ethyl acetate fraction derived from *Cannabis sativa* L. seed on melanogenesis in B16F10 cells

Jae Kwon Kim, Hak Hyun Kim, Jaehyun Kim, Young-Seok Lim, Se-Hui Jung*, and Kooyeon Lee*

Department of Bio-Health Convergence, Kangwon National University, Chuncheon, 24341, Republic of Korea

ABSTRACT

Introduction: Compared with normal skin, hyperpigmentation is characterized by darker spots or patches. Although most hyperpigmentation is harmless, it is undesirable from an aesthetic perspective. Despite the availability of various inhibitors against hyperpigmentation, the development of novel inhibitors with improved efficacy and long-term safety remains a challenge.

OBJECTIVES: In this study, we prepared hemp seed extract and its solvent fractions, and evaluated their biological activity against free radical and melanin synthesis.

Methods: To investigate a potent inhibitory effect of hemp seed extract and its solvent fractions on melanin synthesis, intracellular melanin content and tyrosinase activity in melanoma cells were determined.

Results: Defatted hemp seed extract inhibited tyrosinase activity more efficiently and with better antioxidant activity than fat extract *in vitro*. Both fat and defatted extract had no significant effect on melanoma cell proliferation. The defatted extract inhibited melanogenesis in melanoma cells by inhibiting tyrosinase activity ($p < 0.001$). Comparative evaluation of radical scavenging and anti-tyrosinase activities of hemp seed solvent fraction showed that the ethyl acetate fraction has the highest potential for inhibition of melanogenesis. Quantitative analysis of melanin content and tyrosinase activity in melanoma cells following treatment with solvent fractions suggested the presence of biologically active ingredients in the ethyl acetate fraction, which suppressed melanogenesis ($p < 0.001$). Further, the treatment of melanoma cells with ethyl acetate fraction induced dose-dependent inhibition of melanin synthesis and tyrosinase activation.

Conclusion: Thus, natural compounds present in the ethyl acetate fraction of defatted hemp seed extract improve the efficacy of melanogenesis inhibition with little toxicity, suggesting a potential functional role as a cosmetic whitening agent.

Keywords: hemp seed, melanogenesis, tyrosinase activity, fractionation

Asian Journal of Complementary and Alternative Medicine. Volume 08 Issue 1

Published on: 16/08/2020

***Author for Correspondence:** Kooyeon Lee, Department of Bio-Health Technology, College of Biomedical Science, Kangwon National University, Chuncheon, 24341, Republic of Korea; lky@kangwon.ac.kr; Tel.: +82-33-250-6477; Tel.: +82-33-255-6477

Se-Hui Jung, Department of Bio-Health Technology, College of Biomedical Science, Kangwon National University, Chuncheon, 24341, Republic of Korea; sh0603@kangwon.ac.kr

Cite this article as: Kim JK, Kim HH, Kim J, Lim YS, Jung SH, Lee K. *Inhibitory activity of ethyl acetate fraction derived from Cannabis sativa L. seed on melanogenesis in B16F10 cells*. Asian Journal of Complementary and Alternative Medicine, Vol 8 (2), 17-24:2020.

INTRODUCTION

Melanogenesis is a multistep process for synthesis of melanin pigment by melanocytes located at the epidermal-dermal junction to protect cells against UV rays, dyscrinism and mental stress [1,2]. Melanin is involved in the determination of human skin and hair color; however, abnormal regulation of melanin synthesis leads to several skin disorders such as melasma, freckles, age spots, solar lentigo, and post-inflammatory hyperpigmentation and other hyperpigmentation syndromes

[3]. Melanin is synthesized from the amino acid tyrosine via a multistep enzymatic process, which is triggered by translational upregulation of tyrosinase through ROS generated by UV irradiation and α-MSH.[4, 5] Therefore, tyrosinase inhibition has been shown to prevent skin hyperpigmentation by overproduction of melanin.[6] Skin whitening drugs including, hydroquinone, azelaic acid, arbutin, and kojic acid, have been generally used to prevent skin pigmentation by inhibiting tyrosinase activity [1,4,6]. However, the market demand for functional materials with improved safety, stability, and efficacy is enormous due to side effects of chemical drugs including cytotoxicity, dermatitis, and skin cancer, as well as poor efficacy. Isolation of skin whitening ingredients in natural products is an attractive strategy because natural products contain numerous biologically active molecules with antioxidant, anti-inflammatory, and antimicrobial properties [7,8].

Recently, *Cannabis sativa L.* emerged as an attractive natural product based on its traditional use as a source of food, fiber, medicine, and psychoactive drugs [9,10]. *C. sativa L* is available as drug and non-drug types. The drug type of cannabis is known as marijuana, which contains tetrahydrocannabinol (THC) in concentrations ranging between 1% and 20%, high enough to affect psychoactivity [9]. The non-drug type of cannabis is known as hemp, which has a psychotropic component THC at concentrations less than 0.2%. Recent interest in hemp research has increased due to various beneficial effects, including antioxidant, anti-inflammatory, anti-aging and antimicrobial functions, compared to minimal psychotropic effects [10]. In particular, hemp seed has been an important source of nutrition in China for thousands of years. It is currently 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 human body [9,10]. Further, it has been used in traditional medicine for the prevention of constipation, to lower cholesterol, improve cardiovascular health, promote immunomodulation, and in the treatment of gastrointestinal disease [11]. Furthermore, the hemp seed has potential applications in the cosmetic industry because of strong antioxidant and anti-aging effects.[9] However, the effects of hemp seed extract on melanin synthesis in melanocytes have yet to be elucidated.

In this study, we prepared defatted hemp seed extract and solvent fractions via serial fractionation of hemp seed extract and investigated the inhibitory effects of the defatted extract or solvent fractions on melanin biosynthesis in B16F10 melanoma cells. We identified ethyl acetate (EtOAc) fraction as a major portion containing the natural inhibitor and further characterized its effect on inhibition of melanin biosynthesis.

EXPERIMENTAL SECTION

Materials

The hemp seed was purchased from Biona Organic (UK). It was dried under ambient conditions before use in this study. Reagents such as 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid (ABTS) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and enzymes such as mushroom tyrosinase were obtained from Sigma-Aldrich (St. Louis, MO, USA). Potassium persulfate ($K_2S_2O_8$) and 3,4-dihydroxy-L-phenylalanine (L-DOPA) were purchased from Alfa Aesar (Haverhill, MA, USA).

Extraction and fractionation of hemp seed

Hemp seed (60 g) was pulverized using a pulverizer (HBL-3500S, Samyang Electronics, Korea), and then defatted with 100% petroleum ether (520 mL) at room temperature for 2 h as described previosly.[9] Defatted hemp seed was extracted with 70% ethanol (EtOH) three times (630 mL, each time) at room temperature for 3 d. 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 2.46 g EtOH crude extract. This crude extract was suspended in dH_2O (25 mL), and successively fractionated with n-Hexane (Hex), methylene chloride (CH_2Cl_2), ethyl acetate (EtOAc), n-butanol (BuOH) to yield Hex (75.2 mg, 3.1%), CH_2Cl_2 (192.5 mg, 7.8%), EtOAc (139.5 mg, 5.7%), BuOH (658.4 mg, 26.8%), as well as H_2O residue (1.40 g, 56.9%), as described previously [12]. The resulting extracts and fractions were freeze-dried and stored at -80°C prior to use.

Determination of ABTS free radical scavenging activity

In order to evaluate the antioxidant activity of hemp seed extracts and fractions, the ABTS radical scavenging activity was determined as described previously [13]. To generate the ABTS radical, 10 mL of 7 mM ABTS was mixed with 176 μL of 140 mM potassium peroxydisulfate in dH_2O and incubated in the dark at room temperature for 16 h prior to use. The ABTS radical solution was diluted with absolute methanol to obtain an absorbance near 0.7 at 734 nm. Aliquots of 100 μL of each extract or fractions in the indicated concentration range of 2 to 200 μg/mL were added to 100 μL of the diluted ABTS radical solution and incubated for 10 min in the dark at room temperature. The absorbance was then measured at 732 nm using a SpectraMax M5 Multi-Mode microplate reader (Molecular Devices, Sunnyvale, CA, USA). The ABTS radical scavenging activity was calculated as follows:

ABTS radical scavenging activity (%) = $1-(A_{sample}/A_{control})$ X 100 (1)

In vitro tyrosinase inhibition

The inhibitory effect of hemp seed extracts and fractions on tyrosinase activity was assessed by the amount of dopachrome synthesized from the catalytic reaction of tyrosinase [,.14]. Briefly, each 50 µL of each extract or fractions in the indicated concentration range were mixed with 50 µL of 50 U/mL mushroom tyrosinase in 50 mM phosphate buffered saline (PBS; 8.1 mM Na_2HPO_4, 1.2 mM KH_2PO_4, pH 6.8, 2.7 mM KCl, 138 mM NaCl) in a 96-well plate and incubated for 30 min at room temperature. Then, 100 µL of 1 mM L-DOPA was added to each well, followed by 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.

Cell culture

B16F10 murine melanoma cells were cultured in DMEM 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 under humidified 5% CO_2.

Cell viability

The viability of B16F10 cells was determined using an MTT assay as described previously [11]. Briefly, B16F10 cells were seeded in 24-well plates at a density of $1X10^4$ cells per well. After 24 h, cells were treated with indicated concentrations of hemp seed extracts or fractions for 48 h. The cells were then incubated with MTT solution for 4 h, and the reduced formazan crystals were dissolved in DMSO. 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 described previously with some modifications.[15] The melanoma cells were cultured in a 6-well plate for 24 h. They were treated with indicated concentrations of hemp seed extract or fractions for further 48 h in the presence of 100 nM α-MSH. After washing twice with chilled Dulbecco's phosphate buffered saline supplemented with calcium chloride and magnesium chloride (D-PBS, Gibco), the resulting cells were detached by incubation with trypsin-EDTA solution. After centrifugation at 1,000 rpm for 3 min, the cell pellet was dissolved in 150 µL of 1 M NaOH containing 10% DMSO for 1h at 60°C for 1 h. The melanin content was determined by absorbance at 405 nm using the microplate reader.

Determination of cellular tyrosinase activity in melanoma cells

Tyrosinase activity in B16 cells was examined based on the amount of dopachrome produced from the catalytic reaction of intracellular tyrosinase [16]. Briefly, melanoma cells were cultured in a 6-well plate for 24 h, followed by treatment with different concentrations of hemp seed extract or fractions for 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 (RIPA) buffer (Sigma-Aldrich) containing protease and phosphatase inhibitors. After centrifugation of cell lysate collected from each well at 15,000 g for 15 min, 100 µl of 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 the microplate reader. Data were normalized with protein concentration determined by bicinchoninic acid assay.

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 a Dunnett's post hoc test. The threshold for statistical significance for all analyses was $P < 0.05$ (two-tailed).

RESULTS AND DISCUSSION

Defatted extract from hemp seed scavenges ABTS radical and inhibits tyrosinase activity more efficiently than fat extract

Sequential extraction with petroleum ether (fat extract) and 70% ethanol (defatted extract) were performed in order to assess the potential inhibitory activity of natural compounds in the seed. To evaluate the antioxidant activity of two hemp seed extracts, we determined the ABTS radical scavenging activity in the concentration range between 2 and 200 µg/mL. Arbutin was used as a positive control. Fat extract had no significant effect on ABTS radical scavenging activity at the tested concentration, while the defatted ethanol extract showed concentration-dependent radical scavenging activity, which was complete at 200 µg/mL (Figure 1A).

We also compared the inhibitory effect of fat and defatted extracts on mushroom tyrosinase activity by measuring the rate of dopachrome synthesis catalyzed by tyrosinase. Defatted

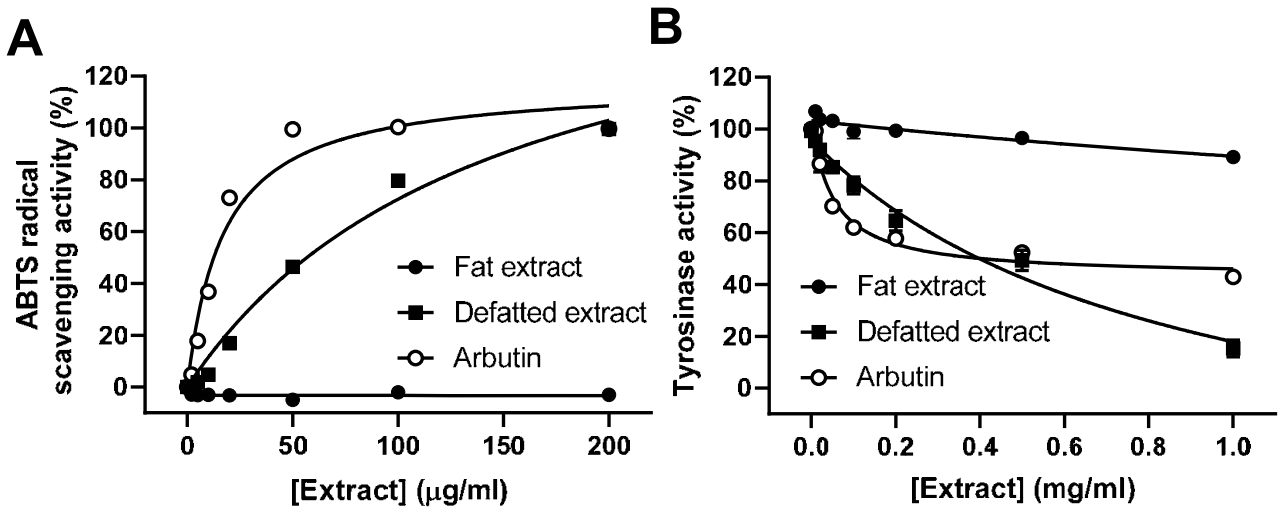

Figure 1. Comparative analysis of fat and defatted extracts of hemp seed for ABTS radical scavenging (A) and anti-tyrosinase (B) activities. (A) ABTS radical scavenging activity was determined by measuring the absorbance of mixture of ABTS radical and indicated concentrations of fat or defatted extracts; arbutin was used as the positive control. (B) Tyrosinase activity was determined via L-DOPA oxidation assay in the presence of fat or defatted extract at the indicated concentrations. Data are expressed as the mean ± SD of three independent experiments.

ethanol extract inhibited 85% of tyrosinase activity at 1 mg/mL, while fat extract inhibited only 11% of tyrosinase activity at the same concentration (Figure 2B). Furthermore, defatted ethanol extract showed higher tyrosinase inhibition than arbutin used as a positive control. These results suggest that the hemp seed contains bioactive ingredients inhibiting tyrosinase activity, and most bioactive ingredients were present in the defatted extract but not in the fat component.

Defatted extract of hemp seed prevents melanin synthesis in melanoma cells

Next, we investigated the potential inhibitory activity against melanin synthesis in melanoma cells. First, the effect of fat and defatted hemp seed extracts in the presence of α-MSH on the melanoma cell proliferation was investigated. High concentration of fat and defatted extracts (1 mg/mL) prevented the proliferation of B16F10 cells by less than 25% ($p < 0.050$), while low concentration of the extracts had no significant effect (Figure 2A). However, the fat extract was not appropriate for cell treatment due to very low solubility in the aqueous phase, because it appeared at the top of the culture medium, but not on the cell surface, when treated with cells. These results indicated that the defatting process is necessary for the extraction of bioactive molecules from hemp seed.

Then, we investigated the inhibitory effect of defatted extract on melanogenesis stimulated by α-MSH in melanoma cells. Treatment with α-MSH induced approximately 230% increase in the intracellular melanin content of B16F10 cells (Figure 2B). Defatted hemp seed extract inhibited the α-MSH-mediated elevation of melanin content in a concentration-dependent manner, with maximal inhibition (89%, $p < 0.001$) at 0.5 mg/mL (Figure 2B). Since tyrosinase is a key enzyme in melanogenesis, we investigated the effect of defatted extract on tyrosinase activity in B16F10 cells. Results showed that the defatted extract also inhibited α-MSH-induced tyrosinase activation in a concentration-dependent manner, with maximal inhibition (70%, $p < 0.005$) at 0.5 mg/mL (Figure 2C). These results indicated that defatted extract of hemp seed inhibited α-MSH-induced melanin synthesis by inhibiting the intracellular tyrosinase activity in B16F10 cells.

Inhibitory potential of hemp seed fractions against melanogenesis

To identify which solvent fractions have a potential against melanogenesis, the radical scavenging and anti-tyrosinase activities of different solvent-soluble fractions of hemp seed were analyzed. First, the radical scavenging activities of different solvent fractions were determined at the concentration range between 2 and 200 μg/mL. All fractions showed a concentration-dependent increase in ABTS radical scavenging activity (Figure 3A). In particular, ABTS radical scavenging activities of n-Hex, CH_2Cl_2, and EtOAc fractions were above 90% at 200 μg/mL concentration. The ABTS radical scavenging activity was in the following order:

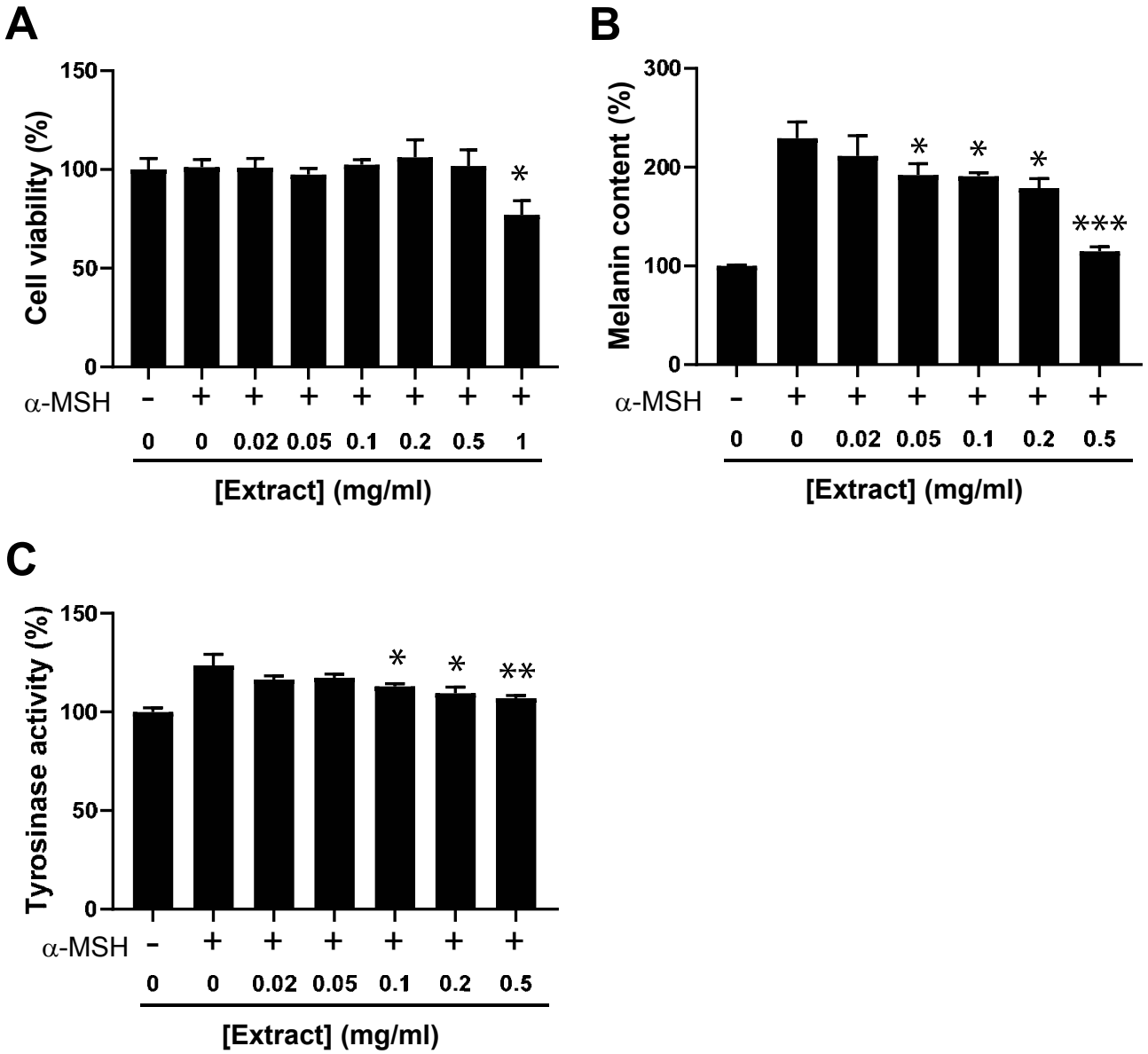

Figure 2. Effects of defatted hemp seed extract on B16F10 cell proliferation and its melanin synthesis. B16F10 cells were treated with 0.1% DMSO as vehicle or with 0.02, 0.05, 0.1, 0.2, and 0.5 mg/mL defatted extract for 48 h. (A) Melanoma cell proliferation was determined by MTT assay. (B) Melanin content in B16F10 cells was determined using a photometric method as described in the Experimental Section. (C) Cellular tyrosinase activity was determined by measuring dopachrome synthesized in the enzymatic reaction of cell lysates with L-DOPA. Data are expressed as the mean ± SD of three independent experiments (*p < 0.05, **p < 0.01, ***p < 0.001 vs. control group; treated with only α-MSH).

EtOAc > n-Hex > CH_2Cl_2 > n –BuOH > dH_2O. Further, the inhibitory effects of different solvent fractions on tyrosinase activity increased in a concentration-dependent manner: the maximal inhibitory activities of CH_2Cl_2, n –BuOH, dH_2O fractions were below 35%, but those of EtOAc and n-Hex fractions were above 70% (Figure 3B). The inhibitory activity was in the following order: EtOAc > n-Hex > CH_2Cl_2 > n –BuOH > dH_2O. These results suggested that the lipophilic fractions, including n-Hex, CH_2Cl_2, and EtOAc fractions showed higher radical scavenging activity and better inhibitory activity than the hydrophilic n–BuOH and dH_2O fractions.

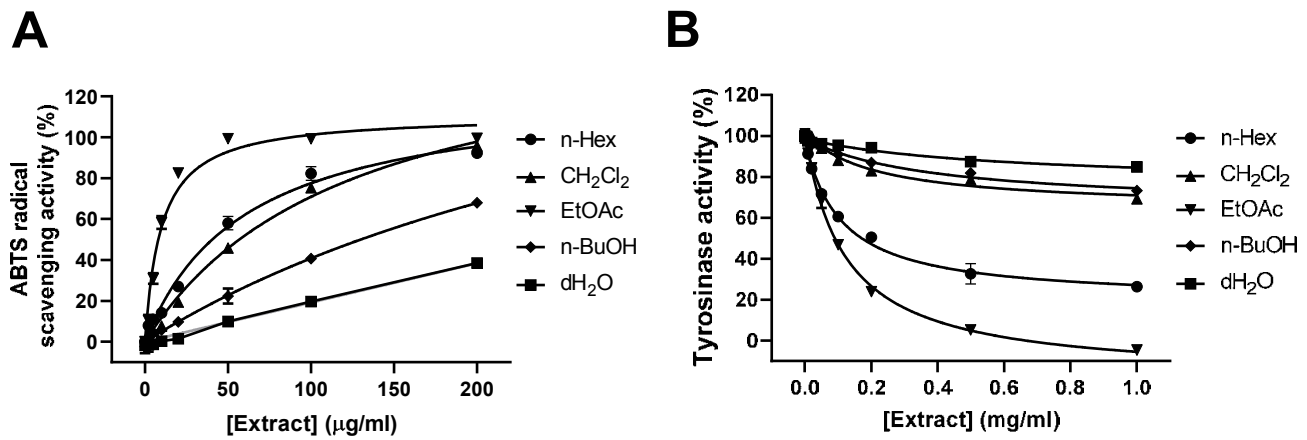

Figure 3. Effects of solvent fractions on ABTS radical scavenging and anti-tyrosinase activities. (A) ABTS radical scavenging activity was determined by measuring the absorbance of mixtures of ABTS radical and indicated concentrations of solvent fractions. (B) Tyrosinase activity was determined by L-DOPA oxidation assay in the presence of solvent fractions at the indicated concentrations. Data are expressed as the mean ± SD of three independent experiments.

Inhibitory activity of hemp seed fractions against melanin synthesis

Next, to identify biologically active solvent fractions underlying the potent anti-melanogenesis, we determined the inhibitory activity of different solvent fractions against melanin synthesis in melanoma cells. First, the effects of hemp seed fractions on melanoma cell proliferation were investigated by treating B16F10 cells with 50 μg/mL each of different solvent fractions in the presence of α-MSH. Results showed that none of the fractions had any significant effect on melanoma cell proliferation (Figure 4A).

We then investigated the inhibitory effect of solvent fractions on α-MSH-stimulated melanogenesis by treating B16F10 cells with 50 μg/mL each of different solvent fractions. Treatment with α-MSH induced approximately 320% increase in intracellular melanin content of B16F10 cells (Figure 4B). Inhibitory activities of n-Hex and CH_2Cl_2 fractions were found to be 27% and 45%, respectively (Figure 4B, $p < 0.001$). The EtOAc fraction showed the highest inhibitory activity ($p < 0.001$) against melanogenesis, but n-BuOH fraction showed only 9% inhibitory activity. Further, the inhibitory effects of solvent fractions on tyrosinase activation mediated by α-MSH were determined. Tyrosinase activation mediated by α-MSH was partially reversed by n-Hex (33%, $p < 0.001$) and CH_2Cl_2 fractions (62%, $p < 0.001$) as shown in Figure 4C. The EtOAc fraction completely inhibited α-MSH-induced tyrosinase activation ($p < 0.001$), whereas the n-BuOH fraction did not (Figure 4C), which are consistent with the results of melanogenesis. These results indicated that EtOAc fraction of hemp seed inhibited α-MSH-induced melanin synthesis by inhibiting the intracellular tyrosinase activity in B16F10 cells. Furthermore, these results suggested that the bioactive ingredients inhibiting melanin synthesis were lipophilic and were mainly found in the EtOAc fraction.

The inhibitory activity of EtOAc fraction against α-MSH-mediated intracellular melanin synthesis and tyrosinase activation was confirmed by treating cells with EtOAc fractions ranging from 5 to 100 mg/mL in the presence of α-MSH. The EtOAc fraction reduced melanin content in a concentration-dependent manner, with maximal inhibition (88%, $p < 0.001$) at 100 μg/mL concentration (Figure 5A). Further, the EtOAc fraction inhibited α-MSH-mediated tyrosinase activation in response to increasing concentrations, with maximal inhibition (85%, $p < 0.001$) observed at 100 μg/mL concentration (Figure 5B). These results suggested that the EtOAc fraction of hemp seed inhibited α-MSH-induced melanogenesis in melanoma cells by inhibiting the intracellular tyrosinase activity. Furthermore, most of the bioactive ingredients inhibiting melanin synthesis occur mainly in the EtOAc fraction. However, further investigations are necessary to identify the bioactive ingredients suppressing melanin synthesis.

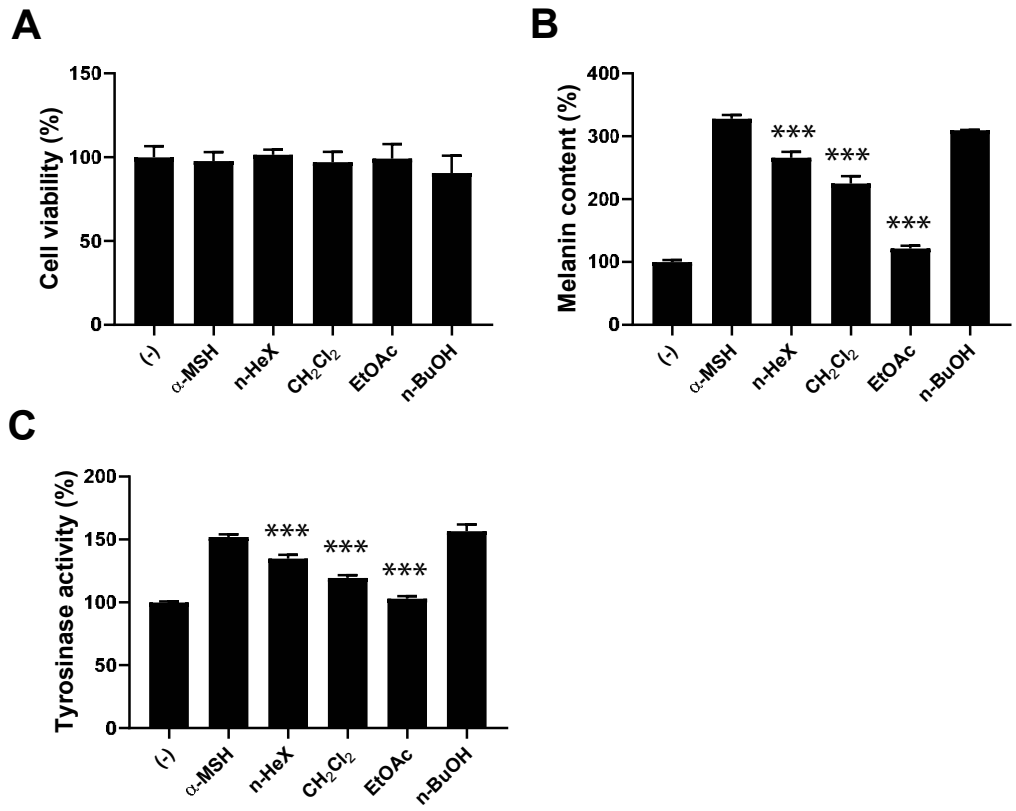

Figure 4. Effects of solvent fractions on cell proliferation and melanin synthesis in B16F10 cells. B16F10 cells were treated with 0.1% DMSO as vehicle or with 50 μg/mL of each solvent fraction for 48 h. (A) Melanoma cell proliferation was determined by MTT assay. (B) Melanin content in B16F10 cells was determined using a photometric method as described in the Experimental Section. (C) Cellular tyrosinase activity was determined by measuring dopachrome resulting from the enzymatic reaction of cell lysates with L-DOPA. Data are expressed as the mean ± SD of three independent experiments (***$p < 0.001$ vs. control group; treated with only α-MSH).

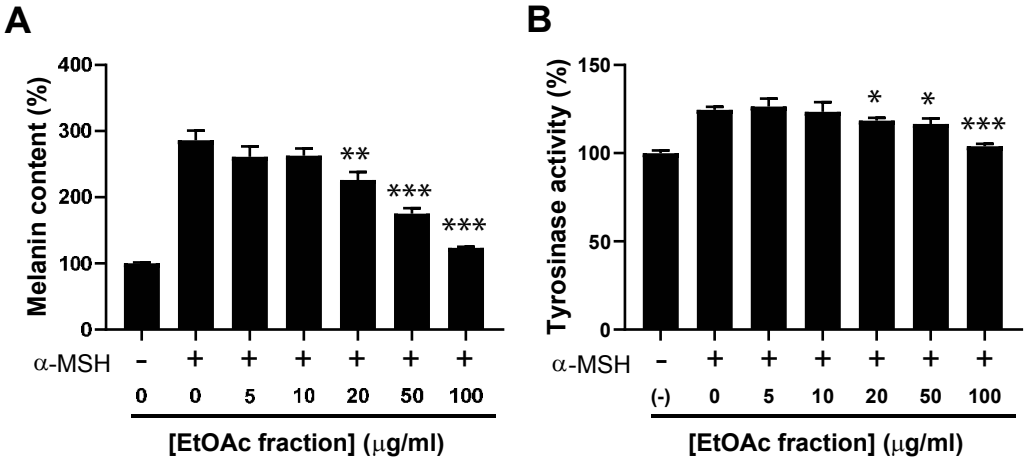

Figure 5. Effects of EtOAc fraction on intracellular melanin synthesis and tyrosinase activity in B16F10 cells. B16F10 cells were treated with 0.1% DMSO as vehicle or with 5, 10, 20, 50, and 100 μg/mL EtOAc fractions for 48 h. (A) Melanin content in B16F10 cells was determined using a photometric method as described in the Experimental Section. (B) Cellular tyrosinase activity was determined by measuring dopachrome generated by the enzymatic reaction of cell lysates with L-DOPA. Data are expressed as the mean ± SD of three independent experiments (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ vs. control group; treated with only α-MSH).

CONCLUSION

In this study, we demonstrated that defatted hemp seed extract scavenges ABTS radicals, and exhibits a potent inhibitory effect on melanin biosynthesis without significant cytotoxicity. Bioactive ingredients existing in the hemp seed and inhibiting melanogenesis was isolated in the EtOAc fraction. Furthermore, we found that the EtOAc fraction suppresses melanogenesis in melanoma cells by inhibiting the cellular tyrosinase activity. Here, we isolated a natural compound inhibitor from hemp seed extract to prevent hyperpigmentation, although molecular identification and characterization of the compounds inhibiting melanogenesis are still necessary.

ACKNOWLEDGMENTS

This research was partially supported by grants from the Research and Development of Chuncheon Hemp (Chuncheon city, Korea) and 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).

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

REFERENCES

1. Jung SH, Kim J, Eum J, et al. Velutin, an Aglycone Extracted from Korean Mistletoe, with Improved Inhibitory Activity against Melanin Biosynthesis. *Molecules.* Jul 12 2019;24(14).
2. Lim J, Nam S, Jeong JH, et al. Kazinol U inhibits melanogenesis through the inhibition of tyrosinase-related proteins via AMP kinase activation. *British Journal of Pharmacology.* Mar 2019;176(5):737-750.
3. Chiang HM, Chien YC, Wu CH, et al. Hydroalcoholic extract of Rhodiola rosea L. (Crassulaceae) and its hydrolysate inhibit melanogenesis in B16F0 cells by regulating the CREB/MITF/tyrosinase pathway. *Food Chem Toxicol.* Mar 2014;65:129-139.
4. Lee SY, Baek N, Nam TG. Natural, semisynthetic and synthetic tyrosinase inhibitors. *J Enzyme Inhib Med Chem.* 2016;31(1):1-13.
5. Peng HY, Lin CC, Wang HY, Shih Y, Chou ST. The melanogenesis alteration effects of Achillea millefolium L. essential oil and linalyl acetate: involvement of oxidative stress and the JNK and ERK signaling pathways in melanoma cells. *PLoS One.* 2014;9(4):e95186.
6. Momtaz S, Lall N, Basson A. Inhibitory activities of mushroom tyrosine and DOPA oxidation by plant extracts. *South African Journal of Botany.* Nov 2008;74(4):577-582.
7. Cazarolli LH, Zanatta L, Alberton EH, et al. Flavonoids: prospective drug candidates. *Mini Rev Med Chem.* Nov 2008;8(13):1429-1440.
8. Cushnie TP, Lamb AJ. Recent advances in understanding the antibacterial properties of flavonoids. *Int J Antimicrob Agents.* Aug 2011;38(2):99-107.
9. Yan X, Tang J, dos Santos Passos C, et al. Characterization of Lignanamides from Hemp (Cannabis sativa L.) Seed and Their Antioxidant and Acetylcholinesterase Inhibitory Activities. *J Agric Food Chem.* Dec 16 2015;63(49):10611-10619.
10. Zhou YF, Wang SS, Lou HX, Fan PH. Chemical constituents of hemp (Cannabis sativa L.) seed with potential anti-neuroinflammatory activity. *Phytochemistry Letters.* Feb 2018;23:57-61.
11. Purushotham G, Padma Y, Nabiha Y, Venkata Raju RR. In vitro evaluation of anti-proliferative, anti-inflammatory and pro-apoptotic activities of the methanolic extracts of Andrographis nallamalayana Ellis on A375 and B16F10 melanoma cell lines. *3 Biotech.* Dec 2016;6(2):212.
12. Kuk EB, Jo AR, Oh SI, et al. Anti-Alzheimer's disease activity of compounds from the root bark of Morus alba L. *Arch Pharm Res.* Mar 2017;40(3):338-349.
13. Re R, Pellegrini N, Proteggente A, Pannala A, Yang M, Rice-Evans C. Antioxidant activity applying an improved ABTS radical cation decolorization assay. *Free Radic Biol Med.* May 1999;26(9-10):1231-1237.
14. Chang TS, Ding HY, Tai SSK, Wu CY. Mushroom tyrosinase inhibitory effects of isoflavones isolated from soygerm koji fermented with Aspergillus oryzae BCRC 32288. *Food Chemistry.* 2007;105(4):1430-1438.
15. Huang HC, Hsieh WY, Niu YL, Chang TM. Inhibitory effects of adlay extract on melanin production and cellular oxygen stress in B16F10 melanoma cells. *Int J Mol Sci.* Sep 19 2014;15(9):16665-16679.
16. Baron R, Zayats M, Willner I. Dopamine-, L-DOPA-, adrenaline-, and noradrenaline-induced growth of Au nanoparticles: assays for the detection of neurotransmitters and of tyrosinase activity. *Anal Chem.* Mar 15 2005;77(6):1566-1571.