Anti-melanogenic and anti-oxidant activities of ethanol extract of *Kummerowia striata*: *Kummerowia striata* regulate anti-melanogenic activity through down-regulation of TRP-1, TRP-2 and MITF expression

Jae Yeon Leec,1, Young-Rak Cho1, Ju Hyoung Park3, Eun-Kyung Ahnc, Wonsik Jeongc, Hyoung Seok Shinb, Mi-Sun Kimb, Seung Hwan Yangd, Joa Sub Ohha,⁎

a Department of Pharmacy, College of Pharmacy, Dankook University, 119 Dandae-ro, Cheonan, 330-714, Republic of Korea
b HANSOLBIO Co., Ltd., Halla Sigmavalley 545, Dunchon-daero, Jungwon-gu, Seongnam-si, Gyeonggi-do, Republic of Korea
c Bio-Center, Gyeonggi Institute of Science and Technology Promotion, 147 Gwanggyo-ro, Yeongtong-gu, Suwon-si, Gyeonggi-do, 443-270, Republic of Korea
d Department of Biotechnology, Chonnam National University, Yeosu, Chonnam, 59626, Republic of Korea

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ABSTRACT

*Kummerowia striata* (*K. striata*) is used as a traditional medicine for inflammation-related therapy. To determine whether it has beneficial anti-melanogenic and anti-oxidant activities, we investigated the biological activities of the ethanol extract of *Kummerowia striata* (EKS) using a variety of *in vitro* and cell culture model systems. The anti-melanogenic activity was assessed in B16F10 melanoma cells in terms of melanin synthesis and *in vitro* tyrosinase inhibitory activity. The anti-oxidant assays were performed using 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2ʹ-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS). EKS showed strong anti-oxidant activities in DPPH and ABTS assays. The mRNA transcription levels and protein expression levels of tyrosinase, tyrosinase-related protein 1, tyrosinase-related protein 2, and microphthalmia-associated transcription factor decreased in a dose-dependent manner with EKS treatment. Additionally, EKS did not affect cell viability at different concentrations used in this study, indicating that the mechanism of action of EKS-mediated inhibition of melanin synthesis does not involve cytotoxicity. Also, we confirmed that p-coumaric acid and quercetin are important compounds for anti-melanogenesis and antioxidant properties of EKS. Collectively, our findings demonstrate for the first time that EKS possesses anti-melanogenic and anti-oxidant activities. Further evaluation and development of EKS as a functional supplement or cosmetic may be useful for skin whitening and reducing wrinkles.

1. Introduction

Studies on external aging have reported that the skin plays a major role in identifying human aging [1,2]. The aging of the skin can be divided into two types: chronologic aging, which is accompanied by the progressive deterioration of the structure and function of the skin over time, and the other is exogenous aging (photoaging) wherein skin texture changes because of long-term exposure to sunlight [3,4]. Free radicals and reactive oxygen species are the most important components that fuel skin aging. They induce oxidative stress in skin cells, and the substances generated during this process cause increased melanin production and wrinkles.

Melanin is synthesized by the oxidation of tyrosinase or L-3,4-dihydroxyphenylalanine (L-DOPA) via the activity of tyrosinase and tyrosinase-related protein 1 (TRP-1). Melanocytes play an important role in protecting the skin from radiation damage [5,6]. The biosynthesis of L-DOPA, L-3,4-dihydroxyphenylalanine; TRP-1, tyrosinase-related protein 1; MITF, microphthalmia-associated transcription factor; ABTS, 2,2ʹ-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt; DPPH, 2,2-diphenyl-1-picrylhydrazyl; EKS, ethanol extract of *K. striata*; DMEM, Dulbecco’s Modified Eagle Medium; FBS, fetal bovine serum; COSY, correlation spectroscopy; HSQC, heteronuclear single quantum coherence; HMBC, heteronuclear multiple bond correlation; ESI, electrospray ionization; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide; DMSO, dimethyl sulfoxide; α-MSH, α-Melanocyte-stimulating hormone; BHA, butylated hydroxyanisole; RT-PCR, reverse transcription-polymerase chain reaction; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis

⁎ Corresponding author.
E-mail address: jsoh@dankook.ac.kr (J.S. Oh).
1 These authors contributed equally to this work.

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melanin in melanocytes is mediated by the enzyme tyrosinase that regulates the formation of L-DOPA via hydrolysis of tyrosine and the formation of DOPA quinone via oxidation of DOPA [7,8]. Additionally, microphthalmia-associated transcription factor (MITF) is a key transcription factor regulating the transcription of melanogenic enzymes (i.e., tyrosinase, TRP-1, and TRP-2) [9,10]. Tyrosinase and TRP-1 are transcriptionally regulated by MITF, which plays an important role in the melanin synthesis pathway [11,12].

Kummerowia striata (Thunb. ex Murray) Schindl (K. striata) is an annual plant indigenous to eastern Asia, including Korea, China, and Japan. The plant has long been used as a traditional medicinal herb in anti-inflammation therapy. The aim of this study was to analyze the potential anti-melanogenic and anti-oxidant activities of an ethanol extract of K. striata (EKS) and its two compounds. The anti-oxidant activities were assessed in terms of their free radical scavenging activities by using 2,2’-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) assays. The anti-tyrosinase activities were assessed using the tyrosinase inhibition assay. We found that EKS and its compounds are promising candidates for use in cosmetic products for skin whitening and reducing wrinkles.

2. Materials and methods

2.1. Cell culture conditions

Murine B16F10 melanoma cell lines were purchased from the American Type Culture Collection (Manassas, VA, USA). The cells were cultured in Dulbecco’s Modified Eagle Medium (DMEM, Gibco, San Jose, CA, USA) supplemented with 10% fetal bovine serum (FBS) and penicillin/streptomycin (Gibco, USA) in a humidified atmosphere containing 5% CO$_2$ in air at 37°C.

2.2. Reagents

The following antibodies were purchased from commercial sources: anti-tyrosinase, anti-TRP-1, anti-TRP-2, and anti-MITF (ThermoFisher Scientific, Rockford, IL, USA); anti-β-actin antibodies, and mouse and rabbit IgG-horseradish peroxidase conjugates (Cell Signaling, Beverly, MA, USA).

2.3. Preparation of Kummerowia striata extract

The aerial parts of K. striata (Thunb. ex Murray) Schindl (K. striata) were collected at Gimpo, Gyeonggi, South Korea in September 2013 and identified by Professor Joa Sub Oh, College of Pharmacy, Dankook University, Cheonan, South Korea. A voucher specimen (G63) has been deposited at the Bio-center, Gyeonggido Business & Science University, Cheonan, South Korea. A voucher specimen (G63) has been collected at Gimpo, Gyeonggi, South Korea in September 2013.

2.4. General procedures

1D and 2D (1H-1H correlation spectroscopy (COSY), heteronuclear single quantum coherence (HSQC), and heteronuclear multiple bond correlation (HMBC)) NMR spectra were measured on a Bruker Ascend III 700 MHz NMR spectrometer (Rheinstetten, Germany) with tetramethylsilane as an internal standard. Chemical shifts were expressed as δ values. Electrospray ionization (ESI) mass spectra were obtained on a LTQ Orbitrap XL (Thermo Scientific) mass spectrometer. Open column chromatography was performed using silica gel (Kiesel gel 60, 70–230 mesh and 230–400 mesh; Merck) and ODS-C18 gel ODS-A (12 nm S-7 μm, YMC GEL, Japan). Thin-layer chromatography was performed using pre-coated silica gel 60 F254 (0.25 mm, Merck) and pre-coated silica gel 60 RP-18 F254S (0.25 mm, Merck), respectively. All chemicals and solvents were of analytical grade and used without further purification.

2.5. Cell viability assay

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay was performed to determine the effect of EKS on cell viability. B16F10 mouse melanoma cells were cultured in 96-well plates (1 × 10$^5$ cells/well) and treated with EKS for 24 h. In total, 100 μL of serum-free medium containing 10% MTT solution (5 mg/ml) was added and incubated for 3 h. The medium was removed and washed twice with phosphate buffered saline (PBS). Thereafter, 100 μL of dimethyl sulfoxide (DMSO) was added to each well and dissolved in a shaker. The absorbance was measured at 540 nm using an ELISA reader (Molecular Device, USA).

2.6. Cellular melanin contents

B16F10 cells were cultured in 6-well plates at a density of 1 × 10$^5$ cells per well and incubated for 24 h. Melanogenesis was induced with 100 nM α-Melanocyte-stimulating hormone (α-MSH). The cells were treated with arbutin (a positive control) and EKS at different concentrations and cultured for 72 h. After washing with PBS twice, lysis buffer [100 mM sodium phosphate (pH 6.8) and 0.1 mM phenylmethylsulfonyl fluoride (PMSF), 1% Triton X-100] was added and dissolved at ~80°C for 30 min. After cell harvesting, 1 N NaOH containing 10% DMSO was reacted at 65°C for 1 h to dissolve the pellet, and the absorbance was measured at 405 nm.

2.7. Tyrosinase inhibition assay

Tyrosinase inhibitory activity was measured by using the method described by Yagi et al. [15]. The reaction was carried out in 0.1 M potassium phosphate buffer (pH 6.5) containing 1.5 mM L-tyrosine and 1250 unit/ml mushroom tyrosinase. The reaction mixture was incubated at 37°C for 20 min. The test samples were assayed for tyrosinase inhibition by measuring its effect on tyrosinase activity using an ELISA reader at 490 nm. Arbutin was used as a positive control.

2.8. DPPH radical scavenging activity assay

DPPH radical scavenging activity was measured by using the method described by Blois [16] and Ozgen et al. [17]. Briefly, 100 μL of DPPH solution dissolved in methanol was added to 100 μL of EKS, which was diluted to the required concentration, and the reaction was carried out at room temperature for 30 min. Absorbance was measured at 517 nm using an ELISA reader (Molecular Device, USA). The
antioxidant butylated hydroxyanisole (BHA) was used as a positive control, and the IC₅₀ value of EKS was determined.

2.9. ABTS radical scavenging activity

ABTS radical scavenging activity was measured by using a previously described method [18, 19]. ABTS⁺ was formed by mixing 7 mM ABTS solution and 2.45 mM potassium persulfate (K₂S₂O₈) solution with ABTS: K₂S₂O₈ (2: 1 ratio) for 12–16 h to form a cation (ABTS⁺). The absorbance value at 734 nm was 1.35 ± 0.05. In total, 100 μL of the diluted solution and 100 μL of EKS diluted to the required concentrations were reacted at room temperature for 6 min, and the absorbance was measured at 734 nm using an ELISA reader. BHA, an antioxidant, was used as the positive control, and the IC₅₀ value of EKS was determined.

2.10. Reverse transcription-polymerase chain reaction (RT-PCR)

B16F10 melanoma cells were seeded in 6-well plates at a density of 1 × 10⁶ cells/well and treated with EKS (100–400 μg/mL) for 72 h. To extract total RNA, Trizol reagent (Invitrogen, Thermo Fisher Scientific, Inc.) was added to each well to lyse the cells and 200 μL of chloroform was added. Then, the mixture was centrifuged at 13,000 rpm for 20 min at 4 °C, and the supernatant was mixed with isopropanol for 30 min at −70 °C. After centrifugation at 13,000 rpm for 20 min at 4 °C, the supernatant was removed, and 70% EtOH-diethylpyrocarbonate water was added to each tube. After centrifugation at 13,000 rpm for 5 min at 4 °C, the supernatant was removed and dried at room temperature. Thereafter, 1 μg of extracted total RNA was reverse transcribed to single stranded cDNA with oligo dT using Taq DNA polymerase and SuperScript™ III First-Strand Synthesis System (Invitrogen, Thermo Fisher Scientific, Inc.). The cDNA was amplified in MyGene™ Series Peltier Thermal Cycler Model MG96 G (LongGene Scientific Instruments, Hangzhou, China) using specific primers and AccuPower® PFU PCR premix (Bioneer Corporation, Daejeon, Republic of Korea). The PCR cycling conditions were 5 min at 95 °C followed by 30 cycles of 30 s at 95 °C, 30 s at 60 °C, 1 min at 72 °C, and final extension for 10 min at 72 °C. After amplification, the PCR products were electrophoresed on 1.5% agarose gel containing ethidium bromide and analyzed using a UV transilluminator.

2.11. Western blot analysis

B16F10 melanoma cells were cultured in DMEM supplemented with 10% FBS at a density of 1 × 10⁶ cells/well and treated with EKS (100–400 μg/mL) for 72 h. After removing the culture medium, EKS (100–400 μg/mL) diluted in the medium was treated for 72 h. The cells were washed with PBS, harvested using RIPA buffer (Sigma Aldrich), and centrifuged at 15,000 rpm for 15 min at 4 °C. The total proteins were extracted from the cells and quantified using the Bradford method. The proteins were separated by 8% sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to nitrocellulose membrane (Whatman, Dassel, Germany). The membrane was blocked with 5% BSA for 1 h at room temperature and incubated overnight with primary antibody at 4 °C. The membrane was then washed and incubated with horseradishperoxidase-conjugated secondary antibodies for 1 h at room temperature. The membrane was washed and detected by SuperSignal® West Pico Chemiluminescent Substrate (ThermoFisher Scientific, Rockford, IL, USA).

2.12. Statistical analysis

Statistical analysis was performed using a Student’s t-test with Microsoft Excel 2007 (Microsoft Corporation, Redmond, WA, USA). The results are presented as the means ± standard deviation, and p < 0.05 was considered to indicate a statistically significant difference.

Fig. 1. Effects of ethanol extract of Kummerowia striata on tyrosinase inhibition, cell viability, and melanin synthesis (A) Tyrosinase inhibitory activity of ethanol extract of Kummerowia striata (EKS) was analyzed by measuring the amount of dopachrome generated in the reaction. Arbutin was used as a positive control. (B) Melanin content in B16F10 cells stimulated with 100 nM α-MSH. The melanin content was calculated as a percentage of the content in control. (C) B16F10 mouse melanoma cells were treated with EKS (25–400 μg/ml) and arbutin (400 μg/ml). Cytotoxicity of EKS and arbutin were determined using MTT assay. Values represent the mean ± SD of three independent replicates. Statistical significance is indicated as *P < 0.05, **P < 0.01, compared with non-treated sample or α-MSH-treated cells.
3. Results

3.1. Anti-melanogenesis effects of EKS

To determine the anti-melanogenic activities of EKS, we employed a mushroom tyrosinase assay using L-tyrosine as the substrate and mushroom tyrosinase as the enzyme source. Since tyrosinase is a key enzyme catalyzing the rate-limiting step in melanin biosynthesis, we first measured the tyrosinase inhibitory ability of EKS. As shown in Fig. 1A, EKS exerted a significant inhibitory effect on tyrosinase activity in a dose-dependent manner. These results suggest that EKS displayed inhibitory effects on mushroom tyrosinase activity, and the inhibitory effect of EKS was similar to that of arbutin used as positive control. Next, to determine the effect of EKS on melanin synthesis, the melanin content of EKS-treated B16F10 melanoma cells was quantified. As shown in Fig. 1B, EKS treatment decreased the melanin content in melanoma cells in a dose-dependent manner, indicating that the decrease in cellular melanin might be due to the inhibition of tyrosinase activities. In addition, investigating the effect of EKS on B16F10 melanoma cell viability showed that EKS had no significant cytotoxic effect on B16F10 cells at the concentration used (Fig. 1C). These findings clearly show that EKS exerts anti-melanogenic effects through inhibition of tyrosinase activity and melanin synthesis in B16F10 melanoma cells without inducing cytotoxicity.

3.2. Anti-oxidant activity of EKS

The scavenging activity of EKS was determined using DPPH free radicals (Fig. 2A). EKS exhibited higher antioxidant activity (scavenging activity = 50.22%, IC₅₀ = 98.71 μg/ml). The scavenging capacity of EKS was also assessed using ABTS radical cation (Fig. 2B). The ABTS radical scavenging capacity of EKS was similar to that of the positive control BHA (scavenging capacity = 99.53%, IC₅₀ = 24.64 μg/ml). These results indicate that EKS has a higher antioxidant activity.

3.3. Effect of EKS on the expression of melanin synthesis genes

To investigate the molecular mechanisms by which EKS regulates melanogenesis further, we examined the changes in expression of melanogenic genes and proteins, such as tyrosinase, TRP-1, TRP-2, and MITF, which play pivotal roles in melanogenesis [20]. As shown in Fig. 3A, the mRNA expression of tyrosinase, TRP-1, TRP-2, and MITF was triggered by α-MSH; however, EKS treatment significantly decreased the mRNA expression (100–400 μg/ml). Moreover, EKS showed
Next, the effect of EKS on the expression of melanogenesis-related proteins was assessed by western blot analysis. As shown in Fig. 3B, EKS treatment markedly inhibited α-MSH-induced expression levels of tyrosinase, TRP-1, TRP-2, and MITF in B16F10 cells. Taken together, these findings demonstrate that the inhibitory effects of EKS on melanogenesis in B16F10 cells might be mediated through down-regulation of melanogenic genes and proteins, such as tyrosinase, TRP-1, TRP-2, and MITF.
3.4. \( p \)-coumaric acid and quercetin from EKS exhibited potent anti-melanogenic and anti-oxidant activities

Next, we attempted to identify and purify the functional compounds present in EKS that exhibit potent anti-melanogenic and anti-oxidant properties. The various compounds were identified as luteolin, \( p \)-coumaric acid, rosmarinic acid, quercetin, genistein, and (\(+\) )-catechin. Among them, \( p \)-coumaric acid and quercetin inhibited tyrosinase, synthesis of melanin and exhibited scavenging capacity similar to arbutin, a well-known depigmenting agent, in a dose-dependent manner (Fig. 4). These results indicate that \( p \)-coumaric acid and quercetin are important compounds for anti-melanogenic and anti-oxidant properties.
of EKS.

4. Discussion

In this study, we aimed to analyze the anti-melanogenic and anti-oxidant potential of EKS and its two compounds p-coumaric acid and quercetin, and found that EKS possesses potent anti-melanogenic and anti-oxidant activities.

Skin pigmentation is mostly caused by melanocytes in the basal layer of the skin, following stimulation by UV radiation. Stimulated keratinocytes secrete α-MSH (a small peptide hormone) [21]. Thus, UV exposure induces melanin production resulting in hyperpigmentation [22]. Recently, many researchers have focused on the development of new and effective whitening compounds, because cosmetics with whitening effects constitute a large part of the cosmetics market. Studies on the inhibition of melanin biosynthesis revealed that arbutin, kojic acid, and many other natural products inhibit melanogenesis and hyperpigmentation. However, the side effects of arbutin [23] and kojic acid [24] have recently been reported, and the use of these substances may be restricted. Therefore, there is a need to develop safe skin whitening compounds with minimal adverse effects. Recent studies have focused on the development of skin whitening cosmetics from natural materials.

Several studies have demonstrated the various biological activities of K. striata extracts. In particular, K. striata is pharmaceutically effective for inflammation and oxidation [25,26]. However, the effects of kojic acid and other natural products inhibit melanogenesis and hyperpigmentation. In this study, we aimed to analyze the anti-melanogenic and anti-oxidant activities of EKS.

Tyrosinase, TRP-1, and TRP-2 play important roles in melanin biosynthesis [27]. Thus, the mechanism underlying the effect of skin whitening agents involves inhibition of melanin production by decreasing tyrosinase activity [28]. The expression of tyrosinase, TRP-1, and TRP-2 genes is known to be regulated by MITF [29]. In the present study, we aimed to analyze the anti-melanogenic activities of EKS by performing RT-qPCR analysis on a group of patients and volunteers with various skin types. EKS showed strong antioxidant activity similar to BHA, which was used as the positive control (Fig. 2). The active compounds present in EKS were isolated and confirmed as p-coumaric acid and quercetin. Although various biological activities, including cardioprotective [30], anti-inflammatory [31,32], anti-mutagenic [33] anti-oxidant [34,35], and anti-melanogenic [36] activities have been reported for p-coumaric acid and quercetin from various plants, this is the first study to report that p-coumaric acid and quercetin from EKS possess anti-melanogenic and anti-oxidant activities.

In this study, the anti-melanogenic and anti-oxidant activities of EKS were evaluated. EKS exerted anti-melanogenic and anti-oxidant activities via regulation of tyrosinase activity and α-MSH-induced melanin synthesis in B16F10 melanoma cells, without inducing cytotoxicity. The main active compounds were p-coumaric acid and quercetin. Thus, our findings demonstrate for the first time that EKS may be utilized as a potential depigmenting and anti-oxidant agent.

Conflicts of interest

The authors declare no conflict of interest.

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