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Research paper

Leukodin isolated from *Artemisia capillaris* inhibits alpha-melanocyte stimulating hormone induced melanogenesis in B16F10 melanoma cells

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ABSTRACT

Introduction: The demand for treatment of hyperpigmentation disorders are on the rise. *Artemisia capillaris* is a traditional herbal plant widely used in skin protective remedies. In the present study, the inhibitory effect of *Artemisia capillaris* ethanol extract on the production of melanin is examined, and the active compound was isolated from the crude extract and identified.

Methods: The structure of the purified active compound of *A. capillaris* ethanol extract (ACE) was elucidated by NMR spectroscopy. ACE at the concentration of 6.25, 12.5, 25, and 50 μg/ml and active compound at the concentration of 37.5, 75, and 150 μg/ml were treated alpha-melanocyte stimulating hormone (α-MSH) induced in B16F10 melanoma cells. Melanin contents, tyrosinase activity, and protein expression of melanogenesis-related proteins were analyzed in ACE or active compound treated or untreated control.

Results: ACE significantly inhibited melanogenesis induced by α-MSH and tyrosinase activity without cell cytotoxicity in a dose-dependent manner. Western blot demonstrated that ACE downregulated the expression of melanocyte-specific proteins such as tyrosinase, tyrosinase-related protein-1 (TRP-1), and tyrosinase-related protein-2, which catalyzes the rate-limiting oxidation of tyrosine to melanin. The active compound was finally identified as leukodin. It inhibited melanin pigment synthesis and tyrosinase activity in B16F10 melanoma cells without cytotoxicity. In addition, the leukodin decreased TRPs expression in a dose-dependent manner.

Conclusions: Bioactivity-guided fraction identified leukodin is the active compound in ACE extract. Leukodin suppressed melanin synthesis through inhibition of the expression of melanogenic enzymes.

1. Introduction

Melanin is skin pigment endogenously synthesized in melanocytes. Melanin formation keeps skin defense against ultraviolet (UV) radiation or other stress through various signaling pathways [1]. However, excessive production and accumulation of melanin in the skin cause hyperpigmentation disorders, including melasma, freckles, lentigo, and skin cancer [2].

There are two types of melanin in human skin: eumelanin (dark-brown pigment) synthesized from L-dopachrome and pheomelanin (yellow-reddish pigment) synthesized in the presence of sulfhydryl compounds in melanosomes [3]. Eumelanin synthesis is regulated primarily via activation of the melanocortin-1 receptor (MC1R) by its agonists α-melanocyte-stimulating hormone (α-MSH) and adrenocorticotropic hormone (ACTH) while pheomelanin synthesis is simulated via agonist stimulating protein (ASP) [4] with presence of 3- or 5-cysteinyl DOPAs [5]. Exposure of epidermal melanocytes to UV-B light activates α-MSH production in keratinocytes. α-MSH binds to the melanocortin-1 receptor (MC1R), subsequently increasing the cyclic adenosine monophosphate (cAMP) level, resulting to activation of protein kinase A (PKA), which stimulates microphthalmia-associated transcription factor (MITF) expression via the cAMP response element-binding protein (CREB) phosphorylation [6]. MITF upregulates tyrosinase (TYR), tyrosinase-related protein-1 (TRP-1), and TRP-2

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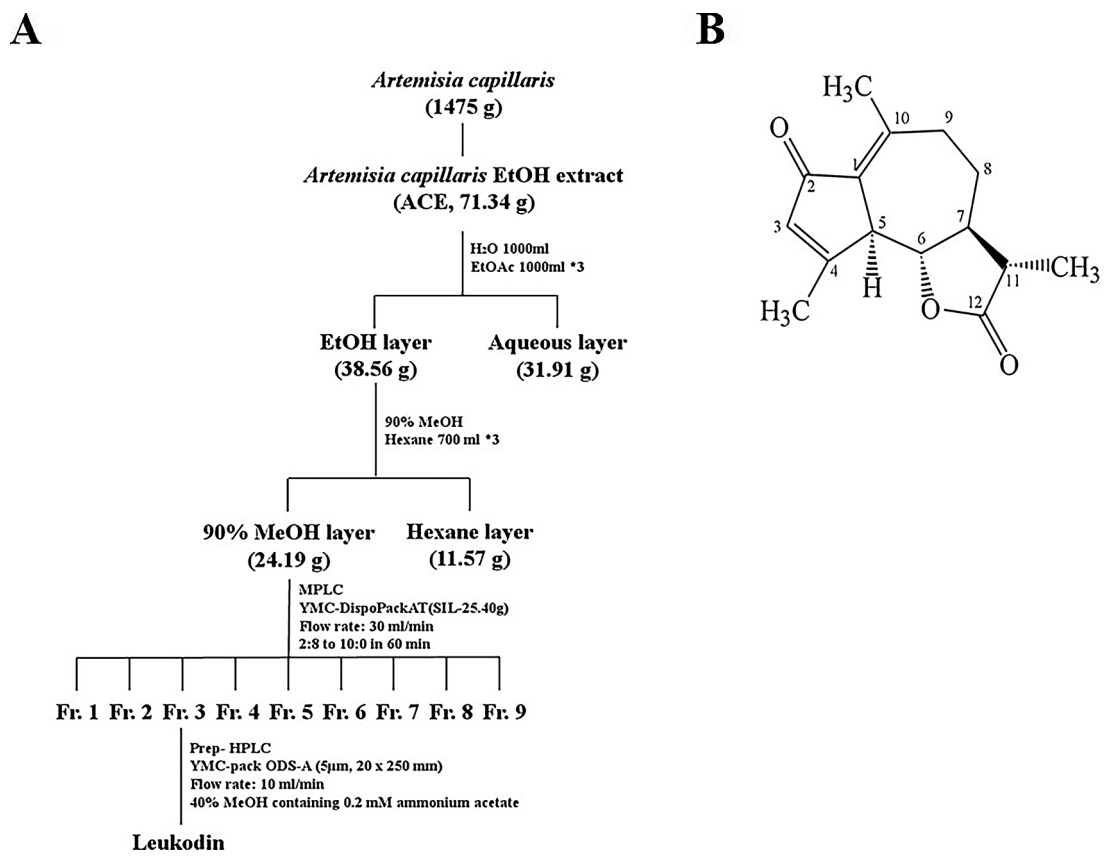


Fig. 1. Isolation procedure of active substance from an ethanol extract of *Artemisia capillaris* (A). Chemical structure of leukodin (B).

expressions which promoting melanin synthesis in melanogenesis pathway [3]. TYR is the key enzyme to hydroxylate tyrosine to L-3,4-dihydroxyphenylalanine (L-DOPA), which oxidized to DOPAquinone [7]. In the absence of cysteine, DOPAquinone undergoes spontaneous cyclic oxidation to yielding in DOPAchrome. TRP-2 then converts L-DOPAchrome to 5,6-dihydroxyindole-2-carboxylic acid (DHICA), which is catalyzed by TRP-1 to form eumelanin [8]. MITF is considered a key transcription factor of melanogenesis; therefore, many studies have been performed to control the expression of MITF to inhibit melanogenesis.

Natural products, which regulate skin pigmentation without toxicity, have been evaluated as candidates for skin whitening agents. Over the last decade, countless researches were done in order to find effective agents in diverse fields from natural products [9,10]. *Artemisia capillaris*Thunberg is an herbal plant widely used for its various beneficial properties [11]. Many studies had been verified the effects of *A. capillaris* on various therapeutic applications such as hepatitis, obesity, inflammation, antimicrobial agent, hemostasis, hypertension, skin cancer, and skin inflammation [12,13]. Previous studies reported that the 4,5-??-dicaffeoylquinic acid purified from ethanolic extract of *A. capillaris* can inhibit pigment formation, whereas isofraxidin 7-O-(6′-O-p-coumaroyl)-??-glucopyranoside enhance pigmentation [14,15]. However, no studies have been investigated on melanin synthesis or tyrosinase activity of *A. capillaris* ethanol extract (ACE) with an active compound in B16F10 mouse melanoma cells. Therefore, in this study, the inhibitory effect of ACE on melanin biosynthesis was evaluated in α-MSH induced B16F10 cells, and the active compounds was identified as leukodin, (3*S*,3aS,9aS,9bS)-3,6,9-trimethyl-3,3a,4,5,9a,9b-hexahydroazuleno[4,5-*b*]furan-2,7-dione, through purification and structure determination. This is the first report on the active compound with anti-melanogenic activity in *A. capillaris*. Furthermore, the possible mechanism of ACE and leukodin on the inhibitory effect of α-MSH induced melanin synthesis were investigated.

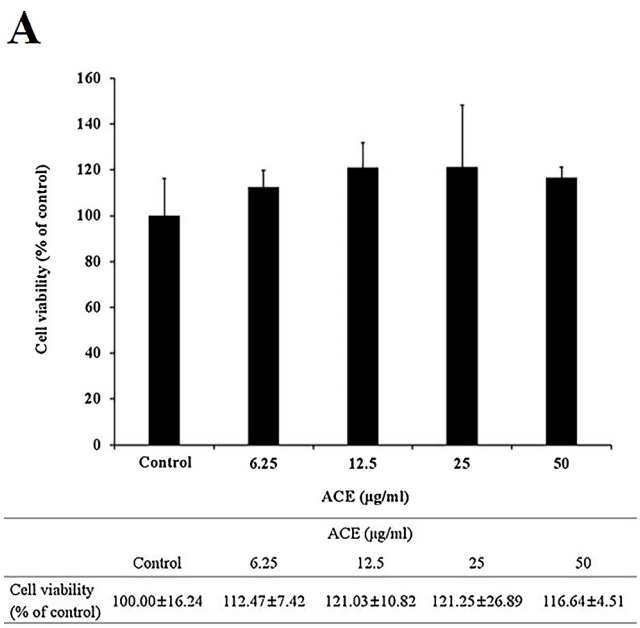
2. Materials and methods

2.1. Chemicals and antibodies

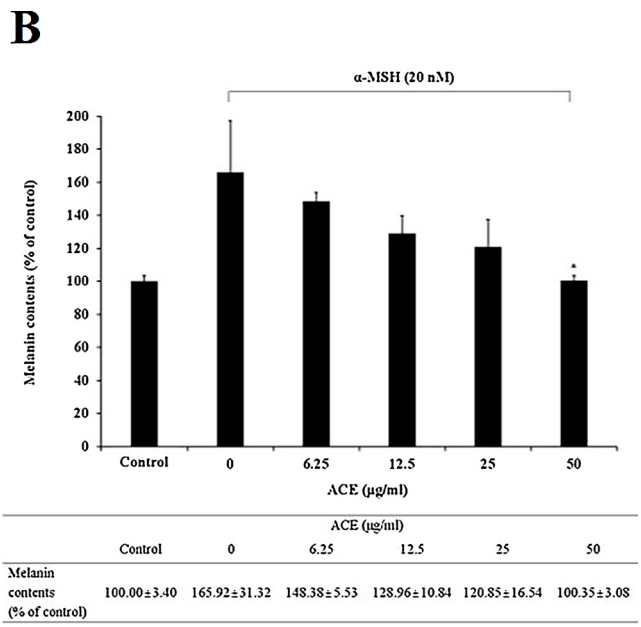
Alpha–melanocyte stimulating hormone (α-MSH), 3-(4,5,-dimetylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), and L-3,4,-dihydroxyphenylalanine (L-DOPA) were obtained from Sigma Aldrich (St. Louis, MO, USA). Primary antibodies for tyrosinase (sc-73244), TRP-1 (sc-58438) and TRP-2 (sc-74439) antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz Biotechnology, CA, USA).

2.2. Isolation of active compound and structure determination

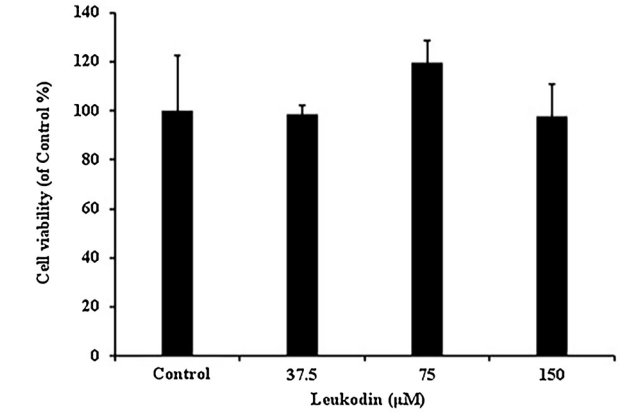
Artemisia capillaris was purchased from Hwasun-bul-minari Company (Hwasun, South Korea). Dried *A. capillaris* (1475 g) was extracted with 100% ethanol for 3 days at room temperature. The filtered extract was concentrated with a rotary vacuum evaporator (EYELA: Rotary evaporator, Tokyo, Japan) and subsequently freeze-dried to yield an ethanol (EtOH) extract (71.343 g). The EtOH extract was then resuspended in water and partitioned into ethyl acetate (EtOAc). The EtOAc layer (38.56 g) was thoroughly evaporated under vacuum and partitioned with 90% methanol (MeOH) and n-hexane. The 90% MeOH layer (24.19 g) was fractionated with Water-medium-pressure liquid chromatography system (MPLC, Milford, MA, USA) using a YMC-DispoPackAT (SIL-25 40 g), eluted with EtOAc and n-hexane mixture in a gradient mode (EtOAc:n-Hexane, 2 : 8 to 10 : 0 in 60 min). The flow rate was 30 ml/min and the elution was monitored at UV 254 nm A total of nine fractions eluted were collected, concentrated, and evaluated anti-melanogenic activity. The 3nd fraction was further purified with Waters prep-high performance liquid chromatography system (HPLC, Milford, MA, USA) using a YMC ODS A column (YMC-pack ODS-A; 5 μm, 20 × 250 mm). The column was eluted with 40% MeOH containing 0.2 mM ammonium acetate at a flow rate of 10 ml/min. The



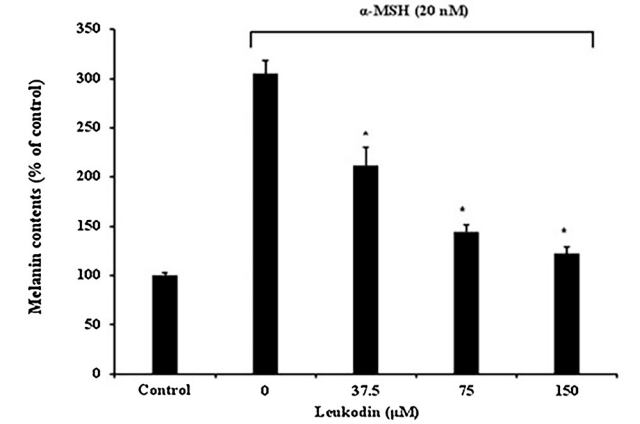
	ACE (μg/ml)				
	Control	6.25	12.5	25	50
Cell viability (% of control)	100.00±16.24	112.47±7.42	121.03±10.82	121.25±26.89	116.64±4.51



	ACE (μg/ml)					
	Control	0	6.25	12.5	25	50
Melanin contents (% of control)	100.00±3.40	165.92±31.32	148.38±5.53	128.96±10.84	120.85±16.54	100.35±3.08



	Leukodin (μM)			
	Control	37.5	75	150
Cell viability (% of control)	100.00±16.24	98.56±3.54	119.62±9.16	97.71±13.29



	Leukodin (μM)				
	Control	0	37.5	75	150
Melanin contents (% of control)	100.00±2.62	304.94±13.23	211.78±18.21	144.30±7.57	122.25±6.90

Fig. 2. Effects of *Artemisia capillaris* ethanol extract (ACE) or leukodin on cell viability and melanin synthesis. B16F10 melanoma cells were treated with various concentrations of ACE (0, 6.25, 12.5, 25 and 50 μg/ml) and leukodin (0, 37.5, 75 and 150 μM) for 48 h. Cell viabilities of ACE (A) and leukodin (C) were determined using the MTT assay. Effects of ACE (B) or leukodin (D) on α-MSH-stimulated melanin synthesis in melanoma cells. B16F10 melanoma cells were incubated with α-MSH (20 nM) containing ACE (0, 6.25, 12.5, 25, and 50 μg/ml) or leukodin (0, 37.5, 75 and 150 μM) concentrations. Each percentage value in treated cells was calculated with respect to the control cells and presented as the mean ± SD from three individual experiments. $^{*}P < 0.05$, compared with α-MSH alone-treated control.

compound was monitored by ultraviolet absorbance at 254 nm After elution, the fraction was dried to give 2.1 mg of purified compound.

2.3. Identification of an active compound

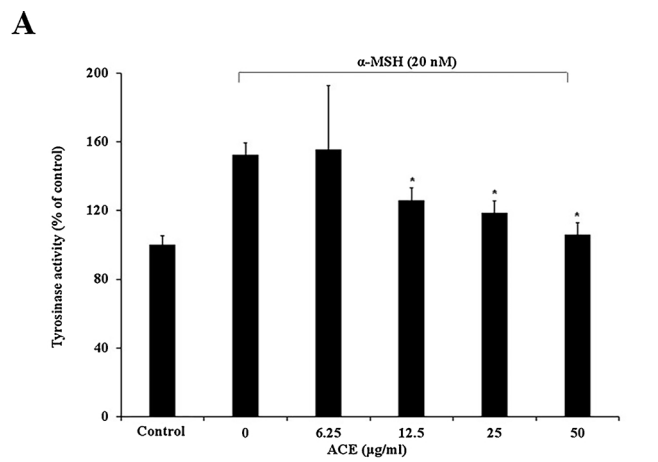
Chemical structure of purified compound was analyzed by nuclear magnetic resonance (NMR, 1D, 2D). ^{1}H- and ^{13}C-NMR spectra were obtained on an Advance DP X 500 MHz NMR spectrometer (Bruker), recorded in a deuterated chloroform ($CDCl_3$) solution, and compared with reported data [16–18].

Leukodin showed the following characteristics: C15H18O3

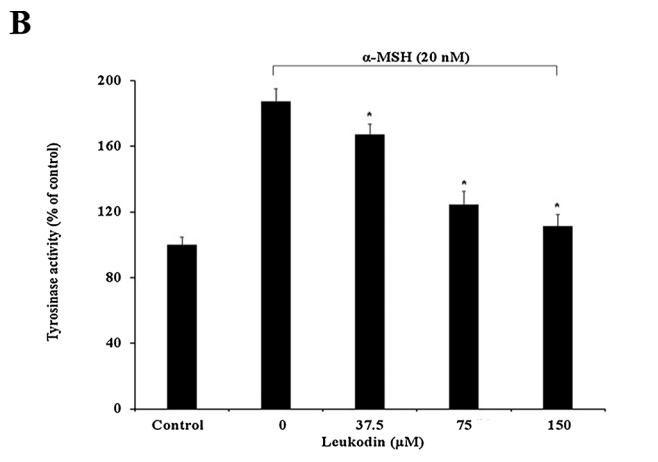
Proton (^{1}H) NMR (CD_3OD, 400 MHz): δ 6.137 (1H, C-3), 3.594 (1H, C-5), 3.663 (1H, C-6), 2.088 (1H, C-7α), 1.998 (1H, C-8α), 1.395 (1H, C-8β), 2.521 (1H, C-9α), 2.324 (1H, C-9β), 2.362 (1H, C-11), 1.205 (1H, C-13), 2.398 (1H, C-14), 2.291 (3H, C-15). 13C NMR (CD_3OD, 100 MHz): δ 133.2 (C-1), 198.4 (C-2), 135.8 (C-3), 173.7 (C-4), 53.6 (C-5), 85.9 (C-6), 57.1 (C-7), 26.8 (C-8), 38.4 (C-9), 155.2 (C-10), 42.0 (C-11), 180.1 (C-12), 12.4 (C-13), 21.8 (C-14), 19.9 (C-15).

2.4. Cell culture

B16F10 mouse melanoma cells were obtained from the American Type Culture Collection (Manassas, VA, USA). The cells were cultured in alpha-modified Eagle's medium (α-MEM; Hyclone, Logan, UT, USA) containing heat-inactivated 10% fetal bovine serum (FBS; Hyclone, Logan, UT, USA) and 1% penicillin-streptomycin mixture (10,000 U/

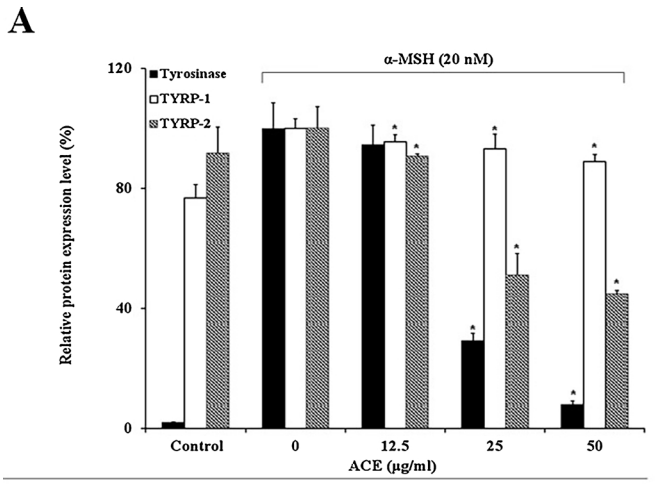


	ACE (μg/ml)					
	Control	0	6.25	12.5	25	50
Tyrosinase activity (% of control)	100.00±5.28	152.29±7.10	155.38±37.28	125.88±7.20	118.59±6.92	105.90±7.13

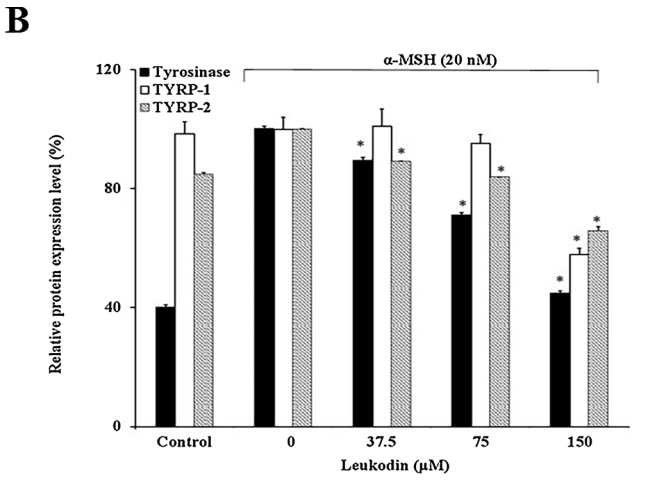


	Leukodin (μM)				
	Control	0	37.5	75	150
Tyrosinase activity (% of control)	100.00±4.69	187.38±7.54	167.18±6.23	124.57±7.85	111.40±7.24

Fig. 3. Effects of ACE (A) or leukodin (B) on the cellular tyrosinase activity. Cells were treated with ACE (0, 6.25, 12.5, 25 and 50 μg/ml) or leukodin (0, 37.5, 75 and 150 μM) and stimulated with α-MSH (20 nM) for 48 h. Each percentage value for treated cells is reported relative to that of the control cells and presented as the mean ± SD from three individual experiments. *P < 0.05, compared with α-MSH alone-treated control.



Relative protein expression level (%)	ACE (μg/ml)				
	Control	0	12.5	25	50
Tyrosinase/β-actin	1.93±0.17	100.00±8.57	94.53±6.59	29.19±2.42	7.84±1.24
TYRP-1/β-actin	76.88±4.53	100.00±3.34	95.58±2.29	93.26±4.89	88.95±2.39
TYRP-2/β-actin	91.71±8.68	100.00±7.42	90.71±0.90	51.13±7.20	44.81±1.24



Relative protein expression level (%)	Leukodin (μM)				
	Control	0	37.5	75	150
Tyrosinase/β-actin	39.91±0.57	100.00±0.15	89.43±0.57	71.02±1.45	44.71±0.72
TYRP-1/β-actin	98.50±6.92	100.00±6.75	104.10±9.93	95.18±5.38	57.85±3.80
TYRP-2/β-actin	84.86±0.77	100.00±0.10	46.09±0.31	83.82±0.20	65.71±2.41

Fig. 4. Effects of ACE (A) or leukodin (B) on the expression of melanogenesis-related proteins. Cells were treated with ACE (0, 12.5, 25 and 50 μg/ml) or leukodin (0, 37.5, 75 and 150 μM) and stimulated with α-MSH (20 nM) for 48 h. Western blot analysis was conducted to evaluate the expression level of tyrosinase, TRP-1, and TRP-2 compared to the total β-actin were calculated, and the α-MSH stimulated value was taken as 100%. Each percentage value for treated cells is reported relative to that of the control cells and presented as the mean ± SD of three individual experiments. *P < 0.05, compared with α-MSH alone-treated control.

100 μg/ml, Gibco Life Technologies, Carlsbad, CA, USA) in a humidified atmosphere incubator containing 5% CO_2 at 37 °C.

2.5. Cell viability assay

The cell viability was determined using MTT method [19]. B16F10 melanoma cells (5×10^3 cells/well) were cultured in a 96-well microplate. After overnight incubation, cells were treated with *Artemisia capillaris* ethanol extract (ACE) or leukodin and then incubated for 24 h in an atmosphere of 5% CO_2 at 37 °C. MTT solution (5 mg/ml) was treated to each well and incubated for 4 h at 37 °C. The supernatants were removed, and dimethyl sulfoxide (DMSO) was added to each well and mixed thoroughly to dissolve the formazan crystals. Cell viability was determined by measuring the optical density of each well at 570 nm using a microplate reader (Tecan, Mannedorf, Switzerland). Cell viability is expressed with respect to the untreated control.

2.6. Measurement of melanin contents

Melanin content was determined by the method described previously [20]. B16F10 melanoma cells (3.2×10^4) were seeded in 6-well plates and incubated for 24 h. Then, the cell culture media was changed to α-MSH (20 nM) and ACE (0, 6.25, 12.5, 25 and 50 μg/ml) or leukodin (0, 37.5, 75 and 150 μM) -containing medium and incubated for

48 h, The melanocyte were washed with PBS, and melanin was dissolved using 1 N NaOH in 10% DMSO at 60 °C for 2 h. The cellular melanin contents were determined by measuring the absorbance at 405 nm using a microplate reader.

2.7. Cellular tyrosinase activity assay

Cellular tyrosinase activity was estimated using spectrophotometry following the oxidation of DOPA to DOPAchrome [14]. B16F10 melanoma cells (3.2×10^4) were cultured in 6-well plates at 37 °C in an atmosphere of 5% CO_2 for 24 h. Then the cells were treated with α-MSH (20 nM) and ACE (0, 6.25, 12.5, 25 and 50 μg/ml) or leukodin (0, 37.5, 75 and 150 μM) respectively, and cultured further for 48 h. The cells were lysed with radioimmunoprecipitation assay (RIPA) buffer containing 0.1 mM phenylmethylsulfonyl fluoride (PMSF) for 30 min. The cell lysates were clarified by centrifugation at 10,000 *g* for 10 min, and 80 μl of the supernatant was mixed with 20 μl of 0.1% L-DOPA in a 96-well plate. Following incubation at 37 °C for 1 h, the tyrosinase activity was measured at an absorbance wavelength of 475 nm, and then the figure was divided by each total protein concentration to normalize.

2.8. Western blot analysis

B16F10 melanoma cells were cultured in α-MSH (20 nM) and ACE or leukodin-containing medium for 48 h, respectively. The cells were washed with cold PBS and lysed with RIPA buffer (Sigma-Aldrich, St. Louis, MO, USA) containing 0.1 mM PMSF and 1% protease inhibitor cocktail for 30 min. The lysates were centrifuged (10,000 *g*, 10 min), and the protein concentrations were determined using the bicinchoninic acid (BCA) assay kit (Thermo Scientific, MA, USA) according to the manufacturer's protocols with microplate reader. Equal quantities of total proteins were resolved by 10% SDS-PAGE, and transferred to nitrocellulose membranes (Bio-Rad, CA, USA). For Western blotting, the nitrocellulose membranes (GE Healthcare, Buckinghamshire, UK) were blocked in blocking buffer containing 5% skim milk in Tris buffer saline (TBS) containing 0.1% (v/v) tween-20 (TBS-T), and then incubated with primary antibodies specific to tyrosinase, TRP-1, TRP-2, and β-actin at 4 °C for overnight. The blotted membranes were washed with TBS-T and incubated with appropriate anti-mouse or anti-rabbit horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature. The immunoreactive bands were enhanced using chemiluminescence reagents, ECL detection kit (Thermo Scientific) and detected by chemi-luminometer (CLINX Science Instruments Co. Ltd., Shanghai, China). All experiments were performed in triplicate.

2.9. Statistical analysis

All data are presented as means ± standard deviation. Values were compared using Student's *t*-test. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Isolation of active substance from the ACE

The purification procedure is shown in Fig. 1A. The fractionation of the ethanol extraction from *A. capillaris* was followed by successive partitions of the supernatant with methanol and n-hexane solvent. The purification of the methanol fractionation was finally obtained as white crystal. The compound was characterized on the basis of ^1H and ^{13}C NMR (CD_3OD, 400 MHz) and identified as leukodin and in accordance with the literature data [18]. The chemical structure of leukodin is shown in Fig. 1B.

3.2. Effect of ACE and leukodin on cell viability and melanin synthesis in B16F10 melanoma cells

The cytotoxic effect of ACE on B16F10 melanoma cells was measured using the MTT assay and expressed as percent viability. As shown in Fig. 2A cell viability did not change in the presence of ACE in all treatment groups (0, 6.25, 12.5, 25, and at 50 μg/ml) compared to the control. This result indicated that ACE is not cytotoxic to B16F10 cells. To determine the effect of inhibiting α-MSH-induced melanin synthesis by ACE in B16F10 melanoma cells, the α-MSH-stimulated cellular melanin contents were measured following treatment with ACE at 6.2550 μg/ml. The result showed that ACE decreased the cellular melanin contents in a concentration-dependent manner, with an inhibition percentage of 40.24% at the concentration of 50 μg/ml when compared with the α-MSH alone-treated cells (Fig. 2B).

To evaluate the leukodin for B16F10 melanoma cell viability, the cells were treated with the indicated concentrations for 24 h, and then the MTT assay was performed. The concentrations of 37.5, 75, and 150 μM leukodin showed no significant cytotoxic effect on B16F10 melanoma cell proliferation (Fig. 2C). The results indicate that leukodin decreased the melanin content in B16F10 melanoma cells without cytotoxicity in a dose-dependent manner, corresponding to 30.26% inhibition at 37.5 μM, 52.96% at 75 μM, and 59.21% at 150 μM of leukodin treatment, compared to cell treated with α-MSH alone (Fig. 2D). This result suggested that leukodin may be responsible for the depigmenting action of ACE.

3.3. Effect of ACE and leukodin on tyrosinase activity in B16F10 melanoma cells

Melanin biosynthesis is known to be regulated by enzymatic activity, mainly by the tyrosinases. To investigate the possible mechanisms of anti-melanogenic activity of ACE, tyrosinase activity was measured in B16F10 melanoma cells. The tyrosinase activity of α-MSH stimulated cells was significantly higher in comparison to resting cells. ACE reduced α-MSH induced tyrosinase activity in a dose-dependent manner (Fig. 3A). It was found that ACE has significantly diminished tyrosinase activity. ACE at a concentration of 50 μg/ml resulted in 30.92% inhibition of intracellular tyrosinase activity. This result indicates that the depigmenting action of ACE involved the inhibition of tyrosinase activity.

The inhibitory effects of leukodin on α-MSH-induced cellular tyrosinase enzymatic activity were also investigated. Leukodin significantly inhibited α-MSH-induced tyrosinase activity at various concentrations (0, 37.5, 75 and 150 μM) (Fig. 3B), suggesting that leukodin displays dose-dependent anti-melanogenesis activity through the decrease of cellular tyrosinase activity.

3.4. Effect of ACE and leukodin on the expression of tyrosinase-related proteins

To investigate the effects of ACE on the protein expression level of TYR, TRP-1, and TRP-2, proteins that lead to melanogenesis, B16F10 melanoma cells were treated with various concentrations of ACE in the presence of α-MSH (20 nM) and protein levels were determined by Western blot analysis. Treatment with ACE significantly reduced the expression levels of tyrosinase protein levels including TYR, TRP-1, and TRP-2, in a dose-dependent manner (Fig. 4A). ACE at 50 μg/ml reduced the relative protein expression level of TYR, TRP-1, and TRP-2 for 92.2, 11.1 and 55.2% respectively, compared to α-MSH only treated cells. These findings suggest that inhibition of tyrosinase activity by ACE is associated with decreased expression of TYR and TRPs.

Furthermore, Western blot analysis was performed to elucidate the effect of leukodin on the protein expression of melanogenic enzymes. The leukodin reduced tyrosinase protein expression in B16F10 melanoma cells in a dose-dependent manner, corresponding to 10.6%

reduction at 37.5 μM, 29.0% at 75 μM, and 55.3% at 150 μM compared with α-MSH only treated B16F10 melanoma cells ($P < 0.05$) (Fig. 4B). Leukodin at a concentration of 150 μM also reduced the protein expression of TRP-1 with a significant difference, and effectively reduced the protein expression of TRP-2 at various concentrations (37.5, 75, and 150 μM) ($P < 0.05$). These results demonstrate that the anti-melanogenic effect of leukodin is directly related to a reduction in protein expression of enzymes involved in melanin synthesis.

4. Discussion

Hyperpigmentation disorders caused by changes in the melanin content produced by melanocytes which impact on human appearance and health. The demand for developments and improvement in pigmentary therapeutics have increased interest from dermatologist and cosmetic company. However, no current therapies achieve the perfect balance of skin lightening and maintaining cutaneous integrity [21]. Moreover, the various depigmentation agents may cause the side effects such as arbutin, kojic acid, and hydroquinone [22–24].

In the present study, we have investigated whether *Artemisia capillaris* ethanol extract (ACE) has anti-melanogenesis effects in B16F10 melanoma cells and identified the active compounds in ACE. The inhibitory mechanism of anti-melanogenic activity of ACE and active compounds were also elucidated. Firstly, the cytotoxicity of ACE on B16F10 melanoma cells was examined. The results indicated that ACE was not cytotoxic at concentrations 6.25–50 μg/ml (Fig. 2A). Secondly, the effects of ACE on α-MSH-stimulated melanogenesis in melanoma cells were determined. It has been reported that α-MSH leads to enhance the activation of tyrosinase and tyrosinase-related proteins, which, in turn, enhances melanin production [25]. ACE significantly inhibited α-MSH-induced melanin synthesis and cellular tyrosinase activity in a dose-dependent manner (Figs. 2B and 3 A). These results indicate that ACE is a safe material with anti-melanogenic effects.

Previous studies have established that *A. capillaris* contains several compounds, including Artemisia ketones, coumarin derivatives, and flavonoid derivatives, which possess a wide spectrum of pharmacological activity as an herbal remedy [11,14,15,26]. In this study, bioassay-guided isolation led to purification of leukodin for the first time from *A. capillaris*. Leukodin was reported to have been isolated from *Artemisia princeps*, an isomeric guaianolide sesquiterpene, and believed to have anti-allergic properties [16,18]. However, there's no report about the regulatory activity of leukodin for melanogenesis.

Tyrosinase, a key enzyme for melanin pigmentation in mammals, catalyzes a two-step mechanism of melanin as a rate-limiting enzyme [27]. The tyrosinase controls melanin synthesis by catalyzing the hydroxylation of tyrosine to L-DOPA, which is ultimately responsible for the accumulation of melanin pigments [28]. For these reasons, anti-melanogenic agents could inhibit tyrosinase activity with the control of TRPs, resulting in regulation of melanin production.

In this study, leukodin showed remarkable inhibition on melanin synthesis and tyrosinase activity without cell cytotoxicity (> 90% of cell viability at 37.5, 75 and 150 μg/mL). It was discovered that leukodin promotes significantly diminished tyrosinase activity, and at a concentration of 37.5–150 μM, leukodin is shown to inhibit intracellular tyrosinase activity as well (Fig. 3B). This result suggests that the leukodin may be responsible for the depigmenting capabilities of ACE. Furthermore, leukodin significantly reduced protein expression of melanogenic enzymes in a concentration-dependent manner. Thus, the present study indicates that leukodin controls melanogenesis by influencing TYR, TRP-1, and TRP-2 expression (Fig. 4B).

5. Conclusions

In summary, this study demonstrated the high capacity of *A. capillaris* ethanol extract, skin-whitening compounds to reduce melanin synthesis in B16F10 melanoma cells. The reduction in melanin synthesis is by ACE could be due to the inhibition of melanogenic enzymes, including TYR, TRP-1, and TRP-2. Leukodin, an isolated compound from ACE that displays inhibitory effects against melanogenic enzymes. Therefore, ACE and leukodin may be an effective and natural agent for skin-lightening and therapeutic treatment for skin hyperpigmentation disorders. However, further study is required to confirm anti- melanogenesis effects of ACE or leukodin via animal and clinical studies. Moreover, further explorations on developing economical production in large-scale and elucidating the mechanism of action in vivo of this agent are recommended. To our knowledge, this appears to be the first report that *A. capillaris* ethanol extract (ACE) inhibits melanogenesis induced by α-MSH in B16F10 melanoma cells.

Conflict of interest

None.

Acknowledgments

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