Inhibitory effects of *Cnidium officinale* extracts on α-MSH induced melanogenesis

GHANG TAI LEE, JEONG NO LEE, KWANG SIK LEE, JI HEAN JEONG, BYOUNG KEE JO

R&D Center, Coreana Cosmetics Co. Ltd, Cheonan, Korea

Abstract

α-MSH plays an important role in UV induced melanogenesis in human skin. It is believed to exert its effects by binding to α-MSH receptor that in turn activates adenylate cyclase and increase melanocyte proliferation, dendricity and melanogenesis. In this study, we evaluated plant extracts showing the inhibitory activity on α-MSH induced melanogenesis. The *Cnidium officinale* extracts showed high inhibitory activity on α-MSH induced melanogenesis. It (50ug/ml) inhibited the melanin synthesis activated by α-MSH in B-16 melanoma cells.

Also, we isolated active compound from *C. officinale* extracts by Mass spectrophotometer, HPLC. It was identified as Senkyunolide A. It showed the same inhibitory activity as *C. officinale* extracts at the lower concentration.

Finally, Senkyunolide A from *Cnidium officinale* extracts could play as α-MSH antagonist and be used as a strong ingredient for skin whitening cosmetics.

Introduction

α-MSH(Melanocyte-stimulating hormone) is derived from the 31-36 kDa protein proopiomelanocortin(POMC)[3]. In the skin α-MSH acts on melanocytes where it regulates melanogenesis through its activation of tyrosinase, the rate-limiting enzyme in the melanin biosynthesis pathway[1, 5]. It increases the enzyme activity through the second messenger, cAMP. Recent studies have shown that transfection of melanoma cells with constructs expressing the catalytic subunit of cAMP-dependent protein kinase will cause an increase in tyrosinase activity, and this finding provides additional support for the role of cAMP in tyrosinase induction [6]. α-MSH can activate melanogenesis through cAMP pathway and exert its effects by binding to α-MSH receptor that in turn activates adenylate cyclase and increase melanocyte proliferation, dendricity and melanogenesis.

In this study, we selected plant extracts having an inhibitory effect on melanin synthesis in B-16 mouse melanoma cells in the presence of α-MSH. The *Cnidium officinale* extracts showed high inhibitory activity on α-MSH induced melanogenesis in B-16 melanoma cells. It inhibited the melanin synthesis activated by α-MSH by controlling the tyrosinase synthesis.

Cnidium officnale is well known as a crude drug having haemodynamic and analgesic effects in Oriental medicines. Various phthalide derivatives and acidic polysaccharides have been

isolated from this plant. To determine the active compound from *Cnidium officinale* we isolated single compound and identified its structure as senkyunolide A. It showed the same activity as *Cnidium officinale* extracts at the lower concentration. We also checked whether this compound affected the cAMP formation in B-16 melanoma cells. It showed the inhibitory activity on cAMP formation compared to a-MSH treated cells.

Materials and methods

Preparation of *Cnidium officinale* extracts

We prepared the *C. officinale* extracts for anti-melanogenic agent. It was extracted in the condition of absolute alcohol and dried to powder. This powder was dissolved in 1,3 butylene glycol and used for this study.

Melanin synthesis inhibition assay in the presence of α-MSH

Cells were seeded on to 6-well plates at a density of 1 x 10^4 per well and incubated for 6 hours. The cells were then incubated with *Cnidium officinale* extracts at varying concentrations up to 100ug/ml in α-MSH(100nM) for 6 days. The melanin content of each well was determined by spectrophotometer (475nm).

Effects of *Cnidium officinale* extracts on Cell growth

Plant extracts was added to B-16 melanoma cells . After 24hours, medium was removed and replaced with medium containing 1mg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide(MTT) and incubated at 37℃ for 5 hours. The medium was aspirated, and the formazan product was solubilized with dimethyl sulfoxide. Absorbency at 570nm was determined for each well.

Effects of *C. officinale* extracts on a-MSH induced tyrosinase synthesis

Detergent-solubilized melanocyte extracts were subjected to SDS gel electrophoresis as previously described [4]. Cell extracts (about 2mg protein) were electrophoresed on 10% polyacrylamide gels. Total protein was measured by protein assay kit(Bio-Rad Laboratories Inc.). After electrophoresis, gels were placed in renature buffer (50mM Tris-HCl(pH 8.0) and 2.5%(v/v) triton X-100) at room temperature for 1 hour. Gels were then incubated in developing buffer(0.1M sodium phosphate(pH 6.8), 0.2%(w/v) L-DOPA) at 37℃ for 4 hours. Upon visualization of the tyrosinase bands, the gel was removed and dried. Finally, the relative intensity of tyrosinase band in each lane was quantified.

Isolation and Identification of active compound from *Cnidium officinale* extracts

The dried powdered *Cindium Officinale*(3 Kg) was extracted with EtOH (18 L) for 3 days. Evaporation of the EtOH solution to dryness yielded 220 g of residue. The EtOH extract was again partitioned with ethylacetate and water. Evaporation of the ethylacetate fraction to dryness yielded 111.4 g of residue. The residue(111.4 g) was chromatographed on Silica gel using Ethylacetate-n-hexane(10:1→3:1), each fraction being monitored by TLC . The fractions eluted were evaporated to give the residue (13.8g). The residue were purified by prep-LC(Ethylacetate-n-hexane=5:1) to give Senkyunolide A(7.613 g).

Figure 1. The structure of Senkyunolide A

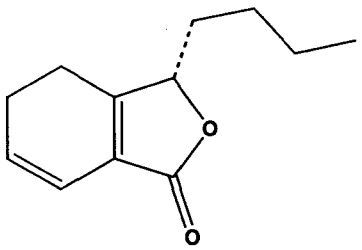
Exact Mass: 192.12

ms(m/z(%))192(17.19), 163(1.91), 135(3.57), 107(100)

^{1}HNMR δ 6.21(dt), 5.91(dt), 4.93(dd) 2.47-1.39(m), 0.9(t)

IR 2932, 2872, 1747, 1241 cm-^{1}

UV (207, 278 nm)



Evaluation of senkyunolide A on α-MSH induced melanogenesis

Cells were seeded on to 6-well plates at a density of 1 x 10^6 per well and incubated for 24 hours. The cells were then washed with PBS and cultured in media containing senkyunolide A at varying concentrations to 1, 2, 5, 10, 20ug/ml in the presence of α-MSH(100nM) for 6 days. After the cultivation, the amount of melanin was determined by spectrophotometer (475nm)

Results

Table1 showed the inhibitory activity of *C. officinale* extracts on α-MSH induced melanogenesis. Melanin content was increased by the treatment of α-MSH(100nM), but *C. officinale* extracts showed the inhibitory activity on melanin synthesis over 50ug/ml(Figure 2). Also, cell viability was measured by means of MTT assay. The cells were treated with *C. officinale* extract at varying concentrations up to 100ug/ml. Little or no effects was found with B-16 melanoma cells.

To examine tyrosinase levels following treatment of α-MSH and/or *C. officinale* extract, we performed zymography. After exposure to *C. officinale* extract alone, there was no change in the level of tyrosinase protein(Data not shown). Synthesis of tyrosinase in response to α-MSH treatment is significantly increased, and it was decreased to background levels after exposure to *C. officinale* extracts (Figure 3).

Senkyunolide A isolated from *C. officinale* extracts showed the anti-melanogenic activity in the presence of α-MSH. It showed the same activity as *C. officinale* extracts at the lower concentration (Figure 4).

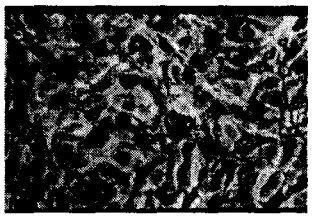
Table 1. Melanin contents and cell viability by *C. officinale* extracts in the presence of α-MSH(100nM)

Concentration of *C. officinale* extracts(ug/ml)	Cell viability (Abs. at 570nm)	Melanin contents (Abs. at 475nm)
0	0.204	0.158 ± 0.0017
0* (α-MSH treated)	0.223	0.418 ± 0.0015
10*	0.213	0.420 ± 0.007
50*	0.201	0.312 ± 0.007
100*	0.198	0.161 ± 0.004

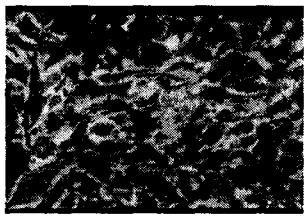
* α-MSH treated cells

Figure 2. Melanin content of B-16 melanoma cells after 6 days of treatment with α-MSH.

Control



α-MSH(100nM)

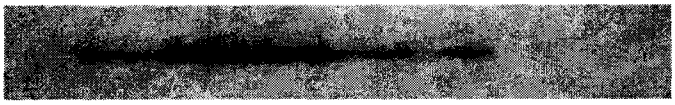


***C. officinale* extracts(10 μg/mℓ)**

***C. officinale* extracts (50 μg/mℓ)**

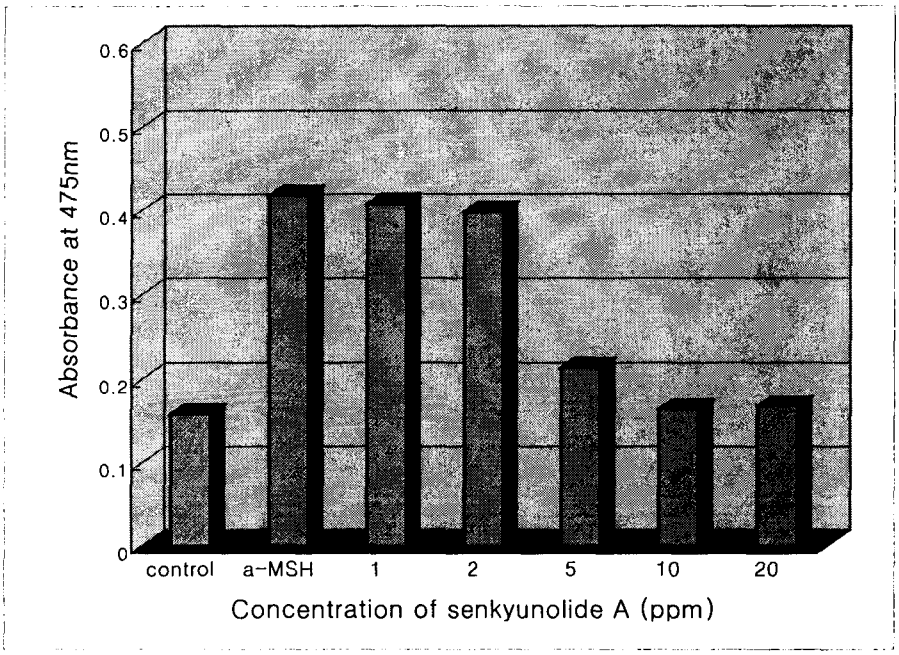
***C. officinale* extracts (100 μg/mℓ)**

Figure 3. Tyrosinase Zymography after the treatment of α-MSH and *C. officinale* extracts



Lane 1: Control, Lane 2: α-MSH(100nM), Lane 3 : *C. officinale* extracts(10 ㎍/㎖)
Lane 4 : *C. officinale* extracts (50 ㎍/㎖) , Lane 5: *C. officinale* extracts (100 ㎍/㎖)

Figure 4. Effects of Senkyunolide A on α-MSH induced melanogenesis



Discussion

We have screened 200 plant extracts for the new whitening agent having anti-melanocyte stimulating hormone. α-MSH can stimulate the synthesis of melanin in melanocytes by binding the MSH receptor. It activated the dendricity, tyrosinae synthesis, cell proliferation of melanocyte and was induced by the UV radiation. In this study we could find *C. officinale* extracts showing anti α-MSH activity. It did not inhibit the tyrosinase(key enzyme of melanogenesis) activity and the melanin synthesis in the absence of α-MSH. But it could decrease the amount of melanin pigment induced by α-MSH and the amount of tyrosinase was also decreased by *C. officinale* extracts in the presence of α-MSH . Zymography showed that *C. officinale* extracts significantly decreased the tyrosinase protein synthesis. So *C. officinale* might affect the α-MSH induced melanin synthesis in upstream level of tyrosinase expression.

Several hypotheses have been put forward to explain the mechanism of *C. officinale* extracts action, including competitive antagonism for α-MSH binding to receptor, inhibition of Microphthalmia(Mi) transcription factor function, cAMP responsive element binding protein(CREB) function or modulation of cAMP synthesis.

We have also isolated active single compound from *C. officinale* extracts and identified it structure. It was identified as senkunolide A and showed the same effects as *C. officinale* extracts at the lower concentration. It may be the main element of the *C. officinale* extracts showing anti melanogenic activity.

References

1. Abdel-Malek Z, Swope VB, Suzuki I, et al: Mitogenic and melanogenic stimulation of normal human melanocytes by melanotropic peptides. *Proc Natl Acad Sci USA* **92**:1789-1793, 1995

2. A Vanny, D Gastaldi and G Giunata, Kinetic investigations on the double enzyme activity of the tyrosinase mushroom. *Annali Di Chimica* **80**:35-60, 1990

3. Eipper BA, Mains RE: Structure and biosynthesis of pro-adrenocorticortropin/endorphin and related peptides. *Endocr Rev* **1**:1-27, 1980

4. Fuller BB, Rungta D, Iozumi K, et al: Hormonal regulation of melanogenesis in mouse melanoma and in human melanocytes. *Ann NY Acad Sci* **680**:302-319, 1993

5. Hunt G, Todd C, Cresswell JE, Thody AJ: α-MSH affect morphology, tyrosinase activity and melanogenesis in cultured human melanocytes. *J Cell Sci* **107**:205-211, 1994

6. Pawel 다 ㅓ, Wong G, Sansone M, Morowitz J: Molecular biology of pigment cells. Molecular controls in mammalian pigmentation. Yale J Biol Med 46: 430-443, 1973