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Inhibition of melanogenesis and melanin transportation by *Gynostemma pentaphyllum*

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Abstract–The extract of *Gynostemma pentaphyllum* was tested to control the melanogenesis in B16 melanoma. Cytotoxic effect by the extract was observed when the dose concentration was higher than 2 mg/L. Most of the inhibitory effect was obtained by the reduced accumulation of extra-cellular melanin. When the extract was dosed as 2 mg/L, the extra-cellular melanin produced was only 24% of the control. Proteome analysis with 2-D PAGE showed that various proteins involved in melanogenesis were down-regulated by *Gynostemma pentaphyllum*. In addition to other proteins related to the intra-cellular melanogenesis, Rab-27b and Rab-38 could explain the remarkable decrease in extra-cellular melanin accumulation by reduced melanin transfer to keratinocyte.

Key words: *Gynostemma pentaphyllum*, Melanogenesis, Inhibition, 2-D PAGE

INTRODUCTION

Melanocytes exist in skin, eyes, ears, the central nervous system, etc. Synthesis of melanin in melanosytes takes place within highly specialized membrane-bound intracellular organelles, the so-called melanosomes, which play important roles in mammals, including the regulation of constitutive pigmentation in the skin, hair, and eyes [1,2]. There are two types of melanin pigments, eumelanin of brown or black pigment and pheomelanin of yellow or red-brown pigment. The synthesis of melanin starts from the conversion of L-tyrosine to DOPA (dihydroxyphenylalanine) by tyrosinase, the enzyme catalyzing the rate-limiting step for the melanin biosynthesis with O_2. DOPA is then oxidized to DOPAquinone that is converted into either eumelanin or pheomelanin by quinine intermediates and high reactive thiol compounds [3]. Because of dendritic process, melanocytes have close relationship with keratinocytes surrounding. Melanin pigments produced by melanocytes can be easily transferred to keratinocytes, which results in the pigmentation of skin, hair, and eyes [4,5].

Melanogenesis and transformation in human skin is an important mechanism for the protection of skin from UV light. Efforts have been made to identify the mechanism of melanin formation and transformation in human skin to control melanogenesis and related side effects [6]. Up to now the regulation of melanogenesis has not been fully understood. Various factors, however, involved in the melanogenic synthesis have been gradually identified. Because of many proteins, including enzymes in the melanogenic pathways, involved simultaneously in melanogenesis, proteome analysis can be an efficient methodology in figuring out melanin formation and transformation.

Gynostemma pentaphyllum is a perennial liana growing mainly in China, Japan, and Korea. It is used as a folk medicine for lowering cholesterol level, regulating blood pressure, and strengthening immune systems. Compared to the expensive ginseng root, gynostemma is a much cheaper source of saponins and possesses great potential to be developed as a new plant drug. The major phytochemicals in gynostemma, including saponins, flavonoids and carotenoids, are believed to be responsible for the health-enhancing effects [7,8]. Because of strong antioxidant activities of phytochemicals in gynostemma, it has been understood that *Gynostemma pentaphyllum* is efficient in skin whitening, hair growth, skin-care and anti-inflammation. Among various effects of *Gynostemma pentaphyllum*, skin whitening efficacy was observed in this study. Inhibitory effect on melanogenesis was observed. Proteome analysis based on the 2-dimensional electrophoresis was applied for the simultaneous examination of protein factors involved in the melanogenesis.

MATERIALS AND METHODS

1. Cell Cultures and Extracts

B16-F1 melanoma cell line originated from the *Mus musculus* (C57BL/6J) was purchased from ATCC (USA). B16-F1 melanoma cells were cultured in DMEM (ATCC) with 1% antibiotic antimycotic (Gibco) supplemented with 1 mM L-tyrosine, 10% fetal bovine serum [9]. Cultures were made in the humidified CO_2-controlled (5%) incubator at 37 °C. Cells were sub-cultured every two or three days.

Gynostemma pentaphyllum grown in Jeju Island, Korea was harvested and dried. Ground *Gynostemma pentaphyllum* was extracted for the 24 hours extraction with 80% ethanol at room temperature. Extract was lyophilized after the ethanol was removed by using a rotary vacuum evaporator. For dose experiments, extract was dissolved in dimethyl sulfoxide (DMSO) and diluted with DMEM.

2. Assays

Melanoma cells were seeded into 96 well plates at 3×10^3 cells/well and allowed 24 hours for surface adhesion. The culture medium was removed after 24 h, exchanged to a new medium containing extracts of *Gynostemma pentaphyllum* with variable concentrations,

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0, 5, 10, 20, 30, 40, and 50 mg/L. Then cultures were maintained for three days at 37 °C in the CO_2 incubator. After the three days of cultivation, the medium was removed. Cells were washed twice with PBS and incubated in MTT solution (0.5 mg/mL) for 5 hours at 37 °C. After the incubation, MTT solution was removed. Cells were rinsed with 200 μL of D-PBS. The formazan crystal formed was dissolved with 200 μL of DMSO for 15 min. The cell viability was determined by measuring the optical density of formazan solution at 540 nm [10].

For the determination of intra-cellular and extra-cellular melanin content, modified Hosoi et al. method [11] was used. Melanoma cells were seeded into six well plates at 1×10^5 cells/well and allowed 24 hours for the surface adhesion. The culture medium was removed after 24 h, exchanged to a new medium containing extracts of *Gynostemma pentaphyllum* with different concentrations, 0, 0.1, 0.3, 1, 3, and 10 mg/L. Then cultures were maintained for three days at 37 °C in the CO_2 incubator. The culture medium was collected after the centrifugation at 1,200×g for 10 minutes. The extra-cellular melanin content was determined by measuring the absorbance of collected medium at the wavelength of 405 nm. For the analysis of intra-cellular melanin content, cells attached to culture plate were washed two times with PBS in order to remove the remaining serum, and then detached by trypsin-EDTA. Cells were collected by centrifugation at 1200×g for 10 min. The intra-cellular melanin was dissolved in 1 N NaOH solution containing 10% DMSO for 60 min at 80 °C. The intra-cellular melanin content was determined by measuring the absorbance of collected medium at the wavelength of 405 nm.

3. 2-Dimensional Polyacrylamide Gel Electrophoresis

10^7 cells washed with PBS were isolated by centrifugation. Proteins were extracted by the lysis of cells. The lysis buffer solution was prepared with 7 M urea, 2 M Thiourea, 4% CHAPS, 1% DTT, 2% Carrier ampholyte, 40 μL/mL PIC, 1 μg/mL Benzonase, 0.002% BPB. Cells in lysis buffer were sonicated for 1 min and maintained for 30 min at room temperature. The solution was centrifuged at 4,000×g for 5 min. The supernatant was collected and preserved at −20 °C. The amount of total protein extracted was measured with modified Bradford method. Bovine γ-globulin was the standard protein [12].

Immobiline Dry strips (13 cm, pI 3-10 NL, Amersham Biosciences, Sweden) were used with IPGphore fixed length strip holder. The strip was rehydrated with 250 μL of the rehydration solution for 12 hours and 100 μg of the sample proteome was injected simultaneously. The rehydration solution was prepared with 7 M urea, 2 M Thiourea, 2% CHAPS, 1% DTT, 2% Carrier ampholyte, 10% glycerol, 0.002% BPB. Isoelectric focusing was done in IPGphore (Amersham Bioscience, Sweden). After 12 h of rehydration, stepwise focusing was made, 1 h at 500 V, 1 h at 2,000 V, then increased to 8,000 V and maintained until no current change was observed [13].

Before the SDS electrophoresis, focused strips were equilibrated in buffer solutions. After the focusing, the strip was immersed in 10 ml of the equilibrium buffer solution (7 M urea, 2 M Thiourea, 2% SDS, 50 mM Tris-HCl, 30% glycerol) with 1% DTT and maintained under the mild shaking for 15 min. Then it was immersed again in the equilibrium buffer solution containing 2.5% iodoacetamide and maintained with mild shaking for 15 min.

10.5% acrylamide homogenous gels (T-13%, C-2.5%, 18×24 cm) were made in 1.5 mm thickness. For stacking, 0.5% low melting agarose was used. The IPG strip was located on the top of the SDS gel with the size marker and the stacking gel was poured. For the stacking, 10 mA was applied for 25 min. For the electrophoresis 35 mA/gel was applied until the bromophenol blue marker striped out.

The gel was washed for 1 h with dH_2O. It was fixed for 1 h in the fixing solution prepared with 50% methanol and 5% acetic acid. After the repeated washing for 1.5 h in dH_2O, the gel was maintained for 5 min in 0.02% sodium thiosulfate solution and then washed twice for 10 min. The staining was made in the refrigerated 0.1% silver nitrate solution for 30 min. After the duplicate washing for a minute, the gel was maintained for 10 min in the developing solution composed of 2% sodium carbonate, 0.014% formaldehyde, and sodium thiosulfate. Once protein spots were recognized, 1% acetic acid was added and maintained 10 min for the fixation. The stained gel was scanned and protein spot images were analyzed with 2D Elite (Amersham Biosciences, Sweden) image analysis software.

RESULTS AND DISCUSSION

1. Inhibitory Effect of *Gynostemma pentaphyllum*

To distinguish the inhibitory effect of *Gynostemma pentaphyllum* from possible cytotoxic effect on melanoma, MTT assays were made with extract of *Gynostemma pentaphyllum*. The extract did not show any cytotoxic activity on melanoma at a concentration up to 2 mg/L (Fig. 1). The cell viability was decreased slightly when the dose concentration was increased from 0.8 mg/L to 2 mg/L. The percentage of cell viability was still higher than 80% without any changes in cell morphology. At a dose concentration of 3 mg/L, however, the viability decreased rapidly to 30%. And cell morphological changes were observed with increased number of round shape cells in cultures.

The inhibitory effects on malanogenesis could be affected by dose

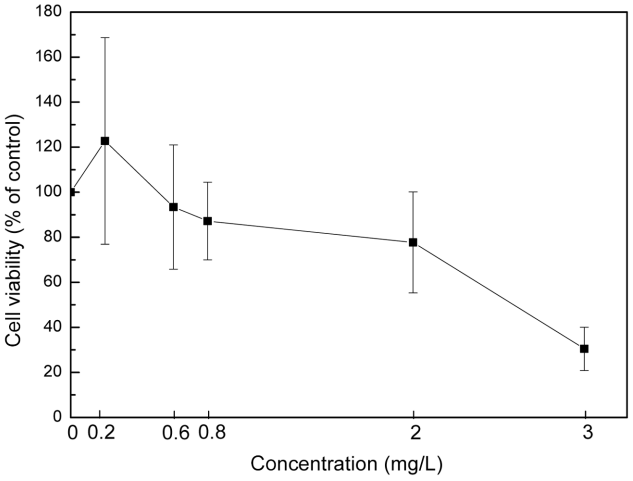

Fig. 1. Effects of *Gynostemma pentaphyllum* extracts on cell viability of B16 melanoma. Cell viabilities were tested after 3 days from dosing in various concentrations of *Morus bombycis* extracts. Cell viability was determined by MTT assay. Results were expressed as % of control (n=3).

concentration in cell cultures of melanoma. Different concentrations of extracts from *Gynostemma pentaphyllum*, lower than 3 mg/L, were prepared and dosed. The content of melanogenesis was measured by the analysis of intra and extra-cellular melanin. The relative amount of total melanin produced to control is shown in Fig. 2. Inhibitory effects were observed. Less melanin was produced as the dose concentration increased. The inhibitory effect was mainly due to the low extra-cellular melanin content. Only the 38% of the melanin produced at 2 mg/L of extract when it was compared to the content of the control. Higher inhibitory effect was observed with the dose concentration of 3 mg/L and higher. At a dose concentration higher than 3 mg/L, it is not clear whether the decrease in melanin production comes from inhibitory effect or cytotoxic effect. The effective dose concentration is important for possible application to cosmetic products. If the effective dose concentration is too high, application could be limited by formulation difficulties and the cost burden. From this point of view, the extract of *Gynostemma pentaphyllum* can be a good anti-malanogenic agent with the low effective concentration.

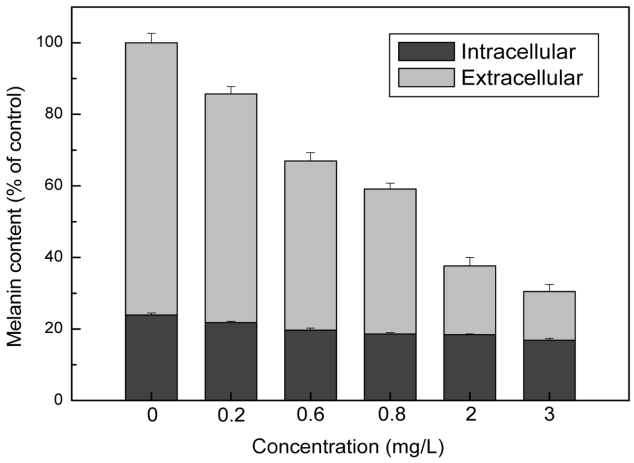

Fig. 2. Effects of *Gynostemma pentaphyllum* extracts on melanogenesis in B16 melanoma. The cells were cultured in various concentrations (0, 0.2, 0.6, 0.8, 2, 3 mg/L) of *Morus bombycis* heartwood extracts for 3 days (n=3).

The inhibitory effect of the extract from *Gynostemma pentaphyllum* on tyrosinase was tested. 2 mg/L of the extract in 100 mL of PBS was reacted within 20 mL of 320 U/mL mushroom tyrosinase and 80 mL of 1.5 M L-DOPA. After 10 minutes reaction at 30 °C, the dopaquinone produced was measured with the spectrophotometer at 475 nm. Arbutin was used as a control. The inhibition rate of tyrosinase activity by 2 mg/L of the extract was 20% which was three times higher than the control, arbutin.

It is not sure how the extract inhibited the extra-cellular production of melanin. The total intra and extra-cellular melanin production could be inhibited. Or, the secretion of melanin produced in melanoma could be strongly interfering with the extract from *Gynostemma pentaphyllum*. Both of inhibitory mechanisms have been regarded as possible with results above until the proteome analysis to investigate the mechanism of inhibitory effect by *Gynostemma pentaphyllum*.

2. Proteome Analysis

Proteome analysis can be an efficient tool to find the cause and effect of inhibitory melanogenesis [14]. By measuring the protein expression profiling of control and effective melanogenesis, roles of proteins involved in inhibitory effect can be identified. Furthermore, possible inhibitory mechanism by *Gynostemma pentaphyllum* could be elucidated. Melanoma cells dosed with 2 mg/L of extract were harvested after 48 hours. SDS-PAGE was made with proteins extracted from cells harvested. For the protein expression pro-

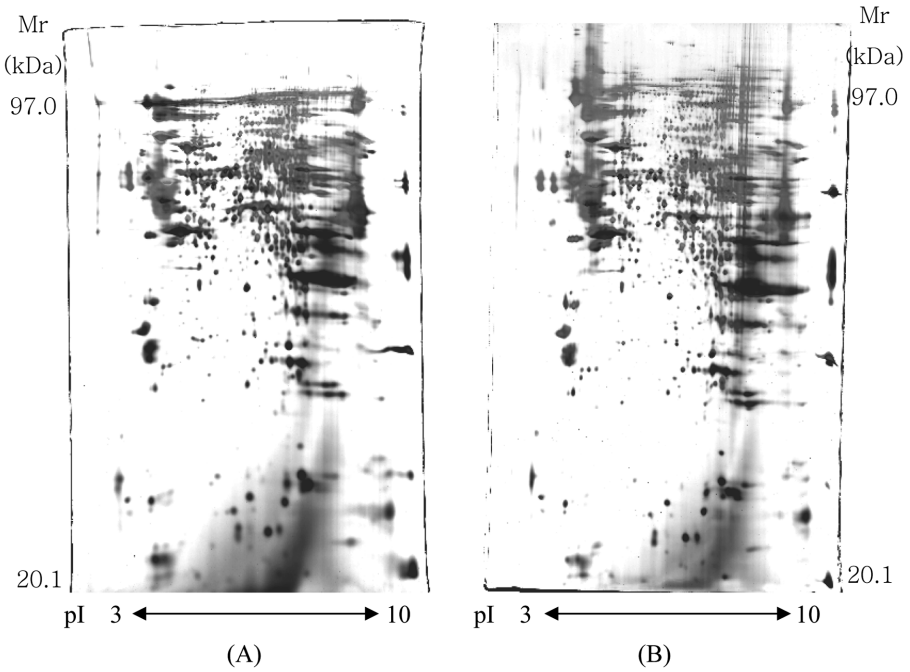

Fig. 3. 2D gel electrophoresis of total protein extracted from B16 melanoma. Cells treated with and without 2 mg/L *Gynostemma pentaphyllum* extract at 48 hours; (A) control, (B) extract treated.

Table 1. List of identified B16 melanoma treated with *Gynostemma pentaphyllum* extract by image master

Spot no.	Protein name	NCBI accession number	Theoretical		Gel-estimated	
			MW kDa	pI	MW kDa	pI
206	Clathrin-coated vesicle/synaptic vesicle proton pump 116 kDa subunit	Q93050	95.8	6.2	96.5	6.3
299	PKC-β	P68404	76.9	6.6	77.1	6.6
347	Pmel 17/gp 100	P40967	70.2	5.4	69.7	5.4
371	Tyrosinase	P14679	60.4	5.7	60.9	5.8
383	TRP2	P40126	59.2	6.7	60.9	6.7
397	saposin precursor	1360694	58.5	5.0	57.8	4.9
441	LAMP 1	P11279	44.7	9.2	46.7	9.3
443	flotillin 1	Q969J8	47.4	7.1	46.5	7.2
462	ocular albinism type 1 protein	P51810	43.9	7.5	43.2	7.5
467	flotillin 2	Q14254	41.7	5.2	42.0	5.2
469	acid ceramidase	Q13510	44.6	7.5	43.6	7.4
499	Rab-27b	O00194	24.6	5.4	24.8	5.4
501	α-MSH	P01193	26.7	8.2	27.3	8.1
503	V-ATPase, subunit E	P36543	26.1	7.7	27.7	7.7
532	type II membrane protein	Q9Y2B0	20.7	4.8	21.5	4.9
534	Rab-38	P57729	23.7	7.6	22.4	7.6

filing, the same SDS-PAGE was made with cells without extract and compared as the control protein expression. Protein spot images in the 18×24 cm gels are shown in Fig. 3. In the control gel, 456 spots were identified and 536 spots were counted in the gel with extract. By comparison image analysis, 293 spots were matched. Among them 186 spots were up-regulated and 107 spots were down-regulated.

3. Inhibitory Mechanism

Among protein spots more than 50% down-regulated, 16 spots related to the melanogenesis were analyzed (Table 1). Clathrin-coated vesicle/synaptic vesicle proton pump subunit and V-ATPase subunit E are known to have important roles in regulating pH of the melanosome, the important regulation for the melanogenesis. Melanin synthesis rate has been known to be controlled by the pH regulation in the melanosome [15,16]. PKC-β (protein kinase C-β), located at the melanosome membrane with tyrosinase, stimulates tyrosinase by phosphorylation of serine residues of tyrosinase cytoplasmic domain C. Tyrosinase and TRP2 (tyrosinase related protein 2) are important enzymes in the melanin synthesis metabolic pathways. TRP2 is essential to the development of the melanosome. Development of the melanosome is facilitated in four steps. TRP2 controls the development of the first step to the second step through endoplasmic reticulum and golgi body. Eventually, melanin is made in melanosome of further expansive third and fourth step [17].

α-MSH (α-melanocyte stimulating hormone) controls the expression of enzyme to control the ratio of eumelanin and pheomelanin. It stimulates eumelanin synthesis by MSH-R with competition to ASP (agouti signal protein) which stimulates the synthesis pheomelanin. MSH-R is known as one of receptors specifically expressed in melanocyte. MSH-R accelerates the synthesis of eumelanin with α-MSH (α-melanocyte stimulating hormone) and ACTH (adenocorticotropic hormone) [18]. Rab-27b and Rab-38 are located on the melanosome membrane. They are known to play an important role in transfer of melanosome to keratinocyte. After the transfer, the melanin appears on the skin through the keratinocyte [19].

The inhibitory effect of melanogenesis by the extract of *Gynostemma pentaphyllum* can be explained in many ways. Maintaining low pH in the melanosome could be failed by the down-regulated proteins, clathrin-coated vesicle/synaptic vesicle proton pump subunit and V-ATPase E2 subunit. The malfunctioning of pH regulation in the melanosome could reduce the melanin synthesis. The reduced melanin synthesis can also be explained by the decrease of tyrosinase and TRP2. They are key enzymes for the metabolism of melanin synthesis. Down-expressed tyrosinase and TRP2 could be related to the reduced production of melanin. The decreased number of PKC-β could also reduce the melanogenesis by reducing the phosphorylation of serine residues in tyrosinase. There are many other down-regulated proteins possibly related to the inhibition of melanogenesis. Their role, however, is not certain at this time and could be elucidated by further studies.

Among various possible explanations related to down-regulated proteins, Rab-27b and Rab-38 can explain the reduced amount of extra-cellular melanin. As shown in Fig. 2, most of the inhibitory effect of melanogenesis by the extract of *Gynostemma pentaphyllum* resulted from the decrease in extra-cellular melanin content. Skin pigmentation by the melanogenesis can be alleviated by the control of melanin transfer to keratinocytes. The inhibitory effect of melanogenesis by the extract of *Gynostemma pentaphyllum* can be achieved by the repression of transfer of melanosome to keratinocytes as well as the reduced melanin synthesis.

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