沙漠研究 **19**-1, 387- 390 (2009)
Journal of Arid Land Studies "Desert Technology IX "
Refereed Paper

Effect of Mediterranean Medicinal Plant Extracts on Melanogenesis Regulation

Kyoko MATSUYAMA[1], Yusaku MIYAMAE[2], Yukiko SEKII[2], Junkyu HAN[1], Manef ABDERRABBA[3], Takahiro MORIO[1], Hideyuki SHIGEMORI[2], Hiroko ISODA[1]

Abstract: We investigated the melanogenesis regulation effect of Mediterranean medicinal plant extracts. The melanin synthesis system is known as melanogenesis, and tyrosinase is its key enzyme. Murine melanoma B16 cells were treated with extracts of medicinal plants', namely, *Capparis spinosa*, *Erica multiflora* and *Thymelaea hirsuta*, and then cell viability and synthesized melanin content were measured. Furthermore, to determine the mechanism of melanogenesis regulation, we performed western blot analysis and DNA microarray. Results show that *C. spinosa* and *E. multiflora* extracts increased melanin content in B16 cells without cytotoxity. We also observed that the tyrosinase protein expression level was clearly increased in cells treated with the extracts. On the other hand, *T. hirsuta* extract significantly decreased the synthesized melanin content in B16 cells without cytotoxity. We also detected ERK1/2 phosphorylation and down-regulation of tyrosinase protein expression level. In addition, we identified that daphnanes-genkwadaphnin, gnidicin and gnidilatidin from *T. hirsuta* extract, have the anti-melanogenesis activity. In this research, we report that Mediterranean medicinal plant extracts have high activities in relation to the stimulation or inhibition of melanogenesis.

Keywords: B16 melanoma, Mediterranean medicinal plant, Melanogenesis

1. Introduction

Pigmentation of the epidermis is caused by melanin synthesis, and melanin plays a major role in the prevention of DNA damage and protection of skin tissues from UV irradiation. The melanin synthesis system is known as melanogenesis, and it is regulated by melanogenic enzymes, such as tyrosinase, tyrosinase-related protein 1 (TYRP-1) and TYRP-2. Among the three enzymes, tyrosinase is the most important enzyme in melanogenesis. Tyrosinase catalyzes the first two steps in melanin synthesis: the hydroxylation of tyrosine to 3-(3, 4-dihydroxyphenyl)-alanine (DOPA) and the oxidation of DOPA to dopaquinone (Kobayashi *et al*., 1998). Thus, melanin production is mainly dependent on tyrosinase expression and activation.

Extracellular signal-regulated kinase (ERK) is a key signaling mediator of cell proliferation and differentiation. Recently, it has been reported that ERK signaling pathway is related to melanogenesis. ERK1/2 phosphorylation is related to the inhibition of melanogenesis (Kim *et al*., 2006).

Medicinal plants are valuable natural products used as raw materials in many fields, including perfumes, cosmetics, aromatherapy, phototherapy, spices and nutrition. Recently, many papers on the biological and pharmacological activity of medicinal plants have been published (Cho et al., 2002, Wang *et al*., 2006). In addition, antimelanogenesis effect of Tunisian herb *Thymelaea hirsuta* extract was initially reported by Kawano *et al*. (2007).

In this study, we investigated the effects of Medditerranean medicinal plant extracts on melanogenesis regulation in the murine melanoma B16 cells.

2. Materials and Methods

2.1 Extraction of Mediterranean medicinal plants

Aerial leaves of Mediterranean medicinal plants (10 g) were extracted with 70% EtOH (100 ml) for 1-2 weeks at room temperature. After extraction, the extract was filter-sterilized using a 0.45 ㎛ pore size filter (Millipore, USA) and then stored at -80 ˚C until use.

[1] Alliance for research on North Africa (ARENA), University of Tsukuba, Ibaraki, Japan
[2] Graduate School of Life and Environmental Sciences, University of Tsukuba, Ibaraki, Japan
[3] Unité de Recherche: Physico-chimie et Moléculaire: Institut Préparatoire aux Etudes Scientifiques et Techniques IPEST, La Marsa, Tunis, TUNISIA

2.2 Measurement of melanin content and cell viability

B16 melanoma cells were seeded onto 100-mm dishes at a density of 5×10^5 cells per dish and cultivated in Dulbecco's modified Eagle's medium (Nissui, Japan) supplemented with 10% fetal bovine serum (Sigma, USA), 4 mM L-gluramine (Sigma, USA), 50 units/ml penicillin and 50 μg/ml streptomycin (Cambrex, USA) at 37 ˚C in a humidified atmosphere of 5% CO_2. After overnight incubation, the medium was replaced with sample-containing medium and incubated for 48 hours. The medium was then removed, and then the cells were washed twice with phosphate-buffered saline and then harvested by trypsinization. The harvested cells were resuspended in growth medium and stained with the ViaCount reagent (Millipore, USA) according to the manufacturer's instructions. Cell viability and cell number was determined with Guava PCA (Millipore, USA) using the ViaCount program for analysis. After determination of cell viability, the cell membrane was dissolved using 0.1% Triton X-100. The synthesized melanin was then purified and precipitated in 10% trichloroacetate. The purified melanin was dissolved by incubation in 8 N NaOH for 2 h at 80 ˚C. The absorbance of the solution was measured at 410 nm.

2.3 Western blotting

B16 melanoma cells were seeded by the method described above. After overnight incubation, the medium was replaced with sample-containing medium followed by incubation for 0, 0.25, 0.5, 1, 2, 4, 8, 12, 24, 48 and 72 hours. After incubation, the total protein was extracted using RIPA buffer (SIGMA, USA) according to the manufacturer's instructions. Fifteen micrograms of extracted protein sample were resolved by SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose membrane, and blotted with primary antibodies for tyrosinase (Santa Cruz Biotechnology, USA), ERK1/2 (SIGMA, USA) and phospho-ERK1/2 (SIGMA, USA). The signal was visualized using Immobilon Western Chemiluminescent HRP Substrate (Millipore, USA), after reaction with HRP-labeled anti-rabbit IgG antibody (Santa Cruz Biotechnology, Inc., USA).

2.4 RNA isolation and DNA microarray

B16 melanoma cells were seeded by the method described above. After overnight incubation, the medium was replaced with sample-containing medium followed by incubation for 1hour. After incubation, the total RNA was isolated using ISOGEN (Nippon Gene, Japan). The total RNA concentration and purity were determined by measuring the optical density, OD_{260} and $OD_{260/280}$ using a UV spectrophotometer.

DNA microarray experiments were performed using GeneChip® Mouse Genome 430 2.0 arrays (Affymetrix, Inc., USA). Total RNA samples were processed following a standard one-cycle eukaryotic target preparation protocol from Affymetrix. Biotinylated cRNA targets were hybridized to GeneChip® array in a rotating hybridization oven. After hybridization, the arrays were stained with streptavidin -phycoerythrin in the GeneChip® Fluidics station and then scanned using Affymetrix GeneChip® Scanner. The array image data were acquired, and the fluorescent signal intensities were quantified using Affymetrix® GCOS v. 1.4 software.

3. Results and Discussion

3.1 Effect of Mediterranean medicinal plant extracts on melanogenesis and cell viability

The effect of *C. spinosa* (**Fig. 1A**), *E. multiflora* (**Fig. 1B**) and *T. hirsuta* (**Fig. 1C**) extract on the melanogenesis of B16 mouse melanoma cells was examined. We observed that B16 mouse melanoma cells treated with *C. spinosa* and *E. multiflora* extract showed an increase in cytoplasmic accumulation of melanin in a dose-dependent manner without cytotoxicity. To examine the mechanism underlying the promotion effect of *C. spinosa* and *E. multiflora* extract on melanin synthesis, we determined the tyrosinase protein expression in B16 cells by western blot analysis. The tyrosinase expression levels of cells treated with the extract were highly upregulated in time-dependent manner compared with the control cells. These results suggest that the melanogenesis promotion effect of *C. spinosa* and *E. multiflora* extract on increased melanin synthesis is due to the up-regulation of tyrosinase, the most important enzyme in melanogenesis.

On the other hand, we observed that B16 mouse melanoma cells treated with *T. hirsuta* extract showed a decrease in cytoplasmic accumulation of melanin in a dose-dependent manner without cytotoxicity. To examine the mechanism underlying the suppressive effect of *T. hirsuta* extract on melanin synthesis, we determined the tyrosinase protein expression and ERK1/2 phosphorylation in B16 cells by western blot analysis. The tyrosinase expression levels of cells treated with the extract were down-regulated compared with the control cells. Although the expression levels of ERK1/2 in cultured B16 cells were not affected by treatment with the extract, the phosphorylation of ERK1/2 was considerably enhanced after 0.5 hour to 5 hour. These results suggest that the suppressive effect of *T. hirsuta* extract on melanin synthesis is due to the down-regulation of tyrosinase and via ERK1/2 phosphorylation.

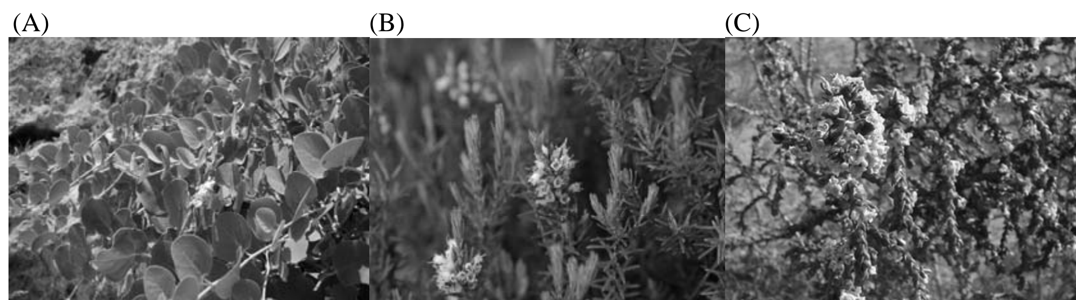

Fig. 1. Mediterranean medicinal plants. (A) *C. spinosa* (B) *E. multiflora* (C) *T. hirsuta.*

3.2 Daphnanes having anti-melanogenesis activity in the *T. hirsuta* extract

To identify the major compound responsible for the activity, leaves of *T. hirsuta* was extracted in MeOH and the extract was partitioned with EtOAc, BuOH and water. We examined the effect of each layer on the melanogenesis of B16 cells and found that the EtOAc layer showed anti-melanogenesis activity. The EtOAc layer was fractionated into 17 fractions (*Th*-EtOAc-1-17) and *Th*-EtOAc-11 possessed the highest antimelanogenesis activity. *Th*-EtOAc-11 was further fractionated into six fractions (*Th*-EtOAc-11-1-6) and *Th*-EtOAc-11-3 strongly inhibited melanogenesis. We identified that daphnanes, genkwadaphnin (**Fig. 2A**), gnidicin (**Fig. 2B**) and gnidilatidin (**Fig. 2C**) included in *Th*-EtOAc-11-3 and these compounds decreased in cytoplasmic accumulation of melanin without cytotoxicity (**Fig. 3**). Moreover, we determined that ginidilatidin, has the strongest effect of anti-melanogenesis activity.

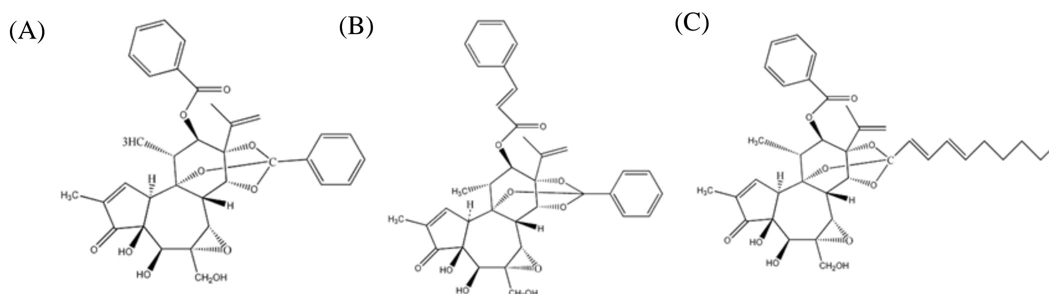

Fig. 2. Structure of major compounds from *T. hirsuta* extract. (A) genkwadaphnin (B) gnidicin (C) gnidilatidin.

3.3 Molecular mechanism of anti-melanogenesis activity by gnidilatidin

To examine the mechanism underlying the suppressive effect of gnidilatidin on melanin synthesis, we determined the tyrosinase protein expression and ERK1/2 phosphorylation in B16 cells by western blot analysis. The tyrosinase expression levels of cells treated with gnidilatidin were significantly down-regulated in time-dependent manner compared with the control cells. The phosphorylation of ERK1/2 was considerably enhanced after 0.25 hours treatment with gnidilatidin and then gradually attenuated in time dependent manner. These results suggest that the anti-melanogenesis effect of gnidilatidin in B16 cells is due to the down-regulation of tyrosinase and via ERK1/2 phosphorylation.

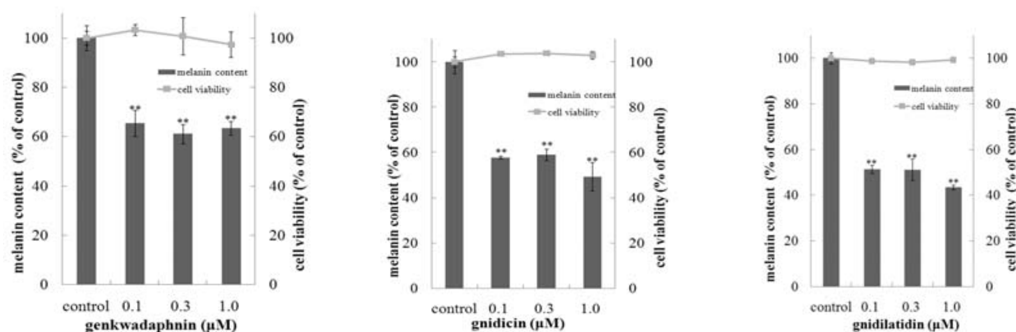

Fig. 3. Effect of daphnanes from *T. hirsuta* extract on melanogenesis and cell viability in B16 murine melanoma cells. Results represent means ± SD of triplicate samples. **Statistically significant ($P < 0.001$) difference between daphnanes treated cells and control.

Furthermore, to evaluate more details of the mechanism underlying the effects of gnidilatidin, we performed DNA microarray analysis and determined comprehensive gene expression level. Of the 34,000 mouse genes examined on the DNA microarray, 606 genes exhibited at least a two-fold change in expression. There was a two-fold increase in gene expression of 394 genes; on the other hand, there was a two-fold decrease in 212 gene's expression. These genes were categorized according to their activity in apoptosis regulation, cell cycle, cell adhesion, cell proliferation and signal transduction.

4. Conclusions

In this research, we found that the extracts of Mediterranean medicinal plants have high activities in relation to the stimulation or inhibition of melanogenesis. *C. spinosa* and *E. multiflora* extract have the melanogenesis stimulation activity. However, we did not identify the major compounds in these plants' extracts. To confirm the melanogenesis stimulation activity by these plant extracts, more analysis should be performed and details of the mechanism should be clarified.

On the other hand, *T. hirsuta* extract have the melanogenesis inhibition activity and we determined that daphnanes contained in *T. hirsuta* extract has strong effect of antimelanogenesis activity. In addition, we found that gnidilatidin, one of dapnanes from *T. hirsuta* extract, has the strongest activity of anti-melanogenesis. Moreover, we clarified that gnidilatidin induced gene variation in relation to cell differentiation in B16 cells briefly and then induced phosphorylation of ERK1/2 and downregulation of tyrosinase in protein expression level, followed by melanin synthesis inhibition.

In conclusion, our results suggest that the extracts of Mediterranean medicinal plants have the ability to maintain skin homeostasis, and can be used as a tanning or whitening agent.

Acknowledgement

This study was supported in part by funds from the Grant-in Aid for Scientific Research (Grant No. 17255011). The photos of flowers were kindly given by Dr. Kawada (ARENA, Japan).

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