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미백제 선발을 위한 *In Vitro* 측정법의 신뢰도

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Reliability of *In Vitro* Assay for Initial Depigmenting Agent Screening

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요 약: 미백제를 선발하기 위해 주로 사용하는 현재의 방법은 *in vitro* 타이로시네이즈 활성 및 항산화능을 측정하는 것이다. 이 결과에 기초하여 다음 단계인 멜라노사이트에서의 멜라닌 생성량을 측정한다. 세포 내의 멜라닌 생성량 측정법은 시간, 인력 및 숙련도가 요구된다. 따라서 초기 선발 방법의 신뢰성이 중요하다. 200개 중국시료 중 측정범위 내에서 세포독성이 없는 34개를 대상으로 세포 내 멜라닌량, 타이로시네이즈 활성, 항산화능의 상관관계를 조사하였다. 조사결과 직선의 상관관계를 확인할 수 없었다. 이 결과는 현재 선발방법의 한계 및 새로운 방법이 필요함을 보여주었다.

Abstract: Initial screening assay for depigmenting agents includes *in vitro* mushroom tyrosinase assay and antioxidant assay. Based on this screening result, melanin synthesis in melanocyte, in screened samples, is further measured. Measuring cellular melanin needs time, human resource, and skills. Therefore initial screening method should be reliable. We examined 34 Chinese herbs, correlated the screening assay methods with cellular melanin. No reliable relationship was observed between factors, indicating the limitation in the use of these assays, probably due to the complexicity of melanogenesis.

Keywords: *depigmenting agents, screening, mushroom tyrosinase, antioxidant, melanin*

1. Introduction

Melanin is the major pigment for color of human skin [1,2]. It is secreted by melanocyte cells in the basal layer of the epidermis[3]. Melanin has a number of important and distinct functions, ranging from its role in the determination of phenotypic appearance, to protective coloration, to balance and auditory processing, to absorption of toxic drugs and chemicals, and to neurological development during embryogenesis[4]. The most important of melanin function is protecting the skin from ultraviolet light, and thus prevention of UV-induced photodamage, photo aging, and photo carcinogenesis[5]. However, increased production and accumulation of melanins characterize a large number of skin diseases, which include acquired hyperpigmentation, such as melasma, post inflammatory melanoderma, solar lentigo. Moreover, as the population ages, dyspigmentation due to photo aging will become more common[6]. Therefore depigmenting agents have become increas-

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ingly important in the cosmetic and medicinal products used for the treatment of hyperpigmentation[7,8]. Many skin lightening products used traditionally, such as linoleic acid, hinokitiol, kojic acid, naturally occurring hydroquinones, and catechols were reported to inhibit melanogenesis but also exhibited side effects[9]. These adverse effects have lead to the search for safer, plant-based skin lightening products. The ideal skin lightening agent for cosmetic products is one that inhibits melanogenesis without cytotoxicity, reduces pigmentation in cells and is of "natural" origin.

The depigmentation effects of herbs can be evaluated based on cell culture assays for screening of novel depigmenting agents[10]. Many tyrosinase inhibitors that suppress melanogenesis have been screened using mushroom tyrosinase inhibition assay with the aim of developing new whitening agents[11,12]. As antioxidants may reduce hyperpigmentation, antioxidant abilities of samples were attempted[13,14]. However, melanin biosynthesis is a complicated process involving many factors[15,16]. Therefore initial screening method should be reliable. In this paper, we report different approaches to achieve depigmenting agents from screening results of 200 Chinese natural plants, include experiments on inhibition of melanin synthesis, antioxidant activity and inhibition of tyrosinase activity. The correlations between antioxidant activity and tyrosinase inhibitory activity, and inhibition of melanin synthesis were also estimated.

2. Materials and Methods

2.1. Materials and Reagents

Mushroom tyrosinase, 3,4-dihydroxy-L-phenylalanine (L-DOPA), arbutin, dimethyl sulfoxide (DMSO) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 1,1-diphenyl-2-picrylhydrazyl radical (DPPH) were purchased from Sigma Chemical Co. (St. Louis, U.S.A). Dulbeco's modified Eagle's mediumn (DMEM), fetal bovine serum (FBS) trypsin-EDTA, phosphate buffered saline (PBS), penicillin / streptomycin were purchased from Invitrogen Corp. (CA, U.S.A).

2.2. Cell Culture

B16F10 murine melanoma cells were purchased from American Type Culture Collection (ATCC). B16 melanoma cells were cultured in DMEM that was supplemented with 10 v/v% FBS, 100 units/mL of penicillin and 100 units/mL of streptomycin at 37 ℃ in a humidified, CO_2-controlled (5 %) incubator. The cells were sub-cultured every three days until a maximal passage number of 30 were achieved.

2.3. Assays: Viability, Melanin, Mushroom Tyrosinase, Cellular Tyrosinase and Radical Scavenging Activity

Cell viability assay was determined using MTT assay. Cells were seeded into a 96-well plate at a density of 2.5×10^3 cells per well. After 24 h incubation, the culture medium was removed and new medium containing the test substance was added to each well in quintuplicate. After 2 days incubation, cell viability was assayed by MTT solution. The percentages of viable cells in each well were calculated with respect to the optical density (OD) value of living cells of the control group[17].

To measure cellular melanin, cells were seeded into a 6-well plate (Falcon, U.S.A) at a density of 6×10^4 cells per well. After 24 h of cultivation, the medium was replaced with fresh medium containing various concentrations of compounds. After 2 days incubation, the adherent cells were washed with PBS and detached from the plate by trypsin-EDTA. The cells were collected in a test tube and washed twice with PBS. Melanin of cells were extracted by mixture of NaOH 1 N in 10 % DMSO at 80 ℃ for 1 h. The melanin content was determined at 475 nm using an enzyme-linked immuno sorbent assay (ELISA) micro plate reader[18].

Mushroom tyrosinase activity was assayed on a 96-well plate at 37 ℃. Reaction mixtures consist of 100 μL of sample, 125 U mushroom tyrosinase, 40 μL of 5 mM L-DOPA, and 0.1 M PBS (pH 6.8). After 20 min, absorbance was measured as described above. Kojic acid was used as a standard agent[18].

Cellular tyrosinase activity was assayed as DOPA oxidase activity. B16F10 cells were seeded in 6-well plates at a density of 6×10^4 cells per well and cultured for 24 h. After being treated with samples for 48 h, the

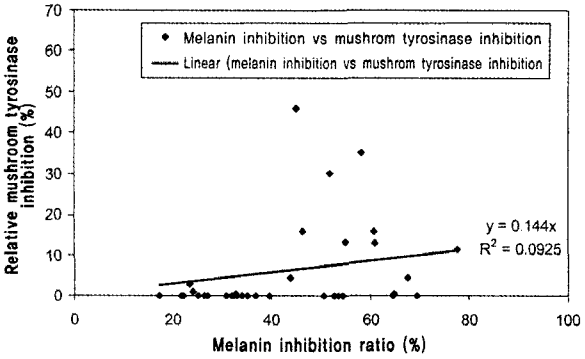

Figure 1. Relation between mushroom tyrosinase inhibitory activity and melanin inhibition in B16F10 melanoma cells.

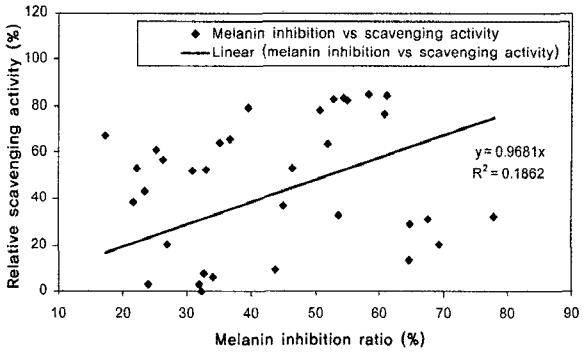

Figure 2. Relation between scavenging activity and melanin inhibition in B16F10 melanoma cells.

cells were washed with PBS and lysed with lysis buffer (0.1 M phosphate buffer pH 6.8 containing 1 % Triton X-100). The cells were then disrupted by sonication for 1 h at 4 ℃, and lysates were clarified by centrifugation at 13,000 rpm for 20 min. After quantifying the protein content using a protein assay kit (Bio-Rad, U.S.A.), the cell lysates were adjusted to the same amount of protein with a lysis buffer. Reaction mixtures consisting of 40 μg of protein, 40 μg of 5 mM L-DOPA and 0.1 M PBS (pH 6.8) were assayed on a 96-well plate at 37 ℃. After 30 min incubation, the absorbance was measured at 475 nm using an ELISA reader[18].

The radical scavenging activity of a sample was determined by the DPPH method. A sample was dissolved in a DMSO with different concentrations. Reaction mixtures consisting of 100 μL of sample and 100 μL of DPPH solution were assayed on a 96-well plate. The absorbance at 517 nm of the solution was measured after 30 min[19].

3. Resullts and Discussion

3.1. Effect of Antioxidant Activity on Melanin Inhibition

Oxidative stress may be induced by increasing generation of reactive oxygen species (ROS) and other free radicals. UV radiation can induce formation of ROS in skin such as singlet oxygen and superoxide anion radical, promoting biological damage in exposed tissues via ion-catalyzed oxidative reactions. These ROS enhance melanin biosynthesis, damage DNA, and may induce proliferation of melanocytes. It is known that ROS scavengers or inhibitors such as antioxidants may reduce hyperpigmentation.

DPPH assay is used as a popular method for screening free radical scavenging activity or antioxidant activity of plant extracts. In screening test of Chinese plants, the antioxidant activity does not show good correlation with melanin inhibitory activity (Figure 1). On cell-based system using B16F10 melanoma, 34 / 200 samples showed potential effect on melanin inhibition (compared with arbutin) without toxicity. However, fourteen of them did not show antioxidant activity. Moreover, many samples showed high antioxidant activity but they did not inhibit melanin synthesis or stimulate melanin synthesis or increase cell cytotoxicity (data not shown).

3.2. Effect of Mushroom Tyrosinase Inhibition on Melanin Inhibition

Tyrosinase inhibitors are of great interest and have become increasingly important in medicinal and cosmetic products for whitening in relation to hyperpigmentation. To screen tyrosinase inhibitors, mushroom tyrosinase activity test is the most common method because of its simplicity. In screening test of Chinese plants, mushroom tyrosinase inhibition does not show good correlation with melanogenesis inhibitory activity. There are many herbs showed high inhibition in melanin synthesis without any inhibition in mushroom tyrosinase activity (Figure 2). The reason of the no good correlation is that mushroom tyrosinase is different from mammalian tyrosinase because of its unique requirements for substrate and cofactor as well as its different

Table 1. Characteristics of Chinese Plant

Number	Name	Part	Characteristics of sample (%, Relative)*			
			Mushroom tyrosinase inhibition	DPPH scavenging activity	Cellular tyrosinase inhibition	Cellular melanin inhibition
1	*Hamenaria sp.*	Fruit	0.52	7.93	3.50	32.60
2	Chinese herb 1	Fruit	0.00	83.40	43.00	54.32
3	*Gastrodia sp.*	tuber	0.00	0.00	25.30	32.30
4	*Fritillaria sp.*	Fruit	0.00	13.67	-23.66	64.61
5	*Styrax sp.*	Resin	0.00	20.35	37.33	26.87
6	*Myrsine sp.*	Fruit	0.00	53.35	-4.80	22.12
7	*Alpinia sp.*	Seed	0.00	3.33	20.00	31.95
8	*Acacia sp.*	Bark	0.00	82.73	-63.00	52.76
9	*Terminaliae sp.*	Seed	13.24	82.32	81.00	54.93
10	Chinese herb 2	Tuber	15.88	76.85	61.00	60.77
11	*Amomum sp.*	Seed	0.52	29.14	68.00	64.78
12	*Nigella sp.*	Seed	4.39	9.31	21.00	43.78
13	*Saxifraga sp.*	Tuber	12.93	84.28	56.00	61.00
14	Chinese herb 3	Resin	0.00	20.45	-31.00	69.38
15	*Santalum sp.*	Powder	0.00	57.00	-13.00	26.30
16	*Cinnamomum sp.*	Bark	0.00	79.50	-32.00	39.43
17	*Holarrhena sp.*	Seed	0.00	67.41	-17.35	17.30
18	*Aucklandia sp.*	Branch	1.15	3.37	-61.00	23.92
19	*Zingiber*	Rhizome	0.00	32.90	-35.00	53.54
20	*Cuminum sp.*	Seed	4.49	31.31	-52.00	67.51
21	*Syzygium sp.*	Seed	0.00	61.17	39.00	25.14
22	*Canavalia sp.*	Seed	0.00	78.50	21.10	50.54
23	Chinese herb 4	Flower	0.00	52.60	19.16	33.00
24	*Carthamus sp.*	Flower	2.90	43.30	14.40	23.40
25	*Rosa sp.*	Flower	15.80	53.40	-41.00	46.30
26	*Jasminum sp.*	Flower	0.00	38.40	12.36	21.70
27	*Rosa sp.*	Flower	0.00	65.50	-8.92	35.70
28	*Thamnolia sp.*	Bulb	0.00	52.30	-32.00	30.80
29	*Acorus sp.*	Tuber	0.00	64.30	51.00	35.10
30	*Polygonum sp.*	Bulb	11.40	32.20	40.00	77.80
31	Chinese herb 5	Seed	30.30	63.40	-15.00	51.80
32	Chinese herb 6	Leaf	46.00	36.90	19.00	45.00
33	Chinese herb 7	Resin	35.17	84.90	5.78	58.23
34	Chinese herb 8	Branch	0.00	6.30	-42.04	34.00

*Relative activities were expressed compared with same concentration of kojic acid (mushroon tyrosinase), and vitamin C (DPPH). Cellular melanin and cellular tyrosinase were expressed as those of cells treated with 200 mg/L of arbutin.
Chinese herbs (1 to 8): The scientific names were not identified.

sensitivity to inhibitors. Any skin-lightening compound that inhibits tyrosinase or other enzymes must penetrate the melanocyte in order to perform its activity. The cellular surroundings may also influence the effect of the product. This may lead to false-positive response. There is also a risk of negative response if the product has an indirect action on tyrosinase or exerts its activity on any other step of the melanin formation.

Another method is using cellular tyrosinase from cultured cells such as murine melanoma or melanocytes cells, human melanocytes. Because of using cultured cells, cellular tyrosinase assay is time-consuming and high cost. Moreover, in screening test of Chinese plants, cellular tyrosinase inhibitory activity also did not show good correlation between with melanin inhibition activity (Figure 3).

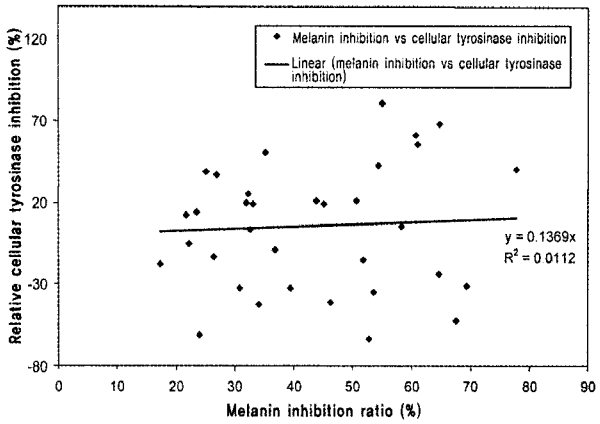

Figure 3. Relation between cellular tyrosinase inhibitory activity and melanin inhibition in B16F10 melanoma cells.

From these results, no good relation was observed between melanin content and results of screening assays.

Melanogesis is complex process. A complex set of both extracellular and intracellular signals control the development and function of the pigment cells. Beside the inhibition of tyrosinase activity, the melanin inhibition can be achieved by inhibiting the transcription, glycosylation and the maturation of melanogenic enzymes, or the transfer of melanin to surrounding keratinocytes[6]. Many factors can regulate melanogenesis including various cytokines, growth factors and inflammatory mediators. In mouse, there are more than 125 loci involved in the melanogenesis. The total number of alleles at these loci has now exceeded 800. Genes that regulate mammalian pigmentation act in the tissue, the cellular, the sub cellular, and/or the environmental level. Protein products of these genes acting as enzymes, structural proteins, transcriptional regulators, transporters, receptors, and growth factors have a wide array of functions and cellular targets[20]. Moreover, keratinocytes and fibroblasts actively regulate melanocyte function with respect to growth, morphology, pigmentation, and other characteristics of differentiation [21]. Melanocytes cultured alone may not accurately reflect the effects of putative bioactive compounds *in vivo* because they do not consider possible interactions with keratinocytes.

4. Conclusion

Assay for inhibition of mushroom tyrosinase, cellular tyrosinase, and antioxidant activity are common methods to find novel depigmenting agents. However, in results from screening Chinese plants, poor correlations between melanin content and antioxidant, mushroom tyrosinase, cellular tyrosinase were observed. These poor correlations may be due to the complexicity of melanogenesis. Therefore, there remains a need for novel approach that presents a potent correlation with melanogenesis inhibition.

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