研究ノート

Study on Melanin Synthesis Inhibiting Substance in Coffee Extracts
− To Aim at Advanced Utilization of Coffee −

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Abstract

Detection sensitivity was increased four-fold *via* investigation of optimal measurement conditions for melanin biosynthesis inhibitory activity *in vivo* employing mouse B16 melanoma cells. The identical methodology was utilized to evaluate an isolation protocol for the coffee-originated melanin biosynthesis inhibitory substance. The MeOH component of the MeOH extract partitioned with hexane demonstrated optimal melanin biosynthesis inhibitory activity. *In vivo* experiments involving guinea pigs with the aforementioned substance were conducted, which afforded observation of the melanin pigmentation inhibitory effect *via* administration of coffee extract transdermally or orally prior to ultraviolet exposure.

Key words: coffee, melanin biosynthesis inhibition, cosmetic whiting reagent

Introduction

The various physiological activities of coffee in methanol (MeOH) and hydrothermal extracts were investigated. As a result, the unique, potent property of coffee extract (500 μg/ml) in terms of inhibition of melanin biosynthesis was discovered, which is stronger than that of arbutin (125 μg/ml), which was believed to be the strongest melanin biosynthesis inhibitor as reported by Myouga and Tamaki (2002)[1]. Additionally, MTT technology applied to coffee extracts with identical concentrations, although unrefined, revealed toxicity of less than 50% against mouse B16 melanoma cells in comparison with arbutin. These findings suggest that coffee extract is a safe, effective and economical alternative cosmetic whiting reagent; furthermore, the possible expansion of demand exists with the potential use of coffee. Recently, preparatory research was conducted involving determination of the optimal conditions of an *in vitro* inspection plan and further isolation of active substances. Subsequently, the effectiveness of these active species was confirmed by *in vivo* studies with guinea pigs.

Materials and Methods

1. Test Coffee Beans and Preparation of Coffee Extract

Mandarin coffee beans, which produced the best results in a study conducted in 2000, were employed in the preparation of test specimens. Coffee beans were ground moderately in a commercially available coffee grinder; the ground product was percolated with four fold-boiled de-ionized water in order to generate the hydrothermal extract. Four-fold MeOH was added to the remaining residual coffee; subsequently, the filtered solution was utilized in the investigation as coffee MeOH extract.

2. Measurement of Melanin Biosynthesis Inhibition Activity

As a model cell system for melanin biosynthesis, the mouse B16 melanoma cell line (JCRB0202) was selected. Model cell lines were grown in Dulbecco's modified Eagles' medium (Gibco-RBL) supplemented with 10% fetal bovine serum (Sigma), penicillin G, streptomycin and HEPES in a humidified atmosphere with 5% CO_2 at 37℃. The B16 cells (1 x 10^4 cells/297 μl /well) were incubated for 24 hours; subsequently, 3 μl of each coffee extract was introduced prior to subsequent incubation. Cells were lysed following rinsing with PBS twice and melanin was extracted *via* ultrasonic treatment in 100 μl of 2 N NaOH. Melanin extract solution was centrifuged at 14,000 rpm for 5 minutes. Light absorbance of supernatant solution was measured (wavelength, 405 nm). Melanin biosynthesis inhibition activity (in %) was measured against commercially available synthesized melanin as a control.

3. Investigation of Optimal Incubation Period for B 16 Cell Melanin Biosynthesis

Following incubation of B16 cells and melanin extraction, light absorbance of each sample was measured at 405 nm. The level of melanin biosynthesis was observed per day.

4. Investigation of Isolation Method of Coffee-originated Melanin Biosynthesis Inhibitor

MeOH coffee extraction was solvent-dispensed with equal volumes of chloroform or hexane (3 repetitions). Extracts, which were concentrated and solidified on a rotary evaporator, were dissolved in dimethylsulfoxide (DMSO). Hydrothermal coffee extract solution was prepared in an identical manner employing ethyl acetate. Melanin biosynthesis inhibitor substance in each allotment was assessed in terms of melanin biosynthesis

inhibition activity as well as dry weight.

5. *In vivo* Experiment Involving Guinea Pigs

The dorsal surfaces of 5-week-old Weiser-maplee guinea pigs (Kiwa Test Animal Research Institute) were shaved; subsequently, each animal was exposed to ultraviolet rays for 6 minutes (1.25 mW/cm^2 per day) over 3-day intervals. After ultraviolet exposure, coffee extract solution was applied to the exposure site (1.0 or 0.5 mg/cm^2 per day) for more than 2 weeks; moreover, a separate group of animals underwent application of an identical amount of MeOH (transdermal administration group). Water, which was supplemented with a coffee extract solution (40 or 20 mg per day), was administered orally to another group of animals (oral administration group). In addition, for each administration group, a corresponding group of animals received coffee extract over a 2-week interval prior to ultraviolet exposure; animals were observed for 1 week following a 3-day period of ultraviolet exposure. Each group consisted of 4 guinea pigs. Color measurement was conducted with a $L^{\star}a^{\star}b^{\star}$ color system colorimeter (ZE2000, Japan Electronics) via measurement of $L^{\star}$ level (color scale) on 5 occasions per 1 site; averages values were obtained. Differences in $L^{\star}$ values prior to and following administration ($\Delta L^{\star}$ value) were used to evaluate change in skin pigmentation. Average body weight of guinea pigs changed from 302.5 g to 447.1 g during the experiment.

Results and Discussion

1. Optimization of Measurement of Melanin Biosynthesis- inhibiting Activity Employing B16 Cells

Disadvantages of conventional measurement methodology for melanin biosynthesis-inhibition activity include difficulty associated with detection of measurement differences due to the small capacity (300 μl) of 48-well micro-plates. Measurement of B16 cell daily melanin biosynthesis demonstrated that the greatest biosynthesis activity occurs on the 5th day, an extension of one additional day beyond the conventional incubation period (Fig. 1). Utility of cells on the 5th incubation day and application of 100 μl of 2 N NaOH (one-third of the conventional amount) afforded a four-fold increase in detection sensitivity.

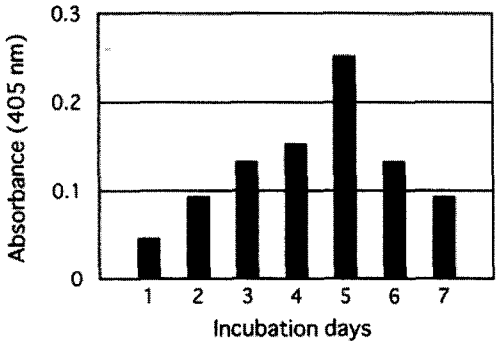

Fig. 1. Daily change of melanin synthesis in mouse B16 melanoma cells.

2. Investigation of Isolation Method of Coffee-originated Melanin Biosynthesis Inhibitor

The isolation method of coffee-originated melanin biosynthesis inhibitor and results are outlined in Fig. 2. Melanin biosynthesis inhibitory activity of the hydrothermal coffee and residual MeOH extracts were 38.2% and 31.9%, respectively; moreover, extract dry weights per 10 ml were 4.21 g and 1.84 g, respectively. These values are comparable to those obtained in our previous study. Hydrothermal extracts were partitioned with ethyl acetate. Dry weight of the water component was 3.26 g, whereas the dry weight of the ethyl acetate portion was 0.86 g; however, activities were dispersed (18.8% and 20.8%, respectively). When MeOH extracts were partitioned with chloroform, activities of the MeOH and chloroform components were 13.3% and 39.6%, respectively; the dry weights of these components were 1.36 g and 0.38 g, respectively. Therefore, the majority of active

substances were transferred to the chloroform phase. When MeOH extracts were partitioned with hexane, activities of the MeOH and hexane components were 61.6% and 0%, respectively, with some condensation; the dry weights of these components were 0.40 g and 0.13 g, respectively. On the basis of these results, we concluded that the MeOH component derived from the MeOH extract partitioned with hexane demonstrated the highest relative biosynthesis activity per unit; therefore, this phase was employed as an optimal sample for coffee-originated melanin biosynthesis inhibitor in subsequent experiments.

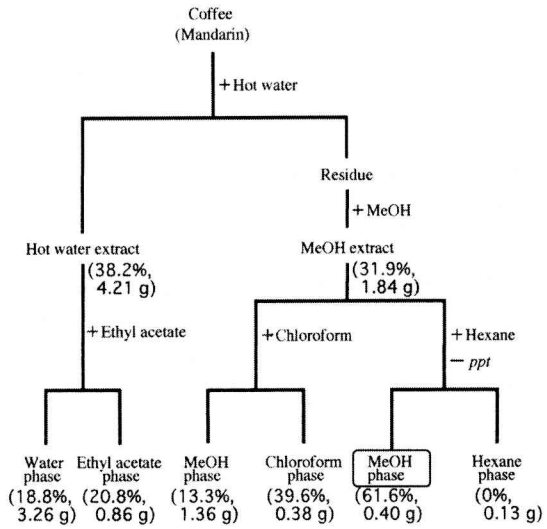

Fig. 2. Isolation of melanin synthesis inhibiting substance from coffee. The upper and lower numbers in the parenthesis indicate melanin synthesis inhibiting activity and dry weight per 10 ml, respectively.

3. In vivo Experiment Involving Guinea Pigs

In the group characterized by 2-week-transdermal coffee extract application prior to ultraviolet exposure, $\Delta L^{\star}$ values in 1.0 and 0.5 mg/cm^2 per day were 0.55 and 2.17, respectively, after 3 days of exposure. In contrast, the MeOH administration group demonstrated a reading of 4.80; moreover, a significant difference was indicated by t-test (p=0.05). This trend extends to the 7 days following exposure; $\Delta L^{\star}$ value in 1.0 and 0.5 mg/cm^2 per day were 2.68 and

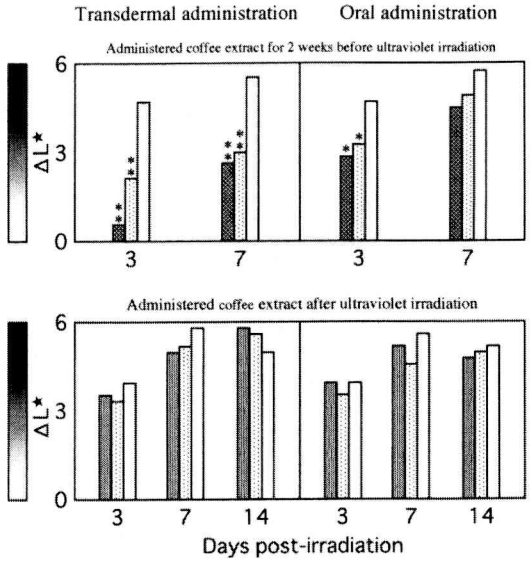

Fig. 3. Results of *in vivo* tests using guinea pigs.
Transdermal administration : ▨; 1.0 mg/cm^2/day, □; 0.5 mg/cm^2/day, □; MeOH
Oral administration : ▨; 40 mg/day, □; 20 mg/day, □; MeOH
**; Significant (p=0.01) *; Significant (p=0.05)

3.09, respectively; conversely, the MeOH group demonstrated a significantly higher value of 5.65 (p=0.01) (Fig. 3). The identical trend was observed in the oral administration group prior to ultraviolet exposure. $\Delta L^{\star}$ values at 40 and 20 mg per day were 2.91 and 3.33, respectively, after 3 days of exposure. On the other hand, the MeOH group displayed a significantly higher value of 4.82 (p=0.05). The identical trend lacking statistical significance continued for 7 days following exposure. However, when coffee extracts were administered after ultraviolet exposure, neither the transdermal nor the oral administration group exhibited significant differences between the coffee administration and the MeOH administration groups (Fig. 3). This result suggests that the melanin biosynthesis inhibitory substance in the MeOH portion of hexane-partitioned MeOH coffee extract exerts a preventive effect against melanin pigmentation triggered by ultraviolet exposure, but no therapeutic effect. In particular, the fact that coffee extract with high concentration

(40 mg/day administration is comparable to 2,000 ml/day consumption of coffee for a 60-kg person) demonstrated a recognizable preventive effect against melanin pigmentation despite oral application should is worthy of attention. In the group receiving transdermal administration following ultraviolet exposure, higher $\Delta L^{\star}$ value was observed in the coffee-administered group in comparison to the control group 14 days after exposure. This phenomenon can be explained by skin pigmentation with brown pigment remaining in the coffee extract. Pigmentation was evident in three of eight animals, appearing from the 5^{th} day following exposure. Therefore, isolation of effective substances and pigments or alternative administration methods requires future examination.

Acknowledgment

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Reference

1) Myouga, H. and Tamaki, M. (2002) "Screenig for biological activity of coffee extracts" *Bulletin of Musashigaoka College*, **10**, 21-27.