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Inhibitory Effects of Phytoncide Solution on Melanin Biosynthesis

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To determine the component-activity relationships of phytoncide solutions on inhibitory activity in melanin biosynthesis, four types of phytoncide solution (A-type, AB-type, D-type, and G-type) were evaluated for inhibition of mushroom tyrosinase activity and melanin synthesis on murine B-16 melanoma cells and a human reconstituted skin model. The A-type, AB-type, D-type, and G-type of phytoncide solution treatment resulted in significant inhibition of tyrosinase activity. The amount of melanin was increased by treatment with phytoncide solutions in a concentration-dependent manner on murine B-16 melanoma cells without affecting cell growth. Furthermore, phytoncide solutions also suppressed melanin synthesis in a concentration-dependent manner on a human reconstituted skin model. These effects of A-type solution were superior to those of other solutions.

Key words: phytoncide solution; tyrosinase activity; melanin synthesis; B-16 melanoma cells; human reconstituted skin model

Hyper-pigmentation is one of the distresses of skin because it reflects aging. It is induced by extrinsic irritation, disorders of homeostasis, and genetic factors. Melanin production is principally responsible for the color of mammalian skin, and it plays an important role in the prevention of sun-induced skin injury. Melanin pigment is a heteropolymer of indole compounds synthesized within melanocytes in the epidermis. Inhibitory compounds as to melanogenesis are useful as skin-whitening agents and are used in medicines, pharmacologicals, and cosmetics and as treatment for hyper-pigmentation. In particular, many Asian have considerable interest in depigmentation. By blocking the pathway of melanin biosynthesis, depigmentating agents can inhibit melanogenesis and treat pigmentation. Melanin is formed through a series of oxidative reactions from tyrosine by tyrosinase. Tyrosinase is a key enzyme catalyzing a step in the biosynthesis pathway of melanin. It has been reported that regulation of tyrosinase activity has an inhibitory effect on pigmentation.[1)] In screening tests of depigmentating agents, most active ingredients have been targeted on inhibition of tyrosinase activity. Therefore, tyrosinase inhibitors are most promising for treating pigmentation and are frequently used as skin-whitening agents in the cosmetics industry. These observations led us to search for naturally occurring tyrosinase inhibitors.

Phytoncides are volatile substances released from trees and plants as protective mechanisms against harmful insects, animals and micro-organisms. Trees and plants synthesize phytoncides as secondary metabolites of photosynthesis. Moreover, it is reported that phytoncides have various effects, such as reducing stress response in stroke-prone spontaneously hypertensive rats,[2)] the GABA receptor expression effect,[3)] human NK cell activity,[4)] and an anti-oxidative effect.[5)] It has been reported that the physiological effects of phytoncides contribute to the improvement of various disorders, including accelerated aging, allergies, multiple sclerosis, and Parkinson disease.[6,7)] The major ingredients of phytoncides are terpenoids, known as flavors of trees and plants. On the other hand, it has been reported that phytoncide solutions contain highly volatile terpenoids, whose major ingredients are monoterpenoids, including α-pinene, careen, and myrcene.[8–10)] It has been reported that terpenoids showed anti-oxidative effects and anti-microbial effects.[5)] Terpenoids exist in essential oils of plants, forming polymeric structures of isoprene, including monoterpenoids and sesquiterpenoids. Terpenoids have been featured since around 1980 as novel nutrients called phytochemicals. The physiological effects of terpenoids have been well studied in a medical content. It is reported that monoterpenoids have disinfection effects,[11)] an analgesic effect,[12)] and anticonvulsant and antioxidant effects,[13)] and it has also been reported that sesquiterpenoids enhance the effects of anti-cancer agents.[14)] Moreover, it was reported that phytoncide solutions provide refreshing effects and antibacterial effects, and inhalation of them is known as forest bathing and aromatherapy. We have reported suppressive effects on *umu* gene expression of SOS response activity induced by chemical mutagens and UV irradiation,[15)] and reductions in cell damage induced by oxidative stress, UVA, UVB, hydroxyperoxide and *t*-butyl-hydroxyperoxide, stimulation of collagen synthesis against UVA irradiation, and inhibition of matrix metalloproteinase-1 activity induced by UVA in human normal dermal fibroblasts and a human reconstituted skin model[16)] of phytoncide solutions. However, no studies have been conducted to investigate the potential of phytoncide solutions in inhibiting tyrosinase activity and melanin synthesis. Because we focused on the anti-

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oxidative effects of phytoncide solutions, we expected that phytoncide solutions suppress the tyrosinase activity by their anti-oxidative activity.

In this study, we examined the inhibitory effects of four types of phytoncide solution: the A-type (from trees such as *Cinnamomum camphora*), the AB-type (from highly bacteriocidal plants such as *Sasa veitchii*), the D-type (from flowering grasses such as *Phyllostachys pubescens*), and the G-type (from non-allergenic plants such as *Chamaecyparis obtuse*) of various plants selected based on their characteristics, on tyrosinase activity and melanogenesis in cultured mouse melanoma cells, and in a human reconstituted skin model. The aim of this study was to clarify the potential of phytoncide solutions for prevention of melanogenesis. Recent advances in medical treatment and equipment have resulted in a worldwide increase in the elderly population. This tendency has given rise to a growing demand for effective skin-care agents and supplements to maintain health and beauty. We found in this study that phytoncide solutions prevented melanogenesis upon direct application to skin due to its excellent anti-oxidative properties.

Materials and Methods

Materials. Four types of phytoncide solution, the A-type, AB-type, D-type, and G-type, were gifts from Phyton-Tao 118 (Osaka, Japan). Four types of phytoncide solution, the A-type (from trees such as *Cinnamomum camphora*), the AB-type (from highly bacteriocidal plants such as *Sasa veitchii*), the D-type (from flowering grasses such as *Phyllostachys pubescens*), and the G-type (from non-allergenic plants such as *Chamaecyparis obtuse*) of various plants, were selected based.[15] Mushroom tyrosinase and synthetic melanin were purchased from Sigma Chemical (St. Louis, MO). L-3,4-Dihydroxyphenylalanine (DOPA) and Advanced Protein Assay Reagent were purchased from Wako Pure Chemical Industries (Osaka, Japan).

Cell culture. Mouse melanoma cells (B-16) were obtained from the Riken BioResource Center (Ibaraki, Japan), and were cultured with Dulbecco's modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) at 37 °C in a humidified atmosphere of 5% CO_2 in air. The human reconstituted skin model, MelanoDerm (MEL300A, a co-culture of normal human melanocytes from Asian donors and normal human keratinocytes), was purchased from Kurabo (Osaka, Japan). The human skin model was cultured following the manufacturer's instructions.

Assay of tyrosinase activity using mushroom tyrosinase. Four types of phytoncide solution (the A-type, AB-type, D-type, and G-type) were prepared at various concentrations as sample solutions. One ml of a 1.5 mM L-3,4-dihydroxyphenylalanine (DOPA) solution, 0.1 ml of dimethyl sulfoxide (DMSO) with and without sample, and 1.8 ml of McIlvain buffer (pH 6.8) were mixed. The mixtures were preincubated at 25 °C for 10 min. Then 0.1 ml of an aqueous solution of mushroom tyrosinase (1,000 U/ml) was added, and the reaction was monitored by measuring the absorbance at 475 nm. A control reaction was conducted with DMSO. The percentage of inhibition of tyrosinase activity was calculated as follows: inhibition (%) = (A − B)/A × 100, where *A* represents the difference in the absorbance of the control sample between incubation times of 0.5 and 1.0 min, and *B* represents the difference in the absorbance of the test sample between incubation times of 0.5 and 1.0 min. The results were the mean of three concurrent readings.

Assay of melanin content in B-16 melanoma cells. Melanin content was measured by a modification of Matsuda *et al.*[17] B-16 melanoma cells (3.0×10^5 cells) were planted with 3.0 ml of medium in a 25-cm^2 flask. After 1 d of culture, 3.0 ml of flesh medium containing four types of phytoncide solution (the A-type, AB-type, D-type, and G-type) at concentrations of 0.05% and 0.1% were added to the flask. After sample solutions were added, B-16 melanoma cells were cultured for 6 d. Then B-16 melanoma cells were treated with 0.25% trypsin, and the cells were harvested as a pellet by centrifugation at 1,000 × *g* for 5 min, and then washed twice with phosphate-buffered saline. After further centrifugation, the supernatant was removed by careful decanting, and the precipitated cells were sonicated in 250 µl of 0.1% TritonX-100/phosphate buffer saline (PBS (−)). The lysate was solubilized by treatment with 500 µl of 10% DMSO in 1 N NaOH aqueous solutions at 80 °C for 2 h. The absorbance of these solutions was measured at 470 nm. Synthetic melanin was used as a standard. The protein content was measured with Advanced Protein Assay Reagent.

Assay of melanin content in a human reconstituted skin model. The human reconstituted skin model was placed in 6-well plates and incubated for 1 h at 37 °C in a humidified atmosphere of 5% CO_2 in air. Then 50 µl of each sample solution containing four types of phytoncide solution (the A-type, AB-type, D-type, and G-type) at concentrations of 0.2% and 0.5% were applied to the surface of the human reconstituted skin model. The human reconstituted skin model was incubated for 3 weeks, and fed with 5 ml of fresh medium every other day. After incubation for 3 weeks, the human skin model was homogenized in 450 µl of 1.0% sodium dodecyl sulfate (SDS) containing 0.05 mM ethylenediaminetetraacetic acid (EDTA) and 10 mM Tris HCl (pH 6.8). To each homogenate, 20 µl of 5 mg/ml, proteinase K was added. After incubation at 37 °C for 18 h, the homogenate was rendered basic by adding 50 µl of 500 mM sodium carbonate, and then 10 µl of 30% hydrogen peroxide was added. The samples were maintained at 80 °C for 30 min. The mixture was extracted with 100 µl of chloroform:methanol (2:1) mixture. After centrifugation at 10,000 × *g* for 10 min, the optical density at 405 nm was determined. Synthetic melanin was used as standard.

MTT Assay of cultured human reconstituted skin models. The viability of a cultured human reconstituted skin model was determined by reduction of MTT (3-(4,5-dimethylthiazole 2-yl)-2,5-diphenyl tetrazolium bromide) to formazan. After incubation for 3 weeks, the human reconstituted skin model was placed in a 24-well plate. Then 300 µl of 1.0 mg/ml MTT solution was added to each well, and the tissue was incubated for 3 h at 37 °C in a humidified atmosphere of 5% CO_2 in air. After incubation, the tissues were washed with PBS (−), and 2.0 ml of isopropanol containing 0.04 M HCl was added to each well. The plate was shaken gently at room temperature for 2 h. The absorbance of extracts was measured at 570–660 nm.

Statistical analysis. The tyrosinase activity, melanin content, protein content, and MTT assay data were expressed as mean ± standard error (S.E.). Statistical significance of differences between various groups was evaluated by one-way analysis of variance (ANOVA test), followed by the Tukey-Kramer multiple comparisons test. A difference between experimental groups was considered statistically significant when the p value was <0.01.

Results

Inhibitory effects of phytoncide solutions on mushroom tyrosinase activity

To determine whether phytoncides have inhibitory effects on tyrosinase activity, we examined the inhibitory effects of four types of phytoncide solution on mushroom tyrosinase activity. The four types of phytoncide solution showed inhibitory effects on tyrosinase activity in a dose-dependent manner (Fig. 1). The A-type, AB-type, D-type, and G-type of phytoncide solution inhibited tyrosinase activity by 83%, 74%, 69%, and 26% at a concentration of 10%, respectively. In addition, the 50% inhibition concentration (IC_{50}) values of A-type, AB-type, and D-type were 1.7%, 2.7%, and 4.8% respectively, while that of the G-type was

estimated to be more than 10%. These results indicate that the A-type, AB-type, and D-type of phytoncide solution suppress tyrosinase activity more strongly than the G-type.

Inhibitory effects of phytoncide solutions on melanin synthesis in B-16 melanoma cells

To investigate the inhibitory effects of phytoncide solutions on melanogenesis, we measured the melanin content of B-16 melanoma cells after treatment with four types of phytoncide solution at concentrations of 0.05% and 0.1%. The treatment with the A-type, AB-type, and D-type of phytoncide solution decreased the melanin content as compared with non-treated cells in a dose-dependent manner (Fig. 2A). The A-type, AB-type, and D-type of phytoncide solution at a concentration of 0.1% reduced the melanin content to 31%, 43%, and 52% of that in non-treated cells respectively. On the other hand, the A-type and AB-type of phytoncide solution equally reduced the melanin content at concentrations of 0.05% and 0.1%. In contrast, the G-type of phytoncide solution did not reduce the melanin content. To identify the inhibition of cell growth by phytoncide solutions in B-16 melanoma cells, we determined the protein content of B-16 melanoma cells (Fig. 2B). Phytoncide solutions did not appear to inhibit the protein content at concentrations below 0.1%. These results indicate that the phytoncide solutions had inhibitory effects on melanin synthesis at non-cytotoxic concentrations below 0.1%.

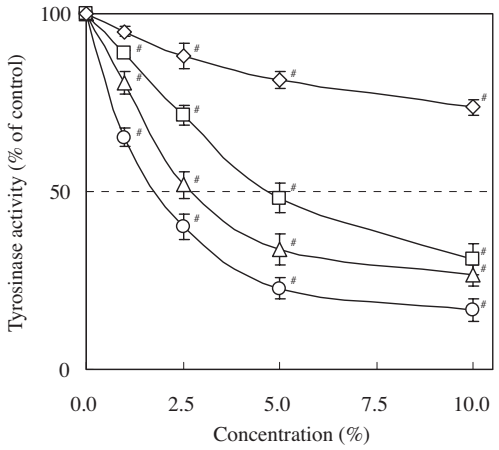

Fig. 1. Inhibitory Effects of the A-Type, AB-Type, D-Type, and G-Type of Phytoncide Solution on Mushroom Tyrosinase Activity.
A-Type (○), AB-type (△), D-type (□), and G-type (◇) phytoncide solutions were mixed with McIlvain buffer (pH 6.8), an L-DOPA solution, and a tyrosinase solution. Tyrosinase activity was determined as described in "Materials and Methods." Each value represents the mean ± SE for three experiments. #Significantly different from the control group at $p < 0.01$.

Inhibitory effects of phytoncide solutions on melanogenesis in human reconstituted skin model

To investigate effects of phytoncide solutions on natural skin pigmentation, we used human reconstituted skin model MEL300A containing normal human melanocytes. Four types of phytoncide solution were applied to MEL300A at the concentrations of 0.2% and 0.5%, and 3.0% Macroscopic darkening of MEL300A was inhibited by the adding of A-Type and AB-Type of phytoncide solutions at a concentration of 0.5% (Fig. 3). Under microscopic observation, the A-Type and AB-Type of phytoncide solutions inhibited more darkened melanocytes of the human reconstituted skin model compared with the other types (Fig. 4). In contrast, macro- and microscopic observations of tissues treated with D-Type and G-Type of phytoncide solutions did not appear to show fewer darkened melanocytes.

To evaluate quantitatively the inhibitory effects of phytoncide solutions on cellular melanin content in human reconstituted skin model, the amount of melanin in MEL300A was measured (Fig. 5A). Four types of phytoncide solution showed a significant suppression of melanin synthesis on MEL300A, in a dose-dependent manner. After 3 weeks culture, 0.5% A-type, AB-type, and D-type of phytoncide solution significantly reduced

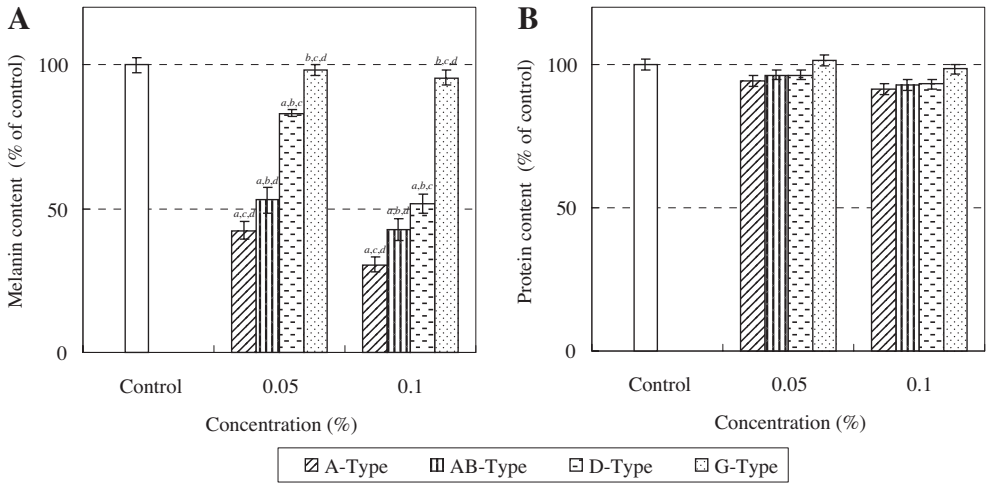

Fig. 2. Inhibitory Effects of the A-Type, AB-Type, D-Type, and G-Type of Phytoncide Solution on Melanin Synthesis in B-16 Melanoma Cells.
B-16 melanoma cells (3.0×10^5 cells) were incubated with 3.0 ml of medium containing various concentrations of the A-type, AB-type, D-type, and G-type phytoncide solutions in 25-cm^2 flasks for 6 d. Quantification of melanin synthesis (A) and measurement of protein content (B) were performed as described in "Materials and Methods." Data are expressed as percentages of control. Bars represent mean ± SE for three independent experiments. [a]Significantly different from control and the G-type group at $p < 0.01$. [b]Significantly different from the A-type group at $p < 0.01$. [c]Significantly different from the AB-type group at $p < 0.01$. [d]Significantly different from the D-type group at $p < 0.01$.

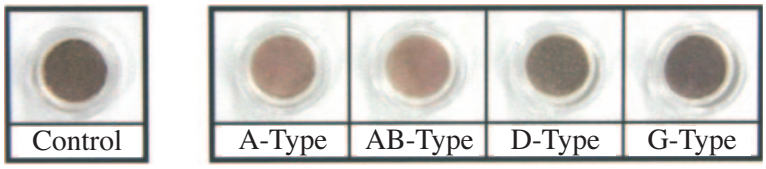

Fig. 3. Macroscopic View of a Human Reconstructed Skin Model Treated with the A-Type, AB-Type, D-Type, and G-Type of Phytoncide Solution at a Concentration of 0.5%.

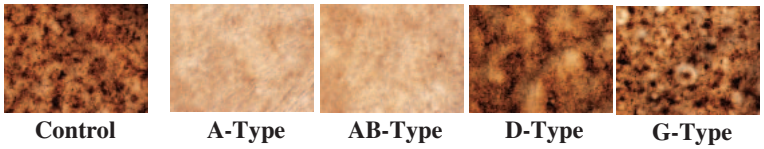

Fig. 4. Microscopic View of a Human Reconstructed Skin Model Treated with the A-Type, AB-Type, D-Type, and G-Type of Phytoncide Solution at a Concentration of 0.5% (100×).

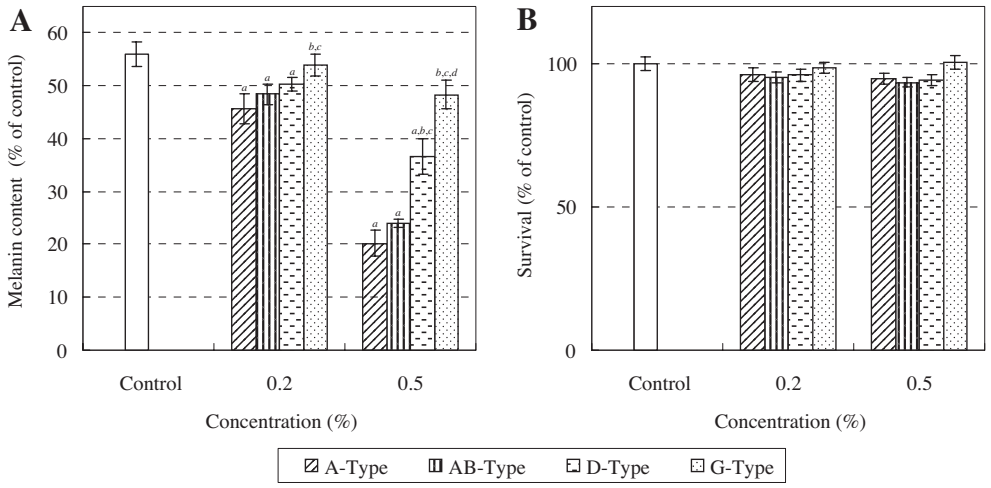

Fig. 5. Inhibitory Effects of the A-Type, AB-Type, D-Type, and G-Type of Phytoncide Solution on Melanin Synthesis and Cytotoxicity in a Human Reconstructed Skin Model.

The human skin model was incubated for 3 weeks. Quantification of melanin synthesis (A) and viability (B) of the human skin model, which was evaluated by MTT assay, as described in "Materials and Methods." Bars represent mean ± SE for three independent experiments. [a]Significantly different from control and the G-type group at $p < 0.01$. [b]Significantly different from the A-type group at $p < 0.01$. [c]Significantly different from the AB-type group at $p < 0.01$. [d]Significantly different from the D-type group at $p < 0.01$.

the melanin content, to 36%, 43%, and 65% of that in the non-treated cells, respectively. These inhibitory effects of A-type and AB-type phytoncide solutions were more effective on melanin synthesis than the D-type. In contrast, the G-type of phytoncide solution slightly inhibited melanin synthesis at a concentration of 0.5%. Further, we evaluated cell viability in a human skin model, that had been topically treated with different concentrations of phytoncide solution, by MTT assay. No significant decrease in cell viability was observed when the tissue was treated with up to 0.5% of each sample (Fig. 5B). These results suggest that topical treatment of tissue with four types of phytoncide solution decreased melanin synthesis without affecting cell viability. In addition, phytoncide solutions did not affect cell viability on MTT assay when the tissue was treated with 0.2% and 0.5% each type of phytoncide solution.

Discussion

In the present study, the inhibitory effects of mushroom tyrosinase activity and melanin synthesis were examined in B-16 melanoma cells and a human reconstructed skin model culture system to address the whitening effects of four types of phytoncide solution on biological systems. Treatment of B-16 melanoma cells with four types of phytoncide solution led to significant inhibition of melanin biosynthesis (Fig. 2). Furthermore, the phytoncide solutions inhibited mushroom tyrosinase activity (Fig. 1). Therefore, the inhibitory effects of phytoncide solutions on melanin biosynthesis may have been due to direct inhibition of tyrosinase activity.

We also evaluated the effects of phytoncide solutions on a human reconstructed skin model, epidermal equivalents containing melanocytes. Its inhibitory effect on melanin synthesis was confirmed by quantitative measurement of melanin as well as macro- and microscopic observations of tissue pigmentation. The phytoncide solutions inhibited melanin synthesis in a human reconstructed skin model (Figs. 3–5). Several researchers have indicated that human epidermal equivalents containing melanocytes and keratinocytes provide a convenient and reliable alternative to animal testing in evaluating the regulation of mammalian pigmentation.[18,19] Our results indicate that phytoncide solutions

also affect melanogenesis in this human reconstructed skin model. Furthermore, this effect was observed without any detectable cytotoxicity to the human reconstructed skin model.

As for the component-activity relationships, the A-type, AB-type, D-type, and G-type of phytoncide solution had distinctly different inhibitory effects on melanogenesis, according to their component patterns. The A-type and AB-type of phytoncide solution were superior to other types in terms of all the inhibitory effects of melanin biosynthesis on B-16 melanoma cells and on a human reconstructed skin model (Figs. 2–5). In contrast to the other types, the A-type and AB-type solutions contain greater concentrations of phenolic compounds, cedrol, isoeugenol, α-amyl cinnamaldehyde, β-acorenol, guaical, and acids, propanic acid, cinnamic acid, and hexadecanoic acid.[15] It has been reported that various compounds containing the hydroxy group of phenolic compounds have anti-oxidative effect.[20,21] An anti-oxidative effect is possible that inhibits the oxidative reaction catalyzed by tyrosinase in the pathway of melanin biosynthesis. These components and their combinations might be one of the important factors responsible for the enhanced inhibition of tyrosinase activity and melanogenesis in B-16 melanoma cells and a human reconstructed skin model. In addition, the A-type, AB-type, and D-type of phytoncide solution contain a greater concentration of terpenoids than the G-type.[15] There are several reports on the antioxidant effects of terpenoids.[13,22] These terpenoids and their combinations might also be important factors responsible for the enhanced inhibition of tyrosinase activity and melanin biosynthesis in B-16 melanoma cells and a human reconstructed skin model.

In summary, this study suggests that four types (the A-type, AB-type, D-type, and G-type) of phytoncide solution showed inhibitory effects on tyrosinase activity and melanin biosynthesis. Melanin and tyrosinase play roles in the process of melanogenesis, which is a common phenomenon in damaged skin. These findings indicate that these phytoncide solutions can be used as skin-whitening agents as effective inhibitors of tyrosinase activity and melanin biosynthesis. In conclusion, we expect that these phytoncide solutions can be utilized not only in the field of cosmetics, but also in other fields. However, phytoncide solutions may not exhibit their expected effects *in vivo* if adversely affected by factors such as transdermal absorption, distribution, and metabolism once inside the human body. Further studies with mammalian cells *in vitro* and *in vivo* are needed to determine the efficacy of phytoncide solutions in the prevention of human melanogenesis.

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