

Comparison of *in vitro* and *in vivo* phototoxicity tests with S-(-)-10,11-dihydroxyfarnesic acid methyl ester produced by *Beauveria bassiana* KACC46831

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Abstract. *Beauveria bassiana* is a fungi that is well-known for demonstrating a resistance to environmental change. To confirm whether S-(-)-10,11-dihydroxyfarnesic acid methyl ester (DHFAME) produced by *Beauveria bassiana* KACC46831 causes phototoxicity when used for cosmetic purposes due to its anti-tyrosinase activity, we conducted *in vitro* and *in vivo* phototoxicity tests. There were no significant changes or damage observed in the compound-treated group with regards to skin phototoxicity, while 8-methoxypsoralen, which served as a positive control, induced toxic effects. The *in vitro* 3T3 neutral red uptake assay, an alternative assessment, was used for further confirmation of the phototoxicity. The results showed that DHFAME did not exhibit phototoxicity at the designated concentrations, with or without UV irradiation in the 3T3 cells. These results indicated that the methyl ester produced by *Beauveria bassiana* KACC46831 does not induce phototoxicity in the skin. Therefore, the results of the present study indicate that DHFAME shows potential for use as a cosmetic ingredient that does not cause skin phototoxicity.

Introduction

Beauveria bassiana is well-known for its broad spectrum for hosts and has relative resistance to environmental

change (1). For centuries, adult *Bombyx mori* infected with *Beauveria bassiana* have been used as an oriental medicine for the treatment of stroke, hives and diabetes (2). To the best of our knowledge, *Beauveria bassiana* has a limited virulence in humans. Notably, only a few cases of invasive disease and keratitis have been documented, despite the widespread use of the organism (3). Entomopathogenic fungi, including *Beauveria bassiana*, *Cordyceps sinensis*, *Cordyceps militaris*, and *Paecilomyces tenuipes*, from a variety of resources have been employed for the treatment of atopic dermatitis, athlete's foot and dandruff (4). The use of this agent as a biological control has received increasing attention as *Beauveria bassiana* is used to exterminate a wide variety of pests (1,5,6). The anti-bacterial activity of entomopathogenic fungi against food-borne bacterial growth has also been investigated (7).

Although these entomopathogenic fungi have been shown to possess valuable properties, including immune-modulation, anti-diabetic, anti-stress and antitumor activities (8), their application in the cosmetics industry has not been thoroughly studied. However, investigation into the whitening effects of fungal fermentation products has been performed (9), and the results of those studies indicated that phototoxicity tests are important for obtaining approval and authorization for the use of test compounds as functional cosmetic ingredients. Since there are numerous methods used to measure the toxicity of substances applied to the skin and skin-related tissues, various trials have been conducted to assess the biological effects of the cosmetic/cosmeceutical ingredients that are being approved (10). However, *in vitro* methods, such as the 3T3 neutral red uptake (NRU) phototoxicity test (11) and local lymph node assay (12), are increasingly being used instead of animal models due to the ethical aspects involved. Emerging applications of insect extracts (or fractions) are employed to broaden the applicability of their biochemicals as cosmetics/cosmeceuticals. However, whether the agents produced by entomopathogenic fungi have adverse effects on exposed skin and eyes has yet to be determined. However, surplus reactions to cosmetics are frequent in patients with

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allergic contact dermatitis. A number of adverse outcomes, such as irritation, sensitization and acute/chronic toxicity, can be evaluated using *in vitro*, *in vivo*, semi-*in vivo*, and *ex vivo* animal models (13–15). The individual components or constituents should not exert toxic effects on the skin and should only be passed and approved in cases in which no eye lens damage/change is observed in animals or clinical trials for the development of cosmetics (16).

In the present study, the phototoxicity of S-(–)-10,11-dihydroxyfarnesic acid methyl ester (DHFAME) was evaluated using an *in vitro* phototoxicity test and an *in vivo* animal model to determine whether the compound is safe for development in cosmetic applications.

Materials and methods

Chemicals. 8-Methoxypsoralen (8-MOP; M3501), polyethylene glycol (P3265), chlorpromazine (CPZ; C0982), and neutral red (N4638) were purchased from Sigma-Aldrich Chemical Co., Ltd. (St. Louis, MO, USA). All media and compositions were commercially available.

Animal care and use. Seven-week-old Hartley guinea pigs, weighing 319.6–372.9 g, were purchased from Samtako Bio Korea (Osan, Korea) and used for the skin irritancy and phototoxicity tests, respectively. The animals were fed a commercial diet (Purina Korea, Inc., Seoul, Korea) and provided with water *ad libitum* throughout all the experiments. The study protocols complied with the guidelines of the International Association for the Study of Pain Committee for Research and Ethical Issues (17), and strictly adhered to the internal guidelines of the Kyungpook National University Animal Ethics Committee. All animals were acclimatized to the laboratory environment for ~1 week prior to commencement of the experiments. Five animals were allocated to each group.

Isolation and preparation of agent. DHFAME was produced by *Beauveria bassiana* KACC46831. Briefly, the fermentation medium consisted of 3% sucrose, 2% corn steep liquor, 0.05% potassium phosphate dibasic, 0.1% potassium phosphate monobasic and 0.05% $\text{MgSO}_4 \cdot 6\text{H}_2\text{O}$. The medium was prepared in a 5l-mini jar fermentor (Hankook Fermentor, Seoul, Korea) and sterilized at 121°C for 30 min, subsequently it was chilled for inoculation of 5% culture. The fermentation was then carried out for 3 days, and subsequently the fermentation broth was centrifuged at 10,000 \times g for 10 min and the supernatant was added as previously described (18). The precipitate was then applied to an HP column chromatogram and high-performance liquid chromatography was performed with a reverse column (Waters, Milford, MA, USA) and a peak was obtained at a retention time of 7.662 min by a detector at 254 nm (2998 PDA; Waters). The peak was identified as S-(–)-10,11-dihydroxyfarnesic acid methyl ester by nuclear magnetic resonance and mass spectroscopy (18). A voucher specimen of the methyl ester produced by *Beauveria bassiana* KACC46831 has been deposited in the Laboratory of Food Enzyme Biotechnology, Kyungpook National University (Daegu, Korea).

***In vitro* 3T3 NRU test.** The *in vitro* 3T3 NRU phototoxicity test was carried out as described previously (11) and by the

OECD guideline 432 (19). Briefly, 96-well plates (REF353072; BD Falcon, Franklin Lakes, NJ, USA) were seeded with 1.0×10^4 cells/ml (total 100 μl) 3T3 cells, and subsequently incubated at 37°C in a humidified 5% CO_2 incubator for 24 h. Following the removal of the media and the washing of cells with Earle's balanced salt solution (EBSS), the cells were exposed to various dilutions (three replicate wells per concentration) of the test materials (100 μl) in EBSS for 60 min. The cells were treated with an initial range of nine concentrations ranging from 0 to 100 μM CPZ (as a positive control) or 0 to 250 μM DHFAME. Following incubation for 24 h in a CO_2 incubator at 37°C, duplicate plates were either exposed to UVA/visible light at 5 J/cm^2 (LF-206.LS; UVitec Strasbourg, France) or kept in the dark for 50 min. Following irradiation, the media were discarded from all the plates and the cells were washed with culture medium. The cells were then reincubated in culture medium overnight. On day 3, the medium was removed and the cells were washed with pre-warmed buffer and added to 100 μl of neutral red medium (50 $\mu\text{g}/\text{ml}$, serum-free). Samples were then incubated for 3 h in a CO_2 incubator at 37°C, and subsequently 150 μl of neutral red extraction solution (distilled water:ethyl alcohol:acetone = 49:50:1) was added to the plates. The plates were then agitated and the optical density was measured at 540 nm using a spectrophotometer (Perkin Elmer Wallac, Inc., Turku, Finland).

***In vivo* phototoxicity test.** An *in vivo* phototoxicity test was conducted using Hartley guinea pigs. The animals were divided into an untreated, three experimental (10, 30 and 100 mg/ml of DHFAME) and a positive control group that was treated with 8-MOP. Each group contained five guinea pigs (7-week-old males, weighing 319.6–372.9 g). The untreated group was exposed to polyethylene glycol. For the three experimental groups, 0.5 ml/site of the solution was applied. The treated skin was then irradiated with UV light at a distance of 10 cm for 10 min using UV irradiation apparatus (UVITEC LF-206.LS) with a UV lamp (365 nm). The left site was designated as the light irradiation site, whereas the right site was not irradiated. After 2, 4 and 24 h of irradiation, any skin erythema, eschar and swelling was scored relative to the control. Transdermal administration was carried out by removing the fur in a 4 \times 6 cm^2 area with an electric hair cutter and then applying the test sample to two regions (each 2 \times 2 cm^2). The test groups were treated with 0.5 ml of DHFAME at concentrations of 10, 30 and 100 mg/ml, whereas 0.5 ml of a 0.1% 8-MOP solution was applied to each side of the test site as a positive control (20). The non-irradiation site was shielded by aluminum tape.

Statistical analysis. Data are presented as the means \pm standard deviation. Statistical analysis was carried out by Probit analysis using the SPSS 9.0 program (SPSS, Inc., Chicago, IL, USA). $P < 0.05$ was considered to indicate a statistically significant different following analysis using Pearson's goodness-of-fit test.

Results and Discussion

Throughout the evaluation of active components that exhibit whitening activities for application as a cosmetic from natural

Table I. Comparison of the phototoxicity test evaluating the effects of S-(-)-10,11-dihydroxyfarnesic acid methyl ester (DHFAME) produced by *Beauveria bassiana* KACC46831.

| Criteria | Total scores | Distilled water | DHFAME, mg/ml | | | 0.1% 8-MOP |
|----------------------|--------------|-----------------|---------------|-----|-----|------------|
| | | | 10 | 30 | 100 | |
| Non-irritating | 0.0-0.5 | Yes | Yes | Yes | Yes | |
| Minimally irritating | 0.6-1.2 | | | | | |
| Obviously irritating | 1.3-2.5 | | | | | Yes |
| Extremely irritating | 2.6-5.0 | | | | | |

8-MOP, 8-methoxypsoralen.

resources, *Beauveria bassiana* KACC46831 was found to produce a potent compound during liquid culture. The compound was identified as DHFAME and found to exert anti-tyrosinase activity *in vitro* and *in vivo* [(12) and data not shown].

In a previous study, we examined whether the agent had the ability to ameliorate skin inflammation, including atopic dermatitis (18). Initially, insect biomaterials were obtained and processed into biomaterials using a variety of methods. Subsequently, microbial fermentation, biotransformation, supercritical extraction or chemical modification techniques were employed to convert the raw extracts into a cosmetic, cosmeceutical, neutraceutical or hit/lead drug. Therefore, the development of anti-tyrosinase agents from medicinal insect extracts was tested, which revealed that the methyl ester had potent whitening activity (18). To determine the toxicity of the agent, an acute toxicity test was conducted for the application of cosmetic ingredients.

3T3 NRU phototoxicity was first tested *in vitro* according to the OECD 432 guideline. For the assay, CPZ was selected as a positive control, as the OECD guideline suggests that this drug exhibits phototoxicity by UV irradiation in 3T3 cells. As shown in Fig. 1A, 3T3 cells showed characteristic features of growth in the presence of various concentrations of CPZ without UV in a concentration-dependent manner. In particular, 50 and 100 μ M CPZ exhibited 88 and 21.5% cell viability, respectively, when compared to the control (Fig. 1; dotted and straight lines). When the cells were treated with UV and 10 μ M CPZ, the growth was decreased significantly by <37.4% (Fig. 1A). Cell viability was 0% in response to treatment with 15 μ M CPZ with UV (Fig. 1B; comparison of upper and lower panels). The probable toxicity rate of CPZ was 1.000, whereas the rates of PIF and MPE were 12.016 and 0.781, respectively. This finding suggested that CPZ treatment results in phototoxicity to UV irradiation. Under these conditions, various concentrations of DHFAME were compared to the positive control. As shown in Fig. 1C, a higher concentration of DHFAME did not cause a notable decrease in cell viability with or without UV (dotted and straight lines, respectively) at <250 μ M. Moreover, the cell morphology did not change unexpectedly at the designated concentration (Fig. 1D). The phototoxicity irritancy factor (PIF) and mean photo effect (MPE) of CPZ was 12.016 and 0.781, respectively, indicating that the probable phototoxicity rate was 1.000 and

that DHFAME did not induce phototoxicity in this *in vitro* 3T3 NRU phototoxicity test (data not shown). Conversely, the PIF of DHFAME was <1.000 and the MPE was 0.060, indicating that the probable phototoxicity rate was 0.003 (data not shown).

To determine whether DHFAME exhibited phototoxicity *in vivo*, DHFAME produced by *Beauveria bassiana* KACC46831 was soaked on the skin of guinea pigs and the toxicity was determined compared to guinea pigs treated with 8-MOP. The lesions were examined at 2, 4 and 24 h after application of DHFAME to evaluate phototoxicity. In particular, erythema and eschar were determined by observation with the naked eye using the following scale: 0, no erythema; 1, extremely slight; 2, well-defined; 3, moderate to severe; and 4, severe erythema to slight eschar formation.

Phototoxicity was subsequently evaluated by analyzing the skin exposed to UV irradiation. Following fur removal, guinea pig skin was treated with DHFAME and 8-MOP, and the degree of erythema was determined using the aforementioned scale. For up to 4 h after UV irradiation, similar erythema symptoms were observed. After 24 h, the DHFAME-treated groups showed no symptoms of toxicity in the skin, whereas the 8-MOP group (0.1% as a positive control) showed moderate to severe erythema (Fig. 2). To measure edema, the following scale was used: 0, no edema; 1, extremely slight; 2, well-defined; 3, moderate to severe; and 4, severe edema. The results showed that DHFAME did not cause erythema or eschar, whereas 8-MOP resulted in slight edema (Fig. 2). A final score was then determined by assessing the total scores for erythema, edema and crust as follows: 0.0-0.5, almost no phototoxic resistance; 0.6-1.2, weakly phototoxic; 1.3-2.5, clearly and highly phototoxic; and 2.6-5.0, highly and severely phototoxic. As shown in Table I, the three samples (10, 30 and 100 mg/ml) were associated with scores of only 0.0-0.5, suggesting that the agent tested in the experiment was non-irritating. However, treatment with 8-MOP was a clearly irritating compound that resulted in erythema, eschar, and edema (Fig. 2). After 2 to 4 h of UV irradiation, a slight redness was observed in all agent-treated groups, but this redness disappeared after 24 h. Conversely, the groups treated with 8-MOP developed erythema and edema, indicating that the overall condition of the phototoxicity test was achieved. Taken together, these findings indicate that 8-MOP treatment induced erythema, edema, and/or eschar in a concentration-dependent manner, whereas DHFAME had no effect.

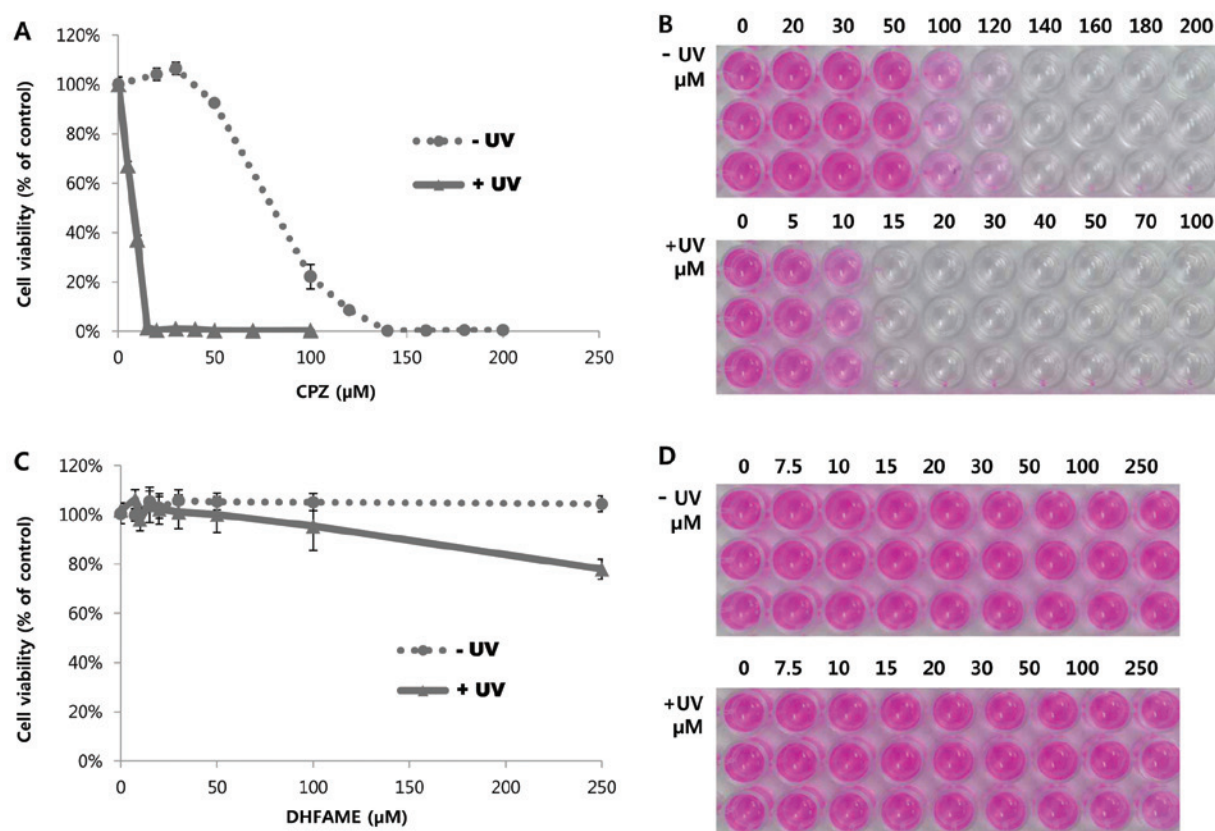


Figure 1. *In vitro* 3T3 neutral red uptake (NRU) phototoxicity tests of S-(-)-10,11-dihydroxyfarnesic acid methyl ester (DHFAME) produced by *Beauveria bassiana* KACC46831. (A and C) The *in vitro* 3T3 NRU phototoxicity test was carried out and characteristic features of growth in the presence of various concentrations of CPZ without UV were identified in a concentration-dependent manner (dotted and straight lines). (B) The cells were killed by UV irradiation with CPZ, (D) whereas the cells remained alive following UV irradiation with DHFAME.

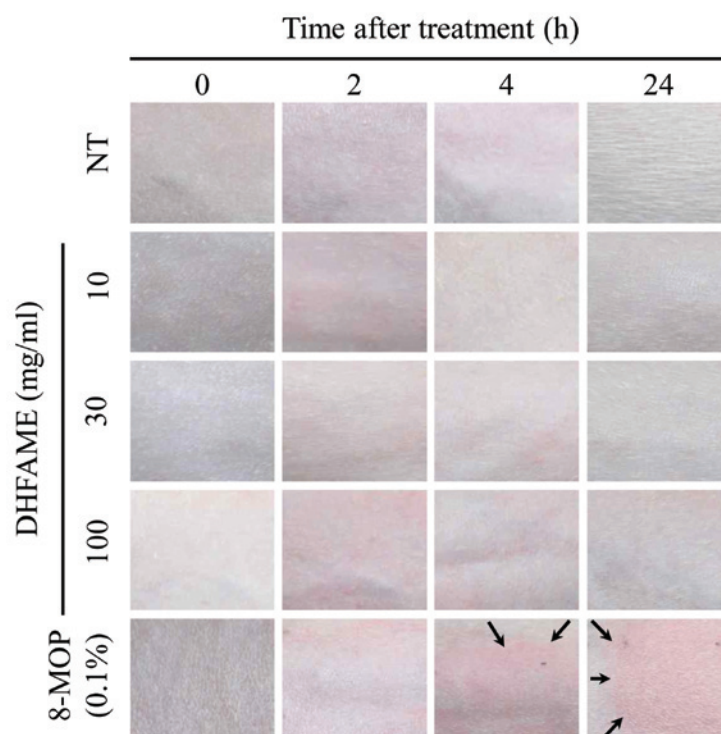


Figure 2. *In vivo* phototoxicity tests of S-(-)-10,11-dihydroxyfarnesic acid methyl ester (DHFAME) produced by *Beauveria bassiana* KACC46831. The images show the shaved backs of guinea pigs. The positive control group treated with 8-MOP showed a toxic response at 2 h after UV irradiation, and gradually degenerated. The arrows indicate swelling and erythema with phototoxicity and the dotted boxes show that 8-methoxypsoralen (8-MOP) was applied to the area. Data are a classical set of five independent experiments.

In summary, the present study investigated whether DHFAME has the potential to cause skin phototoxicity. None of the investigated concentrations of DHFAME were found to irritate the skin or were phototoxic, indicating that DHFAME may be useful in the cosmetic or cosmeceutical industry and for other applications. Although DHFAME was derived from an entomopathogenic fungus, its potential mode of action and toxicity require further evaluation.

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