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ROS Scavenging and Anti-Wrinkle Effects of Clitocybin A Isolated from the Mycelium of the Mushroom *Clitocybe aurantiaca*

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Clitocybin A, an isoindolinone from *Clitocybe aurantiaca*, was investigated to assess its anti-wrinkle properties, through reactive oxygen species (ROS)-scavenging and elastase inhibitory activities, procollagen synthesis, and matrix metalloproteinase-1 (MMP-1) expression, in human primary dermal fibroblast-neonatal (HDF-N) cells. Clitocybin A exhibited no significant cytotoxicity up to 10 ppm in HDF-N cells, with cell viability and cell proliferation activity greater than 94.6% and 91.9%, respectively. Strong and concentration-dependent ROS radical scavenging activities of clitocybin A were observed following irradiation with UVB at 30 mJ/cm^2. Furthermore, clitocybin A treatment of cells at 0.1, 1, and 10 ppm exhibited decreased elastase activity, in a concentration-dependent manner, by 1.97%, 6.6%, and 8.31%, respectively, versus the control group. The effects of clitocybin A on procollagen synthesis and MMP-1 expression were investigated. Clitocybin A treatment of cells at 1, 5, and 10 ppm increased procollagen synthesis, by 67.9%, 74.4%, and 112.9%, respectively, versus the control group. At these concentrations, MMP-1 expression decreased significantly following UV irradiation. Together, these findings suggest that clitocybin A may be an effective ingredient for use in anti-wrinkle cosmetic products.

Keywords: Clitocybin A, anti-wrinkle, reactive oxygen species, elastase, procollagen synthesis, matrix metalloproteinase

Introduction

Skin aging is a complex biological process, with multiple underlying influences, including genetically programmed intrinsic (cellular metabolism, hormone and metabolic processes) and extrinsic (chronic light exposure, pollution, ionizing radiation, chemicals, toxins) factors [1]. Wrinkle formation is a representative feature of skin aging and is characterized by reduced skin elasticity and degeneration of the extracellular matrix, such as collagen, which is produced by fibroblasts in the dermis [2]. In particular, important factors in the causation of wrinkles include loss of cytokines [3, 4] and collagen [5–7] and activation of matrix metalloproteases (MMPs) and human neutrophil elastase [8–12]. Oxidative stress, initiated by reactive oxygen species (ROS) generation, is also an important cause of wrinkles [13–15]. Among these factors, matrix degradation is the most important. UV irradiation generates ROS that can induce the expression of MMPs that then degrade the collagen matrix system in the dermis [16]. Thus, reasonable targets for protecting against wrinkle formation include increasing collagen synthesis and regulating the MMP expression induced by ROS and collagen synthesis.

Clitocybin A is an isoindolinone compound isolated from a mycelium extract of the wild Korean mushroom, *Clitocybe aurantiaca*. Fig. 1 presents the chemical structure of clitocybin A. This compound was reported to eliminate superoxide

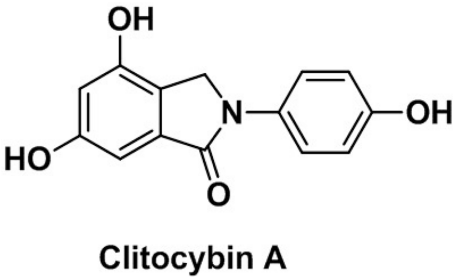

Fig. 1. Chemical structure of clitocybin A.

and ABTS radicals, at IC_{50} values of 10.3 and 6.4 μM, respectively, and effectively inhibits DNA degradation induced by H_2O_2 [17]. Moon *et al*. [18] observed that clitocybin A strongly inhibited cell aging and apoptotic cell death and investigated the underlying molecular mechanism(s). Kim *et al*. [19] reported the potential of clitocybin A as an ingredient for anti-wrinkle cosmetics and established conditions of a fermentation process for its mass production.

This study was conducted to examine the potential of clitocybin A, isolated from *C. aurantiaca*, as an anti-wrinkle cosmetic ingredient. The anti-wrinkle effects of clitocybin A were evaluated with regard to ROS scavenging and elastase inhibitory activities, procollagen synthesis, and MMP-1 expression in human primary dermal fibroblast-neonatal (HDF-N) cells.

Materials and Methods

Isolation of Clitocybin A

The culture broth of *C. aurantiaca* was sequentially extracted with *n*-hexane and Ethyl acetate. The Ethyl acetate layer was subjected to a series of chromatographic steps, resulting in the isolation of clitocybin A. The detailed procedure has been described in our previous work [17].

Cells and Cell Culture

Normal HDF-N cells, originating from human neonatal foreskin, were used for the wrinkle repair efficacy test. After inoculation of cell cultures, the cells were incubated using fibroblast basal medium (FBM; Lonza CC-3131, USA) containing 0.1% insulin, 0.1% rhFGF, 0.1% gentamicin, and 2% FBS, at 37°C in a 5% CO_2 incubator.

Cell Viability Assay

HDF-N cells were plated in 96-well plates at 5×10^4 cells/well and incubated for 24 h. Then, the cells were starved for 12 h and incubated for 24 h with clitocybin A in fresh medium (medium supplements excluded). After 24 h of incubation, cell viability was measured by diluting cell proliferation reagent WST-1 (Roche, CC-3131, Switzerland) reacting agent to 1/10 of the initial concentration in medium without supplements. Next, each well was treated with 100 μl of the solution, reacted for 1 h, and the absorbance was measured at 450 nm.

Cell Proliferation Assay

HDF-N cells were plated in 96-well plates at 5×10^4 cells/well and incubated for 24 h. Next, the cells were starved for 12 h and incubated after adding clitocybin A and fresh medium (medium supplements excluded). At 48 h of incubation, WST-1 reacting solution was diluted to 1/10 of the concentration in medium without supplements. Each cell was treated with 100 μl of the solution and reacted for 1 h, and the absorbance was measured at 450 nm.

Reactive Oxygen Species-Scavenging Activity

HDF-N cells were plated in 96-well plates at 5×10^4 cells/well and incubated for 24 h, followed by clitocybin A treatment, and 3 h further incubation at 37°C and 5% CO_2. After incubation, the medium was removed and 50 μM of 2′7′-dichloro dihydrofluorescin diacetate (DCF-DA; Sigma, USA), diluted with HBSS, was added prior to 1 h of incubation. After washing twice with HBSS, cells were irradiated (or not) with UVB at 30 mJ/cm^2 and then incubated for a further 2 h. Fluorescence intensities were measured at excitation and emission wavelengths of 483 and 535 nm, respectively, using a fluorescence microplate reader.

Elastase Inhibitory Activity

Tris-HCl (0.2 M, pH 8.0) with 0.1% Triton X-100 (Sigma, T8787, USA) was added to the incubated HDF-N cells. The cells were subject to ultrasonic disruption, and the supernatant was obtained by centrifugation (1,000 ×*g*, 20 min). The supernatant was quantified, and the enzyme activity was measured along with total protein quantity. To determine the elastase inhibition activity, 200 μg/ml of homogenized fibroblast elastase, 0.2 M Tris-HCl buffer, and samples were added in varying concentrations. After the addition of 50 mM *N*-succinyl-Ala-Ala-Ala-*p*-nitroanilide (Sigma, S4760, USA), a specific substrate of elastase, it was incubated at 37°C, and the absorbance was measured at 450 nm. The elastase inhibitory activity was compared with that of a positive control, phosphoramidon (Enzo, BML-Pl111, USA).

Procollagen Synthesis

To measure procollagen synthesis, HDF-N cells were plated in 48-well plates at 2×10^4 cells/well and incubated for 24 h. The medium was removed and cells were starved for 24 h. The cells were treated with clitocybin A that had been diluted in FBM culture medium (medium supplements excluded) at varying concentrations and incubated for 24 h. Next, the medium was collected and the procollagen quantity was measured using a procollagen type I c-peptide EIA kit (Takara, MK101, Japan). Cells attached to the bottom were washed with PBS and lysed with 1 N

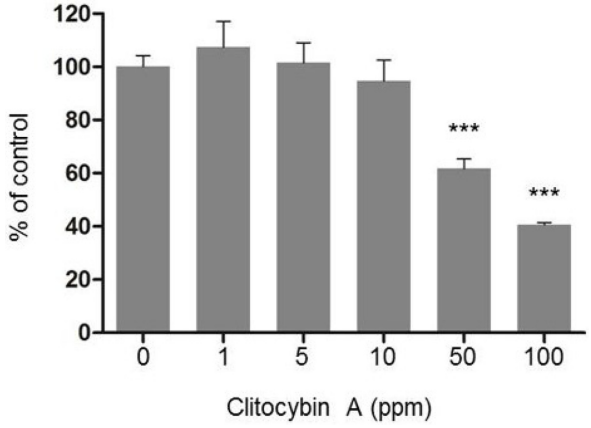

Fig. 2. Cytotoxicity of clitocybin A against HDF-N cells.
Cell viability was determined using the WST-1 assay. HDF-N cells were cultured in serum-free medium for 12 h and treated with clitocybin A at 0, 1, 5, 10, 50, and 100 ppm for 24 h. Cell viability was assessed after exposure to the different concentrations of clitocybin A. ***$p < 0.005$, vs. the control group.

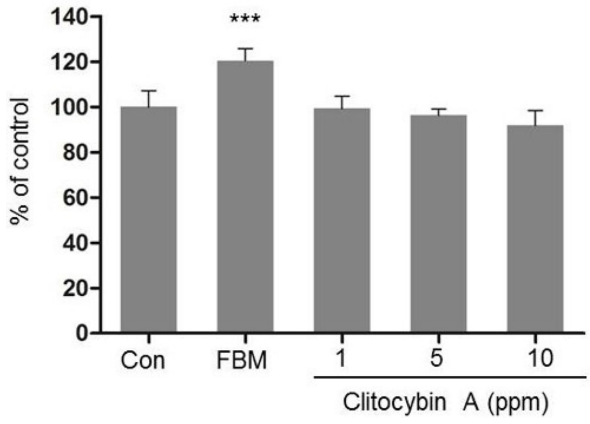

Fig. 3. Effects of clitocybin A on HDF-N cell proliferation.
Cell proliferation was assessed using the WST-1 assay. HDF-N cells were cultured in serum-free medium for 12 h and treated with clitocybin A at 1, 5, and 10 ppm for 48 h. Cell proliferation was determined after exposure to the different concentrations of clitocybin A. ***$p < 0.005$, vs. the control group.

NaOH, and the total protein was quantified to determine procollagen synthesis.

Inhibition of MMP-1 Expression

HDF-N cells were plated in 24-well plates at 2×10^4 cells/well. After incubation, the medium was removed and the cells were washed with DPBS. After the addition of 200 ml DPBS, the cells were irradiated with UVA at 5 J/cm^2 plus UVB at 40 mJ/cm^2. The cells were then incubated for 24 h. The culture solution was used to measure MMP-1 using a human total MMP-1 ELISA kit (R&D Systems, DY901, USA). The measured MMP-1 amount was corrected by the total protein quantity. All experiments were repeated three times and average values are reported. The inhibition of MMP-1 expression was calculated by the equation below.

Inhibition of MMP-1 expression (%) = 100 [(control absorbance – sample absorbance)/(control absorbance)]

Total protein was quantified by the Lowry method using a Bio-Rad (Korea) DC protein kit.

Statistical Analysis

Experiments were carried out with four replications, and all experimental data are presented as the mean ± SD. For statistical analyses, Student's *t*-test was used. *p*-Values were determined to assess significance.

Results

Cell Viability

The in vitro cytotoxicity of clitocybin A was tested in HDF-N cells and was expressed as percentages against the control that was treated with culture medium without clitocybin A (Fig. 2). When clitocybin A was used at 1, 5, 10, 50, and 100 ppm, the cell viability was 107.5 ± 9.6%, 101.5 ± 7.5%, 94.6 ± 7.9%, 61.8 ± 3.6%, and 40.6 ± 0.7%, respectively, compared with vehicle-treated cells. These findings demonstrate the safety of clitocybin A, which did not exhibit cytotoxicity up to 10 ppm.

Cell Proliferation Activity of Clitocybin A in HDF-N Cell

In determining cell proliferation activity, clitocybin A concentrations were selected to avoid cytotoxicity, as determined above. HDF-N cells were treated with clitocybin A at 1, 5, and 10 ppm and the cell growth rate was examined after 48 h (Fig. 3). Cell proliferation activity determined at 1, 5, and 10 ppm was 99.4 ± 5.4%, 96.3 ± 2.9%, and 91.9 ± 6.4%, respectively, and did not differ significantly from the control. However, the growth rate of FBM full medium-treated cells, used as the positive control, increased to 120.5 ± 5.3%, which was a significant difference. Based on these findings, clitocybin A was considered to have little effect on cell proliferation.

ROS-Scavenging Activity of Clitocybin A

The ROS-scavenging activity was determined to evaluate the protective effects of clitocybin A against oxidative damage (Fig. 4A). Cells irradiated with UVB at 30 mJ/cm^2 had a significantly increased level of ROS compared with non-irradiated cells. However, when treated with clitocybin

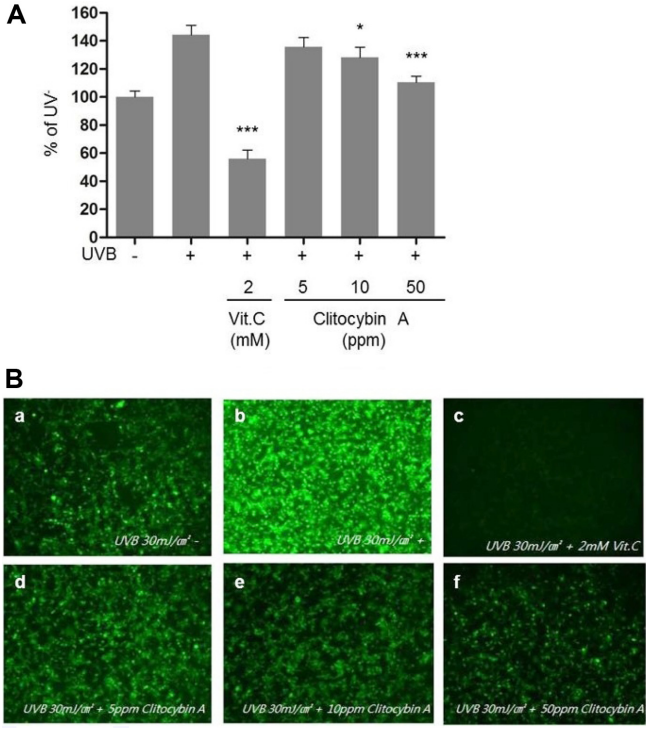

Fig. 4. ROS-scavenging activity of clitocybin A.
HDF-N cells were preloaded with DCF-DA (50 μM) in the medium for 1 h and exposed to UVB (30 mJ/cm^2) for 2 h. (**A**) At the end of exposure, ROS-scavenging activity in the cells was determined using a microplate reader. (**B**) ROS-scavenging activity was examined under a fluorescence microscope. a: UVB non-irradiated control. b: UVB-irradiated control (30 mJ/cm^2). c: Cells exposed to UVB at 30 mJ/cm^2 and treated with 2 mM of vitamin C. d, e, f: Cells exposed to UVB at 30 mJ/cm^2 and treated with 5, 10, and 50 ppm of clitocybin A. *p < 0.05, ***p < 0.005, vs. the UVB-irradiated control group.

A at 5, 10, and 50 ppm, ROS levels in the irradiated cells were decreased in a concentration-dependent manner, by 19.7%, 36.5%, and 76.5%, respectively, versus irradiated cells. When treated with 2 mM vitamin C (Sigma, A7506) as a positive control, ROS levels decreased by 199.3%, versus the irradiated cells. Fig. 4B presents the corresponding fluorescence imaging results.

Elastase Inhibitory Activity of Clitocybin A

Elastase inhibitory activity was measured using *N*-succinyl-*tri*-alanyl-*p*-nitroaniline as a substrate in HDF-N cells (Fig. 5). Clitocybin A treatment of cells at 0.1, 1, and 10 ppm decreased the elastase activity in a concentration-dependent manner, by 1.97%, 6.6%, and 8.31%, respectively, versus the control group, whereas the positive control, phosphoramidon, decreased elastase activity by 51.8% at 0.1 μM.

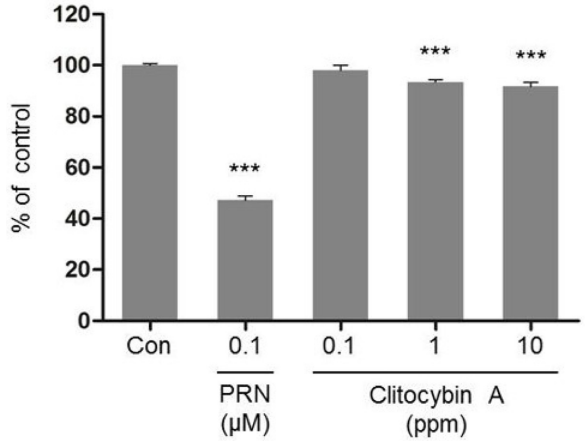

Fig. 5. Elastase inhibitory activity of clitocybin A.
Elastase inhibitory activity was measured using *N*-succinyl-*tri*-alanyl-*p*-nitroaniline as a substrate in HDF-N cells. The cells were treated with 0.1, 1, and 10 ppm of clitocybin A and phosphoramidon (PRN, 0.1 μM), a positive control. ***p < 0.005, vs. the control group.

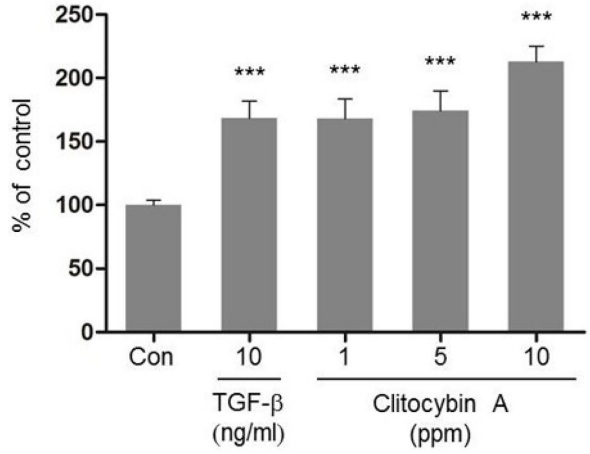

Fig. 6. Procollagen synthesis activity of clitocybin A.
Procollagen synthesis was measured using procollagen type I c-peptide in HDF-N cells. The cells were treated with 1, 5, and 10 ppm of clitocybin A and TGF-β (10 ng/ml), a positive control. ***p < 0.005, vs. the control group.

Effect of Clitocybin A on Procollagen Synthesis

Procollagen synthesis is an important factor that directly affects the improvement of wrinkles. Thus, the influence of clitocybin A on procollagen synthesis was investigated. As shown in Fig. 6, clitocybin A stimulated the procollagen synthesis capacity, with statistically significant values of 167.9 ± 15.3%, 174.4 ± 15.5%, and 212.8 ± 12.3% at 1, 5, and 10 ppm, respectively, versus the control group. When treated with the positive control, TGF-β (10 ng/ml), the

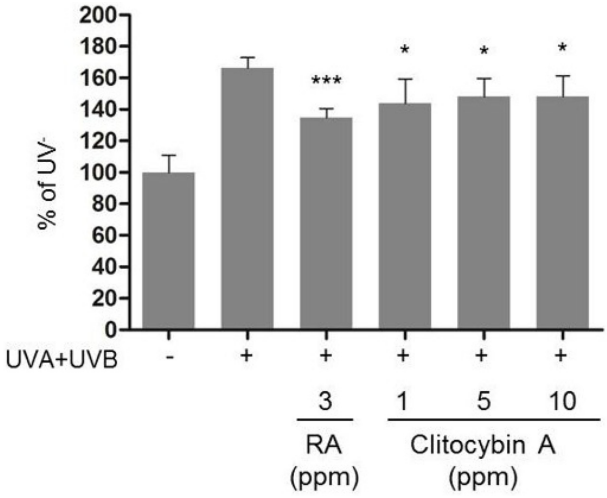

Fig. 7. Inhibitory activity of clitocybin A on matrix metalloproteinase-1 (MMP-1) expression.
The level of MMP-1 expression in HDF-N cells was determined using a commercially available ELISA kit. Cells were exposed to UVA (5 J) + UVB (40 mJ) and treated with 1, 5, and 10 ppm of clitocybin A and retinoic acid (RA, 3 ppm), a positive control. *$p < 0.05$, ***$p < 0.005$, vs. the UV-irradiated control group.

value of procollagen synthesis was 168.4% versus the control.

Inhibition of Matrix Metalloproteinase-1

As a mechanism by which wrinkles form in natural aging and photoaging, the activity level of the collagen-degrading enzyme MMP-1 was evaluated with an ELISA kit. Fig. 7 presents the results. UV-irradiated cells had increased MMP-1 expression, by 66.4%, compared with non-irradiated cells. However, when treated with 1, 5, and 10 ppm of clitocybin A, MMP-1 expression levels were reduced significantly, by 33.9%, 27.6%, and 27.5%, respectively, compared with the UV-irradiated cells. As a control, retinoic acid (Sigma, R2625) treatment at 3 ppm decreased MMP-1 expression by 47.6%, compared with UV-irradiated cells.

Discussion

Skin aging is a major issue nowadays, involving processes of photo-aging due to industrial pollution and global warming. Repeated exposure to UV radiation accelerates skin aging, leading to the formation of peroxyl free radicals, which break down to form malondialdehyde, which subsequently cross-links and polymerizes collagen [20]. This leads to depletion of the dermal extracellular matrix and chronic changes in skin structure, which in turn result in wrinkle formation.

Recently, more research has focused on naturally occurring anti-aging agents and many plant-derived products have been investigated. Various phytomolecules, such as carnosic acid, curculigoside, curcumin, glycyrrhizic acid, mangiferin, mirkoin, asiaticoside, rosmarinic acid, tectorigenin, and tyrosol, have been reported to inhibit tyrosinase, hyaluronidase, elastase, and collagenase; to scavenge free radicals from skin cells; to prevent trans-epidermal water loss; and to contribute to skin protection from wrinkles [21].

Previously, we have screened many natural products for their anti-collagenase, anti-elastase, and antioxidant activities. We found that naphthalene derivatives, known as syriacusins A and B, isolated from *Hibiscus syriacus*, reduced the expression of MMP-1/2 and induced the expression of type-1 procollagen at the protein level in UV-irradiated cultured HDFCs [22]. We also investigated the relationship between iridoid glycosides, isolated from *Hedyotis diffusa*, and human neutrophil elastase activity [23]. Recently, we isolated four novel human neutrophil elastase inhibitors, two sesquiterpenes and two labdane diterpenes, from the fruiting bodies of *Ramaria formosa* [24, 25]. Of these compounds, the mechanism by which methyl 3β,18-dihydroxy-8S-13,14,15,16-tetranorlabdan-12-oate (a labdane diterpene) inhibited human neutrophil elastase was a mixed-type non-competitive inhibition [25].

In this study, we sought to assess the anti-wrinkle properties of clitocybin A, an isoindolinone isolated from *C. aurantiaca*. We found that clitocybin A exhibited no significant cytotoxicity up to 10 ppm in HDF-N cells, and observed cell viability and cell proliferation activity greater than 94.6% and 91.9%, respectively. Additionally, the anti-wrinkle capacity of clitocybin A, as determined from ROS-scavenging and elastase inhibitory activities, procollagen synthesis, and MMP-1 expression in HDF-N cells, was statistically significant and concentration-dependent. In conclusion, clitocybin A may be an effective ingredient in functional cosmetics to prevent or alleviate skin wrinkles induced by ultraviolet rays, given its ROS radical-scavenging capacity, effects on procollagen synthesis, and the inhibitory effects on elastase and MMP-1 expression levels, which directly affect the elasticity of skin tissues.

Acknowledgments

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