

Protecting Effect of Phytoncide Solution, on Normal Human Dermal Fibroblasts against Reactive Oxygen Species

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Abstract: Four types of phytoncide solutions (A-Type, AB-Type, D-Type and G-Type) was evaluated for reduction of cell damage induced by oxidative stress, ultraviolet A (UVA), ultraviolet B (UVB), hydroxyperoxide (H₂O₂) and *t*-butyl-hydroperoxide (*t*-BHP); stimulation of collagen synthesis against UVA irradiation; and inhibition of matrix metalloproteinase-1 (MMP-1) activity induced by UVA in human normal dermal fibroblasts and human reconstituted skin model. The A-Type, AB-Type, D-Type and G-Type of phytoncide solutions pretreatment resulted in significant protection against cell damage induced by UVB, UVA, H₂O₂ and *t*-BHP. The amount of type I collagen following UVA irradiation was increased by treatment with phytoncide solutions in a concentration-dependent manner. On the other hand, phytoncide solutions also suppressed the excess MMP-1 irradiated UVA in a concentration-dependent manner. These effects of G-type solution were superior to those of other types solutions.

Key words: Phytoncide solution, oxidative stress, collagen content, matrix metalloproteinase-1 (MMP-1), normal human dermal fibroblasts

1 INTRODUCTION

Aging of skin is a complex biological phenomenon consisting of two components: intrinsic aging and photoaging caused by environmental exposure, primarily to ultraviolet (UV) light, but decreased metabolic function and accumulated oxidative damage induced by reactive oxygen species (ROS) are responsible for cutaneous inflammatory disorders and skin aging^{1,2)}.

Wrinkling of the skin, including dryness, roughness, and pigmentation, is a common phenomenon in aged skin. Dermal-epidermal junctions are also weaker. Striking changes are observed in the dermis, in which there is a loss of thickness. There is a decrease in the number of elastic fibers and in skin elasticity. These phenomena are accompanied by a decrease in dermal cell activity, including a decrease in fibroblast proliferation and low cellular communication by cytokines. Aged skin especially shows a decrease in collagen production. In the case of photoaging, the collagen content is decreased, because of increased degradation and decreased synthesis. Decreased collagen

affects the dermal architecture and the stretching and elasticity of skin, which causes fine wrinkling³⁾.

Normal human dermis consists primarily of an extracellular matrix (ECM) of connective tissue. Three major extracellular components have been recognized that contribute to the physiological properties of the skin. Specifically, fibers consisting of collagen, an abundant ECM protein that accounts for about 80% of the dry weight of the skin, provide tensile properties to the dermis, so as to allow the skin to serve as a protective organ against external trauma⁴⁾. Type I collagen is the most abundant ECM protein in skin connective tissue, which is responsible for conferring strength and resiliency. Type I collagen is synthesized primarily by fibroblasts residing within skin connective tissue. Type I collagen is synthesized as a soluble precursor, type I procollagen, which is secreted from fibroblasts and proteolytically processed to form insoluble collagen fibers. Thus processes that alter the relative proportions of Type I collagen can result in clinical manifestations that are recognized as part of the cutaneous aging

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Accepted April 4, 2009 (received for review March 10, 2009)

Journal of Oleo Science ISSN 1345-8957 print / ISSN 1347-3352 online

<http://www.jstage.jst.go.jp/browse/jos/>

process.

More recently, changes in collagen metabolism have been brought into focus as a major factor leading to photoaging. Specifically, it has been found that accumulation of elastic material is accompanied by concomitant degeneration of the surrounding collagenous meshwork, and evidence implicating matrix metalloproteinases (MMPs) as mediators of collagen damage in photoaging has been presented⁵⁻⁷. MMPs comprise a family of degradative enzymes consisting of at least 14 different members with rather broad substrate specificity⁸. Many of these proteases can degrade native collagen fibers, denatured collagen, elastic fibers, various proteoglycans, and fibronectin, among other components of the dermis. It has been demonstrated *in vitro* that UV irradiation of fibroblasts in culture enhances the expression of these proteolytic enzymes^{9,10}. Of those, matrix metalloproteinase-1 (MMP-1, collagenase), is thought to play a critical role in the remodeling of extracellular matrix, and so is the most important MMPs in the degradation of the dermal extracellular matrix by photoaging. Among human MMPs reported previously, MMP-1 which is an interstitial collagenase, is mainly responsible for the degradation of dermal collagen in human skin aging process^{11,12}.

Phytoncides are natural antibiotic volatile compounds emitted by trees and plants as a protective mechanism against the harmful insects and animals or micro-organism. The inhalation of phytoncides is known as forest bathing and aromatherapy. Chemical and pharmacological studies have shown that some species produce active principles that exert anti-gastropathic, anti-inflammatory, anti-oxidant activity¹³⁻¹⁵. It has been reported that physiological effects of phytoncides contribute to the improvement of various disorders including accelerated aging, allergies, multiple sclerosis, and Parkinson disease^{16,17}. Moreover, the anti-oxidative potentials of phytoncides and its effect on reducing oxidative stress response in stroke-prone spontaneously hypertensive rats was reported^{18,19}. Additionally, phytoncide enhances the activity of the human NK cells²⁰. We have previously reported that the suppressive effects on *umu* gene expression of SOS response activity induced by chemical mutagens and UV irradiation of phytoncide solutions²¹. On the other hands, it has been reported that phytoncide solutions contains highly volatile terpenoids, whose major ingredients are monoterpenoids such as α -pinene, careen, and myrcene^{18,19,21}. It has been reported that terpenoids showed anti-oxidative effects and anti-microbial effects²². However, no studies have been conducted to investigate the potential of phytoncide solutions on the protection against ROS on normal human dermal fibroblasts. This study examined the suppressive effects of these chemical components (hereinafter referred to as phytoncide solutions) released from plants, widely distributed in nature.

In the present study, we show that four different types of phytoncide solutions: A-Type (from trees), AB-Type (from highly bacteriocidal plants), D-Type (from flowering grasses) and G-Type (from non-allergenic plants), isolated from a combination of various plants selected based on their characteristics, effectively reduced cell damage induced by physical (UVA and UVB) and chemical (H_2O_2 and *t*-BHP) oxidative stresses, suppressed the UVA-induced decrease in type I collagen, and inhibited matrix metalloproteinase-1 (MMP-1) production induced by UVA irradiation in normal human dermal fibroblasts and human reconstituted skin model. The aim of this study was to clarify the potential of phytoncide solutions for prevention of the effects of aging and wrinkle formation. Recent advances in medical assistance 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 wrinkle formation with direct application to skin by its excellent anti-oxidative properties.

2 EXPERIMENTAL

2.1 Materials

Four types of phytoncide solution, A-type, AB-type, D-type, and G-type, were a gift from PHYTON-TAO 118 Inc. (1-1 Sakuragaoka Yao, Osaka, 581-0869, Japan). Four types of phytoncide solution, A-Type (from trees such as *Cinnamomum camphora*), AB-type (from highly bacteriocidal plants such as *Sasa veitchii*), D-type (from flowering grasses such as *Phyllostachys pubescens*), and G-type (from non-allergenic plants such as *Chamaecyparis obtuse*) of various plants, selected based on their characteristics²¹. Hydroxyperoxide (H_2O_2) and *t*-butyl-hydroperoxide (*t*-BHP) were obtained from Wako Pure Chemical Industries (Osaka, Japan).

2.2 Cell culture

Normal human dermal fibroblasts (NHDFs) were purchased from Kurabo Industries Ltd. (Osaka, Japan), and cultured with Kurabo's modified Medium 106S, containing 2% fetal bovine serum (FBS), cells were cultured with a 5% CO₂ incubator at 37°C. The fibroblasts were assayed for reduction of cell damage, and suppression of collagen decrease, and MMP-1 activity, as described below.

2.3 Reduction of cell damage induced by oxidative stress on normal human dermal fibroblasts

NHDFs were inoculated in 96-well microplates at a density of 2.0×10^4 cells/well, and cultivated for 24 h. After 24 h of pretreatment with phytoncides solution at several concentrations, cells were exposed to UVB (30 mJ/cm²) or UVA

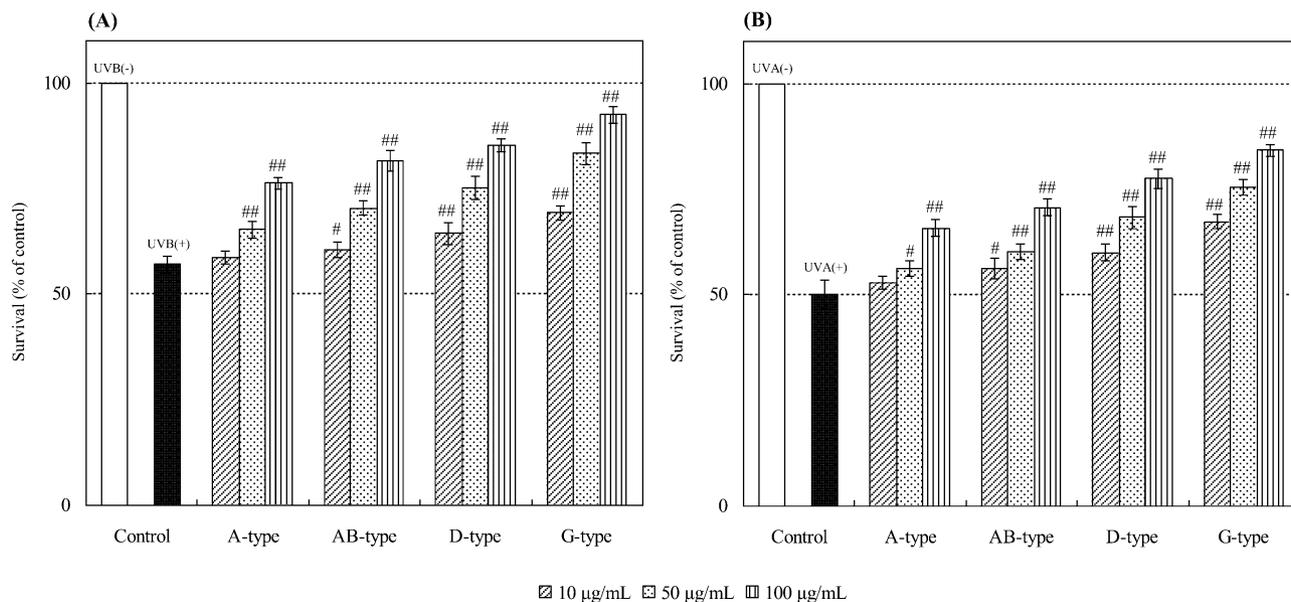


Fig. 1 Reduction of A-Type, AB-Type, D-Type and G-Type of Phytoncide Solutionin on Cell Damage Induced by Oxidative Stress Caused by Physical Reactive Oxygen Species, UVB (A) and UVA (B) Irradiation. NHDF Cells were irradiated with UVB (30 mJ/cm²) or UVA (10 J/cm²) irradiation in HBS after pretreatment with phytoncide solutions. Cell viability was evaluated by MTT assay. Each value represents the mean \pm S.E. of three experiments, and values were significantly different from the damaged group, UVB(+),or UVA(+), at $P < 0.05$ (#) and $P < 0.01$ (##).

(10 J/cm²) in Hank's buffered solution (HBS). The viability of the cells was evaluated by MTT assay after 24 h of cultivation. Control cells were cultivated without samples. The viability of the cells was evaluated by MTT assay after 72 h of cultivation (Fig. 1A). The MTT assay was based on the Mosmann method. In a case of H₂O₂ or *t*-BHP exposure, pre-treatment with phytoncides solution at several concentrations in the following, cells were exposed, cell viability was estimated by MTT assay at 2 h after exposing to H₂O₂ (1 mM), or *t*-BHP (0.1 mM) (Fig. 1B).

2.4 Suppression of collagen decrease and MMP-1 production stimulated by UVA irradiation on normal human dermal fibroblasts

NHDF cells was inoculated into 96-well microplates (2.0 \times 10⁴ cells/well) and cultivated for 24 h. After culture, the medium was changed to serum-free Medium 106S containing phytoncide solution at several concentrations and cultivated for 24 h. Control cells were cultivated without phytoncides solution. After pretreatment, cells were exposed to UVA (5 J/cm²) in HBS. The supernatants of each well were collected after 24 h of cultivation, and the amount of procollagen type I pN-peptide and interstitial MMP-1 activity were measured using a type I pN-peptide assay kit (TaKaRa, Kyoto) and human MMP-1 activity ELISA Kit (AnaSpec, Inc. USA).

2.5 Suppression of collagen decrease and MMP-1 production stimulated by UVA irradiation on human reconstructed skin model culture

Human reconstructed skin model (EFT-200) were purchased from Kurabo Industries Ltd. (Osaka, Japan), and cultured with Kurabo's modified EFT-200-MM-ASY, skin model were cultured with a 5% CO₂ incubator at 37°C. Human reconstructed skin model (EFT-200) was cultivated for 18 h. After culture, culture medium was changed to an assay medium, and 50 μ L of sample solution containing phytoncide solution at several concentrations was applied to the surface of the tissue. After pretreatment for 24 h, skin model were exposed to UVA (40 J/cm²) in HBS. The skin model was cultivated for 48 h. The viability of the skin model was evaluated by MTT assay after 48 h of cultivation. The supernatants of each well were collected after 48 h cultivation, and the amount of procollagen type I pN-peptide and MMP-1 activity were measured by using the former kits.

2.6 Statistical analysis

The proliferation, collagen content, and MMP-1 activity data were expressed as the mean \pm standard error (S.E.), and subsequent inspection of means was evaluated by Student's t-test between two groups at significance levels of $p < 0.05$ and $p < 0.01$.

3 RESULTS

3.1 Reduction of cell damage induced by oxidative stress

Treatment of NHDF cells with four types, A-type, AB-type, D-type, and G-type, of phytoncide solution significantly protected against cell damage induced by physical ROSs, UVB irradiation and UVA irradiation in a dose-dependent manner (Fig. 1). The A-type, AB-type, D-type, and G-type of phytoncide solutions suppressed the UVB-irradiation-induced cell damage by 32%, 41%, 55%, and 68%, at a concentration of 100 $\mu\text{g}/\text{mL}$, respectively (Fig. 1A). The A-type, AB-type, D-type, and G-type of phytoncide solution suppressed the UVA-irradiation-induced cell damage by 45%, 57%, 66%, and 82%, at a concentration of 100 $\mu\text{g}/\text{mL}$, respectively (Fig. 1B). These protective effects of phytoncide solution against cell damage induced by UVA irradiation were superior to those by UVB irradiation. Pretreatment of NHDF cells with four types, A-type, AB-type, D-type, and G-type, of phytoncide solution significantly protected against cell damage induced by chemical ROSs, H_2O_2 exposure and *t*-BHP exposure in a dose-dependent manner (Fig. 2). The A-type, AB-type, D-type, and G-type of phytoncide solution at 100 $\mu\text{g}/\text{mL}$ suppressed the H_2O_2 -exposure-induced cell damage by 31%, 37%, 41%, and 46%, respectively (Fig. 2A). The A-type, AB-type, D-type, and G-type of phytoncide solution showed the *t*-BHP-exposure-induced cell damage by 33%, 35%, 42%, and 48%, respectively (Fig. 2B). These protective effects of phytoncide solu-

tion against cell damage induced by physical ROSs were superior to those by Chemical ROSs. The G-type of phytoncide solution protected cell damage induced by physical and chemical ROSs stronger than other types of phytoncide solution.

3.2 Suppression of UVA-induced decrease in collagen and excess production of MMP-1 on normal human dermal fibroblasts

Pretreatment of NHDF cells with four types of phytoncide solution showed significant suppression of the decrease in collagen induced by UVA irradiation, in a dose-dependent manner (Fig. 3A). The A-type, AB-type, D-type, and G-type of phytoncide solution at a concentration of 100 $\mu\text{g}/\text{mL}$ significantly suppressed the UVA-irradiation-induced decrease in collagen, by 42%, 57%, 79% and 107%, respectively. Pretreatment of NHDF cells with four types of phytoncide solution showed significant suppression of the excess production of MMP-1 induced by UVA irradiation, in a dose-dependent manner (Fig. 3B). A-type, AB-type, D-type, and G-type of phytoncide solution at a concentration of 100 $\mu\text{g}/\text{mL}$ showed a significant 62%, 69%, 81% and 96% suppression of the excess production of MMP-1 induced by UVA irradiation, respectively. On normal human dermal fibroblasts, the G-type of phytoncide solution suppressed the decrease in collagen and excess production of MMP-1 activity induced by UVA irradiation stronger than other

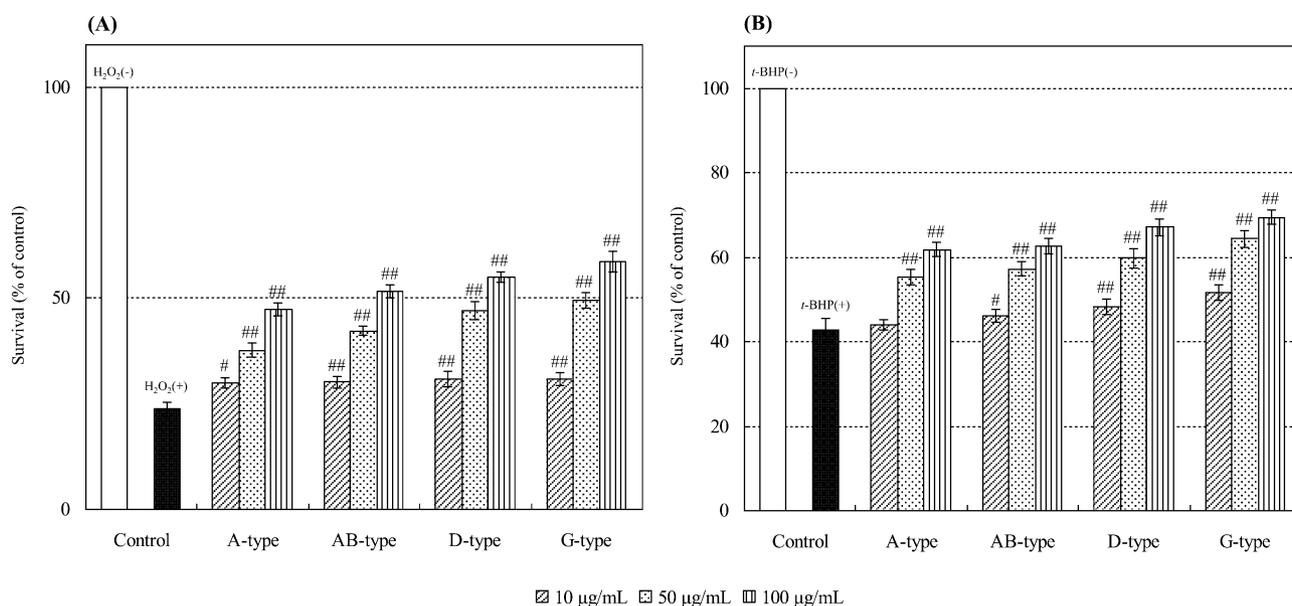


Fig. 2 Reduction of A-Type, AB-Type, D-Type and G-Type of Phytoncide Solution in on Cell Damage Induced by Oxidative Stress Caused by Chemical Reactive Oxygen Species, H_2O_2 (A) and *t*-BHP (B) exposure.

NHDF Cells were incubated with H_2O_2 (2 mM) or *t*-BHP (0.1 mM) in HBS after pretreatment with phytoncide solutions. Cell viability was evaluated by MTT assay. Each value represents the mean \pm S.E. of three experiments, and values were significantly different from the damaged group, H_2O_2 (+), or *t*-BHP (+), at $P < 0.05$ (#) and $P < 0.01$ (##).

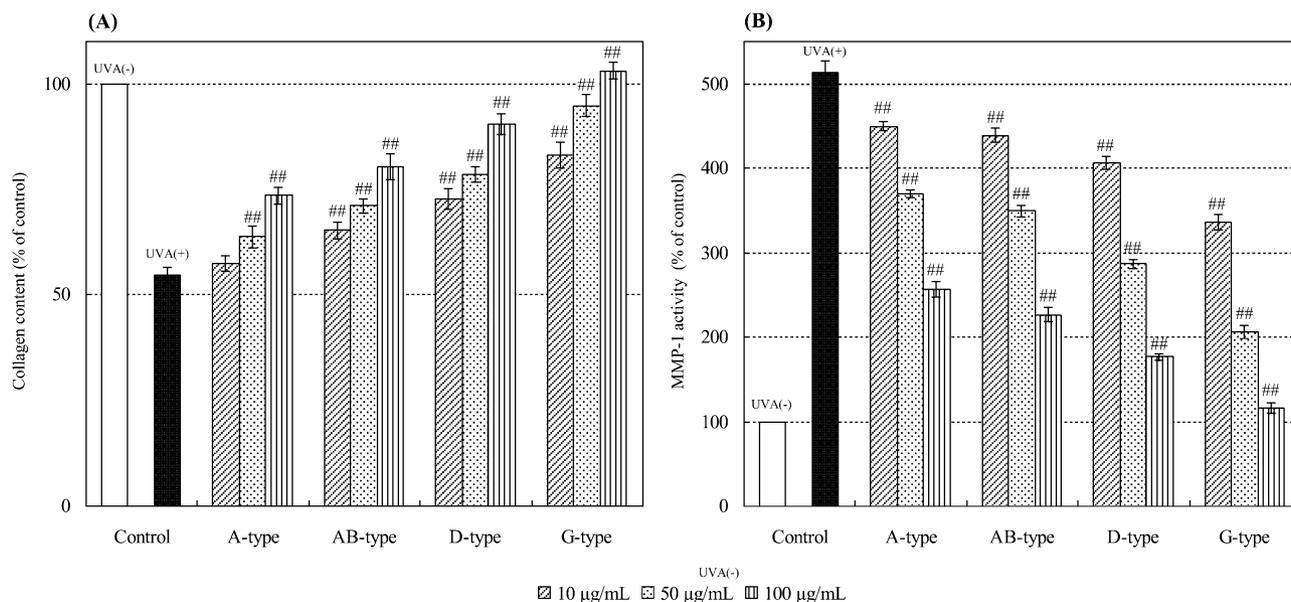


Fig. 3 Suppression of A-Type, AB-Type, D-Type and G-Type of Phytoncide Solutionin on Collagen Decrease (A) and MMP-1 Production (B) Induced by UVA Irradiation in Normal Human Dermal Fibroblasts.

NHDF cells were exposed to UVA (5 J/cm²) in HBS after pretreatment with phytoncide solutions. The amounts of procollagen type I pN-peptide and MMP-1 in the supernatants was measured after 24 h of cultivation. Each value represents the mean ± S.E. of three experiments, and values were significantly different from the damaged group, UVA(+), at $P < 0.05$ (#) and $P < 0.01$ (##).

types of phytoncide solution.

3.3 Suppression of UVA-induced decrease in collagen and excess production of MMP-1 on human reconstituted skin model

Pretreatment of human reconstructed skin model, EFT-200 with four types of phytocide solution showed significant suppression of the decrease in collagen induced by UVA irradiation, in a dose-dependent manner (Fig. 4A). The A-type, AB-type, D-type, and G-type of phytoncide solution at a concentration of 1 mg/ml significantly suppressed the UVA-irradiation-induced decrease in collagen, by 50%, 48%, 64% and 91%, respectively. Pretreatment of NHDF cells with four types of phytocide solution showed significant suppression of the excess production of MMP-1 induced by UVA irradiation, in a dose-dependent manner (Fig. 4B). A-type, AB-type, D-type, and G-type of phytoncide solution at a concentration of 1 mg/mL showed a significant 42%, 38%, 67% and 94% suppression of the excess production of MMP-1 induced by UVA irradiation, respectively. On human reconstructed skin model, the G-type of phytoncide solution suppressed the decrease in collagen and excess production of MMP-1 activity induced by UVA irradiation stronger than other types of phytoncide solution.

4 DISCUSSION

In the present study, the reductive effect of cell-damage induced physical and chemical reactive oxygen species (ROS) and the suppressive effect of the decrease in collagen and MMP-1 production induced by UVA irradiation were examined in normal human dermal fibroblasts and human reconstructed skin model culture system to address the anti-aging effects of four types of phytoncide solution in biological systems. Pretreatment of normal human dermal fibroblasts with four types of phytoncide solution led to significant protection against cell damage induced by physical (UVB, UVA) and chemical (H₂O₂ and *t*-BHP) ROSs (Fig. 1 and Fig. 2). It has been established that UVB produces superoxide anion radicals mainly by stimulation of the mitochondrial respiratory chain reaction²³. Superoxide anion radicals spontaneously convert to H₂O₂, and then to hydroxyl radicals in the presence of metal ions, Fe²⁺, and Cu⁺²⁴. The substance involved in cell damage during H₂O₂ exposure was the hydroxyl radical. Blocking effects of phytoncide solutions could also be caused by the possible involvement of their antioxidant and scavenging properties. Phytoncide solutions have antioxidant and radical scavenging activities^{18,19}. Therefore, the suppression of cell damage, decrease on collagen contents, excess production of MMP-1 activity by phytoncide solutions could arise from their scavenging ability to trap superoxide anions, hydroxyl radicals, lipid peroxy radicals and singlet oxygen

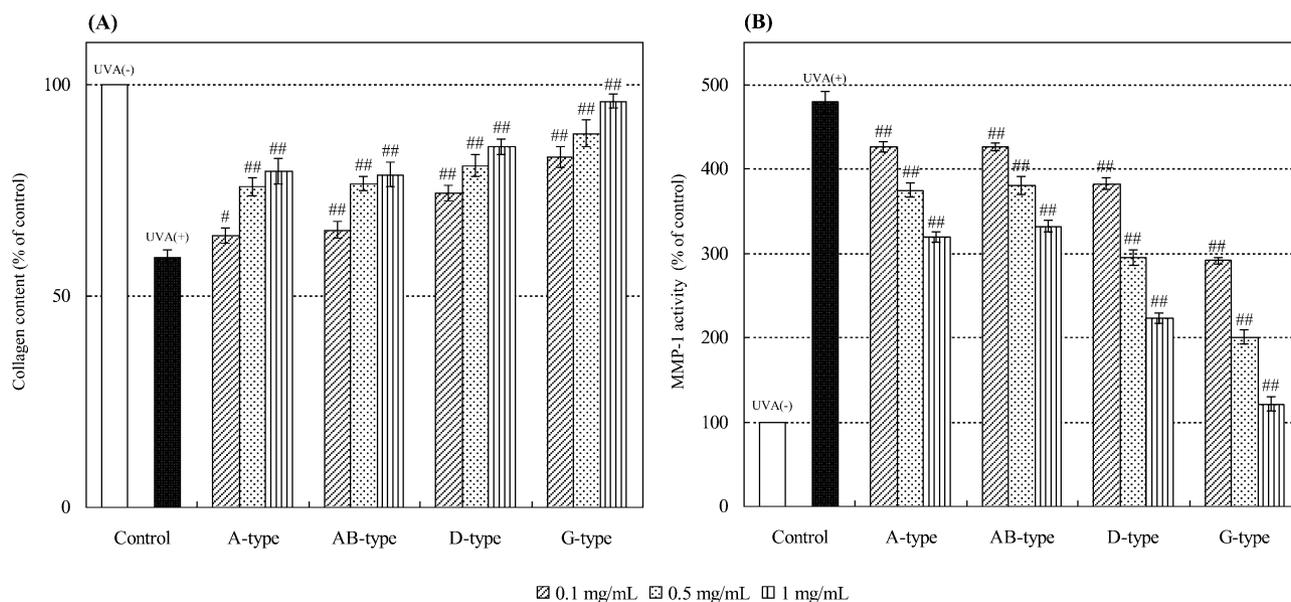


Fig. 4 Suppression of A-Type, AB-Type, D-Type and G-Type of Phytoncide Solutionin on Collagen Decrease (A) and MMP-1 Production (B) Induced by UVA Irradiation in Human Reconstructed Skin Model.

Human reconstructed skin model was exposed to UVA (5 J/cm²) in HBS after pretreatment with 50 μ L of phytoncide solutions. The amounts of procollagen type I pN-peptide and MMP-1 in the supernatants was measured after 24 h of cultivation. Each value represents the mean \pm S.E. of three experiments, and values were significantly different from the damaged group, UVA(+), at $P < 0.05$ (#) and $P < 0.01$ (##).

originating from ROSs into the cells.

It is acknowledged that alteration in the quantity and character of collagen is a critical factor in the process of wrinkle formation. UV (UVB and UVA) irradiation of the skin causes changes in the metabolism of collagen matrix. UVA irradiated-fibroblasts have been found to show decreased collagen production and increased MMP-1 production²⁵. Repetition of these reactions causes a significant decrease in dermal collagen, leading to the formation of very fragile dermis and wrinkles. Phytoncide solutions significantly suppressed the excess decrease in type I collagen and the production of MMP-1 following UVA irradiation in normal human dermal fibroblasts and human reconstructed skin model (Fig. 3 and Fig. 4). It has been established that singlet oxygen produced in cells by UVA irradiation up-regulates MMP-1 gene transcription²⁶. Also, phytoncide solutions reduced the damage to normal human dermal fibroblasts exposed to UVA. These results suggest that phytoncide solutions suppressed MMP-1 production by quenching singlet oxygen, superoxide, and free radicals, and thus suppressed the decrease in type I collagen caused by inhibition of MMP-1 production in normal human fibroblasts and human reconstructed skin model.

As for the component-activity relationships, A-Type, AB-Type, D-Type, and G-Type phytoncide solutions had distinctly different anti-aging effects, protection against cell damage induced by physical and chemical oxidative

stress, suppressing the excess decrease in type I collagen induced by UVA irradiation, and inhibition of excess MMP-1 production induced by UVA irradiation, according to their component patterns. G-Type of phytoncide solution was superior to other types of phytoncide solutions in terms of all anti-aging effects in normal human fibroblasts and human reconstructed skin model. In contrast to the other types, the G-Type solution contains a greater concentration of alcohols such as *Iso*-borneol, *p*-Menthane-3,8-diol and 3,7-Dimethyl-1,7-octanediol, α -hexyl cinnamaldehyde and coumarin²¹. These components and their combinations could be one of the important factors that is responsible for the enhanced protection against cell damage induced by physical and chemical oxidative stress, suppressing the excess decrease in type I collagen induced by UVA irradiation, and inhibition of excess MMP-1 production induced by UVA irradiation in normal human fibroblasts and human reconstructed skin model.

In summary, this study suggests that the four types (A-Type, AB-Type, D-Type and G-Type) of phytoncide solutions showed protective effects against physical and chemical oxidative stress, and a suppressive effect against excess type I collagen decrease and MMP-1 production induced by UVA irradiation. Type I collagen and MMP-1 play a role in the process of wrinkle formation, which is a common phenomenon of aged skin. These findings indicate that these phytoncide solutions were anti-aging agents in

skin care. In conclusion, we expect that these phytoncide solutions with such characteristic properties will 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 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 for the prevention of human aging.

ACKNOWLEDGMENT

We would like to thank Mr. Yasunori Mihara and PHYTON·TAO 118 Inc, for their generous gift of the phytoncide solutions.

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