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A Study on Resveratrol on the Antioxidative and Whitening Cosmeceutical Ingredients

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ABSTRACT

Objectives: Resveratrol is a variety of physiological active substances and is an excellent ingredient in antioxidant, anti-inflammatory and anti-aging action. Therefore, I would like to find out the possibility of adding cosmetics.

Method: The cytotoxicity test using MTT solution was carried out to check the safety of reverse control. Also DPPH assay was performed to measure antioxidant activity in resveratrol itself, and ROS was measured to measure antioxidant activity in cells. NO production was measured to observe anti-inflammatory action, and tyrosinase activity measurement and melanin production were measured to measure skin whitening activity.

Findings: we were able to confirm the safety of resveratrol's cytotoxicity. At concentration of 1, 10, 100 μg/mL of Resveratrol, the DPPH radical scavenging activity was observed to show high concentration - dependent activity of free radical scavenging. And In order to check the antioxidant properties within the cell, the ROS (reactive oxygen specifications) using DCF-DA were observed to reduce the effects. The addition of resveratrol 1, 10, 100 μg/mL on RAW 264.7 macrophages stimulated with lipopolysaccharide resulted in NO produce inhibition in concentration dependent and a strong inhibition rate of 53% at 100 μg/mL concentration. Tyrosinase active action was found to inhibited dose dependant. Melanin produce was also prevented by dose dependant.

Applications: These results suggest that the active oxygen - induced skin inflammation process is delayed as much as possible, so that it can be utilized as a functional cosmetic material having an effect on skin diseases and skin aging.

Keywords: *Resveratrol, Antioxidant, DPPH, ROS, Nitric oxide, melanin*

Introduction

Resveratrol (3,5,4'-trihydroxy-trans-stilbene) is included in poly-phenol compounds found in various natural substances such as grapes, peanuts, and berries. Trans resveratrol is an antibiotic produced in the vine against the invasion of fungi, and is often found in grape shells and seeds[1]. The amount of resveratrol that is contained in the shell varies depending on the variety, region, and how much mold is exposed[2]. It is well known for its phytosterone and antioxidant[3], and in response to the wound, it is a naturally phyto-alexin, produced by several spermatophytes, likes vines[4]. Long-term studies have shown that polyphenols are known as natural antioxidants[5], especially protecting against oxidative damage caused by human active oxygen[6]. Resveratrol, which belongs to these polyphenols compounds, has been used in various treatments for heart disease, and has been found to have excellent anti-cancer[7], anti-oxidant[8], anti-aging effects[9]. Recently, skin whitening has become

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an excellent ingredient[10]. As such, Resveratrol is an ingredient in plant systems that has been studied for various physiological and pharmacological effects.

Living things that need oxygen forms are formed by biochemical reactions during metabolic processes and by external environments. This is known as the cause of various diseases such as inflammation, skin aging, and cancer, as it acts as oxidative stress and destroys DNA, substrate and enzymes in the body, causing serious damage to tissue[11,12]. However, free radicals are not necessarily harmful to the human body. For example, the hydroxide arrow produced as a result of the decomposition of hydrogen peroxide in the body acts as a disinfectant by indiscriminately attacking the hospital. The problem is that it attacks even the molecules that the human body needs. Therefore, substances that have antioxidant efficacy, such as compounds that can kill active oxygen species or substances that suppress the production of peroxide, are expected to be treated for various diseases that are caused by oxidative stress as well as age retardant. Antioxidants produced in the body include catalase, an enzyme that breaks down the calcium hydroxide, and other glutathion and peroxidase. The active site contains Selenium (Se), which can reduce the concentration of active oxygen if the intake of selenium helps to break down hydrogen peroxide in the body. There are also three types of enzymes (SODs) that convert ions of excess oxidation into oxygen and hydrogen peroxide. Their enzymes contain metal ions such as copper, manganese, and zinc. Currently, BHA (butylated-hydroxy-anisole), PG (progyl-gallate), BHT(butylated- hydroxy-toluene) and TBHQ (t-butylhydroquinone) have been used for synthetic antioxidants, but there is a growing interest in natural antioxidants as safety issues arise, such as avoidance of compounds and toxic effects in heavy use[13].

Therefore, the possibility of natural antioxidants and anti-inflammatories using resveratrol belonging to polyphenol compounds is monitored to see if they can be expected to be cosmetic materials from a spice point of view.

Reagents and Method

Meterial: Resveratrol, L-DOPA, (3-(4,5-dimethyliazol-2-yl)-2, 5-diphenyl thrazolium bromide (MTT), Mushroom Tyrosinase, L-tyrosine were obtained from Sigma-Aldrich, Inc. 2',7'-dichlorofluorescin di-acetate (DCF DA) was purchased from the Molecular Probe Co. RAW 264.7 cells and B16 F10 melanin cells were purchased from Seoul National University's Cellular Bank.

Cell Culture: RAW 264.7 macrophages and B16 F10 melanin cells were grown at a concentration of 37°C with 10% phthalate serum and a 5% concentration of phenicillin/streptomysin (100 IU/50 μg/mL).

Cytotoxicity Measurement Using MTT: To confirm the cytotoxicity of Resveratrol, the MTT method was applied. RAW 264.7 cell was used, and divided 1 X 10^4 cells per well in 96 well plates, cultivated for 24 hours, added sample by concentration, and cultivated at 37℃, CO_2 incubator for 72 hours. After 72 hours, the cultivation solution was removed, and 1mL of 500 μg/mL of MTT solution dissolved in Krebs solution (mM : KCl 2.7, NaCl 137, $MgCl_2$ 0.5, Na2HPO 0.4, HEPES [pH 7.4] 10, $CaCl_2$ 1.8, glucose 5) to each well and cultivated for 4 hours in dark place. Then, the supernatant was removed, and 200 μL of D.M.S.O. was added to dissolve MTT formazan. After completely dissolving MTT formazan for 15 minutes in room temperature, the absorbance was measured in 570nm.

DPPH Radical Scavenging Activity Measruement: 180μL of 0.1 mM DPPH (1,1-diphenyl-2-picrylhydrazyl) solution dissolves in ethanol to 96 well plates resveratrol prepared in each concentration was added 20 μL each, cultivated for 30 minutes in 37℃ in the dark were processed to absorbance measurement in 517nm using FL 600 spectrofluorometer (BioTek, Winooski, VT, USA).

DPPH radical scavenging activity(%) = 100 - {(Absorbance of added/Absorbance of non-added) ×100}

Intracellular Oxidation Stress Measurement: This experiment statistical analysis was performed using the SPSS Window Version 17.0 (SPSS Inc., Illinois), and the significance was tested by Student's t-test. Was carried out three times or more independently under the same conditions noted in Mean ± standard deviation (Mean ± SD).

Intracelluar Nitric Oxide Measurement: RAW 264.7 cell was divided 1ml to each well as 1 X 10^6 cells/mL to 24 well plates. After mixing 100 μL of cell culture supernatant and 150 μL of Griess reagent to 96 well plates, and reacted 5 minutes and used ELISA reader(BioTek, Winooski, USA) to measure absorbance in 540nm. To create calibration curve, the study used sodium nitrite($NaNO_2$) as standard for comparison.

Measuring in-vitro Tyrosinase Activity: As for the activity habit of tyrosinase, the study used L-DOPA and L-tyrosine. L-DOPA was dissolved with 2 ㎎/mL of phosphate buffer (PBS 0.1 M, pH 6.8), and the concentration of tyrosinase was 25 units/mL. To 90 μL of tyrosinase, 10 μL of resveratrol dissolved in different concentration was put into eppendorf tube, mixed, divided into 40 μL to 96 well plates, added 200 μL of L-DOPA (2 mg/mL), let it react for 1 hour in 37℃, and measured absorbance in 475 nm. 0.3 ㎎/mL of L-tyrosine was completely dissolved with potassium phosphate buffer (PBS 0.1 M, pH 6.8) and the tyrosinase concentration was 100 units/mL. To 90 μL of tyrosinase, 10 μL of resveratrol dissolved in different concentration was put into eppendorf tube, mixed, divided into 40 μL to 96 well plates, added 200 μL of L-tyrosine (0.3 ㎎/mL), let it react for 1 hour in 37℃, and measured absorbance in 475 nm.

Measured Melanin Product Inhibition: After dividing B16F10 melanin cell into 3 mL to 6 well plates, it was cultivated for 12 hours in phenol red-free DMEM solution include 10 % FBS. And the sample with different concentration were cultivated for 10 minutes in 37℃ for preprocessing, and processed 1 μM of α-MSH(melanocyte stimulating hormone) and cultivated for 72 hours in 37℃. After cultivation, 100 μL of 10mM phosphate buffer pH 6.8 containing 1 % Triton was added, shook for 5 minutes, moved to tube and centrifuged for 5 minutes in 10,000 rpm. Then, 100 μL of 1 N NaOH and 100 ml of purified water was added to cell pellet, and cultivated in 60℃ for 1 hour to completely dissolve melanin, moved 200 μL to 96 well plates to measure absorbance of 405 nm. The experiment was conducted 4 times repeatedly in same condition, and obtained average to calculate melanin produced from each well using calibration curve from melanin standard.

Data Analysis and Statistical Verification: Result of experiment was displayed in average ± S.D and the experiment outcome was verified by non-paired student's t test.

Results and Review

Cytotoxicity Measurement: To determine the cell survival rate of Resveratrol, cytotoxicity was observed using the MTT measurement method using RAW 264.7 cells. All concentrations tested for 72 hours using resveratrol 1, 10, and 100 μg/mL were not toxic[Figure 1]. Therefore, we were able to confirm the safety of resveratrol's cytotoxicity. These results are similar to the results of cell survival of more than 90% at the highest concentration of 100 μM in the study conducted by Ivan M. Petyaev[14].

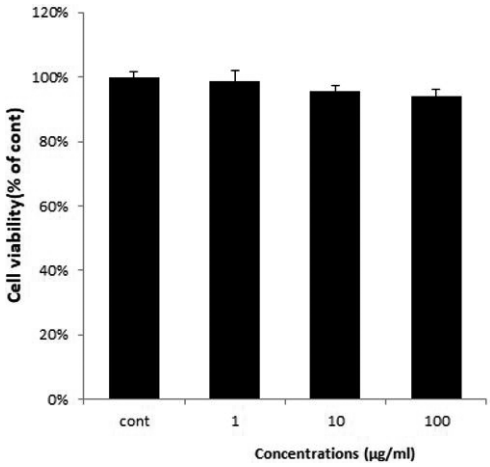

Figure 1: Cell toxicity of Resveratrol in RAW 264.7 cells. No toxicity appeared at all concentrations. Results are averages ± SD from four times experiments

In Vitro anti-oxidation: Resveratrol is a member of the polyphenolic compounds and is known for its plant-like hormone phytrogen, and is reported to have an antioxidant effect in particular[15]. The results of observing the cellular protection and antioxidation effect of resveratrol in a study at Jo et al. [16] showed higher antioxidant efficacy than control L-ascorbic acid. Polyphonic sound is an element closely related to the antioxidant action, which is considered to be part of Resveratrol's own anti-oxidant efficacy and thus was measured by DPPH radiological. At concentration of 1, 10, 100 μg/mL of Resveratrol, the DPPH radiological activity was observed to show high concentration - dependent activity of free radical scavenging[Figure 2].

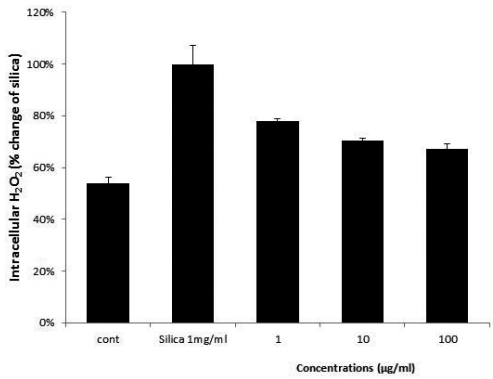

Figure 2: Antioxidant activity of Resveratrol in DPPH Assay. Dose dependent on activity of antioxidant. Results are averages ± SD from four times experiments

Reactive-oxygen species (ROS) scavenging activity of RAW 264.7 macrophage: Aerobic organisms, through their respiration, obtain oxygen, which is absolutely necessary, as energy. In the process, they constantly produce active oxygen such as super-oxide radical, hydroxyl-radical and hydrogen- peroxide[17]. These free radicals are known to damage the lipids, proteins, sugars and DNA that make up the cells, causing various diseases and aging such as skin and heart diseases, cancer, digestive disorders, inflammation, rheumatoid and autoimmune diseases[18,19]. Resveratrol, which belongs to a polyphenolic compound with a rich antioxidant efficacy, was verified in a previous experiment to verify the activity of DPPH free radiological removal in the test tube, and In order to check the antioxidant properties within the cell, the ROS (reactive oxygen specifications) using DCF-DA were observed to reduce the effects. ROS was inhibited by 33% at the highest concentration of 100 μg/mL in cells induced by 1 mg/mL of silica[Figure 3]. These results are consistent with the DPPH radiological denotential results.

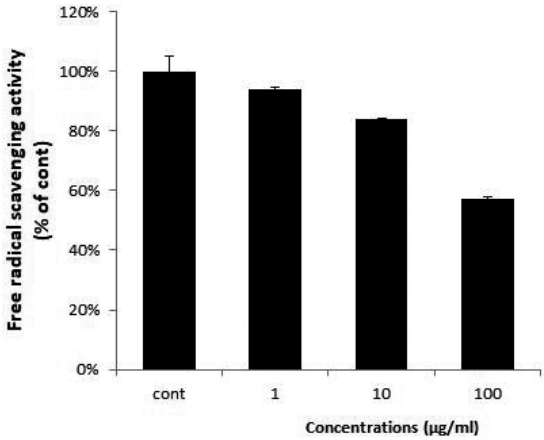

Figure 3: Antioxidant activity of Resveratrol 1 mg/mL silica-induced ROS production in Raw 264.7 macrophage. ROS produce was inhibited at all concentrations. Results are averages ± SD from four times experiments

Nitric Oxide Product Inhibition Activity of RAW 264.7 Cell: When inflammation occurs during the oxidation process, nitric oxide, interleukin-6, prostaglandin E_2, tumor necrosis factor -α. nitric oxide is synthesized by nitric oxide synthase in L-arginine in tissues and cells and is known to function as immune function regulation, vasodilation, neurotransmission, and blood coagulation[20,21]. However, the accumulation of nitric oxide due to excessive active oxygen production has a deleterious effect on human body. These nitric oxide induce excessive activity of macrophages which are involved in the immune function of the human body, and cause inflammatory diseases by causing excessive activation of inflammation by a necessary defense function *in vivo*[22]. And Excessive inflammation also directly affects skin aging and wrinkles. The mediators of the reaction are known as nitric oxide, active oxygen, prostaglandin (PG) and cytokines. In previous studies, resveratrol has been reported to exhibit anti-inflammatory activity at low concentrations. It has been shown to inhibit the production of inflammation cytokine and nitric oxide such as IL-6 and TNF-a from macrophages[23,24].

The addition of resveratrol 1, 10, 100 μg/mL on RAW 264.7 macrophages stimulated with lipopolysaccharide resulted in NO produce inhibition in concentration dependent and a strong inhibition rate of 53% at 100 μg/mL concentration[Figure 4]. These results suggest that the active oxygen - induced skin inflammation process is delayed as much as possible, so that it can be utilized as a functional cosmetic material having an effect on skin diseases and skin aging.

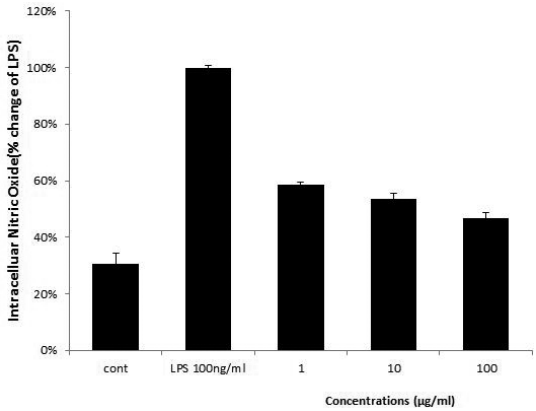

Figure 4: Effects of Resveratrol on NO production by 1 μg/mL lipopolysaccharide in Raw 264.7 macrophage. NO production was inhibited at all concentrations. Results are averages ± SD from four times experiments

Whitening Effect: Melanin is produced by oxidation of tyrosinase, an enzyme called tyrosinase, in the melanocyte of melanocyte melanocyte when exposed to ultraviolet rays. The enzymes such as catalase, peroxidase, dopa-chrome tautomerase and glutathione reductase and IF(interferon), prostaglandin, It is known that mediators such as cyclooxygenase and metal ions likes copper and zinc are involved[25]. tyrosinase is an enzyme that binds to Cu^{2+} and is a polyphenol oxidase widely

distributed in plants, microorganisms, and humans. It is an enzyme that represents a major regulatory step in melanin synthesis, such as limiting the rate of melanin synthesis[26]. Melanin binds to phospholipids or proteins to form melanin granules. It enters the keratinocyte and excretes out of the skin by keratinization. This action melanin protects the skin from ultraviolet rays[27]. This melanin acts as a protective agent to remove toxic substances in the human body, but excessive production causes hyperpigmentation such as spots and freckles in the human body, promotes skin aging, and causes skin cancer[28]. Currently, the mechanism of melanogenesis is relatively clear, and tyrosinase is involved in the conversion of tyrosine to dopaquinone (DOPA-quinone) during the production process. Indirectly regulates the biosynthesis of the melanin pigment and inhibits tyrosinase activity in most whitening studies[27,29]. The inhibition of tyrosinase activity by L-DOPA as an active substrate was controlled by resveratrol dependent on the concentration and was inhibited by 42% at 100 µg/mL[Figure 5]. The inhibition on tyrosinase activation was also strongly inhibited in dose dependent, and 43% inhibition at 100 µg/mL[Figure 6]. To investigation the effect of resveratrol on melanin-pigmentation synthesis at the cellular level, melanin-pigmentation production was observed by adding MSH to B16F10 melanocyte. Resveratrol 100 µg/mL significantly inhibited melanin production by MSH to 26% [Figure 7].

These results are similar to those obtained by Lee et al[29]. In the inhibition of 28.2% melanogenesis in B16 F10 melanocyte. Therefore, resveratrol is expected to be very useful as a functional whitening cosmetic material.

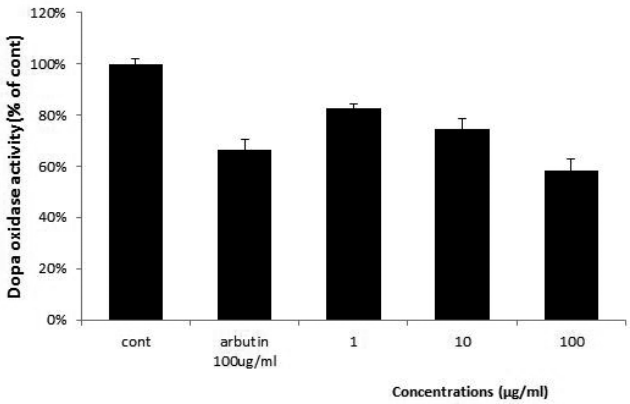

Figure 5: Effect on Resveratrol on L-dopa induced tyrosinase active. Results are averages ± SD from four times experiments

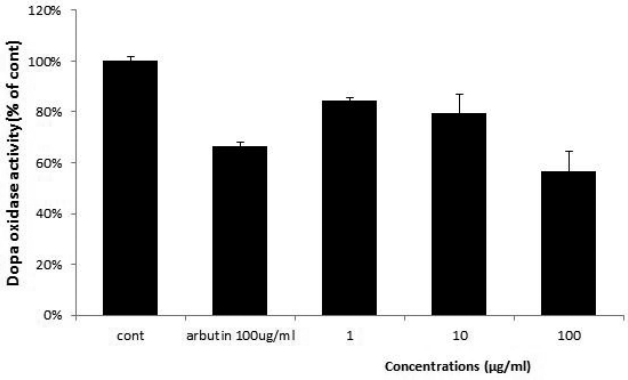

Figure 6: Effect on Resveratrol on L-tyrosine inducible tyrosinase active. Results are averages ± SD from four times experiments.

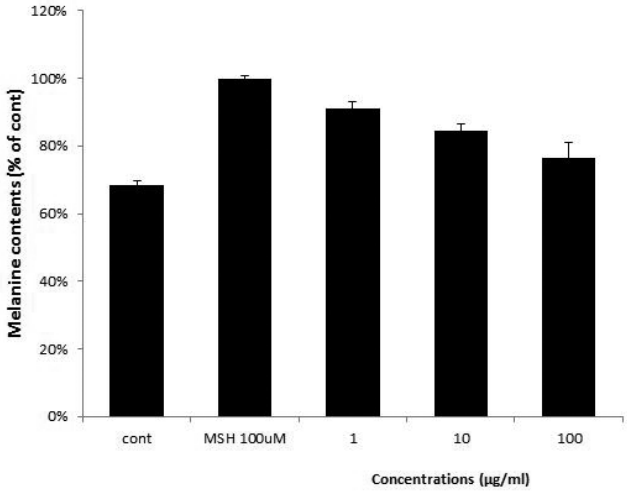

Figure 7: Effects of Resveratrol on melanin production by 1 µM MSH in B16F10 melanocyte. Results are averages ± SD from four times experiments.

Conclusion

In this study, we observed the anti - inflammatory and antioxidant effect of resveratrol, a polyphenolic compound, and observed the possibility of it as a cosmetic additive component. Since RAW 264.7 cells did not show toxicity at dose of Resveratrol 1, 10, 100 µg/mL, safety of the cosmetic products can be confirmed. Resveratrol concentrations of 1, 10, 100 µg/mL were shown to inhibit D.P.P.H. activity and showed free radical scavenging activity. In particular, antioxidant activity was inhibited by about 43% at 100 µg/mL concentration. It was observed that resveratrol belonged to a polyphenol compound rich in antioxidant efficacy and that DPPH free radical erasing activity was confirmed in the test tube, and DCF-DA produced ROS

(reactive oxygen specs) to inhibit antioxidant activity in cells. 1 mg/mL of silica was used as an ROS-generated stimulator at resveratrol 1, 10 and 100 µg/mL. Resveratrol inhibited ROS generation by concentration. Especially, the antioxidant activity was about 33% at 100 µg/mL The addition of resveratrol 1, 10, 100 µg/mL to Raw 264.7 macrophage stimulated with Lipopolysaccharide resulted in Nitric Oxide production inhibition in a concentration dependent and a strong inhibition rate of 53% at 100 µg/mL concentration. The inhibition of tyrosinase activity by L-DOPA and L-tyrosine was inhibited by resveratrol in a dose dependent and was inhibited 42% and 43% at 100 µg/mL. To investigate the effect of resveratrol on melanin synthesis at the cellular level, MSH was added to investigate the production of melanin. Resveratrol 100 µg/mL significantly inhibited melanin production by 26%.

These results suggest that resveratrol can delay the skin inflammation process by active oxygen to maximize the skin diseases and functional cosmetic material with skin whitening effect.

Ethical Clearance: Not required

Source of Funding: Nil

Conflict of Interest: Nil

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