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A Study on Coumarin as a Cosmetic Ingredient

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화장품 성분으로서의 Coumarin에 관한 연구

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Abstract : This study investigated the effects of coumarin, a flavonoid known for various physiological activities like antiviral, anticancer, and antibacterial properties, on anti-oxidants and anti-inflammatory processes, aiming to explore its application in functional cosmetics. The results are as follows: Cell toxicity experiments using RAW 264.7 cells showed no significant cytotoxicity for coumarin at any concentration, indicating its safety for skin application. Observing coumarin's antioxidant activity through DPPH radical scavenging showed concentration-dependent effectiveness, though not significantly varied with concentration. The inhibition of silica-induced ROS production in cells was concentration-dependent. Both NO production inhibition and histamine release measurements showed concentration-dependent suppression. These findings suggest that Coumarin can be effectively used as a natural ingredient in cosmetic development for its antioxidant and anti-inflammatory properties.

Keywords : Coumarin, anti-oxidant activity, anti-inflammatory activity, cosmetic, cosmeceutical

요 약 : 본 연구는 플라보노이드 계열의 물질로써 항바이러스, 항암, 항균작용 등 다양한 생리활성을 나타내는 물질로 알려져 있는 coumarin의 항산화 및 항염증에 미치는 영향을 관찰함으로써 피부에 적용할 수 있는 기능성 화장품의 원료로써의 활용방안을 모색하고자 하였다. 연구의 결과는 다음과 같다. RAW 264.7 세포를 이용하여 세포독성 실험을 실시한 결과 coumarin은 실험에 적용한 모든 농도에서 이렇다 할 세포독성을 나타내지 않았다. 따라서 본 실험에 적용된 농도 범위 내에서는 피부에 적용하였을 때 안전한 물질이라 사료된다. coumarin 물질 자체의 항산화 작용을 알아보기 위하여 DPPH radical 소거능을 관찰한 결과 농도 의존적으로 소거능을 나타내었지만 농도에 따라 큰 차이는 나타내지 않았다. 세포내에서 생성되는 hydrogen peroxide을 DCF-DA 형광물질을 이용하여 ROS를 측정한 결과 silica에 의해 생성된

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ROS의 억제효과가 농도 의존적으로 나타났다. coumarin의 항염증에 미치는 영향을 관찰하기 위해 Nitric oxide 생성억제와 histamine release를 측정한 결과 모두 농도 의존적으로 억제하였다. 이상의 결과를 종합해 볼 때 Coumarin이 화장품 소재개발에 있어서 항산화와 항염증에 관련된 천연물로써 효과적으로 활용이 가능할 것으로 사료된다.

주제어 : 쿠마린, 항산화작용, 항염증작용, 화장품, 기능성화장품

1. Introduction

Despite entering an era of aging, oxidative stress and chronic inflammation are key factors accelerating human aging and reducing lifespan and quality of life, not just major chronic diseases[1]. Reactive oxygen species (ROS), significant in forming oxidative stress, also induce various inflammations in the human body[2,3]. ROS, constantly produced in aerobic organisms during normal metabolic processes and necessary for cellular function[4], can attack cell membranes, DNA, proteins, lipoproteins when oxidative stress persists, leading to tissue damage. This is connected to the signaling of inflammatory responses, inducing systemic chronic inflammation, thus promoting cardiovascular diseases, cancer, dementia, diabetes, autoimmune diseases, and aging[5,6]. Additionally, ROS can be generated by UV exposure, accelerating photoaging by inducing oxidative stress and destroying cell membrane lipid peroxidation and transmembrane signaling pathways[7]. Oxidative stress in the skin triggers various reactions, particularly inflammatory responses [8]. Nitric oxide, a key inflammatory mediator, increases in production when inflammation activates inducible nitric oxide synthase (iNOS) expression in various cells. Cyclooxygenase (COX)-2, important in inflammation, rapidly expresses in response to inflammatory signals, producing the inflammatory mediator PGE_2[9]. Histamine, known to mediate itching in inflammation, is involved in local immune responses, physiological functions in the gut, and acts as a neurotransmitter in the brain and spinal cord[10]. Although inflammation is a vital immune response to protect the body against bacteria, physical or chemical stimuli, chronic excessive inflammation can cause pain, swelling, redness, fever, etc[11,12]. Atopic dermatitis, a chronic inflammatory skin disease characterized by itching, involves various inflammatory cells like CD4+, CD8+, CD25+ T cells, eosinophils, and mast cells, expressing cutaneous lymphocyte-associated antigen (CLA)[13,14]. Various cytokines (IL-4, IL-13) and chemokines are involved in this process, with associated receptors expressed in various cells [15,16].

This study uses coumarin, a flavonoid present in strawberries, apricots, cherries, known for antiviral, anticancer, antibacterial activities[17,18]. Previous studies, like those by Duck-Sool Kim[19], have shown the anti-inflammatory activity of coumarin derivatives, inhibiting cytokine interleukin-6, while Lee et al.[20] observed significant anticancer effects of coumarin derivatives. Flavonoids, known to eliminate ROS and have excellent antioxidant activities, are studied in various fields like medicine, food, cosmetics[21]. Given the rising interest in anti-aging, the focus on natural plant-derived substances with antioxidant and anti-inflammatory effects is increasing. This study observes the antioxidant, anti-inflammatory, and whitening effects of coumarin, considering its application in functional cosmetics.

2. Research Method

2.1. Reagent and Cell culture

Coumarin, 3-(4,5-dimethyliazol-2-yl)-2, 5-diphenyl tetrazolium bromide(MTT), L-DOPA, Mushroom Tyrosinase, purchased from Sigma-Aldrich, Inc.(St. Louis. Mo. USA). DCF-DA (2',7'-dichlorofluorescin diacetate) was purchased from the Molecular Probe Co. (Eugene, OR, USA). Raw 264.7 cell and RBL2H3 macrophage were purchased from Seoul National University's Cellular Bank. Raw 264.7 cell and RBL2H3 macrophage were grown at a concentration of 37° C with 10% fetal bovine serum and a 5% concentration of phenicillin/streptomysin (100 IU/ 50 μg/mL).

2.2. Cytotoxicity measurement using MTT

To confirm the cytotoxicity of Coumarin, the MTT method was applied. Raw 264.7 cell was used, and divided 1 X 10^6cells per well in 96 well plates, cultivated for 24 hours, added coumarin by concentration, and cultivated at 37℃, CO_2 incubator for 48 hours. After 72 hours, the cultivation solution was removed, and 1 mL of 500 μg/mL of MTT solution dissolved in Krebs solution (mM :NaCl 137, KCl 2.7, $Na2HPO_4$ 0.4, $MgCl_2$ 0.5, HEPES [pH 7.4] 10, $CaCl_2$ 1.8, glucose 5) to each well and cultivated for 6 hours in dark. Then, the supernatant was removed, and 200 μL of DMSO was added to each well to dissolve MTT formazan. After completely dissolving MTT formazan for 10 minutes in room temperature, the absorbance was measured in 570nm.

2.3. DPPH radical scavenging activity

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

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

2.4. Intracellular oxidation stress measurement

The fluorescence of DCF-DA (2',7'-dichlorofluorecin diacetate) in RAW 264.7 cells was measured by conversion of DCF, which is produced by oxidation and deacetylation with intracellular oxygen radicals (ROS), into a fluorescent substance. After RAW 264.7 cells were suspended in 10 mL of Krebs buffer, followed by 20 μM DCF-DA and incubated in a light-shielding state for 30 minutes. After washing once with a Krebs buffer without DCF-DA, cells were extracted by centrifugation. It was divided into 1 X 10^6 cells/mL, pre-treated with coumarin by concentration, and added 1 mg/mL of silica to induce ROS production for 30 minutes. After centrifugation, the cell microplate was redistributed into 200 μL of Krebs buffer, transferred to 96 well plates, and measured the fluorescence (Ex 485 nm/Em 535 nm).

2.5. Nitric oxide measurement

Raw 264.7 cells were divided into 24 well plates at 10^4 cells/mL, 1 mL per well. In 96 well plate, 100 μL of the cell culture supernatant and 150 μL of the Griess reagent (1 % sulfanilamide in 5 % phosphoric acid + 1 % α-naphthylamide in H_2O) were mixed, and reacted for 5 minutes to measure the absorbance at ELISA microplate reader (Model: MQX40, Bekio). Sodium nitrite ($NaNO_2$) was used as a standard for comparison to prepare a calibration curve.

2.6. Histamine release

After RBL 2H3 cells were divided into 10^4 cells/mL, the coumarin was pretreated for 10 minutes, and 0.5 μM melittin was treated to extricate histamine for 30 minutes. After reactions, the supernatant and the cells were separated by centrifugation, and the cells were

destroyed by an ultrasonic pulverizer, and distilled water was added to adjust the final volume to 2 mL. In each tube, 0.4 mL of 1 N NaOH and 0.1 mL of 1 % OPT (o-phthalaldehyde, 10 mg/mL in absolute methanol) were added, mixed, incubated in room temperature for 4 minutes or more, and the reactions were stopped by adding 0.2 mL of 3 N HCl. The fluorescence was measured by dividing 200 μL into 96 well plate (Ex. 355 nm/Em. 455 nm). The amount of histamine in supernatant was considered as the amount of extricated histamine.

2.7. Data analysis and statistical verification

This experiment statistical analysis was performed using SPSS Window Version 23.0 (SPSS Inc., Illinois, USA), 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), The experiment determined that there was a statistically significant difference when the p value was less than 0.05.

3. Results and discussion

3.1. Measurement of cytotoxicity

To examine the cytotoxicity of coumarin, the cell viability was measured using the MTT assay. Treating Raw 264.7 cells with coumarin concentrations of 25, 50, 100 μg/mL showed no cytotoxicity, with the highest concentration showing 89 % cell viability, suggesting its effectiveness and safety as a cosmetic ingredient.

3.2. DPPH radical scavenging activity

1,1-diphenyl-2-picrylhydrazyl (DPPH) is a method commonly used for antioxidant capacity measurement, especially with phenolic and aromatic amine compounds[22]. DPPH, due to its unstable nature, readily accepts hydrogen atoms, losing its coloration when reacting with antioxidants[23]. This principle is used to measure antioxidant capacity[24]. In vitro experiments with coumarin concentrations of 25, 50, 100 μg/mL showed increased DPPH radical scavenging ability with concentration, but the difference was minimal, suggesting that coumarin, although a known strong antioxidant in the flavonoid group, has limited antioxidant capacity.

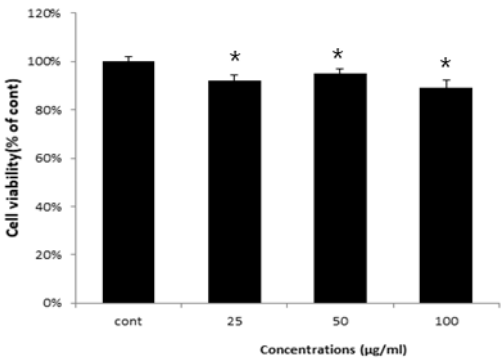

Fig. 1. Cell viability of Coumarin in RAW 264.7 cell. the MTT method was applied. Results are means±SD from 4 separate experiments. (*〈0.05)

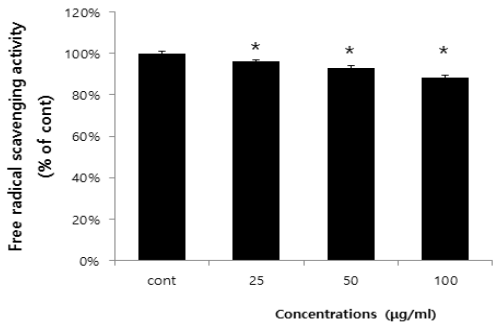

Fig. 2. Anti-oxidant activities of Coumarin in the DPPH radical scavenging activity assay. Results are means±SD from 4 separate experiments. (* p〈0.05)

3.3. Reactive oxygen specifications (ROS) erasing activity in RAW 264.7 cells

Using DCF-DA fluorescent substance, the production of hydrogen peroxide within cells was measured[25,26]. ROS, oxidative agents with unpaired electrons in an unstable state,

are formed internally from normal cellular metabolic processes requiring oxygen or from various external factors[27]. Adequate ROS levels help in antimicrobial actions and cell growth, protecting attacked cells. ROS also promotes the secretion of inflammatory cytokines, increasing matrix metalloproteinases (MMPs) synthesis, accelerating the breakdown of collagen and elastin, leading to skin aging symptoms like wrinkles, pigmentation, dehydration, tone loss[28,29]. Cells have antioxidant defense mechanisms against ROS accumulation and damage, but excessive ROS leads to oxidative stress exposure[30]. In this experiment, silica, known to produce ROS in macrophages and fibroblasts, was used as a stimulant[31]. Observing the inhibitory effect on ROS produced by silica, coumarin showed a concentration-dependent strong inhibition, with the highest concentration (100 μg/mL) showing 22 % antioxidant activity. This aligns with studies showing significant radical scavenging activity of coumarin derivatives found in ash bark, indicating high potential for coumarin's antioxidant efficacy in cosmetic applications.

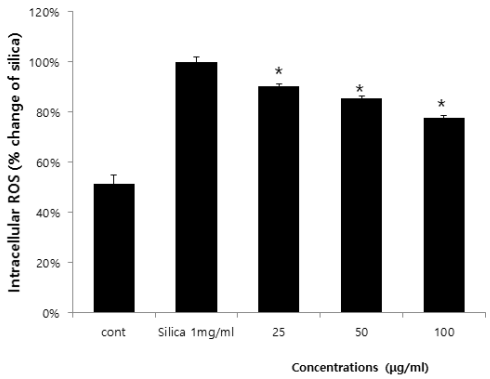

Fig. 3. Effects of Coumarin on ROS generation in RAW 264.7 cells. Data were expressed as % change of silica. Results are means ± SD from 4 separate experiments. (* $p<0.05$)

3.4. Effects of Nitric oxide production in raw 264.7 cells

Nitric oxide (NO), a stable and toxic free radical, is a key vasodilator secreted by endothelial cells and a cellular messenger in all vertebrates, regulating blood flow, thrombosis, and neural activity[33]. Initially known for its role in regulating vascular and muscle tissue relaxation, NO is now studied as an important signaling molecule in antimicrobial and anti-inflammatory actions, particularly in macrophages where NO production mediates inflammation[34]. This study confirmed the potential of coumarin as an anti-inflammatory agent by observing its inhibition of NO production in mouse macrophages (RAW 264.7) treated with lipopolysaccharide (LPS). Following LPS induction, coumarin reduced NO production in a concentration-dependent manner, with the highest concentration (100 μg/mL) showing a 29 % inhibitory effect. This result is similar to previous findings where coumarin derivatives reduced interleukin-6 production in LPS-stimulated macrophages, confirming its potent anti-inflammatory properties. Thus, coumarin shows potential as a cosmetic ingredient for improving atopic and acne-prone skin conditions.

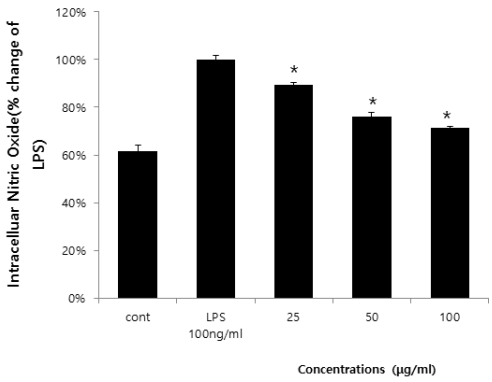

Fig. 4. Effects of Coumarin on Nitric oxide generation in RAW 264.7 cells. Data were expressed as % change of silica. Results are means ± SD from 4 separate experiments. (* $p<0.05$)

3.5. Histamine release active

Histamine, a biological amine synthesized from the amino acid histidine, plays a crucial role in local immune responses, causing inflammation and allergic reactions. It induces mucus secretion in the nasal and bronchial mucosa, contraction of bronchial smooth muscles, and itching and pain at nerve endings[35]. In experiments using RBL 2H3 cells stimulated with Melittin to induce histamine release, coumarin showed a concentration-dependent inhibition of histamine release, with the highest concentration (100 μg/mL) achieving a 29 % reduction. These results indicate that coumarin can effectively inhibit histamine release, suggesting its use as a natural alternative to steroids for treating skin allergies and irritations in people continuously exposed to allergens.

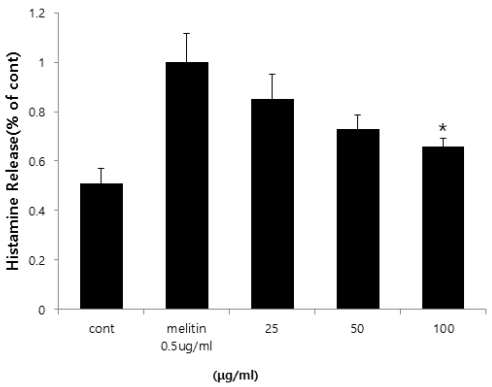

Fig. 5. Effects of Coumarin on Histamine release in RBL 2H3 cells. Results are means ± SD from 4 separate experiments. (* $p<0.05$)

4. Conclusion

This study investigated the effects of coumarin, a flavonoid known for various physiological activities like antiviral, anticancer, and antibacterial properties, on anti-oxidants and anti-inflammatory processes, aiming to explore its application in functional cosmetics. The results are as follows: Cell toxicity experiments using RAW 264.7 cells showed no significant cytotoxicity for coumarin at any concentration, indicating its safety for skin application. Observing coumarin's antioxidant activity through DPPH radical scavenging showed concentration-dependent effectiveness, though not significantly varied with concentration. The inhibition of silica-induced ROS production in cells was concentration-dependent. Both NO production inhibition and histamine release measurements showed concentration-dependent suppression. These findings suggest that Coumarin can be effectively used as a natural ingredient in cosmetic development for its antioxidant and anti-inflammatory properties.

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