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Evaluation of Skin Aging Preventive Effects of Cherry Blossom Petal Extracts Through Antioxidant and Anti-Glycation Activities

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Abstract

Skin aging progresses due to factors such as aging and UV exposure, with oxidative stress and glycation stress being known to be important contributing factors. Reactive oxygen species (ROS) promote collagen degradation through DNA damage and MMP induction, while advanced glycation end products (AGEs) cause collagen cross-linking and decreased skin elasticity. In this study, to evaluate the potential of *Cerasus* petal extract as a skin aging prevention material, we examined its antioxidant activity, anti-glycation activity, and melanin production inhibitory effect using B16 cells. As a result, the extract showed high antioxidant and anti-glycation activity, but its melanin production inhibitory effect was limited. These results suggest that *Cerasus* petal extract may be useful not so much as a whitening material, but rather as a natural material that prevents skin aging through oxidative and glycation stress.

Keywords: Skin Aging; Glycation; Antioxidant; *Cerasus* Petal

Abbreviations

ROS: Reactive Oxygen Species; AGEs: Advanced Glycation End Products; MMPs: Matrix Metalloproteinases.

Introduction

In Japan, which is facing a super-aging society, increasing medical expenses are putting a strain on public finances, leading to growing interest in extending healthy life expectancy, preventing lifestyle-related diseases, and anti-aging. Much of the research on aging reports that UV radiation, oxidative stress, glycation, and chronic inflammation interact with each other to accelerate aging [1]. We are affected by everyday stresses such as ultraviolet radiation, reactive oxygen species, glycation stress, and various chemical substances. Reactive oxygen species (ROS) produced by UV irradiation induce DNA damage and lipid peroxidation [2] and further promote the expression of matrix metalloproteinases (MMPs), causing collagen degradation [2-5]. MMPs cause collagen degradation and elastin degeneration, which leads to wrinkles, sagging, and loss of firmness [3]. In recent years, the accumulation of AGEs has also attracted attention as an important factor in skin aging. Glycation stress is a concept that comprehensively considers the biological stress caused by the loading of reducing sugars and aldehydes, and the subsequent chemical reactions. Glucose and methylglyoxal, for example, react with biological proteins to form AGEs. AGEs, which are produced by in vivo glycation, are the final products of the glycation reaction and are known to cause protein cross-linking and functional denaturation. The accumulation of AGEs is attracting attention as an important

pathological factor because it is involved in the development of diabetic complications such as diabetic retinopathy, nephropathy, and neuropathy, as well as ischemic heart disease and cerebrovascular disease. In particular, in the skin, it is thought to be involved in the progression of skin aging because soft collagen changes into hard and brittle collagen [6]. For this reason, the development of AGEs production inhibitors and degradation accelerators is progressing. AGEs are known to cause collagen cross-linking and a decrease in skin elasticity, and are involved in the progression of skin aging. For this reason, there is growing interest in methods to prevent and suppress glycation stress and oxidative stress. In recent years, AGEs production inhibitory effects and antioxidant effects have been reported in plant and food-derived components, and the development of naturally derived anti-aging materials is attracting attention. Against this backdrop, natural plant-derived materials with antioxidant and anti-glycation activity are attracting attention as materials for preventing skin aging.

Among the many varieties of cherry blossoms (*Cerasus*), Someiyoshino is the most popular in Japan and is cultivated variety of *Cerasus* native to Japan, resulting from natural or artificial crossbreeding between a hybrid of the Yedohigan cherry (*Cerasus spachiana*) and the Oshima cherry (*Cerasus speciosa*), a species endemic to Japan. Genetic studies have shown that Someiyoshino is a clone of a cultivated variety originating from a single tree that resulted from the crossbreeding of an Yedohigan cherry and an Oshima cherry [7]. *Cerasus* trees are widely distributed mainly in the temperate regions of the Northern Hemisphere. Many species of *Cerasus* are found in Asia, with a particularly high concentration in the Japanese archipelago. *Cerasus* petals contain polyphenols and flavonoids, and have been reported to potentially have anti-inflammatory effects and protect epidermal cells from UVB-induced oxidative stress and apoptosis [8-10]. In particular, a positive correlation has been shown between phenol content and radical scavenging activity [11]. On the other hand, there are limited reports on the anti-glycation activity and skin aging prevention function of *Cerasus* petal extract. Therefore, in this study, we evaluated the antioxidant activity, anti-glycation activity, and melanin production inhibitory effect of *Cerasus* petal extract and investigated its potential as a skin aging prevention material.

Material and Methods

Sample Preparation

During the peak blooming season early May of Someiyoshino, petals were collected from a Someiyoshino cherry tree on the grounds of National Institute of Technology, Hachinohe College, in Aomori Prefecture. The petals were washed with water, dried with air, and powdered. 5g of the petal powder was placed in 300mL of ethyl acetate (EtOAc) and extracted by perfusion for 1 hour. The extract was filtered to remove solid matter, and the EtOAc was removed using an evaporator to obtain the extract. Additionally, 5g of the petal powder was placed in 300mL of 80% ethanol (EtOH) and the extraction procedure was performed in the same manner as above to obtain the extract. 0.715g of the EtOAc extract and 1.115g of the EtOH extract were obtained. Both extracts were redissolved in dimethysulphoxicide (DMSO) and used in the tests.

Cell Line

B16 mouse melanoma cells obtained from JCRB were maintained at 37°C and 5% CO_2 under Dulbecco's modified Eagle's medium (DMEM: Nissui Pharmaceutical CO, LTD) containing 10% FBS and 200 μg/mL streptomycin. Cells were used during the logarithmic growth phase. Cell viability with the extract was calculated, with the cell count of the group without extract (0 μg/mL) set as 100%. Student's t-test was used for statistical analysis.

Melanine Suppression Test

The melanin production inhibition test was performed following the method of Ohguchi, et al. [12]. B16 cells were seeded at 4×10^4 cells/mL in 24-well plates and cultured for 24 hours. The medium was removed and replaced with D-MEM medium (± 1 μM α-MSH melanin synthesis inducer) containing EtOAc or EtOH extract, and the cells were re-cultured for 7 days. Both extracts were used as the baseline of 500 μg/mL, which ensures cell viability of 80% or more. The medium was removed and the B16 cells were washed with PBS at 4°C. 20 mM Tris-HCl buffer (pH 7.5) containing 0.1% Triton X-100 was added to the wells and allowed to stand at 37°C for 1 hour. Cell were sampled by pipetting using 20mM Tris-HCl buffer, centrifuged at 10,000 rpm at 4°C, and the supernatant was removed to collect cells. 1N NaOH was added to collected cells and stirred, and cells were dissolved by standing at 60°C for 1 hour. The absorbance of the pellet solution (400 nm) was measured. Kojic acid was used as the positive control agent. Kojic acid is a chemical substance used in cosmetics because it exhibits tyrosinase activity and inhibits melanin production. Melanin content was calculated by setting the state in which B16 melanoma cells were cultured in the absence of α-MSH and the sample as 100%. Statistical analysis was performed in the same manner as described above. The sample concentration was defined as a cell viability of 80% or more. Cell viability was determined as follows: B16 cells were seeded at 5×10^4 cells/mL in a 98-well plate, and the sample was added and cultured for 48 hours. CCK-8 reagent was added and cultured for 3 hours, and the absorbance at 450 nm was measured.

Quantitative Analysis of AGEs

The protein concentration was measured using the ELISA method. The glycation reaction solution, which had been reacted for 8 weeks as described above, was placed in an 8 mm cellulose tube (Fujifilm Wako) and dialyzed in 1 M PBS at 4°C for 48 hours. After dialyzation, the protein concentration of the reaction solution was measured using a DC protein assay kit. The reaction solution was placed in a 96-well plate at a concentration of 100 ng protein/well and allowed to stand at room temperature for 1 hour. The supernatant of the reaction solution was removed, washed with washing buffer (0.001% triton-X, phosphate buffer), and blocking buffer (0.5% gelatin, 50 mM phosphate buffer) was added and allowed to stand for 1 hour. The supernatant of the reaction solution was removed, washed with washing buffer, and 1μM primary antibody PEN-12 (Funakoshi Co., Ltd.) was added and allowed to stand for 1 hour. The supernatant was removed, washed with washing buffer, and 0.06% secondary antibody Mouse-Goat IgG (Funakoshi Co., Ltd.) was added and allowed to stand for 1 hour. A citrate substrate solution containing o-phenylenediamine dihydrochloride (OPD tablets: Fujifilm Wako) was added, and the mixture was allowed to stand for 5 minutes. The absorbance at 492 nm was then measured.

Anti-Glycation Test

Extracts were added to 2 mg/mL human serum albumin (HSA: Sigma-Aldrich, Co.,LLC) and 37 mM glucose and reacted at 50°C for 8 weeks. Fluorescence intensity was measured weekly, and the percentage increase relative to the AGE amount on day 0 was calculated.

Antioxidant Test

The extract was reacted with 0.016% 1,1-diphenyl-2-picrylhydrazyl (DPPH: Tokyo Chemical Industry, Co.,LTD.)/EtOH in 0.1M acetate buffer, and absorbance was measured (510 nm). The antioxidant activity (radical removal rate) was determined based on the percentage decrease in absorbance compared to the reaction with distilled water and DPPH.

Results and Discussion

In this study, we investigated the antioxidant activity, anti-glycation activity, and melanin production inhibitory effect of *Cerasus* petal extract to evaluate its potential as a skin aging prevention material.

This study evaluated whether *Cerasus* petal extracts could inhibit melanin production, one of the causes of skin dullness and blemishes. First, the toxicity of the extracts to B16 cells was evaluated. EtOAc extract showed a toxicity of 114 ± 1.02 % at 1000 μg/mL, and EtOH extract showed a toxicity of 110 ± 2.02 % at 1000 μg/mL. No significant difference was observed between the group without extract administration (0 μg/mL) (Figure 1). After confirming that neither extract showed toxicity to B16 cells, tests were conducted in a concentration range of 1000 μg/mL or less. The rate of α-MSH-induced melanin production was determined, with melanin production in B16 cells under α-MSH induction set as 100%. EtOAc extract yielded 85.5 ± 2.4 % at 500 μg/mL, and EtOH extract yielded 58.0 ± 3.3 % at 500 μg/mL (Figure 2). EtOAc extract did not show significant inhibition of melanin production at any concentration, but a decreasing trend in melanin was observed at 500 μg/mL. EtOH extract inhibited melanin production at concentrations above 31.25 μg/mL (Student's T-test $p<0.05$), and at concentrations above 62.5μg/mL it showed inhibition comparable to kojic acid ($p<0.01$) (Figure 2). EtOH extract showed concentration-dependent inhibition of melanin production (Pearson correlation coefficient r=-0.7286).

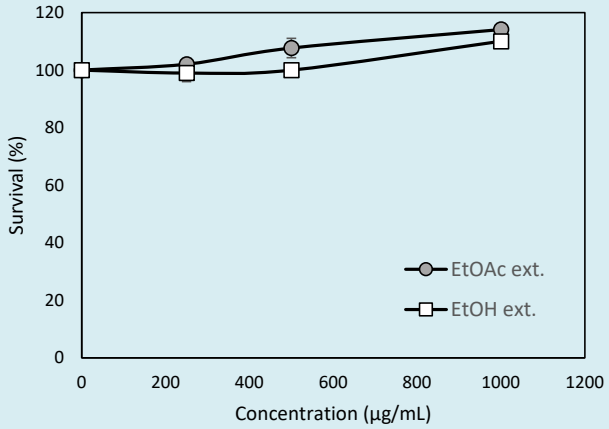

Figure 1: Cytotoxicity of Someiyoshino extract.

The cell growth rate was calculated when the number of viable cells in the untreated cells was set to 100% viability. Student's t-test showed no significant difference.

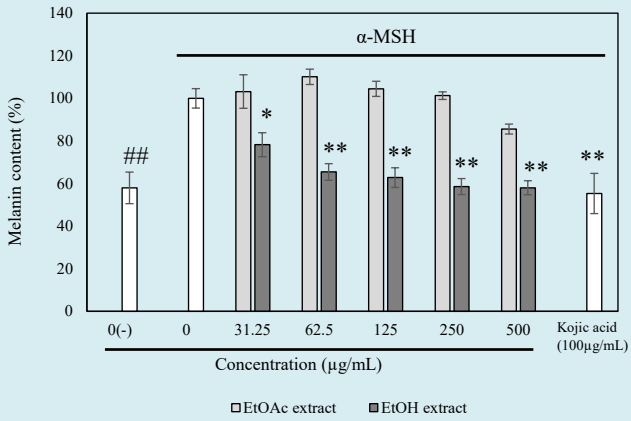

Figure 2: Melanin production inhibitory activity of Someiyoshino extract (EtOAc extract).

There was a significant difference in melanin production in the presence and absence of α-MSH ($p<0.01$). In the EtOH extract, a significant difference was observed at 31.25 μg/mL ($p<0.05$) and at 62.5 μg/mL or higher ($p<0.01$). The Pearson correlation coefficient between concentration of EtOH extract and melanin content rate was r=-0.7286. Statistical significances were represented as follows: *, $p< 0.05$; **, $p< 0.01$ compared to untreated (+) α-MSH; ##, $p< 0.01$ compared to with (+) α-MSH.

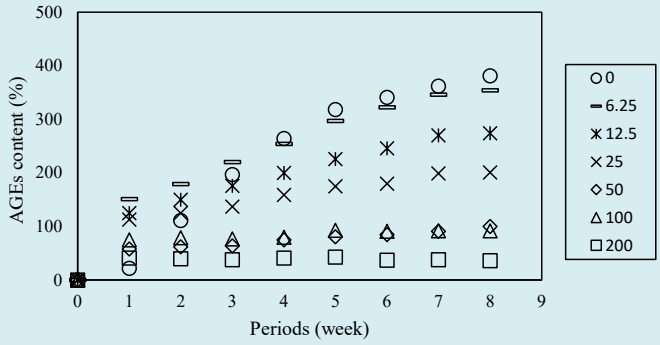

Figure 3: AGEs formation of EtOH extract in HSA/Glucose glycation reaction.

An increase in AGEs over time was observed in the HSA and Glucose controls. The Pearson correlation coefficients were r=0.9721 at 0μg/mL, r=0.9468 at 6.25μg/mL, r=0.9352 at 12.5μg/mL, r=0.8893 at 25μg/mL, r=0.8776 at 50μg/mL, r=0.7172 at 100μg/mL, and r=0.4609 at 200μg/mL.

AGEs are produced by the glycation reaction between HSA and glucose. Among AGEs, there are brown AGEs, which, when produced and accumulated in the skin, lead to skin dullness and blemishes. Since brown AGEs are fluorescent, the amount of brown AGEs produced was calculated by measuring the fluorescence intensity. The amount of AGEs produced on day 0 of the glycation reaction was set to 0 %, and the AGEs production rate was determined. In the group without EtOH extract administration (0 μg/mL), a time-dependent increase in AGEs was observed over 8 weeks (Pearson correlation coefficient r=-9721) (Figure 3), with AGEs increasing to 381 % at week 8 (Figure 3). With 200 μg/mL of EtOH extract, AGEs increased by 38-43 % during the 8 week reaction (Figure 3). Furthermore, the AGEs production inhibition rate by EtOH extract was calculated, with the amount of AGEs produced at 0 μg/mL at week 8 set to 100%. Significant inhibition of AGEs production was shown at 12.5 μg/mL or higher ($p<0.01$) (Figure 4). A 28.4 % inhibition of AGEs formation was confirmed at 200 μg/mL (Figure 4).

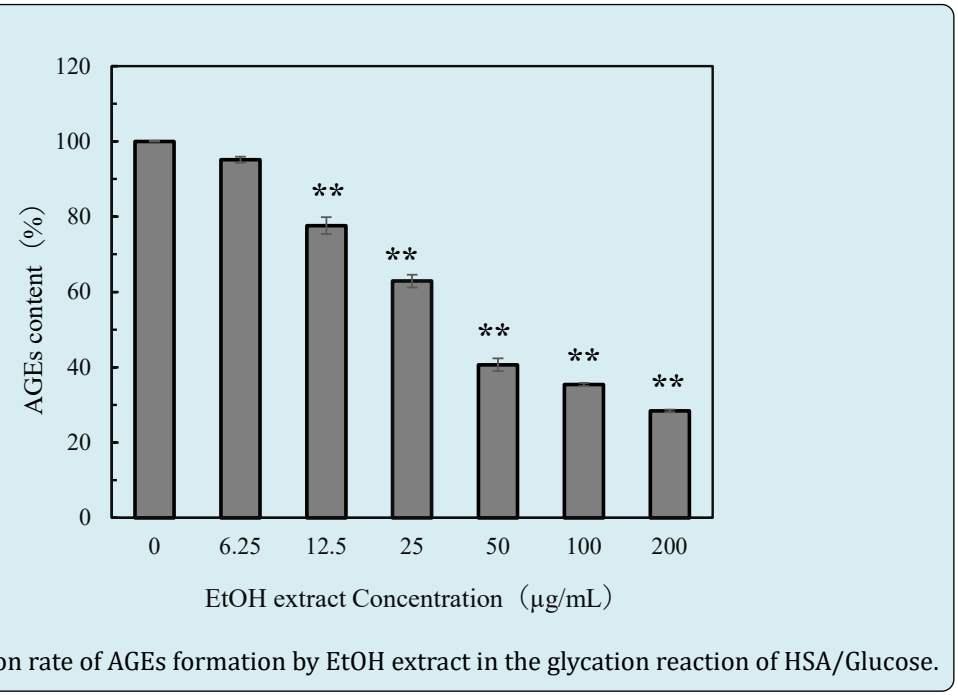

Figure 4: Inhibition rate of AGEs formation by EtOH extract in the glycation reaction of HSA/Glucose.

The AGEs suppression rate by EtOH extract was shown, with the untreated sample at 8 weeks after the glycation reaction representing 100% AGEs formation. Significant suppression of AGEs formation was confirmed with EtOH extract of 12.5 μg/mL or higher ($p<0.01$). Statistical significances were represented as follows: **, $p< 0.01$ compared to untreated.

AGEs are formed through chemical reactions between proteins and sugars. During these reactions, sugars bind to two or more proteins, forming crosslinks. Because crosslinked AGEs cause a loss of skin elasticity, they are considered to be deeply related to skin aging. The amount of pentosidine produced by the glycation reaction between HSA and glucose was set to 100%, and the inhibition of pentosidine production by EtOH extract was investigated. While no statistically significant difference was observed, pentosidine production was suppressed to 88.8 ± 7.0 % with a 1000 μg/mL EtOH extract (Figure 5).

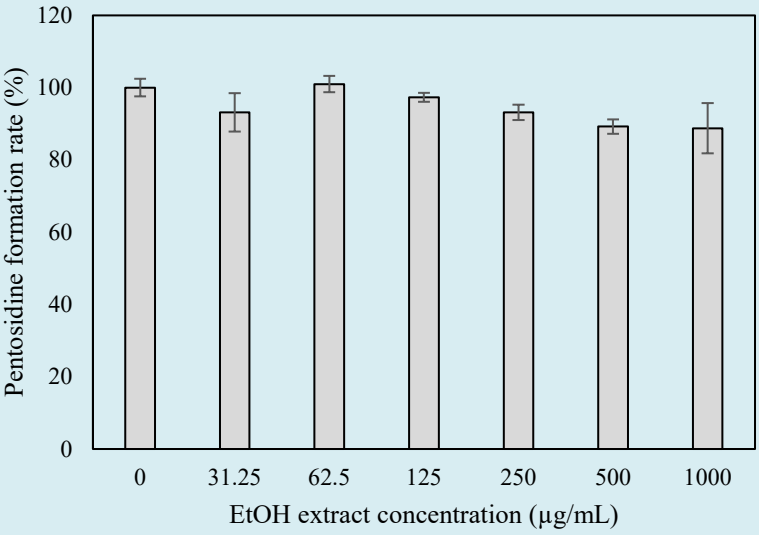

Figure 5: Inhibition of pentosidine formation by EtOH extract.

The suppression of pentosidine production by EtOH extract was shown, with the untreated group (0 μg/mL) set as 100%. No significant difference was observed.

Furthermore, it is known that AGEs are generated by aldehydes derived from lipid peroxides, and lipid peroxides are generated by reactive oxygen species. Therefore, we evaluated the antioxidant activity using the DPPH method and investigated whether it contributes to skin aging through its antioxidant activity. As a result, the EtOH extract showed radical scavenging of 81 ± 1.0 % at 500 μg/mL ($p<0.01$) (Figure 6). It also showed radical scavenging ability of 10 ± 2.3 % at 100 μg/mL ($p<0.05$), but it was confirmed that the antioxidant activity of the *Cerasus* extract is limited to high doses.

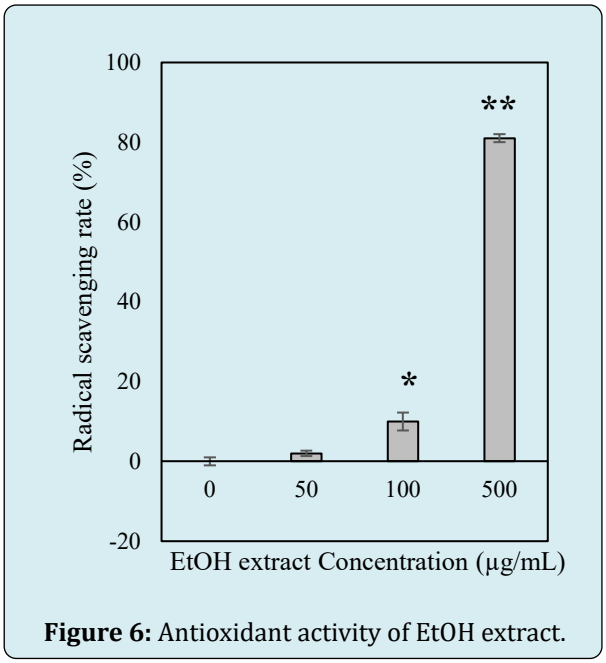

Figure 6: Antioxidant activity of EtOH extract.

DPPH radical scavenging activity was determined by setting the removal rate of DPPH radicals in the untreated extract group to 0%. Significant radical scavenging activity was observed with the 500 μg/mL EtOH extract ($p<0.01$). Statistical significances were represented as follows: *, $p< 0.05$; **, $p< 0.01$ compared to untreated.

Oxidative stress is deeply involved in the formation of age spots and wrinkles in the skin. ROS increases with UV exposure, inducing DNA damage and lipid peroxidation, and has been reported to cause collagen degradation by promoting MMP expression via the AP-1 pathway [2-5]. *Cerasus* extract shows high anti-glycation activity (Figure 2), suggesting that *Cerasus* extract may suppress ROS production and further contribute to preventing skin aging. In addition, *Cerasus* extract showed antioxidant activity (Figure 6). It is known that various proteins, including collagen and elastin, which do not have a corresponding effect on aging, are denatured by glycation [13]. AGEs produced by glycation accumulate, causing yellowing and a decrease in skin elasticity due to collagen cross-linking [6,14-15], and have been reported to be involved in the progression of skin aging. It has also been reported that adding AGEs to cultured pigment cells significantly increases melanin production [16]. Since oxidative stress promotes glycation and increases AGEs [6], it is thought that controlling glycation is deeply involved in skin aging. In a melanin production inhibition test using B16 cells, activity was observed with EtOH extract only. Extraction methods using EtOAc can distribute a large amount of polyphenols. On the other hand, 80 % EtOH can extract a wide range of compounds, including lipids, terpenes, alkaloids, polyphenols, and sugars. Inhibition of melanin production by EtOAc extract was confirmed at concentrations of 500 μg/mL or higher, but inhibition was confirmed at concentrations of 31.25 μg/mL or higher for EtOH extract, which is thought to have extracted many compounds. Since there are reports that a mixed extract of four herbs has stronger glycation inhibitory activity than herb extracts alone [17], the possibility of a combined effect by multiple compounds in the EtOH extract should be considered. *Cerasus* belong to the Rosaceae family, and Rosaceae plants, including *Cerasus*, contain polyphenols, anthocyanins, and flavonols [11]. A correlation has been observed between these substances and antioxidant activity, but it has been reported that flavonols, rather than polyphenols, are the components that contribute to antioxidant activity [11]. It is known that antioxidant activity and melanin production inhibitory activity do not necessarily coincide, and the isolation and identification

of the active components contained in this extract will be a challenge for the future.

Skin aging progresses due to factors such as aging and UV exposure, leading to wrinkles, sagging, and loss of elasticity. Photoaging caused by UV radiation is particularly well-known as a major extrinsic aging factor. In recent years, it has been reported that skin aging is deeply involved not only in melanin accumulation but also in the decline of dermal function due to the accumulation of ROS and AGEs. This study showed that *Cerasus* petal EtOH extract exhibited strong antioxidant and anti-glycation activity, as well as weak pentosidine production inhibitory activity, suggesting its potential to contribute to preventing skin aging by suppressing oxidative and glycation stress. Further research is needed regarding the effects of *Cerasus* extract on skin aging, including anti-glycation tests using collagen models, collagen acidity evaluation using fibroblasts, and MMP expression analysis. *Cerasus* petal EtOH extract may have potential use in cosmetics and health foods designed as anti-aging ingredients for skin and body.

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Ethical Approval

It is not applicable.

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