

# Evaluation Biological Activities of *Diphylleia grayi* Extract as a Natural Cosmetic Ingredient

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## Abstract

**Purpose:** In this study, we investigated the biological activities of *Diphylleia grayi* extract as a natural cosmetic ingredient. **Methods:** The antioxidant activities of *Diphylleia grayi* extract was measured through 2,2-diphenyl-1-(2,4,6-trinitrophenyl)hydrazinyl (DPPH), 2,2'-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid (ABTS) scavenging assay and Fluorescence recovery after photobleaching (FRAP) assay. We further investigated whether *Diphylleia grayi* extract could reduce the intracellular reactive oxygen species (ROS) using dichlorofluorescein diacetate (DCF-DA) fluorescence. To investigate the whitening effect, we conducted tyrosinase inhibition assay and L-DOPA oxidation inhibition assay. Furthermore, we performed collagenase inhibition assay to evaluate the anti-wrinkle activity of *Diphylleia grayi* extract. **Results:** *Diphylleia grayi* extract showed strong antioxidant activities on DPPH, ABTS radical scavenging assay. Furthermore, *Diphylleia grayi* extract decreased the intracellular ROS on skin normal cells. *Diphylleia grayi* extract has also inhibition activities of tyrosinase, L-DOPA oxidation and collagenase. **Conclusion:** Therefore, *Diphylleia grayi* extract was identified as a promising natural cosmetic ingredient which has antioxidant, whitening and anti-wrinkle properties.

**Keywords:** *Diphylleia grayi*, Reactive oxygen species, Antioxidant, Tyrosinase, Collagenase

## Introduction

As the first organ that contact the external environment, skin is exposed to various aging factors including ultraviolet rays (UVR), air pollutants, heavy metals, and cigarette smoke (Krutmann *et al.*, 2017). The prolonged continuous exposure of these exogenous factors to skin enhances the release of reactive oxygen species (ROS). Then, it trigger the activation of matrix metalloproteinases (MMPs) and inflammatory responses, hence damaging the extracellular matrix (ECM) components such as, collagen fibres and natural moisture factors (NMF) (Rinnerthaler *et al.*, 2015). In addition, it is well studied that ROS play an important role of melanin production in human melanocyte (Denat *et al.*, 2014). In fact, it is reported that hydrogen peroxide produced by UV irradiation, activates tyrosinase and promotes melanin synthesis in melanocyte

(Brenner & Hearing, 2007). Although the melanin plays an essential role in protecting the skin against damaging effects of UV radiation, overproduction of melanin is sometimes regarded as considered as a metaphor of aging in Asian countries (Samson *et al.*, 2011). Furthermore, it has been well studied that oxidative stress generated form UV light induces the expression of collagenase and elastase in skin cells, which are involved in wrinkle formation (Pittayapruek *et al.*, 2016). Therefore, cosmetic ingredients which have ROS scavenging activities have been most commonly developed for the purpose of preventing skin aging.

Nature derived ingredients have been applied in cosmetic formulation for many years. As well as they have skin beneficial activities such as antioxidant, anti-inflammation, whitening and anti-wrinkle effects, they become one of the marketing points of some cosmetic products because of their

unique stories (Ribeiro *et al.*, 2015). Recently, consumers' interest in natural ingredients has increased as the risk of chemical ingredients in cosmetics has emerged. Furthermore, the concept of natural and organic cosmetics was stipulated in the Korean Cosmetics Act, increasing the need for effective functional natural materials to replace chemicals (Kim, 2020). Therefore, it is necessary to develop natural-derived cosmetic ingredients that are safe and effective against chemicals.

*Diphylleia grayi* is in the family *Berberidaceae* and it is commonly known as the skeleton flower. Usually, *Diphylleia grayi* has white petals, but when it rains, the color of its petals becomes translucent (Yong *et al.*, 2015). It is native to Japan and has rarely been reported about biological effectiveness. It is reported that podophyllotoxin and diphyllin have been found in *Diphylleia* species and natural-derived podophyllotoxin has been reported to have anti-cancer and anti-viral efficacy (Broomhead *et al.*, 1990). However, it has not been investigated the cosmetic effectiveness of *Diphylleia grayi* extract.

In this study, we firstly report the antioxidant activities of *Diphylleia grayi* ethanol extract. Moreover, we investigated tyrosinase, L-DOPA oxidation and collagenase inhibition activities of *Diphylleia grayi* ethanol extract. Through this research, we evaluated the cosmetic effectiveness of *Diphylleia grayi* extract.

## Methods

### 1. Reagents and chemicals

2,2-Diphenyl-1-picrylhydrazyl and 2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt were purchased from Alfa Aesar (USA) 2',7'-dichlorofluorescein diacetate (DCF-DA), 2,2'-azobis dihydrochloride(AAPH), potassium ferricyanide,  $\alpha$ -MSH, L-Tyrosin, L-DOPA, mushroom tyrosinase, arbutin, azo dye-impregnated collagen and collagenase from *Clostridium histolyticum* were purchased from Sigma Aldrich (USA).

### 2. Preparation *Diphylleia grayi* Extract

The whole plant parts of *Diphylleia grayi* were dried at 45°C for 2 h in an oven and then, it were finely ground into powder. 70% ethanol was poured into the powder and incubates at 140 rpm for one day using the shacking incubator. The samples were then filtered through filter paper to separate the residue.

After that, the filtrated sample was evaporated by the vacuum rotary evaporator. Finally, *Diphylleia grayi* extract was obtained after freeze-drying.

### 3. Cell culture and cytotoxicity of *Diphylleia grayi* extract

HaCaT (human keratinocyte), HDF (human dermal fibroblast), CCD-986sk (human skin fibroblast) were cultured in Dulbecco's modified Eagle's medium (DMEM; WelGENE, Korea) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin at 37°C in a 5% CO<sub>2</sub> incubator. Cytotoxicity of *Diphylleia grayi* extract was evaluated using cell proliferation kit I (Applied Science, Germany). 1 × 10<sup>5</sup> Cells were plated per well in 96-well plates and serial dilution samples were treated with for 48 h. After that, add MTT in medium to a final concentration of 0.5 mg/mL and re-incubated at 37°C. After 4h, MTT formazan was dissolved with DMSO and cell viability was measured at 540 nm using a microplate reader (Spectra Max, USA).

### 4. DPPH radical scavenging assay

DPPH radical scavenging assay was performed as previously described (Lee *et al.*, 2018). 500  $\mu$ L of DPPH solution (0.1 mM) was mixed with 500  $\mu$ L of the sample and incubated 30 min in dark room. The DPPH radical scavenging activity was measured at a wavelength of 517 nm using a microplate reader (SpectraMax i3; Molecular devices, USA).

### 5. ABTS Radical Scavenging Assay

ABTS radical scavenging assay was performed as previously described (Lee *et al.*, 2018). ABTS (7 mM) stock solution with 2.45  $\mu$ M potassium persulfate was prepared and stored in the dark at room temperature for 12–16 h before use. The solution was diluted in PBS and equilibrated to obtain an absorbance of 0.700 at 734 nm. 0.2 mL of the sample and blank were mixed with 0.8 mL of ABTS solution. Finally, the absorbance was measured at 734 nm in a microplate reader (SpectraMax i3; Molecular devices, USA) against a blank after a 5 min of reaction time at room temperature.

### 6. Ferric Reducing Antioxidant Power (FRAP) Assay

Ferric reducing antioxidant power of extract was determined using the method previously described (Lee *et al.*, 2018). Firstly, *Diphylleia grayi* extract was mixed with 0.2 M sodium phosphate buffer (pH 6.6) and 1% potassium ferricyanide. The

mixture was incubated at 50°C for 20 min. After that, 10% trichloroacetic acid (2.5 mL) was then added to stop the reaction and centrifuged at 3,000 g for 10 min. The supernatant was collected and mixed with distilled water and 1% ferric chloride. The FRAP was measured at 700 nm using a microplate reader (SpectraMax i3; Molecular devices, USA).

### 7. Intracellular ROS Detection

Intracellular ROS was detected using DCF-DA fluorescence. Cells were incubated with 10 mM AAPH for 1 h in the presence or absence of sample, and then stained with 10 µM DCF-DA solution for 15 min. After that, the cells were washed with PBS and DCF-DA fluorescence was detected by fluorescence microplate reader (SpectraMax i3; Molecular devices, USA) with excitation and emission of 495 nm and 529 nm, respectively.

### 8. In vitro tyrosinase Inhibition Assay

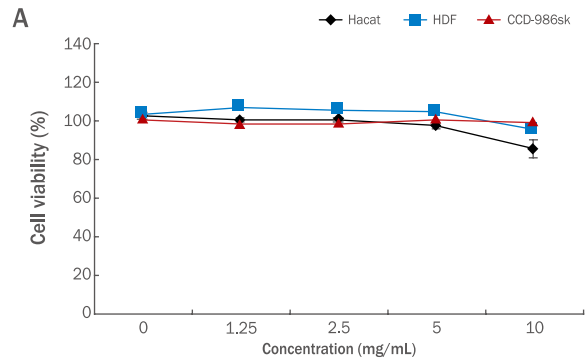
Tyrosinase inhibition assay was performed as previously described (Lee *et al.*, 2018). L-Tyrosine was used as substrate. The reaction mixture consisted of 0.1 M Sodium phosphate buffer (pH 6.5), purified mushroom tyrosinase and the sample. After adding 1.5 mM of L-Tyrosin solution, incubate 10–15 min at 37°C. The tyrosinase inhibition activity was measured at 490 nm using a microplate reader (SpectraMax i3; Molecular devices, USA).

### 9. In vitro DOPA oxidation inhibition assay

The reaction mixture is consisted of 0.1 M sodium phosphate buffer (pH 7.0), purified mushroom tyrosinase and the sample. After adding 0.06 mM of L-DOPA solution, *in vitro* DOPA oxidation inhibition was measured at 475 nm using a microplate reader (SpectraMax i3; Molecular devices, USA).

### 10. Collagenase inhibition assay

Collagenase inhibition assay was performed as previously described (Jiang *et al.*, 2007). 1mg of azo dye-impregnated collagen was washed twice with PBS. After then, mix with 800 µL of 0.1 M Tris-HCl (pH 7.0), 100 µL of 200 units/mL collagenase (stock solution), and 100 µL sample and incubated at 37°C. After 1 h incubating, the reaction mixture was centrifuged at 3000 rpm for 10 min and the absorbance of the supernatant was detected at 550 nm using a microplate reader (SpectraMax i3; Molecular devices, USA).



**Figure 1. Cytotoxicity of *Diphylleia grayi* extract.** Cell viability of the *Diphylleia grayi* extract on normal skin cells.

### 11. Statistical Analysis

All experiments in this study performed more than three times and were presented as average values. Statistically significant was analyzed based on Student's *t*-test and determined to be statistically significant if the *p* value was less than 0.05 (\**p*<0.05, \*\**p*<0.01).

## Results and Discussion

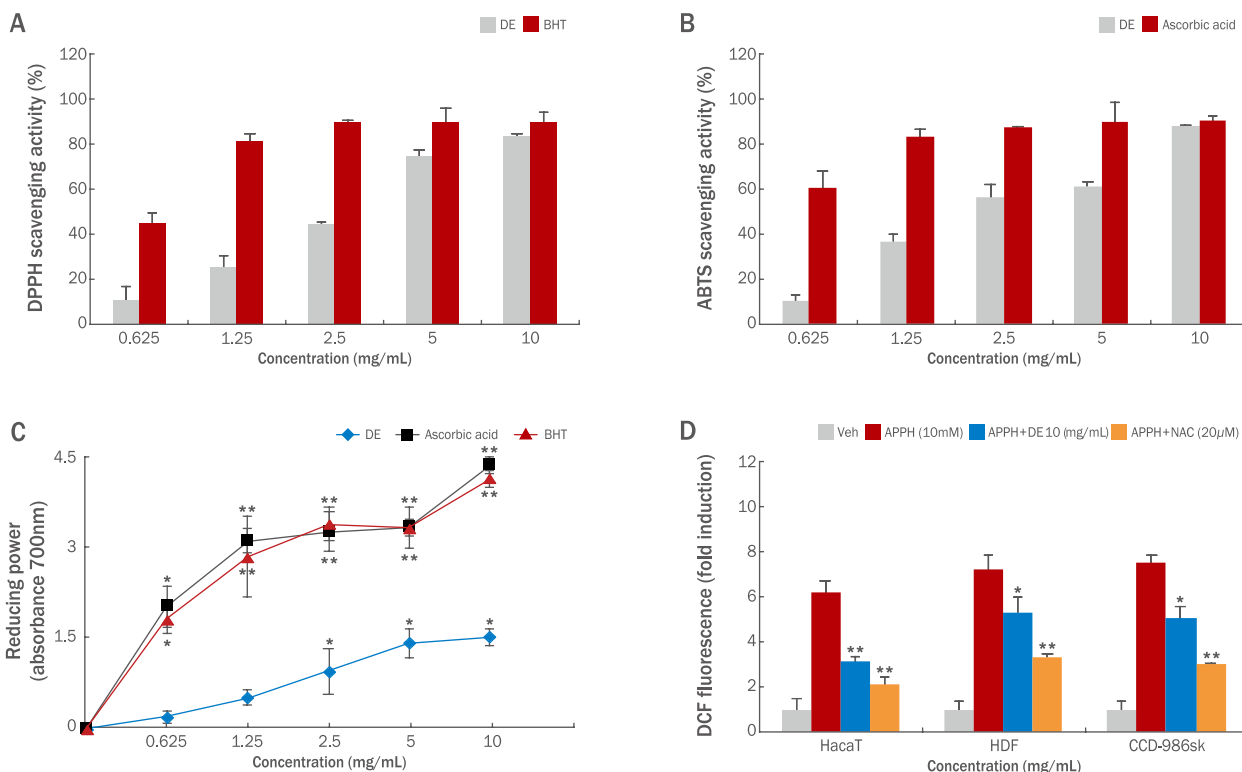
### 1. Cytotoxicity of *Diphylleia grayi* extract

Because cosmetic products are daily exposed to skin, it is important to check the cytotoxicity of ingredients of cosmetics. We investigated the cytotoxicity of *Diphylleia grayi* extract through the MTT assay on normal skin cells. We found that *Diphylleia grayi* extract dose not reduced cell viabilities, even at high dose (10 mg/mL) (Figure 1). Based on this result, we evaluated the cosmetic effectiveness below the concentration of 10 mg/mL.

### 2. Antioxidant activities of *Diphylleia grayi* extract

Oxidative stress in skin plays a major role in aging process. The oxidative stress is generated by reactive oxygen species that can cause significant damage to biomolecules and it leads to disruption of skin barrier and wrinkle formation (Rinnerthaler *et al.*, 2015). Hence, natural products which have antioxidant properties used many in cosmetic formulation to expect the prevention effect of skin aging. Here, we investigated the antioxidant activities of *Diphylleia grayi* extract through DPPH, ABTS radical scavenging assay and FRAP assay and

## Cosmetic Effectiveness of *Diphylleia grayi* Extract



**Figure 2. Antioxidant effects of *Diphylleia grayi* extract.**

(A) DPPH radical scavenging activity of *Diphylleia grayi* extract. (B) ABTS radical scavenging activity of *Diphylleia grayi* extract. (C) FRAP reducing activity of *Diphylleia grayi* extract. (D) The intracellular ROS levels in AAPH-treated normal skin cells in a presence of *Diphylleia grayi* extract. DE, *Diphylleia grayi* extract. The Student's *t*-test was performed to determine statistical significance (\* $p < 0.05$ , \*\* $p < 0.01$ ).

the results are shown at figure 2. The *Diphylleia grayi* extract could effectively decrease DPPH and ABTS radical in a dose dependent manner. The *Diphylleia grayi* extract showed 84.21% DPPH radical scavenging activity at 10 mg/mL. ABTS radical scavenging activity of the *Diphylleia grayi* extract was shown 88.21% at 10 mg/mL. These radical scavenging effects of the *Diphylleia grayi* extract were equivalent to those of similar to those of as positive controls (ascorbic acid and BHT). It is shown the  $IC_{50}$  values of DPPH, ABTS assay in Table 1, FRAP reducing activity of the *Diphylleia grayi* extract was increased in

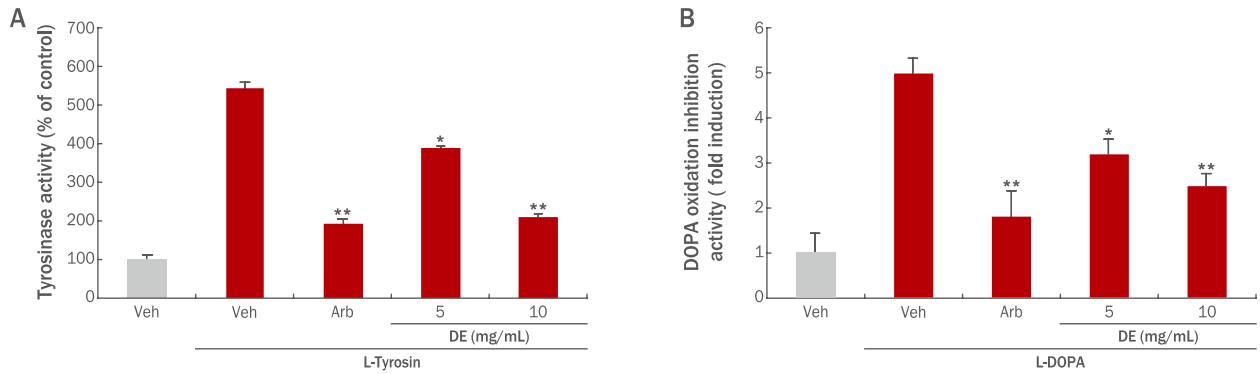
a concentration dependent manner, but it was not as powerful as positive controls.

We further investigated whether the *Diphylleia grayi* extract could reduce intracellular ROS in skin cells using DCF-DA fluorescence. The oxidative stress model was followed as previously reported. The ROS was induced by 2,2'-azobis (2-amidinopropane) dihydrochloride (AAPH) and the intracellular ROS was measured in a presence of *Diphylleia grayi* extract. The *Diphylleia grayi* extract was significantly reduced the oxidative stress in human normal skin cell.

**Table 1. Antioxidant activities of *Diphylleia grayi* extract ( $IC_{50}$  value)**

Samples	$IC_{50}$ value (mg/mL)	
	DPPH scavenging	ABTS scavenging
<i>Diphylleia grayi</i> extract	3.23±0.13**	3.42±0.35**
BHT	0.72±0.02**	-
Ascorbic acid	-	0.56±0.04**

Data are means±S.D. of 3 experiments. \*\* $p < 0.01$  significantly different as compared to negative control group. DPPH, 2,2-diphenyl-1-(2,4,6-trinitrophenyl)-hydrazinyl. ABTS, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid).



**Figure 3. Whitening effects of *Diphylleia grayi* extract.**

(A) *In vitro* tyrosinase inhibition activity of *Diphylleia grayi* extract (B) *In vitro* DOPA oxidation inhibition activity of *Diphylleia grayi* extract. Veh, vehicle, Arb, arbutin (200 µg/mL), DE, *Diphylleia grayi* extract. The Student's *t*-test was performed to determine statistical significance (\* $p < 0.05$ , \*\* $p < 0.01$ ).

Together, the *Diphylleia grayi* extract has strong antioxidant activity at the intracellular and extracellular conditions.

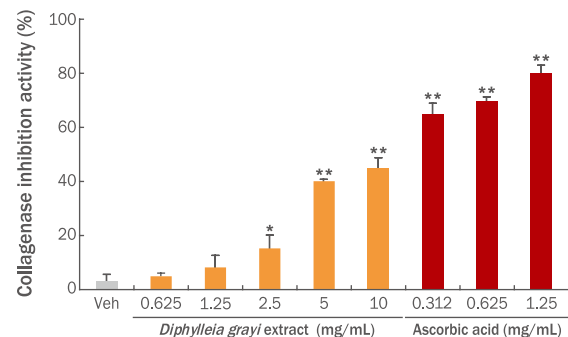
### 3. Whitening effects of *Diphylleia grayi* extract

Tyrosinase is a key enzyme, which catalyzes a rate-limiting step of the melanin synthesis. During the melanogenesis, L-tyrosine and L-DOPA served as substrates of the tyrosinase and they are oxidated to 3,4 dihydroxyphenylalanine (L-DOPA) and DOPAquinone (Shim, 2019). Therefore, inhibition of tyrosinase activity is the most prominent approach for the development of melanogenesis inhibitors (Pillaiyar *et al.*, 2017). In fact, it is well researched that natural products have activities of tyrosinase inhibition. Here, we investigated the whitening effect of *Diphylleia grayi* extract through the *in vitro* tyrosinase inhibition assay and *in vitro* DOPA oxidation inhibition assay and the results are shown at Figure 3. We found that the *Diphylleia grayi* extract inhibited tyrosinase activity at over 5 mg/mL concentration and also decreased L-DOPA oxidation rate. The whitening effect of *Diphylleia grayi* extract appear to related its antioxidant activities. Indeed, it is well researched natural antioxidants have anti-melanogenic activities. Based on these results, we found that *Diphylleia grayi* extract has skin whitening effect.

### 4. Collagenase inhibition activity of *Diphylleia grayi* extract

Collagen is a fibrous protein of conjunctive and connective tissues in the human body. Besides maintaining the skin structure, collagen establishes elasticity and keeps moisture in the skin (Aguirre-Cruz *et al.*, 2020; Kim *et al.*, 2020). Therefore, preventing collagen disruption is considered an

effective way to conserve younger skin in cosmetic industries. Collagenase is responsible for cleavage of collagen and is necessary for remodeling of the extracellular matrix and facilitating the migration of keratinocytes, especially in injured skin (Rohani & Parks, 2015). However, the induction of collagenase by UV light is considered the major reason of photo-aging in the skin (Pittayapruerk *et al.*, 2016). Here, we investigated collagenase inhibition activity of *Diphylleia grayi* extract using the azo dye-impregnated collagen and collagenase from *Clostridium histolyticum*. As shown in Figure 4, addition of increased concentration of *Diphylleia grayi* extract resulted in decreased collagenase activity. The collagenase inhibition activity was measured 45.31% at 10 mg/mL of *Diphylleia grayi* extract.



**Figure 4. Anti-collagenase effects of *Diphylleia grayi* extract.**

Collagenase inhibition activity of *Diphylleia grayi* extract. The Student's *t*-test was performed to determine statistical significance (\* $p < 0.05$ , \*\* $p < 0.01$ ).

## Conclusion

It is well researched that "intrinsic" and "extrinsic" factors results in skin aging (Ryu *et al.*, 2019). The intrinsic skin aging is genetically determined process that generated by naturally occurring free radicals and hormonal changes. On the other hand the extrinsic skin aging is caused by exogenous environment damaging factor such as, UV light, various pollutions (Farage *et al.*, 2008). The extrinsic damaging factor triggers the production of ROS and it is considered the major reason of skin aging (Poljšak *et al.*, 2012). Unlike intrinsic skin aging, extrinsic skin aging could be regulated through the inhibition of oxidative stress so that it has continuously researched to find effective antioxidants in cosmetic industries (Zhang *et al.*, 2018). In this study, we firstly report the antioxidant effect and cosmetic effectiveness of the *Diphylleia grayi* extract. Through the ROS scavenging activity assays (DPPH, ABTS, FRAP) and measuring the oxidative stress reducing effect on skin cells, it has been found that *Diphylleia grayi* extract has strong antioxidant activities. In conjunction with these antioxidant properties of *Diphylleia grayi* extract, we found that *Diphylleia grayi* extract has the effect of inhibiting tyrosinase and collagenase activity. Taken together, *Diphylleia grayi* extract was identified as a promising natural cosmetic ingredient which has antioxidant, whitening and anti-wrinkle properties.

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## Author's contribution

GL designed all experimental investigations. GL, AY and CP collected all data of this manuscript. MSJ and GYL assisted with experimental design. GL oversaw the project, and contributed to all aspects of analysis and experimental design. GL wrote the manuscript with assistance from AY and CP.

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## 국문초록

### *Diphylleia grayi* 추출물의 천연 화장품 원료로서의 생리활성 효능 평가

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**목적:** 본 논문은 *Diphylleia grayi* 추출물의 천연 화장품 소재로서의 생리활성효능을 평가하기 위해 수행 되었다. **방법:** *Diphylleia grayi* 추출물의 항산화 효능을 평가하기 위해 DPPH, ABTS, FRAP 법을 수행하였다. 그리고, *Diphylleia grayi* 추출물의 세포 내 산화적 스트레스 감소 효과를 확인 하기 위하여 DCF-DA 형광을 이용하여 항산화 능력을 평가 하였다. 더불어 *Diphylleia grayi* 추출물의 미백 및 항주름 효능을 확인하고자 타이로시나아제와 콜라게나아제 활성 저해 실험을 수행하였다. **결과:** *Diphylleia grayi* 추출물은 DPPH 와 ABTS 라디칼을 소거하는 강한 항산화 능력을 보였으며, 세포 내에서 발생하는 산화적 스트레스를 효과적으로 감소시켰다. 게다가, 세포독성이 없는 농도에서 타이로시나아제와 콜라게나아제의 활성을 저해함이 확인 되었다. **결론:** 본 연구 결과를 종합하여, *Diphylleia grayi* 추출물은 항산화, 미백, 항주름 효능을 가진 천연화장품 소재임이 확인되었다.

**핵심어:** *Diphylleia grayi* 추출물, ROS, 항산화, 타이로시나아제, 콜라게나아제

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## 中文摘要

### 作为天然化妆品成分 *Diphylleia grayi* 提取物的生物活性评价

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**目的:** 探索作为天然化妆品成分的*Diphylleia grayi*提取物的生物活性。**方法:** 采用DPPH法, ABTS清除法和FRAP法测定*Diphylleia grayi*提取物的抗氧化活性。我们进一步研究了使用DCF-DA荧光法检测*Diphylleia grayi*提取物是否能减少细胞内ROS。为了研究增白效果, 进行了酪氨酸酶抑制测定和L-DOPA氧化抑制测定。此外, 进行了胶原酶抑制试验, 以评价白花前胡提取物的抗皱活性。**结果:** *Diphylleia grayi*提取物对DPPH, ABTS自由基清除试验具有较强的抗氧化活性。此外, *Diphylleia grayi*提取物减少皮肤正常细胞上的细胞内ROS。*Diphylleia grayi*提取物还具有酪氨酸酶, L-DOPA氧化和胶原酶的抑制活性。**结论:** 因此, *Diphylleia grayi*提取物被鉴定为具有抗氧化, 增白和抗皱特性的有前途的天然化妆品成分。

**关键词:** *Diphylleia grayi*, 活性氧, 抗氧化剂, 酪氨酸酶, 胶原酶

