pISSN 1226-2587
J. Soc. Cosmet. Scientists Korea eISSN 2288-9507
Vol. 40, No. 1, March 2014, 29-36 http://dx.doi.org/10.15230/SCSK.2014.40.1.29

철피석곡의 원괴체상구체 및 다신초 추출물의 생리활성에 관한 비교 연구

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(2013년 8월 22일 접수, 2013년 9월 9일 수정, 2013년 10월 14일 채택)

Comparative Study of Protocorm-like body and Multiple Shoots from *Dendrobium Candidum* on Biological Activities

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(Received August 22, 2013; Revised September 9, 2013; Accepted October 14, 2013)

요 약: 철피석곡은 중국에서 전통의약식물로 잘 알려진 난과 식물 중 하나이다. 본 연구에서는 생물반응장치를 이용하여 조직 배양된 철피석곡에 대하여 화장품 성분으로써 응용 가치를 평가하였다. 이미 몇몇 보고된 논문에서 철피석곡은 항암, 상처 치유 그리고 면역기능증진 등의 약리학적 활성에 대한 연구가 진행되었다. 본 연구에서는 생물반응장치를 이용하여 조직 배양된 철피석곡의 원괴체상구체 및 다신초에 대하여 화장품 성분으로서 효능, 효과를 자연에서 자란 철피석곡과 비교 평가하였다. 항산화 활성 비교 실험 결과 철피석곡의 원괴체상구체는 조직배양된 다신초 및 철피석곡 지하부 및 지상부보다 항산화 효과가 우수하였다. 조직배양된 원괴체상구체의 총 페놀 및 총 플라보노이드 성분 또한 다른 다신초나 자연에서 자란 철피석곡 추출물보다 함유량이 높음을 알 수 있었다. 다른 한편으로는 피부 미백과 관련한 효과를 비교 조사한 결과, 조직 배양된 다신초가 자연에서 자란 철피석곡 지상부, 지하부 및 조직 배양된 원괴체상구체보다 타이로 시네이즈 활성 억제 및 멜라닌 생성 억제 효과가 우수하였다. 이러한 결과들은 피부개선을 위한 화장품 성분으로서 조직배양체의 부분별 응용 가능성을 제공할 수 있을 것으로 사료된다.

Abstract: *Dendrobium candidum* is one of the well-known orchid on traditional and rare medicinal herb in China. We artificially cultured protocorm-like body and multiple shoots using the bioreactor culture system from *Dendrobium candidum* and experimented an efficacy as a cosmetic ingredient for skin care. Several studies previously have reported anti-tumor, wound healing and immunological function of *Dendrobium candidum* but other efficacies were not well known. In the present study, we investigated the cosmetic efficacy to know applicable value of protocorm-like body and multiple shoots cultured from *Dendrobium candidum* as a cosmetic ingredient. The biological activities of extracts from protocorm-like body, multiple shoots, aerial part and underground part of *Dendrobium candidum* were investigated. Results were found that extracts of protocorm-like body are superior to other extracts (underground part, aerial part and multiple shoots extracts) on anti-oxidant effect. Also, protocorm-like body extract contained the phenolic and flavonoid compounds more than aerial part, underground part and multiple shoots extracts. In addition, we investigated skin whitening effect related to whitening of skin. In tyrosinase activity and melanin synthesis assay, multiple shoots extract is superior to other extracts (aerial part, underground part and protocorm-like body), on inhibitory effect of tyrosinase activity and melanin synthesis respectively. These results indicate that the protocorm-like body and multiple shoots extracts cultured

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from *Dendrobium candidum* presents a new possibility of being applicable to skin improvement as a cosmetic ingredient.

Keywords: *Dendrobium candidum, anti-oxidation, biological activity, protocorm-like body, multiple shoots*

1. Introduction

Among the commercially important orchids, *Dendrobium* is a huge genus of orchids and accounts for about 80% of the total micropropagated tropical orchids. In these genus of orchid, *Dendrobium candidum* called a 'cheolpiseokgok' in korean is one of the most well-known traditional medicinal plants in china[1]. The stem has been used to treat throat, lung, and ophthalmic disorders. The main bioactive substances of the plant are coumarin, alkaloids, polysaccharides, phenolic and flavonoids[2-5]. The species is difficult to germinate in nature, so many reports have indicated that the populations of *Dendrobium candidum* have been extirpated[6-8]. We artificially cultured protocorm-like body and multiple shoots of *Dendrobium candidum* by using the bioreactor culture system from *Dendrobium candidum*. Previously, several studies have been reported on anti-tumor and immunological function of *Dendrobium candidum* but other efficacies as a cosmetic ingredient for skin improvement were not well known. In this study, we compare aerial and underground parts of *Dendrobium candidum* with protocorm-like body and multiple shoots of tissue-cultured *Dendrobium candidum* about biological activities such as anti-oxidant activity, whitening activity and cytotoxic activity and suggest the possibility of applying it to the cosmetics.

2. Materials and Methods

2.1. Reagents and Cell Culture

Antibodies against tyrosinase and ß-actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). 1,1-diphenyl-2-picrylhydrazyl (DPPH) and 2,2'-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid (ABTS) were purchased from sigma-aldrich (St. Louis, MO, USA). Other commercially available reagents and solvents were used as received. B16 melanoma cell lines (obtained from American Type Culture Collection) were maintained in Dulbecco's modified Eagle's medium (DMEM, WelGENE, Korea) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Gibco BRL, USA), 100 U/mL penicillin (Gibco BRL, USA) and 100 μg/mL streptomycin (Gibco BRL, USA) at 37 ℃ in a humidified atmosphere containing 5% CO_2.

2.2. Preparation of protocorm-like body and multiple shoots cultured from *Dendrobium candidum*

The tissue cultured protocorm-like body, multiple shoots, aerial and underground parts of *Dendrobium candidum* were provided by Research Center for the Development of Advanced Horticultural Technology in Chungbuk National University. Extraction : After dried, 10 g of tissue culture and different parts of *Dendrobium candidum* were immersed in 900 g with mixed solvent of 70% ethanol and 30% 1,3-butyleneglycol for sufficient time and mixed enough for 72 h in room temperature using agitator. Then, the solution was filtered with a filter paper (5C, TOYO, Japan), followed by the removal of ethanol from the filtrate at 50 ~ 60 ℃ to obtain the extract of protocorm-like body, multiple shoots, aerial and underground parts of *Dendrobium candidum*.

2.3. Cell cytotoxicity

Cells (1×10^5 cells/well) were seeded in 10% FBS/DMEM medium and incubated in 5% CO_2 incubator at 37 ℃ after treatment with extracts for indicate times. Measurement of mitochondrial activity to form purple formazan by MTT was used to assess the cytotoxicity of cell following extract treatment: MTT (0.5 mg/mL), one tenth of the original culture volume, was added to each culture and incubated for 3 h at 37 ℃ in 5% CO_2. The purple formazan formed by viable cells was dissolved by the addition of DMSO and absorbance at the dual ranges

of 540 nm and 630 nm was measured by using spectrophotometer.

2.4. Free radical scavenging activity

The DPPH (1,1-diphenyl-2-picrylhydrazyl) assay was done according to the method of Brand-Williams *et al.*[9,10]. The working solution was prepared 0.2 mM DPPH and then stored at −20 ℃ until needed. Then, the absorbance was taken at 540 nm and its anti-oxidative activity was calculated as compared with blank control.

2.5. ABTS radical cation scavenging activity

The procedure followed the method of Roberta R *et al.*[11] with some modifications for ABTS assay. The stock solutions included 7 mM $ABTS^{\cdot+}$ solution and 2.4 mM potassium persulfate solution. The working solution was then prepared by mixing the two stock solutions in equal quantities and allowing them to react for 12 h at room temperature in the dark. The solution was then diluted by mixing $ABTS^{\cdot+}$ solution with methanol. Then, the absorbance was taken at 734 nm and its $ABTS^{\cdot+}$ scavenging activity was calculated as compared with blank control.

2.6. Determination of total phenolic content

The total phenolic content in extracts was determined as described by Lee *et al.*[12]. with modifications. To 0.05 mL of the extract with 2.55 mL distilled water, 0.1 mL (2 N) folin–ciocalteu reagent was added. The solution was thoroughly mixed and allowed to stand for 6 min before 0.5 mL of 20% (w/v) sodium carbonate solution was added. The color developed after 30 min at room temperature, and the absorbance was measured at 760 nm with a UV visible spectrophotometer (UV-1650PC; Shimadzu, Kyoto, Japan). Measurements were compared to a standard curve for prepared gallic acid solution (Sigma Chemical Co., St. Louis, MO, USA).

2.7. Determination of total flavonoid content

The total flavonoid content in extracts was determined as described by Lee *et al.*[12]. To 0.25 mL of the methanolic root extract or a (+)-catechin standard solution (Sigma chemical Co., St. Louis, MO, USA), 1.25 mL distilled water and 0.075 mL 5% (w/v) of sodium nitrate solution were added. After 6 min, 0.15 mL of 10% (w/v) aluminum chloride solution was added, and the mixture was allowed to stand for 5 min before 0.5 mL of 1 M sodium hydroxide solution was added. The absorbance was measured at 510 nm with a UV visible spectrophotometer (UV-1650PC; Shimadzu, Kyoto, Japan). The absorbance measurements were integrated by comparison with an external standard calibration curve.

2.8. Tyrosinase inhibition assay

Tyrosinase activity was performed using a modification of the method reported by Vanni A *et al.*[13]. The reaction mixtures were prepared by adding 10 μL of 2000 unit mushroom tyrosinase to 170 μL of extracts dissolved in 0.1 M sodium phosphate buffer (pH 6.5). After incubation at 37 °C for 10 min and then adding 20 μL of 1.5 mM L-tyrosine. Absorbance was measured using an UV spectrophotometer at 490 nm.

2.9. Measurement of Melanin Content in B16 melanoma cells

Measurement of melanin content was performed using a modification of the method reported by Funasaka Y *et al.*[14]. B16 melanoma cells were cultured in DMEM supplemented with 10% FBS in humidified incubator at 37 ℃ under 5% CO_2 in 6 well plate at density of 2×10^4 cells/well. After cells were attached, medium was replaced with DMEM containing 10% FBS, 0.2 μM α-MSH, 2 mM theophylline and samples addition. After 3 days, trypsin was added and suspended cells were collected by centrifugation. Then cell pellets were dried and dissolved in 1 N NaOH. Melanin synthesis inhibition rates were measured 490 nm using ELISA reader.

2.10. Western Blot Analysis

Cells were treated with various dose and lysed in lysis buffer as described previously[15]. After differentiation, cells were lysed in lysis buffer. The lysates were clarified

Figure 1. The *Dendrobium candidum* cultured by the bioreactor culture system and *Dendrobium candidum* of nature. Figure 1Ⓐ and 1Ⓑ is a picture of aerial part and underground part of *Dendrobium candidum*. Figure 1Ⓒ and 1Ⓓ is a picture of protocorm-like body and multiple shoots cultured by the bioreactor culture system.

by centrifugation at 12,000 × g for 15 min at 4 ℃ and protein content was measured by 12.5% SDS-PAGE and blotted to nitrocellulose membrane (0.2 mm, Amersham, Arlington Heights, IL). The membrane was blocked with 5% non-fat skim milk in TBS-T and incubated with the primary and secondary antibodies. Immunoblots were visualized by enhanced chemiluminescence (Amersham, UK), according to the manufacturer's protocol. Densitometric analysis for protein bands was done using NIH/Scion Image program.

2.11. Statistical analysis

Data are presentated as mean ± SD. Comparisons between groups were used to the paired Student's *t*-test. Asterisk (**, $p < 0.01$; *, $0.01 < p < 0.05$) was considered to be statistically significant.

3. Results

3.1. The protocorm-like body and multiple shoots of *Dendrobium candidum* cultured by the bioreactor culture system

Tissue culture protocols have been developed for preservation of this valuable plant. Figure 1A, 1B is the picture of aerial part of *Dendrobium candidum* and underground part of *Dendrobium candidum* grown in natural. Figure 1C, 1D is the protocorm-like body and multiple shoots cultured by the bioreactor culture system using liquid medium. We compare aerial and underground parts of *Dendrobium candidum* with protocorm-like body and multiple shoot of tissue-cultured *Dendrobium candidum* about biological activities.

3.2. Comparative study of the protocorm-like body, multiple shoots, aerial part and underground part of *Dendrobium candidum* on anti-oxidant effect

To compare tissue-culture with natural *Dendrobium candidum* on biological activities, we first studied the antioxidant effect of extracts from protocorm-like body, multiple shoots, aerial part and underground part of *Dendrobium candidum*. Assays were treated with various concentrations of the extract and antioxidant activities were measured using DPPH and ABTS assay as de-

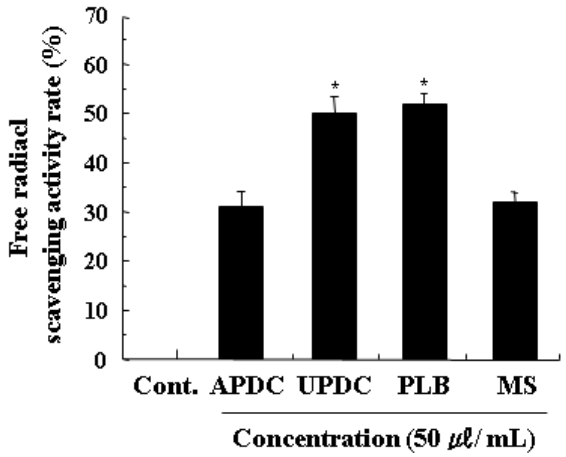

Figure 2. DPPH free radical scavenging activities of extracts from aerial part (APDC), underground part of *Dendrobium candidum* (UPDC), protocorm-like body (PLB) and multiple shoots (MS). Assay were treated with 50 μL/mL concentrations of the extract and free radical scavenging activity measured by DPPH assay as described in Materials and Methods. The results were represented as mean of standard deviation (S.D.) of three independent experiments. *, $0.01 < p < 0.05$ versus control.

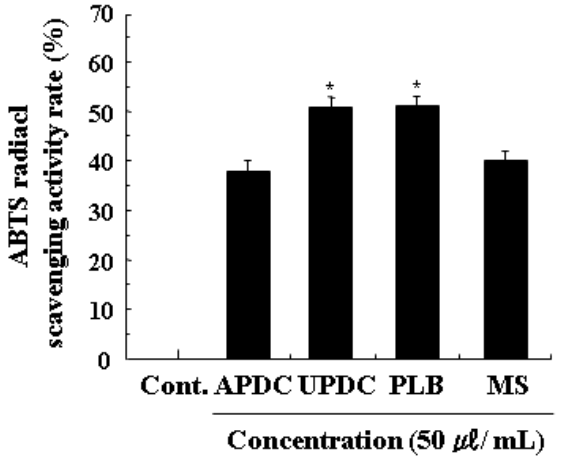

Figure 3. ABTS radical scavenging activities of extracts from aerial part (APDC), underground part of *Dendrobium candidum* (UPDC), protocorm-like body (PLB) and multiple shoots (MS). Assay were treated with 50 μL/mL concentrations of the extract and free radical scavenging activity measured by ABTS assay as described in Materials and Methods. The results were represented as mean of standard deviation (S.D.) of three independent experiments. *, $0.01 < p < 0.05$ versus control.

scribed in materials and methods. As shown in Figure 1 and Figure 2, protocorm-like body and underground part of *Dendrobium candidum* extract more enhanced the anti-oxidative effect than aerial part and multiple shoots extracts in free radical scavenging activity and $ABTS^{+}$ radical scavenging activity. Extracts of underground part and protocorm-like body had free radical scavenging activity (50% and 53% at sample 50 μL/mL) and ABTS radical cation scavenging activity (51% and 52% at sample 50 μL/mL).

3.3. Determination of the total phenolic content and total flavonoid content of the protocorm-like body, multiple shoots, aerial part and underground part of *Dendrobium candidum*

To investigate the phenolic and flavonoid content with anti-oxidant activity, we examined a total phenolic and flavonoid content of extracts from protocorm-like body, multiple shoots, aerial and underground parts of *Dendrobium candidum*. The contents of total phenolic and flavonoid compounds ranged from 336 mg/100 g to 583 mg/100 g and from 182 mg/100 g to 268 mg/100 g respectively. As shown in Table 1, protocorm-like body extract more contained the phenolic and flavonoid compound than aerial part, underground part and multiple shoots extracts.

Table 1. Total Phenolic Content and Total Flavonoid Content of Aerial Part (APDC), Underground Part of *Dendrobium Candidum* (UPDC), Protocorm-like Body (PLB) and Multiple Shoots (MS)

Sample	Total phenolic content (mg/g DW)	Total flavonoid content (mg/g DW)
APDC	3.36 ± 0.042	1.82 ± 0.031
UPDC	4.43 ± 0.038	1.81 ± 0.037
PLB	5.83 ± 0.033	2.68 ± 0.089
MS	4.00 ± 0.061	1.31 ± 0.073

The results were represented as mean of standard deviation (S.D.) of three independent experiments.

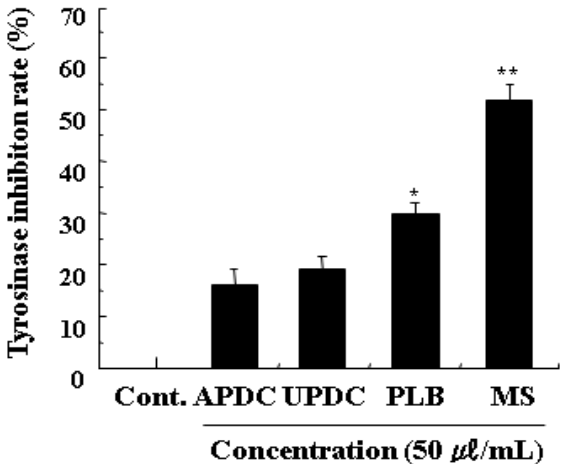

Figure 4. The effect of aerial part (APDC), underground part of *Dendrobium candidum* (UPDC), protocorm-like body (PLB) and multiple shoots (MS) on tyrosinase inhibition activity in cell culture free system. Tyrosinase activities were expressed as a percentage of control. The results were represented as mean of standard deviation (S.D.) of three independent experiments. **, $p < 0.01$; *, $0.01 < p < 0.05$ versus control.

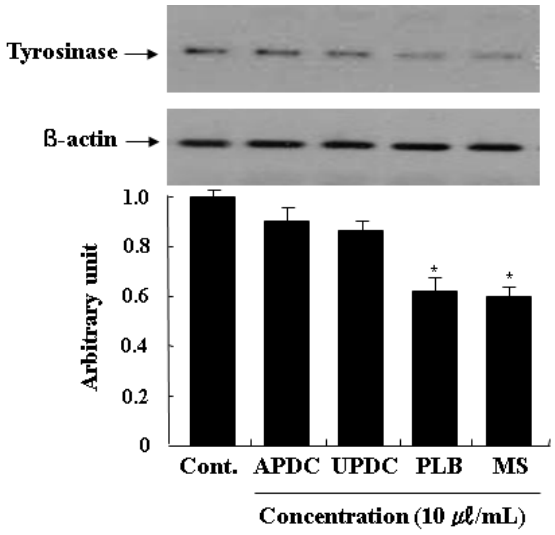

Figure 5. The effect of aerial part (APDC), underground part of *Dendrobium candidum* (UPDC), protocorm-like body (PLB) and multiple shoots (MS) on the protein expression of tyrosinase in B16 melanoma cells. Tyrosinase protein expression was determined by a western blotting analysis using a specific antibody, as described in the Materials and Methods. Bands were subjected to densitometric scanning using the NIH/Scion image software. Cells were treated with indicated concentrations for 24 h. The results were represented as mean of standard deviation (S.D.) of three independent experiments. *, $0.01 < p < 0.05$ versus control.

3.4. Inhibitory effect of the protocorm-like body, multiple shoots, aerial part and underground part of *Dendrobium candidum* on mushroom tyrosinase activity in a cell culture free system and tyrosinase protein expression in a cell based system

To compare the other effect of protocorm-like body, multiple shoots, aerial part and underground part extracts as well as antioxidant effect, we first analyzed inhibition rate of mushroom tyrosinase activity in a cell culture free system. As shown in Figure 4, protocorm-like body extract showed similar inhibition rate compared with underground part and aerial part extract. However, multiple shoots extract showed high inhibition rate compared with that of other extracts. In addition, we investigated whether various extracts inhibits melanin synthesis and tyrosinase expression in B16 melanoma cells as well as mushroom tyrosinase activity in cell culture free system. We performed western blot analysis against the cell lysate obtained from B16 melanoma cells treated various extracts. As shown in Figure 5 and Figure 6, multiple shoots extract more inhibited expression of tyrosinase and melanin synthesis than protocorm-like body, underground part and aerial part extracts and protocorm-like body, multiple shoots, aerial part and underground part did not have cytotoxicity in 10 μL/mL.

4. Discussion

Generally, tissue-cultured plant has a benefit to solve the drawbacks such as a rareness, long time and high cost for growing herb. Although the *Dendrobium candidum* has been well known as a traditional medicine, we investigated whether *Dendrobium candidum* have efficacy as a cosmetic ingredient for skin care in this study. From the results, the tissue-cultured protocorm-like body of *Dendrobium candidum* extracts more enhanced an-

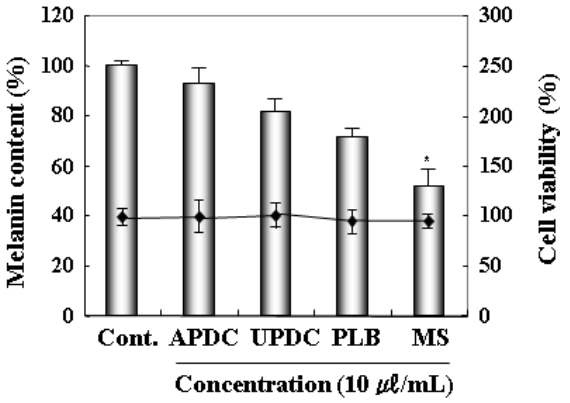

Figure 6. The Effect of aerial part (APDC), underground part of *Dendrobium candidum* (UPDC), protocorm-like body (PLB) and multiple shoots (MS) on the melanin content and cell viability of B16 melanoma cells. B16 melanoma cells were treated with extracts of aerial part (APDC), underground part of *Dendrobium candidum* (UPDC), protocorm-like body (PLB) and multiple shoots (MS) at the concentrations indicated in the Figure 6 and then incubated for 72 h. The melanin content and cell viability were determined as described in the Materials and Methods. The results were represented as mean of standard deviation (S.D.) of three independent experiments. *, $0.01 < p < 0.05$ versus control.

ti-oxidant effect than underground part, multiple shoots and aerial part extracts on investigation by DPPH and ABTS assay. On the other side, multiple shoots extract more enhanced whitening effect than protocorm-like body, aerial part and underground part of *Dendrobium candidum* through inhibition of tyrosinase and melanin synthesis. These results showed clearly that the tissue-cultured protocorm-like body has higher effective antioxidant activity in comparison with natural *Dendrobium candidum* and tissue-cultured multiple shoots. It is considered because of contain high concentration of flavonoid and phenolic compounds. In the whitening effect associated with tyrosinase and melanin synthesis, we thought that it is not influence of the flavonoid and phenolic compounds but bioactive substances as alkaloids and polysaccharides newly created in the course of plant tissue culture. Therefore, protocorm-like body of *Dendrobium candidum* has more potential benefits to anti-oxidant effect for skin care than other parts (underground part, aerial part and multiple shoots) respectively. On the other hand, multiple shoots has more potential benefits to whitening effect for skin care than protocorm-like body, underground part and aerial part of *Dendrobium candidum*. Although future work will be required to elucidate whether this effects occurs in comparative experiment by clinical test, these findings provide possible cosmetic ingredient for skin care as anti-oxidant effect and whitening effect of skin.

Acknowledgments

This study was supported by a grant of the Korea Healthcare technology R&D Project, Ministry for Health, Welfare & Family Affairs, Republic of Korea (Grant No : HN10C0007).

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