J. Soc. Cosmet. Scientists Korea
Vol. 37, No. 3, September 2011, 219-226

에키네시아 조직 배양체 추출물의 생리활성에 관한 연구

박 창 민† · 정 민 석 · 고 두 진 · 백 기 엽* · 최 종 완

(주)한국화장품제조 기술개발연구소, *충북대학교
(2011년 7월 29일 접수, 2011년 9월 16일 수정, 2011년 9월 19일 채택)

Study on Biological Activities of Adventitious Roots Cultured from *Echinacea angustifolia*

Chang-Min Park†, Min-Seok Joung, Du-Jin Ko, Kee-Yoeup Paek*, and Jong-Wan Choi

R&D Center, Hankook Cosmetics Manufacturing Co., Ltd., 76-1, Yongseong-Ri, Samseong-Myeon, Eumseong-Gun, Chungcheongbuk-Do 369-834, Korea
*Research Center for The Development of Advanced Horticultural Technology, Chungbuk National University
(Received July 29, 2011; Revised September 16, 2011; Accepted September 19, 2011)

요 약: 일반적으로 쌍떡잎식물 국화과 허브의 한 종인 에키네시아는 미국 북미 대평원에 서식하는 야생 식물로 수세기 동안 감기나 또 다른 바이러스 감염에 의한 질병에 대하여 면역 기능을 증진시키는 전통 식물 약제로 널리 사용되어 왔다. 본 연구에서는 생물반응장치를 이용하여 조직 배양된 에키네시아 식물체에 대하여 화장품 성분으로써 응용 가치를 평가하였다. 이미 몇몇 보고된 논문에서 에키네시아는 조직재생, 상처 치유, 그리고 염증 억제 등의 약리학적 활성에 대한 연구가 진행되었다. 본 연구에서는 생물반응장치를 이용하여 조직 배양된 에키네시아 추출물에 대하여 화장품 성분으로서 효능, 효과를 평가하였다. 실험 결과 조직배양된 에키네시아 추출물은 항산화 효과 및 단백분해효소 발현 억제 효과가 자연 상태의 에키네시아 추출물과 비교하여 우수한 결과를 보였다. 이러한 결과들은 피부보호를 위한 화장품 성분으로서 응용 가능성을 제공할 수 있을 것으로 사료된다.

Abstract: The *Echinacea*, which has been commonly known as a species of composite herb of dicotyledonous plant, has been used in native American traditional medicine for the treatment of diseases like colds or other infections in North America. We artificially cultured the adventitious roots of *Echinacea angustifolia* using the bioreactor culture system from *Echinacea angustifolia* and evaluated the efficacy as a cosmetic ingredient for skin care. Several studies previously have reported neogenesis, wound healing and inflammatory inhibition effect of *Echinacea angustifolia* but other efficacies were not well known. In the present study, we investigated the cosmetic efficacy to know applicable value of adventitious roots cultured from *Echinacea angustifolia* as a cosmetic ingredient. The adventitious roots extract of *Echinacea angustifolia* has superior anti-oxidant effect and matrix metalloproteinase-2 inhibitory effect, compared to natural *Echinacea angustifolia*. These results indicate that the adventitious roots extract cultured from *Echinacea angustifolia* presents a new possibility of being applicable to skin care and anti-wrinkle products as a cosmetic ingredient.

Keywords: *Echinacea angustifolia, matrix metalloproteinase, anti-oxidant, collagenase*

1. Introduction

Echinacea is a traditional perennial medicial herb in North America. *Echinacea* is a genus in the *Asteraceae* family. There are nine different species of *Echinacea* and they are herbaceous perennial. The three *Echinacea* species which have been found the most in herb products are *Echinacea angustifolia*, *Echinacea purpurea*

† 주 저자 (e-mail: cmpark@ihkcos.com)

and *Echinacea pallida*[1]. American Indians have long used *Echinacea angustifolia* as a herbal remedy against colds, wound, sores, snakes and insects bites, especially as a herbal tea to strengthen the immune system[2]. *Echinacea* has great values in herbal medicines because of its pharmacological activities.

Several studies previously have reported anti-oxidant and immuno-enhancing effects of *Echinacea angustifolia* extract[3,4]. Nowadays, the abilities of *Echinacea* are well known by many studies. *Echinacea* has the capacity to activate human macrophages and stimulate the phagocytosis[5,6]. It also has a mild antibacterial and fungicidal activity and increases fibroblasts helping to stimulate new tissue development. Furthermore, the roots of *Echinacea* help activate different immune system mechanisms for the human body[7,8]. In the present study, we artificially cultured *Echinacea angustifolia* adventitious roots using the bioreactor culture system and compared the adventitious roots cultured from *Echinacea angustifolia* with the natural *Echinacea angustifolia* in order to investigate if *Echinacea angustifolia* cultured by the bioreactor culture system can be used as a cosmetic ingredient for skin care products.

2. Materials and Methods

2.1. Reagents and Cell Culture

Antibodies against MMP-1, pro-collagen type I and β-actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). echinacoside, 1, 1-diphenyl1-2-picrylhydrazyl (DPPH), nitroblue tetrazolium (NBT) 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. Mouse fibroblast cell lines NIH-3T3L1 (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 Adventitious Roots Cultured from *Echinacea angustifolia*

Echinacea angustifolia adventitious roots were obtained from Research Center for The Development of Advanced Horticultural Technology, Chungbuk National University. Culture: The initial medium pH was adjusted to 6.0 before autoclaving (at 121 ℃ and 1.2 kg cm^{-2} pressure for 15 min), and the cultures were kept under darkness at 25 ℃. These adventitious roots were sub-cultured monthly. Adventitious roots (10 g L^{-1}, 2 cm long) were inoculated in 500-mL shake flasks containing 200 mL half strength MS medium with 5 % (w/v) sucrose and IAA, IBA, or NAA. Extraction: After dried, 3 g of *Echinacea angustifolia* adventitious roots were immersed in 97 g of 70 % ethanol solution and 30 % 1,3-butyleneglycol (1,3-BG) for sufficient time and mixed enough for 48 h in 45 ℃ using agitator. Then, the solution was filtered through a filter paper, followed by the removal of ethanol from the filtrate at 50 ~ 60 ℃ to obtain *Echinaceaangustifolia* adventitious roots extract of 80 %.

2.3. UV Irradiation

Cells were plated in 6 well cell culture dishes and incubated at 37 ℃ under humidified 5 % CO_2 and 95 % air in culture medium until 70 % to 80 % confluent. Then cells were exposed 5.8 J/cm^2 (UVA) from high intensity UV lamp (UVGL-58, San Gabriel, CA91778 U.S.A).

2.4. Cell Cytotoxicity by MTT Assay

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 *Echinacea angustifolia* adventitious roots extract 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.

2.5. Free Radical Scavenging Activity

The DPPH(1,1-diphenyl1-2-picrylhydrazyl) assay was done according to the method of Brand-Williams *et al.* [9]. 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.

2.6. Superoxide Radical Scavenging Activity

The procedure followed the method of F. Liu *et al.* [10] with some modifications for NBT assay. Superoxide anion scavenging activity was measured using xanthine-xanthine oxidase system as a source of superoxide and nitroblue tetrazolium (NBT). Then, the absorbance was taken at 540 nm and its SOD-like activity was calculated as compared with blank control.

2.7. ABTS Radical Cation Scavenging Activity

The procedure followed the method of R. Roberta *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.

2.8. Collagenase Activity Assay

The procedure followed the method of Wunsch et al. [12] with some modifications for collagenase activity assay. Sample is mixed with Substrate buffer 900 uL [10 mg azocollagen, 50 mM Tris-HCl buffer pH 7.5, 5 mM $Ca(C_2H_3O_2)_2$] and preincubate for 10 min at 25 ℃. Then, 100 uL enzyme (5 ug/mL) mix a substrate contained sample and incubate 20 min at 25 ℃. Then, the absorbance was taken at 540 nm and its collagenase inhibitory activity was calculated as compared with blank control.

2.9. Western Blot Analysis

Cells were treated with various dose and lysed in lysis buffer as described previously[13]. After differentiation, cells were lysed in lysis buffer. The lysates were clarified 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 Scion Image NIH Image program.

2.10. Echinacoside Content in the Adventitious Roots of *E. angustifolia*

A Agilent technologies HPLC equipped with Agilent 1200 series HPLC and variable wavelength detector was used in the analysis. A reversed-phase column (Waters Nova-Pak C18 4 μm 150 mm × 3.9 mm μm) was used. The gradient elution system consisted of 0.1 % phosphoric acid (A) and acetonitrile (B). Separation was achieved using the following gradient: 0 ~ 13 min: 10 ~ 22 % B, 13 ~ 14 min: 22 ~ 40 % B, 14 ~ 14.5 min: 40 ~ 40 %, 14.5 ~ 15 min: 40 ~ 10 % B, 15 ~ 20 min: 10 ~ 10 % B. The column temperature was set at 35 ℃. The flow rate was 1.5 ml/min. The UV detection wavelength was 330 nm. The mean values of three replicates were calculated. Standard solution were prepared by echinacoside (cromadex) 5 mg, dissolved in water 50 mL.

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.

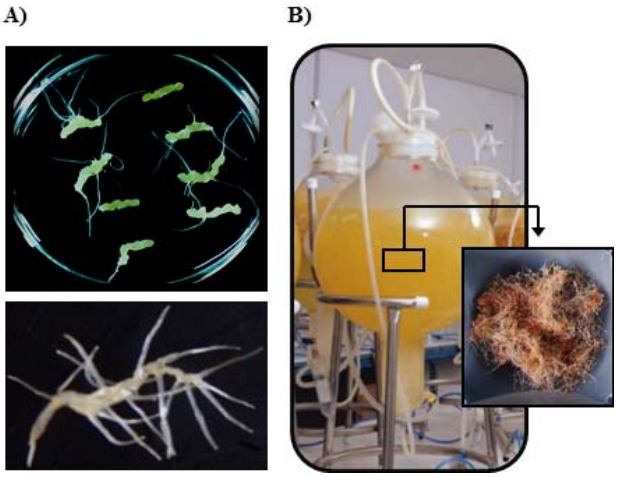

Figure 1. The *Echinacea angustifolia* cultured by the bioreactor culture system. Figure 1A is a picture of induction and growth of *Echinacea angustifolia* adventitious roots culturing by MS (Murashige and Skoog, 1962) medium. Figure 1B is the *Echinacea angustifolia* adventitious roots culturing by the bioreactor culture system using liquid medium and picture of the dried adventitious roots of *Echinacea angustifolia*.

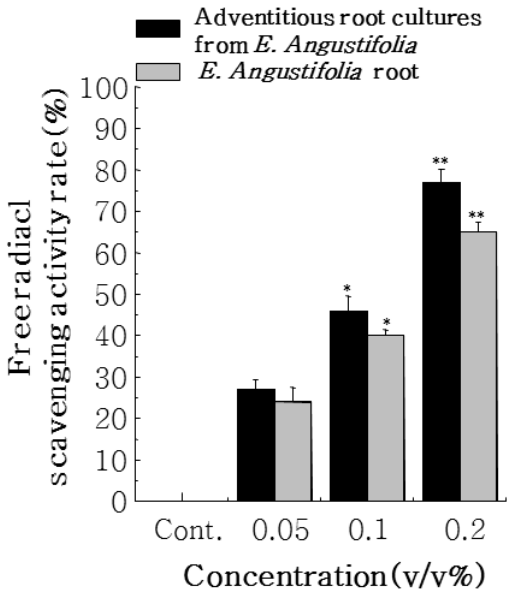

Figure 2. DPPH free radical scavenging activities of the adventitious root extract cultures from *Echinacea angustifolia* and *Echinacea angustifolia* root extract. Assay were treated with various concentrations of the extract and free radical scavenging activity measured by DPPH assay as described in materials and methods. The results were represented as mean of standard deviation (S.D.) of three independent experiments.**, $p < 0.01$; *, $0.01 < p < 0.05$ versus control.

3. Results

3.1. The Aventitious Roots of *Echinacea angustifolia* Cultured by the Bioreactor Culture System

Tissue culture protocols have been developed for preservation of this valuable plant. Figure 1A is the picture of induction of adventitious roots of *Echinacea. angustifolia* by plant hormone after 5 weeks of culture. Figure 1B is the adventitious roots of *Echinacea angustifolia* cultured by the bioreactor culture system using liquid medium. We extract the dried *Echinacea angustifolia* adventitious roots and investigate the efficacy as cosmetic ingredient.

3.2. Antioxidant Activity of the Adventitious Roots Extract of *Echinacea angustifolia* and Roots Extract of *Echinacea angustifolia*

To investigate the effect of the adventitious roots extract of *Echinacea angustifolia*, we first studied the antioxidant effect of *Echinacea angustifolia* adventitious roots extract. Three assays were treated with various concentrations of the extract and antioxidant activities were measured using DPPH, NBT and ABTS assay as described in Materials and Methods. The adventitious roots extract of *Echinacea angustifolia* enhanced the hydroxyl radical scavenging activity and superoxide anion scavenging activity more than roots extract of *Echinacea angustifolia* in a dose dependent manner in DPPH assay (Figure 1) and NBT assay (Figure 2). In DPPH assay and NBT assay, the adventitious roots extract of *Echinacea angustifolia* enhanced scavenging activity about 15 % more than roots extract of *Echinacea angustifolia* in 0.2 % concentration. To certificate the antioxidant effect of the adventitious roots extract of *Echinacea angustifolia*, we investigated $ABTS^{\cdot+}$ radical scavenging activity using ABTS assay. As shown in Figure 3, the adventitious roots extract of *Echinacea angustifolia* significantly enhanced the scavenging activity more than roots extract of *Echinacea angustifolia*. In ABTS assay, the adventitious roots ex

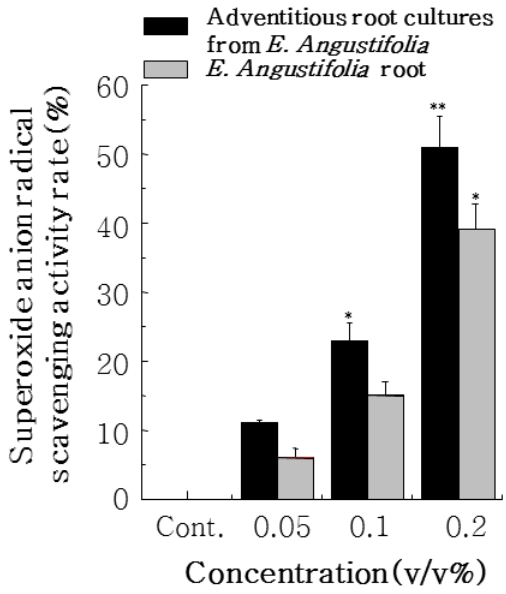

Figure 3. NBT superoxide radical scavenging activities of the adventitious root extract cultures from *Echinacea angustifolia* and *Echinacea angustifolia* root extract. Assay were treated with various concentrations of the extract and free radical scavenging activity measured by NBT assay as described in materials and methods. 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.

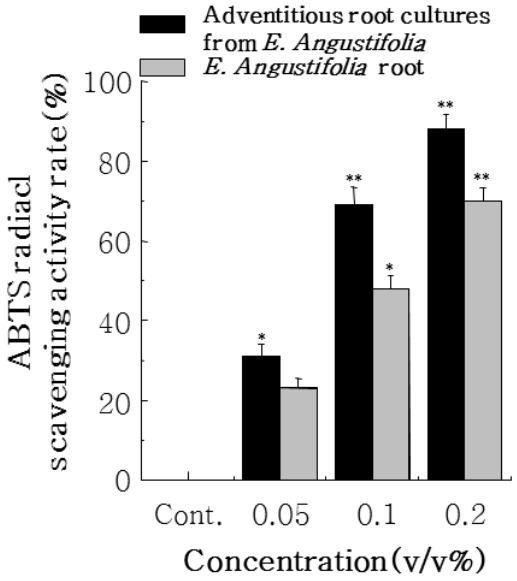

Figure 4. ABTS radical scavenging activities of the adventitious root extract cultures from *Echinacea angustifolia* and *Echinacea angustifolia* root extract. Assay were treated with various concentrations of the extract and free radical scavenging activity measured by ABTS assay as described in materials and methods. The results were represented as mean of standard deviation (S.D.) of three independent experiments.**, $p < 0.01$; *, $0.01 < p < 0.05$ versus control.

tract of *Echinacea angustifolia* enhanced scavenging activity about 20 % more than roots extract of *Echinacea angustifolia* in 0.2 % concentration.

3.3. Cell Cytotoxicity by the Adventitious Roots Extract of *Echinacea angustifolia*

To investigate inhibitory effects of proteinase and collagenase as well as antioxidant effect by the adventitious roots extract of *Echinacea angustifolia*, we first studied the effect of cytotoxicity in NIH3T3-L1 cells treated with the adventitious roots extract of *Echinacea angustifolia* and roots extract of *Echinacea angustifolia* as described in Materials and Methods. Cell cytotoxicity was measured by MTT assay. As shown in Figure 5, the adventitious roots extract of *Echinacea angustifolia* did not have cytotoxicity until 0.2 % concentration for 24 h.

3.4. Inhibitory Effect of Matrix Metalloproteinases by the Adventitious Roots Extract of *Echinacea angustifolia*

To know the other effect of *Rubus coreanus* extracts, we examined expression of matrix metalloproteinase-2 and matrix metalloproteinase-9 enhancing the degradation extracellular matrix (ECM) components related with skin wrinkle in cell lysate as described in Materials and Methods. As shown in Figure 6, *Echinacea angustifolia* adventitious roots extract significantly reduced the expression of MMP-2 more than *Echinacea angustifolia* roots extract as compared with the UV-treated cells at a concentration without any cytotoxicity. On the other hand, matrix metalloproteinase-9 expression was not affected by *Echinacea angustifolia* adventitious roots extract and *Echinacea angustifolia* roots extract.

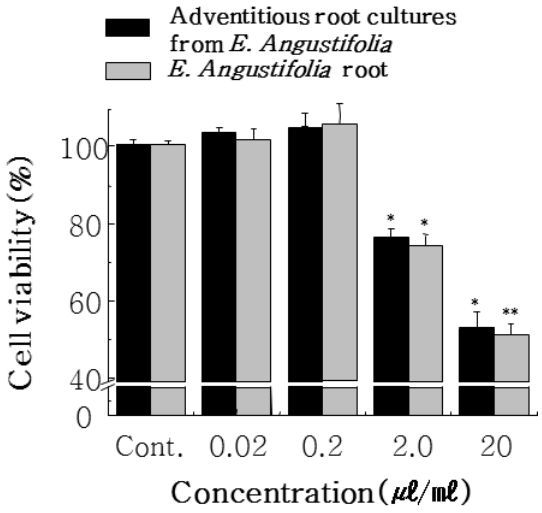

Figure 5. Cell viability of the adventitious roots extract cultured from *Echinacea angustifolia* and *Echinacea angustifolia* root extract. NIH3T3-L1 mouse fibroblast cells were treated with various concentrations and cell viability measured by MTT assay described in materials and methods. The results were represented as mean of standard deviation (S.D.) of three independent experiments. **, $p < 0.01$; *, $0.01 < p < 0.05$ versus control.

3.5. Inhibitory Effect of Collagenase

In addition, we examined whether the adventitious roots extract of *Echinacea angustifolia* have an inhibitory effect of collagenase activity relating with skin wrinkle. As shown in Figure 7, the adventitious roots extract of *Echinacea angustifolia* inhibited the collagenase activity in a dose-dependent manner. Collagenase inhibitory activity of the adventitious roots extract of *Echinacea angustifolia* was enhanced more than *Echinacea angustifolia* roots extract as compared with roots extract of *Echinacea angustifolia*.

3.6. Echinacoside Content in the Adventitious Roots of *Echinacea angustifolia*

Among the bioactive substances of *Echinacea angustifolia*, the Echinacoside which is a kind of derivates of Caffeic acid is exist in great quantities. Therefore, we compared the content analysis of the cultured adventitious roots of *Echinacea angustifolia* with that of the natural *Echinacea angustifolia* extracts. As shown in Table 1, we realized that the adventitious roots of

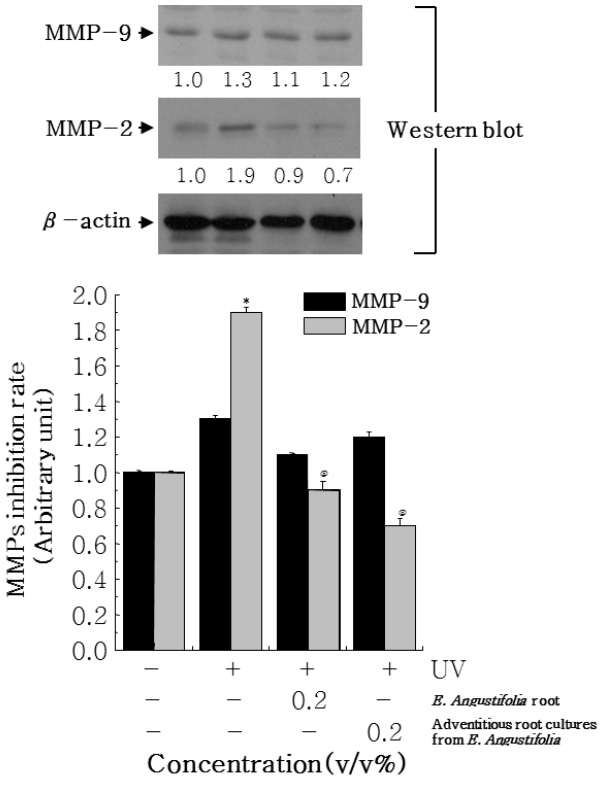

Figure 6. Inhibtion of MMP-2 and MMP-9 by the adventitious roots extract cultured from *Echinacea angustifolia* and *Echinacea angustifolia* root extract in NIH3T3-L1 cells. Cells were lysed in lysis buffer and subjected to western blot for the detection of MMP-2 and MMP-9 as described in materials and methods. NIH3T3- L1 cells were treated with indicated concentration and compared with *Echinacea angustifolia* root extract. Western blot bands were subjected to densitometric scanning using the Scion image NIH image software. The results were represented as mean of standard deviation (S.D.) of three independent experiments. Bars represent the mean ± S. D. * $p < 0.05$ versus control, @ $p < 0.05$ versus UV alone.

Echinacea angustifolia include more Echinacoside than natural *Echinacea angustifolia* extracts through the Table 1.

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 *Echinacea angustifolia* has been well known as herb to remedy of neo-

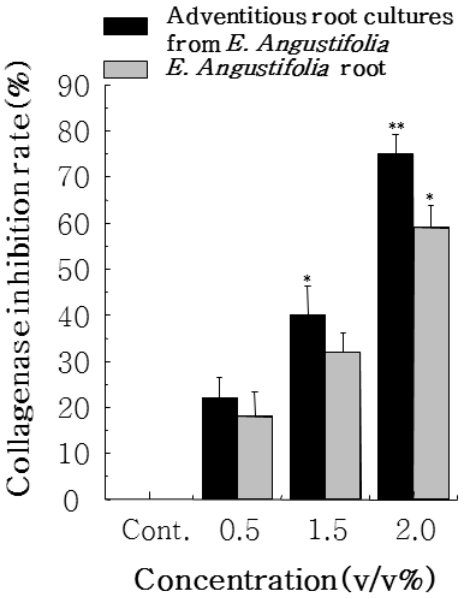

Figure 7. Inhibition of the adventitious roots extract cultured from *Echinacea angustifolia* and *Echinacea angustifolia* root extract on collagenase. Assay were treated with various concentrations of the extract and collagenase inhibitory activity measured by collagenase assay as described in materials and methods. 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.

genesis, wound healing, inflammation, hypertension, diabetes, heart and liver disease as a traditional medicine, we investigated whether the adventitious roots cultured from *Echinacea angustifolia* have efficacy as a cosmetic ingredient for skin care in this study. In summary, we found that the adventitious root extract cultured from *Echinacea angustifolia* enhanced the radical scavenging activity and superoxide anion scavenging activity more than *Echinacea angustifolia* roots extract in measurement using DPPH, NBT and ABTS assay for antioxidant activities. The adventitious root extract cultured from *Echinacea angustifolia* significantly reduced the expression of MMP-2 more than *Echinacea angustifolia* roots extract as compared with the UV-treated cells. Also, the adventitious roots of Echinacea angustifolia include more echinacoside than natural *Echinacea angustifolia* extracts. As a result, we suggest

Table 1. Echinacoside Content of the Adventitious Roots of *Echinacea angustifolia and Echinacea angustifolia Root Extract*

Content \ Extract	The adventitious roots of *Echinacea angustifolia*	*Echinacea angustifolia root extract*
Echinacoside (mg/mL)	0.22 ± 0.005	0.26 ± 0.003

that the adventitious roots cultured from *Echinacea angustifolia* could be cosmetic ingredient for skin care with antioxidant activity, inhibitory effect of collagenase activity and inhibition of proteinase expression related with skin wrinkle.

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 : A103017).

Reference

1. S. S. Percival, Use of echinacea in medicine, *Biochem Pharmacol*, **60**(2), 155 (2000).
2. B. Barrett, Medicinal properties of echinacea: a critical review, *Phytomedicine*, **10**(1), 66 (2003).
3. S. Osowski, M. Rostock, H. H. Bartsch, and U. Massig, Pharmaceutical comparability of different therapeutic Echinacea preperations, *Forsch Komplementarmed Klass Naturheilkd*, **7**(6), 294 (2000).
4. R. A. Burger, A. R. Torres, R. P. Warren, and V. D. Caldwell, Echinacea-induced cytokine production by human macrophages, *Int. J. Immunopharmacol.*, **19**(7), 371 (1997).
5. F. Pellati, S. Benvenuti, L. Magro, M. Melegari, and F. Soragni, Analysis of phenolic compounds and radical scavenging activity of *Echinacea* spp, *J. Pharm. Biomed. Ana*, **35**(2), 289 (2004).
6. B. D. Sloley, L. J. Urichuk, C. Tywin, R. T. Coutts, P. K. Pang, and J. J. Shan, Comparison of chemical components and antioxidants capacity of different echinacea species, *J. Pharm. Pharmacol.*, **53**(6), 849

(2001).
7. E. H. South and J. H. Exon, Multiple immune functions in rats fed echinacea extracts, *Immunopharmacol Immunotoxicol*, **23**(3) 411 (2001).
8. M. Sharma, R. Schoop, and J. B. Hudson, Echinacea as an antiinflammatory agent: the influence of physiologically relevant parameters, *Phytother Res.*, **23**(6), 863 (2009).
9. W. Brand-Williams, M. E. Cuvelier, and C. Berset, Use of free radical method to evaluate antioxidant activity, *Lebensmittel Wissenschaftund Technologie*, **28**, 25 (1995).
10. F. Liu, V. E. C. Ooi, and S. T. Chang, Free radical scavenging activities of mushroom polysaccharide extracts, *Life Sci.*, **60**, 763 (1997)
11. R. Roberta, P. Nicoletta, P. Anna, P. Ananth, Y. Min, and R. E. Catherine, Antioxidant activity appling an improved ABTS radicla cation decolorization assay, *Free Radical Biology & Medicine*, **26**(9), 1231 (1999).
12. E. Wunsch and H. G. Heindrich, On the quantitative determination of collagenase, *Hoppe Seylers Z Physiol Chem.*, **333**, 149 (1963).
13. C. M. Park, M. J. Park, H. J. Kwak, H. C. Lee, M. S. Kim, S. H. Lee, I. C. Park, C. H. Rhee, and S. I. Hong, Ionizing radiation enhances matrix metalloproteinase-2 secretion and invasion of glioma cells through Src/epidermal growth factor receptor-mediated p38/Akt and phosphatidylinositol 3-kinase/Akt signaling pathways, *Cancer Res.*, **66**(17), 8511 (2006).