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라울리아 신초 추출물의 항산화 및 항염 효능에 관한 연구

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Study on Anti-oxidant and Anti-inflammatory Activity of the Tissue-cultured Shoot Clumps from *Raoulia australis*

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요 약: 본 연구에서는 생물반응장치를 이용하여 조직 배양된 라울리아 신초에 대하여 화장품 성분으로써 응용 가치를 평가하였다. 조직 배양한 라울리아 신초에 대한 항산화 및 항염 활성 효과를 연구하였다. 라울리아는 뉴질랜드나 호주에서 자생하는 국화과의 야생초본식물이다. 이미 몇몇 보고 된 논문에서 라울리아는 기관지염, 수막염 그리고 호흡기 질병 등을 유발하는 바이러스에 대한 증식 억제 활성이 있다고 보고되었다. 실험 결과 조직 배양된 라울리아 신초 추출물은 자연 상태의 라울리아 추출물과 비교하여 항산화 활성 및 항염 활성 효과가 우수하였다. 조직 배양된 라울리아 신초 추출물은 자연에서 자란 라울리아 추출물보다 50 μL/mL 농도에서 10~25% 항산화 활성을 증가시켰다. 또한 조직 배양된 라울리아 신초 추출물은 LPS로 유도된 대식세포에서 iNOS와 COX-2의 단백질 발현이 자연에서 자란 라울리아 추출물보다 억제되었다. 본 연구의 결과들로, 조직배양 한 라울리아 신초 추출물은 피부 보호를 위한 천연 화장품 성분으로써 우수한 가능성을 제공할 수 있을 것으로 사료된다.

Abstract: In this study, the shoot clumps extract of tissue-cultured *Raoulia australis* using the bioreactor culture system was tested for use a natural cosmetic ingredient. Tissue-cultured *R. australis* shoot clumps extract was tested anti-oxidant and anti-inflammatory activity for a cosmetic application. *R. australis* is a wild herbaceous plant of the asteraceae growing in New Zealand and Australia. Previous studies have reported anti-viral activity of the inhibitory effects for the growth of viruses induced meningitis, bronchitis and respiratory diseases but other biological effects are unknown. The shoot clumps extract of tissue-cultured *R. australis* showed higher anti-oxidant effect and anti-inflammatory effect than the natural *R. australis* extract. In DPPH, NBT and ABTS assay, the shoot clumps extract of tissue-cultured *R. australis* enhanced radical scavenging activity (up to 10~25% at 50 μL/mL) more than the natural *R. australis* extract. Also, the shoot clumps extract of tissue-cultured *R. australis* inhibited expression of iNOS and COX-2 protein in LPS-stimulated Raw 264.7 macrophages more than the natural *R. australis* extract. From this study, the shoot clumps extract of tissue-cultured *R. australis* displayed strong possibility as a new natural cosmetic ingredient for skin-care products.

Keywords: *Raoulia australis, anti-oxidation, biological activity, anti-inflammation, shoot clumps*

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1. Introduction

Raoulia australis, a wild herbaceous plant of the compositae growing in New Zealand and Australia has been widely used as ground cover plants for landscape in Europe, America and Australia. However, *R. australis* recently has been known to contain raoulic acid[1]. Raoulic acid, known as terpene-based material comprising of 25 carbons, which has been reported on growth inhibition effect on virus activity such as bronchitis, meningitis and respiratory diseases[2]. Generally, the tissue-cultured plant has a benefit to solve the drawbacks such as a rareness, long time and high cost for growing herb. This study has examined whether the shoot clumps extract of tissue-cultured *R. australis* can be utilized as a new cosmetic resource for skin care as a part of our continuous program to find new ingredients for functional cosmetics from natural resources. We artificially cultured the shoot clumps of *R. australis* using the bioreactor culture system for this study[3]. Several studies have reported antiviral activity of *R. australis* but other efficacies as a cosmetic ingredient for skin improvement are unknown previously[4]. Although pharmacological studies have been reported, *R. australis* has not been subjected to a systematic cosmetic evaluation to determine its propriety in practical application. We compared the shoot clumps extract of tissue-cultured *R. australis* with natural *R. australis* extract on biological activities such as anti-oxidant activity, anti-inflammatory activity and cytotoxic activity in order to investigate the possibility of the shoot clumps extract of tissue-cultured *R. australis*. We measured its anti-oxidant and anti-inflammatory activities by hydrogen donating ability, superoxide anion scavenging, hydroxyl radical scavenging[5] and inducible nitric oxide (iNOS), cyclooxygenase2 (COX-2), nitric oxide (NO), and Prostaglandin E_2 (PGE_2) which are known as factor associated with inflammation[6,7]. In the present study, we suggest the possibility of applying it to the cosmetics through the *in vitro* experiments of cosmetic efficacy evaluation.

2. Materials and Methods

2.1. Reagents and Cell Culture

Antibodies against COX2, iNOS and ß-actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). 1,1-diphenyl-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). The mouse macrophage-like cell line, RAW 264.7 macrophages (obtained from Korean Cell Line Bank) 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 shoot clumps extract cultured from *R. australis*

The tissue-cultured *R. australis* shoot clumps were provided by Research Center for the Development of Advanced Horticultural Technology in Chungbuk National University. Extraction : After dried, 10 g the natural *R. australis* or the shoot clumps of tissue-cultured *R. australis* were immersed in 900 g with mixed solvent of 70% ethanol and 30% 1,3-butyleneglycol with 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 natural *R. australis* extract and the shoot clumps extract of tissue-cultured *R. australis*.

2.3. Plant tissue culture process

Seeds were disinfected with 70% ethanol for 10 s followed by surface sterilization with 2% sodium hypochlorite for 10 min. The sterilized seeds were cultured on MS medium with gelrite and sucrose. Cultures were maintained in the culture room at 25 ℃ and 16 h photoperiod with a photon flux density of 50 μM m^{-2} s^{-1} for 4 weeks.

2.4. 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 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-diphenyl-2-picrylhydrazyl) assay followed the method of Brand-Williams *et al*. [7]. 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. ABTS radical cation scavenging activity

The procedure followed the method of Roberta R *et al.* [8] 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.

2.7. Superoxide radical scavenging activity

The procedure followed the method of F. Liu *et al.* [9] 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.

2.8. Determination of total phenolic content

The total phenolic content in extracts was determined as described by Lee *et al.* [10] 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).

2.9. Determination of total flavonoid content

The total flavonoid content in extracts was determined as described by Lee *et al.* [10]. To 0.25 mL of the 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.

2.10. Nitrite production assay

Nitrate in the media was measured by the Griess assay and was used as an indicator of NO synthesis in cells [11]. In brief, an equal volume of the culture supernatants in Raw 264.7 macrophages and Griess solution (1:1 mixture (v/v) of 1% sulfanilamide and 0.1% N-(naphthyl) ethylenediamine dihydrochloride in 5% H_3PO_4 was added into 96-well plates for 10 min at room temperature. Absorbance was measured using an UV spectrophotometer at 540 nm.

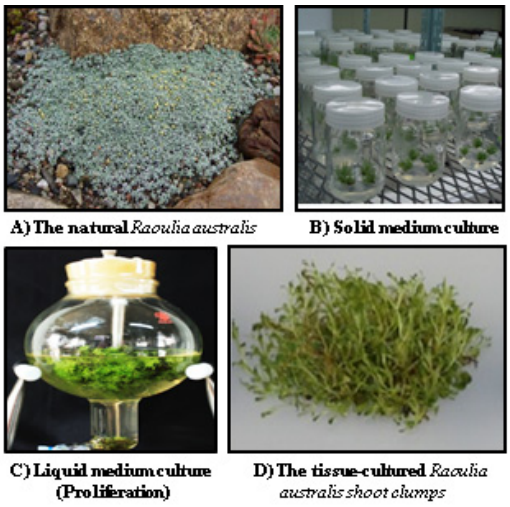

Figure 1. Scheme of the whole procedure of the *R. australis* cultured by the bioreactor culture system. A) and D) is a picture of the natural *R. australis* and the shoot clumps of tissue-cultured *R. australis* by the bioreactor culture system. B) and C) is a picture of proliferation of the shoot clumps of tissue-cultured *R. australis* in solid and liquid medium.

2.11. Prostaglandin E2 release assay

Raw 264.7 macrophages were seeded at a density of 1×10^6 cells into 6 well plates and cultured at 37 ℃ in 5% CO_2. After 1 day, fresh medium containing 10% serum was added to cells, which were treated a various concentrations of sample and then stimulated in the presence of LPS (1 μg/mL) for 24 h. The culture supernatants were used to quantify by the enzyme immunoassay kit according to the protocols of the manufacturers.

2.12. Western blot analysis

Raw 264.7 macrophages were treated with various concentrations and lysed in lysis buffer as described previously. 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 [12].

2.13. Statistical analysis

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

3. Results

3.1. The natural *R. australis* and the shoot clumps of tissue-cultured *R. australis* by the bioreactor culture process

Plant tissue cultures have been developed for preservation of this valuable plant. Natural and tissue-cultured *R. australis* shoot clumps was tested as shown in Figure 1A) and 1D). They were proliferated by the bioreactor culture system using solid medium and liquid medium as shown Figure 1B) and 1C). We compared the natural *R. australis* extract with the shoot clumps extract of tissue-cultured *R. australis* about biological activities.

3.2. Comparative study of anti-oxidant activity on the shoot clumps extract of tissue-cultured *R. australis* and the natural *R. australis* extract

First, we studied the anti-oxidant effect of the shoot clumps extract of tissue-cultured *R. australis* and the natural *R. australis* extract to compare the tissue-cultured *R. australis* shoot clumps extract with the natural *R. australis* extract on biological activities. Three assays were treated with various concentrations and anti-oxidant activities were measured using DPPH, NBT and ABTS assay as described in Materials and Methods. The shoot clumps extract of tissue-cultured *R. australis* more strong anti-oxidant activity than the natural *R. australis* extract in hydroxyl radical scavenging activity, $ABTS^{\cdot+}$ radical scavenging activity and superoxide anion scavenging activity respectively. As shown in Figure 2 and Figure 3, the shoot clumps extract of tissue-cultured *R. australis* more enhanced scavenging activity about 25% and 15%

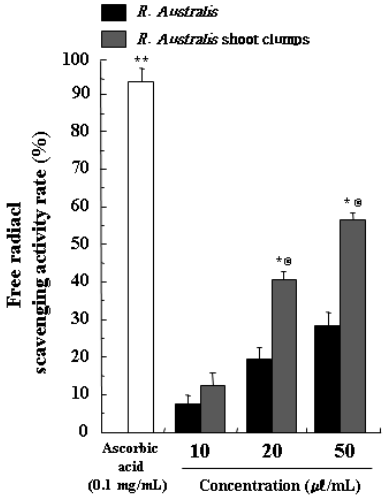

Figure 2. DPPH free radical scavenging activities of extracts from the natural *R. australis* and the shoot clumps extract of tissue-cultured *R. australis*. The results were represented as mean ± standard deviation (S.D.) of three independent experiments. $^{**}p < 0.01$; $0.01 < {}^{*}p < 0.05$ vs. control. $0.01 < {}^{@}p < 0.05$ vs. the treated group with natural *R. australis* extract.

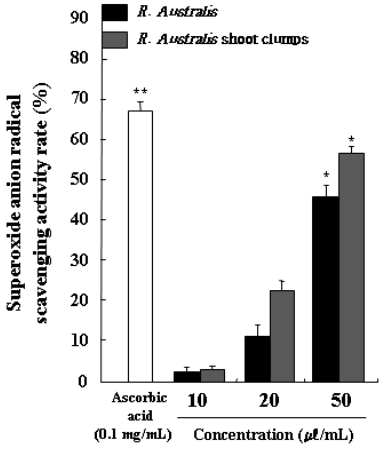

Figure 3. Superoxide anion radical scavenging activities of extracts from the natural *R. australis* and the shoot clumps extract of tissue-cultured *R. australis*. The results were represented as mean ± standard deviation (S.D.) of three independent experiments. $^{**}p < 0.01$; $0.01 < {}^{*}p < 0.05$ vs. control.

than the natural *R. australis* extract in 50 μL/mL concentration. To certificate the anti-oxidant effect of the tissue-cultured *R. australis* shoot clumps extract, we in

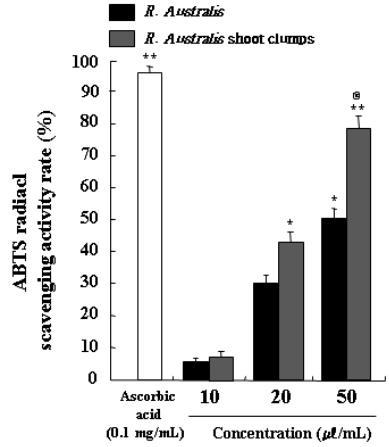

Figure 4. ABTS radical scavenging activities of extracts from the natural *R. australis* and the shoot clumps extract of tissue-cultured *R. australis*. The results were represented as mean ± standard deviation (S.D.) of three independent experiments. $^{**}p < 0.01$; $0.01 < {}^{*}p < 0.05$ vs. control. $0.01 < {}^{@}p < 0.05$ vs. the treated group with natural *R australis* extract.

vestigated $ABTS^{\cdot+}$ radical scavenging activity. As shown in Figure 4, the shoot clumps extract of tissue-cultured *R. australis* enhanced scavenging activity about 28% more than the natural *R. australis* extract at 50 μL/mL concentration on ABTS assay.

3.3. Determination of the total phenolic content and total flavonoid content of the shoot clumps extract of tissue-cultured *R. australis* and the natural *R. australis* extract

The total phenolic and total flavonoid compounds help to inhibit many of the oxidative reactions caused by free radicals such as superoxide and hydroxyl radical. We investigated the amount of total phenolic and total flavonoid compounds of the shoot clumps extract of tissue-cultured *R. australis* and the natural *R. australis* extract. The amount of phenolic compounds in the shoot clumps extract of tissue-cultured *R. australis* (4.72 ± 0.048 mg/g DW) was higher than the natural *R. australis* extract (3.41 ± 0.056 mg/g DW). Furthermore, the amount of flavonoid compounds in the shoot clumps extract of tissue-cultured *R. australis* (2.52 ± 0.051 mg/g DW) was higher than the natural *R. australis* extract

Table 1. The Total Phenolic Content and Total Flavonoid Content of the Natural *R. australis* Extract and the Shoot Clumps Extract of Tissue-cultured *R. australis*

Sample	Total phenolic content (mg/mL)	Total flavonoid content (mg/mL)
R. Austalis extract	3.41 ± 0.06	1.77 ± 0.05
R. Austalis shoot clumps extract	4.72 ± 0.05 **	2.52 ± 0.05 **

The results were represented as mean ± standard deviation (S.D.) of three independent experiments. $^{**}p < 0.01$ vs. the natural *R. australis* extract

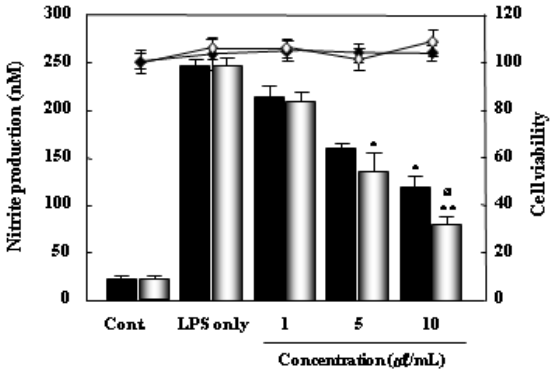

Figure 5. Effects of the natural *R. australis* extract and the shoot clumps extract of tissue-cultured *R. australis* on cell viability and nitrite production in LPS-stimulated Raw 264.7 macrophages. The results were represented as mean ± standard deviation (S.D.) of three independent experiments. $^{**}p < 0.01$; $0.01 < {}^{*}p < 0.05$ vs. LPS alone. $0.01 < {}^{@}p < 0.05$ vs. the treated group with natural *R. australis* extract.

(1.77 ± 0.047 mg/g DW). The phenolic and flavonoid compounds of the shoot clumps extract of tissue-cultured *R. australis* were significantly higher than the natural *R. australis* extract as shown in Table 1.

3.4. Comparative study of anti-inflammatory activity of the shoot clumps extract of tissue-cultured *R. australis* and the natural *R. australis* extract

First, we analyzed total nitrite production and prostaglandin E_2 release in Raw 264.7 macrophages to investigate the anti-inflammatory effect of the natural *R. australis* extract and the shoot clumps extract of tissue-cultured *R. australis*. To evaluate the cytotoxicity of the natural *R. australis* extract and the shoot clumps extract of tissue-cultured *R. australis* against Raw 264.7 macrophages, cytotoxicity was carried out by the MTT assay. The results of this investigation were shown in Figure 5. The natural *R. australis* extract and the shoot clumps extract of tissue-cultured *R. australis* didn't show any cytotoxicity up to 10 μL/mL in the presence of LPS. In order to assess the anti-inflammatory activity of the natural *R. australis* extract and the shoot clumps extract of tissue-cultured *R. australis*, Raw 264.7 macrophages were exposed to LPS in the presence or absence of extracts and nitrite production was measured in the medium. The shoot clumps extract of tissue-cultured *R. australis* inhibited LPS-induced nitrite production in a dose-dependent manner more than the natural *R. australis* extract as shown in Figure 5. The shoot clumps extract of tissue-cultured *R. australis*, especially at 10 μL/mL, significantly reduced the LPS-induced nitrite production. On the other hand, prostaglandin E_2 is another key inflammatory mediator. Therefore, we investigated the shoot clumps extract of tissue-cultured *R. australis* on prostaglandin E_2 production in LPS-stimulated Raw 264.7 macrophages.

The shoot clumps extract of tissue-cultured *R. australis* inhibited LPS-induced prostaglandin E_2 production in a dose-dependent manner more than the natural *R. australis* extract as shown in Figure 6. Comparing to inhibition of nitrite production, the shoot clumps extract of tissue-cultured *R. australis*, especially at 5 μL/mL, significantly reduced the LPS-induced prostaglandin E_2 production.

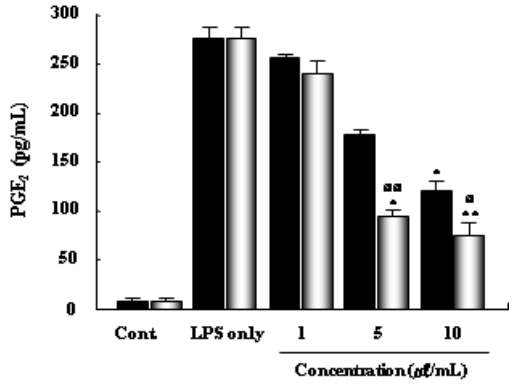

Figure 6. Effect of the natural *R. australis* extract and the shoot clumps extract of tissue-cultured *R. australis* on prostaglandin E_2 release in LPS-stimulated Raw 264.7 macrophages. The results were represented as mean ± standard deviation (S.D.) of three independent experiments. $^{**}p < 0.01$; $0.01 < {}^{*}p < 0.05$ vs. LPS alone. $0.01 < {}^{@}p < 0.05$ vs. the treated group with natural *R. australis* extract.

3.5. Inhibition of iNOS and COX-2 expression in LPS-stimulated Raw 264.7 macrophages

We investigated the anti-inflammatory effects of the shoot clumps extract of tissue-cultured *R. australis* and the natural *R. australis* extract on iNOS and COX-2 protein expression to identify the mechanism using a murine macrophage RAW 264.7 cell model. iNOS and COX-2 protein expression were markedly reduced by the shoot clumps extract of tissue-cultured *R. australis* and the natural *R. australis* extract in Raw 264.7 macrophages as shown in Figure 7. Comparing the shoot clumps extract of tissue-cultured *R. australis* with the natural *R. australis* extract, the shoot clumps extract of tissue-cultured *R. australis*, especially at 5 μL/mL, significantly inhibited COX-2 and iNOS protein expression more than the natural *R. australis* extract.

4. Discussion

The *R. australis* has been well known as ground cover plants for landscape in New Zealand or Australia, but *R. australis* can't grown in Korea because of climate and environment. Therefore, we investigated whether the tissue-cultured *R. australis* shoot clumps have efficacy as a

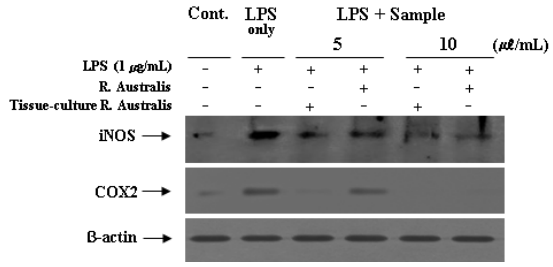

Figure 7. Effects of the natural *R. australis* extract and the shoot clumps extract of tissue-cultured *R. australis* on iNOS and cyclyoxygenase-2 expression in Raw 264.7 macrophages. Cells were pre-incubated for 12 h with indicated concentrations of the natural *R. australis* extract and the tissue-cultured *R. australis* shoot clumps extract stimulated for 12 h with 1 μg/mL of LPS.

cosmetic ingredient for skin care. In this study, we aimed to examine the anti-oxidant effect and anti-inflammatory effect of the shoot clumps extract of tissue-cultured *R. australis* and natural *R. australis*. Generally, tissue-cultured plants have a benefit to solve the drawbacks such as a rareness, long time and high cost for growing herb. Also, the tissue culture plants are significantly different from natural plant about bioactive compounds or contents depending on the culture conditions (Various tissue culture medium, cytokinin, auxin, nitrogen source, air condition and density etc.). Especially, the plant tissue culture conditions involved in levels of plant secondary metabolites such as phenols, flavonoids, polyphenols, tocopherols, and derivatives of cinnamic acid [13,14]. Phenolic compounds and flavonoid compounds are very important plant constituents because of their radical scavenging ability resulting from their hydroxyl groups [15-18]. The results showed different outcome with the shoot clumps extract of tissue-cultured *R. australis* and the natural R. australis extract. The shoot clumps extract of tissue-cultured *R. australis* has more strong anti-oxidant effect than the natural *R. australis* extract on investigation by DPPH, ABTS and NBT assay. It is considered that the shoot clumps extract of tissue-cultured *R. australis* more contained the amount of total flavonoid, total phenol and another plant compounds. These phenolic and flavonoid compounds have anti-oxidant

properties. On the investigation of total phenolic content and total flavonoid content, the shoot clumps extract of tissue-cultured *R. australis* as more total phenolic compound and total flavonoid compound than the natural *R. australis* extract. In addition, we investigated the signaling molecules involved in LPS-stimulated inflammatory response production in murine RAW 264.7 macrophages. We found that the shoot clumps extract of tissue-cultured *R. australis* inhibited LPS-induced NO and prostaglandin E_2 production through suppression of iNOS and COX-2 expression more than the natural *R. australis* extract. These results showed clearly that the shoot clumps extract of tissue-cultured *R. australis* has higher anti-oxidant activity and anti-inflammatory activity in comparison with the natural *R. australis* extract. From this study, the tissue-cultured *R. australis* shoot clumps extract displayed strong possibility as a new natural cosmetic ingredient for skin-care products.

Acknowledgments

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