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복분자 딸기 추출물의 효능에 관한 연구

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The Study on the Effect of *Rubus coreanus* Miquel Extract as a Comsetic Ingredient

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요 약: 통상적으로 식물의 열매, 잎, 뿌리 등의 추출물이 가지는 다양한 효능이 밝혀짐에 따라 점차 천연소재에 대한 관심이 증가하고 있는 추세이다. 본 연구에서는 복분자 딸기로부터 얻은 4종(미숙과, 완숙과, 잎/줄기, 뿌리의 에탄올 추출물)의 각기 다른 추출물에 대한 항산화, 미백, 항주름 효과를 비교하였다. 복분자 딸기의 항산화 및 항염 효과에 대한 몇몇의 보고가 있지만 효능에 대한 연구는 충분하지 않다. 따라서 복분자 추출물의 화장품적 효능·효과를 비교한 결과, 미백효과 및 주름개선효과에 있어, 미숙과부위의 추출물이 다른 부위의 추출물에 비하여 효과가 높은 것으로 관찰되었다. 이 결과를 바탕으로 복분자 딸기의 미숙과는 완숙과나 잎/줄기, 뿌리보다 화장품 원료로 더 높은 응용 가능성을 가지고 있음을 알 수 있었다.

Abstract: Recently plant extracts have obtained increased attention because of their health-related beneficial aspects. In this study, we evaluated anti-oxidation, whitening and anti-wrinkle of four different extracts obtained from *Rubus coreanus* (ethanol extracts of unripe, ripe fruits, leaves/stems, and roots). The ethanol extract of unripe fruits of *Rubus coreanus* generally showed stronger whitening and the anti-wrinkle activities than other extracts of *Rubus coreanus*. As a results, we knew that the ethanol extract of unripe *Rubus coreanus* fruit had more possibility as a whitening and anti-wrinkle ingredient for skin care products.

Keywords: *cosmetics, Rubus coreanus Miquel, whitening, anti-wrinkle, anti-oxidation*

1. Introduction

Rubus coreanus Miquel well known as 'Bok-bun-ja' in Korea is Rosaceae and deciduous prickly shrub. It has been used for centuries as traditional medicine. *Rubus coreanus* Miquel is used for the remedy of impotence, spermatorrhea, allergic diseases, asthma and also has been used as a stomachic and tonic in Korea [1-3]. In the present study, we made a comparative examination of the whitening effect, anti-wrinkle and anti-oxidant effect about each parts of *Rubus coreanus* extracts in order to identify the merit as a cosmetic ingredient.

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2. Materials and Methods

2.1. Reagents and Cell Culture

Rubus coreanus was collected from Chonbuk Kochang in Korea. Antibodies against collagen type I, MMP-1, actin were purchased from Santa Cruz Biotechnology. Soluene 350 was purchased from Perkin Elmer (Massachusetts, USA) and other reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA). All commercially available reagents and solvents were reagent grades and used without any further purification. 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 DMEM supplemented with 10% heatinactivated fetal bovine serum (FBS), penicillin (100 U/mL) and streptomycin (100 g/mL) at 37℃ in a humidified atmosphere containing 5% CO_2 and green tea extract (Bioland, Korea) was used to the positive control.

2.2. *Rubus Coreanus* Extraction

After being dried, 1 kg of unripe fruits, ripe fruits, leaves/stems and roots were immersed in 10 kg of 95% ethanol solution for 2 months. After that, the solution was filtered through a filter paper (5C, TOYO, Japan) and obtain *Rubus coreanus* extracts in each parts by evaporation of ethanol at 50 ~ 60℃ to obtain an extract.

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 to 5.8 J/cm^2 (UVA) from high intensity UV lamp (UVGL-58, San Gabriel, CA91778 U.S.A) for 6 s.

2.4. Inhibition of Auto-oxidation by L-DOPA

The solution containing 135 U/mL of tyrosinase, 0.03% of L-DOPA and 0.1 M potassium phosphate buffer (pH 6.8) in presence of various concentration of samples were incubated for 1 h at 37℃. Then precipitates were collected by centrifugation. After washing in several times with 6 N HCl and distilled water, precipitates dissolved in 2 mL of soluene 350. Then absorbance was measured at 475 nm.

2.5. Inhibition of Melanin Synthesis

B-16 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 2.0×10^4 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 4 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.

2.6. Free Radical Scavenging Activity (DPPH Test)

1, 1-diphenyl-2-picrylhydrazyl (DPPH) is a stable free radical using determination of radical scavenging assay. The radical scavenging activity was determined by a previous report of Haraguchi *et al.*[4]. Then, its antioxidative activity (%) was calculated as compared with blank control.

2.7. Superoxide Dismutase Activity (NBT Test)

Superoxide dismutase (SOD) activity was measured using xanthine-xanthine oxidase system as a source of superoxide and nitroblue tetrazolium (NBT) as a scavenger for this radical. SOD activity was determined as described by K. Furuno[5] by measuring percent inhibition of NBT reduced by SOD.

2.8. Quantitation of Collagen Synthesis

Synthesized collagen from transformed mouse fibroblast NIH3T3L1 was quantitated by the Sirius Red F3BA (BDH, UK). Cells were seeded in 1 mL aliquots in 24-well plate. After 24 h cell culture medium and cells lysate by rapid freeze thawing were dried onto the plate. All plates were incubated at 37℃ for 16 h and then dried for 24 h at 37℃. The wells were filled with 1 mL of 0.1% Sirius Red F3BA in saturated picric acid (w/v%) and the samples stained for 1 h at room temperature. The plate were washed five times with 2 mL of 10 mM HCl for 10 s per wash. The collagen bound stain was extracted with 2 mL of 0.1 M NaOH for 5 min. We used red ginseng (Korea Ginseng Co., Korea) extract to the positive control. Absorbance was then read at 540 nm in microplate reader.

2.9. Gelatin Zymography

Fibroblast cells in subconfluent culture were washed and refreshed with serum-free DMEM medium, and treated for 18 h. The enzymatic activity and molecular weight of electrophoretically separated gelatinolytic enzymes in the conditioned medium of cells were determined by SDS-PAGE as follows. 20 μL of serum free culture medium per sample was prepared in

non-denaturing loading buffer and size fractionated in 10% SDS-polyacrylamide gel impregnated with 0.1% gelatin. The gels were washed with 2.5% Triton X-100 for 1 h at room temperature to remove SDS, rinsed twice with water, and then incubated in a developing buffer (50 mM Tris-HCl buffer, pH 7.4, 20 mM NaCl, 10 mM $CaCl_2$, and 0.1% NaN_3) for 18 h at 37℃. Subsequently, gels were fixed and stained with 10% acetic acid containing 0.5% Coomassie Blue R250. Zons of gelatinolytic activity were detected as clear bands against a blue background. Densitometric analysis was done using Scion Image NIH Image program.

2.10. Western Blot Analysis

Cells were irradiated at various dose and lysed in lysis buffer (20 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM glycerolphosphate, 1 mM Na_3VO_4, 1 μg/mL leupeptin, and 1 mM PMSF). The lysates were clarified by centrifugation at 12,000 × g for 15 min at 4℃, and protein content was measured by the Bradford method. The protein was separated by SDS-PAGE and blotted to nitrocellulose membrane (0.2 μm) (Amersham, Arlington Heights, IL UK). The membrane was blocked with 5% nonfat skim milk in TBST and incubate with the primary and secondary antibodies. Immunoblots were visualized by enhanced chemiluminescence (Amersham, UK) according to the manufacturer's protocol. Conditioned medium from each sample was also subjected to protein analysis of MMP-1, -2, and collagen. For this experiment, culture medium in each tissue culture dish was collected and concentrated using a Centricon 10 microconcentrator (Amicon, Beverly, MA, USA), and 5-fold concentrated conditioned medium (20 μL) was then used for SDS-PAGE analysis. Densitometic analysis for protein bands was done using Scion Image NIH Image program.

2.11. Elastase Activity Assay

Sample is mixed with 1 : 1 [Sample : 1 μg/mL Elastase diluted 0.2 M Tris-HCl (pH 8.0)] and preincubate for 20 min at 25℃. Then, substrate (0.8 mM, succinyl-ala-ala-pro-*p*-nitroanilide) of same volume mix with preincubated mixture and incubate 20 min at 25℃. And we used *Mimosa pudica* (kyung-dong market, Korea) extract, which is same concentration of *Rubus coreanus* for positive control. After that absorbance was measured at 490 nm with UV spectrophotometer.

Table 1. Anti-oxidant Effect of *Rubus coreanus* Extracts by DPPH Test

Anti-hydroxyl radical activity (%)

Extracts \ Conc. (w/w%)		1	0.1	0.01
Rubus coreanus	Unripe fruits	92.3	62.8	48.2
	Ripe fruits	92.1	57.4	18.5
	Leaves/Stems	69.3	42.8	11.4
	Roots	62.1	15.6	9.6
Green tea		92.2	62.2	21.2

Table 2. Anti-oxidant Effect of *Rubus coreanus* Extracts by NBT Test

Anti-superoxide anion activity (%)

Extracts \ Conc. (w/w%)		1	0.1	0.01
Rubus coreanus	Unripe fruits	91.4	71.7	57.6
	Ripe fruits	87.1	64.7	26.9
	Leaves/Stems	64.5	53.2	14.8
	Roots	42.3	13.5	5.6
Green tea		68.8	41.2	22.7

2.12. Collagenase Activity Assay

Each experiment was performed at least three independent conditions and all values are represented as means ± S.D. of triplicates.

3. Results

3.1. Radical Scavenging Activity of *Rubus coreanus* Extracts

To investigate the effect of *Rubus coreanus*, we studied the effect of *Rubus coreanus* extracts on the radical scavenging activity. The ethanol extract of *Rubus coreanus* extracts generally have an anti-oxidant effect. Ripe/unripe *Rubus coreanus* fruits enhanced more the anti-oxidative activity than other extracts of *Rubus coreanus* in DPPH (Table 1) and NBT test (Table 2). Unripe *Rubus coreanus* extract more enhanced radical scavenging activity than green tea known as radical scavengers in 0.01% concentration.

Table 3. Inhibition of Auto-oxidation Effect of *Rubus coreanus* Extracts by L-DOPA

Inhibition of auto-oxidation (%)

Extracts \ Conc. (w/w%)		1
Rubus coreanus	Unripe fruits	43
	Ripe fruits	39
	Leaves/Stems	12
	Roots	17

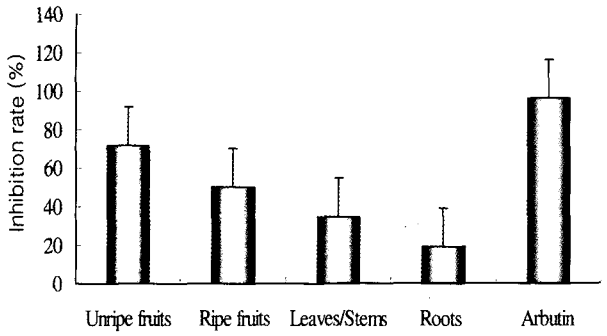

Figure 1. Inhibition of melanin synthesis by *Rubus coreanus* extracts. B-16 melanoma cells were treated on 0.05% concentration. The results were represented as mean of standard deviation (S.D.) of three independent experiments.

3.2. Whitening Effect of *Rubus coreanus* Extracts

We investigated analysis of auto-oxidation by 3, 4-dihydroxyphenylalanine (DOPA) and inhibition of melanization in B-16 melanoma cells for whitening effect of *Rubus coreanus*. The results are given in Table 3 and Figure 1.

As shown in Table 3, ripe fruits extract showed similar inhibition rate compared with unripe fruits extract but not leaves/stems and roots extract. Unripe *Rubus coreanus* fruits extract showed high inhibition rate compared with that of other extracts in 1% concentration.

To investigate whether *Rubus coreanus* extracts inhibit melanin synthesis in B-16 melanoma cells, we analyzed inhibition rate of melanin synthesis by *Rubus coreanus* extracts. Unripe *Rubus coreanus* fruits more enhanced the inhibition rate of melanin synthesis than other extracts of *Rubus coreanus* in 0.05% concentration (Figure 1).

Table 4. Collagen Quantitation of *Rubus coreanus* Extracts by Collagen Synthesis Assay

Collagen synthesis rate (%)

Extracts \ Conc. (w/w%)		0.01	0.005
Rubus coreanus	Unripe fruits	130.1	113.1
	Ripe fruits	110.3	101.4
	Leaves/Stems	-	-
	Roots	-	-
Red ginseng		122.9	111.5

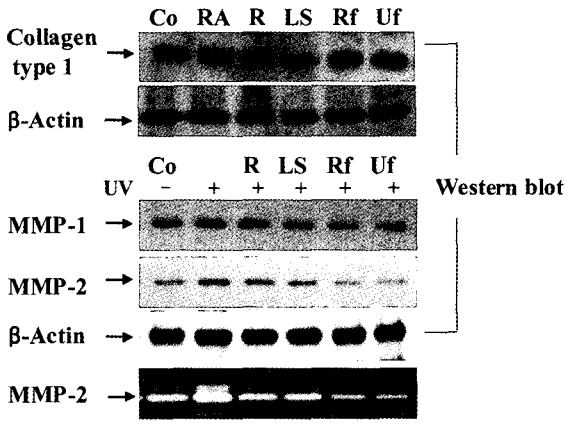

Figure 2. Effect of *Rubus coreanus* extracts on MMP-1, 2 and collagen type I. Bands were subjected to densitometric scanning using the Scion Image NIH Image software. Cells were treated at 0.01% concentration for each extract (RA : retinoic acid, R : roots, LS : leaves/stems, Rf : ripe fruits, Uf : unripe fruits extracts).

3.3. Anti-wrinkle Effect of *Rubus coreanus* Extracts

To study the effect of *Rubus coreanus* extracts, we examined collagen and MMP-1, -2 secreted in culture medium. The ethanol extract of unripe *Rubus coreanus* fruits reduced the expression of MMP-1, -2 and increased the expression of type I collagen secreted in culture medium or cell lysate than other extracts of *Rubus coreanus* (ripe fruits, leaves, stems, and roots). The ethanol extract of unripe *Rubus coreanus* fruit enhanced better collagen synthesis rate than red ginseng extracts and other extracts of *Rubus coreanus* as shown in Table 4.

Also, we examined inhibition of collagenase and elastase activity by extracts of *Rubus coreanus*. Elastase and collagenase inhibitory activity of the

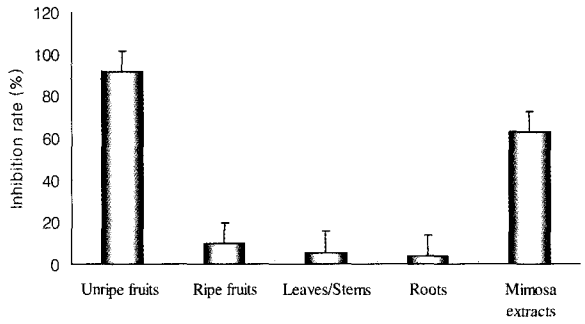

Figure 3. Effect of *Rubus coreanus* extracts on collagenase inhibition assay. Collagenase inhibition rate is measured in 1% concentration on each extracts. The results were represented as mean of standard deviation (S.D.) of three independent experiments.

ethanol extract of unripe *Rubus coreanus* fruits was more enhanced than those of extract of ripe *Rubus coreanus* fruits as shown Figure 3. Otherwise, the leaves, stems and roots extracts did not affect a inhibition of elastase and collagenase.

4. Discussion

In these results, unripe/ripe *Rubus coreanus* fruit extracts are gradually superior to leaves, stems, roots extract in all effect of cosmetic ingredients. Especially, unripe *Rubus coreanus* fruit extracts more reduced the expression of MMP-1, -2 and more induced collagen expression than ripe *Rubus coreanus* fruit extracts at protein levels. Also, unripe *Rubus coreanus* fruit extracts significantly more inhibited collagenase and elastase than ripe fruits, leaves, stems and roots extracts of *Rubus coreanus*.

Therefore, unripe *Rubus coreanus* fruit has more potential benefits applied to cosmetic ingredient for skin care than other parts of *Rubus coreanus* respectively. Although future work will be required to elucidate whether these effects of unripe *Rubus coreanus* fruits extracts occur in comparative experiment by clinical test, these findings provide possible cosmetic ingredient for skin care.

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