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Potential application of acetone extract of *Astragalus sinicus* Linne seed to functional cosmetics

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Abstract−For the functional cosmetic agent using acetone extract of *A. sinicus* Linne seed, the effects of whitening, wrinkling, and safety were investigated. Cell viabilities of Raw 264.7 up to 60 mg/mL did not appear to have any significant direct cytotoxic effect. The melanin concentration was decreased up to 62.1% at 20 mg/mL. When the acetone extract concentration of *A. sinicus* Linne seed was increased from 5 to 20 mg/mL, the inhibitory activity of tyrosinase was sharply increased from 61.3 to 93.8%. However, above 30 mg/mL, it did not increase. The inhibition effects of elastase and collagenase were increased with the extract concentration. Especially, when acetone extract concentration of *A. sinicus* Linne seed was increased from 25 to 200 μg/mL, the inhibition effect of elastase was increased from 60.2 to 97.5%. The inhibition effect of collagenase was increased from 35.0 to 99.0% when increased from 50 to 300 μg/mL. The indexes of pigment and coarseness were 28.56 MI and 18.45R-value, respectively, after 8 weeks of clinical trial using cream pack containing 0.2% of acetone extract of *A. sinicus* Linne seed. The indexes of elasticity and moisture were 64.5Ur/Uf and 55.2AU, respectively, after 8 weeks of clinical trial. These results demonstrate that acetone extract of *A. sinicus* Linne seed may be useful as a potential agent for functional cosmetics.

Key words: *Astragalus sinicus* Linne Seed, Whitening Effect, Anti-wrinkle Effect, Functional Cosmetics

INTRODUCTION

The dominant component of normal skin color is provided by melanin, although there are actually four chromophores that contribute to skin color: hemoglobin, oxyhaemoglobin, carotenoids and melanin [1]. Melanin plays the main role in skin color and pigmentation and up to 10% of skin cells in the innermost layer of the epidermis produce melanin. Upon exposure of the skin to UV radiation, melanogenesis is initiated through tyrosinase [2,3]. Use of tyrosinase inhibitors such as kojic acid and hydroquinone is becoming increasingly important in the cosmetic industry due to their anti-pigmenting effects. Such synthetic agents often result in inflammation of the skin, so alternatives to them are being sought, including naturally occurring compounds. The traditional use of plants against skin disease, and especially for cosmeticeutical purposes, is a common practice in the domestic medicine of many cultures, and may provide leads for better anti-pigmentation compounds [4].

Astragalus Linne is the largest genus in the family Leguminosae. It is annual and perennial herbs or small shrubs with pinnate leaves and pink-purple flowers with some species serving as foodstuffs and pharmaceutical emulsifiers [5]. The roots and leaves of some *Astragalus* species are a very old and well-known drug in traditional medicine, used as antiperspirant, antihypertensive, antidiabetic, diuretic and tonic [6]. Especially, the roots of some *Astragalus* species have shown interesting pharmacological properties including hepatoprotective, immunostimulant, and antiviral activities [7]. However, while the biological activities of *Astragalus* species have been intensively researched, there have been no reports of the skin bioactive effect of *Astragalus sinicus* Linne seed. Previously, the physicochemical features of *A. sinicus* Linne seed were studied in order to identify possibilities of developing new raw materials for functional cosmetic food [8].

In this study, in order to investigate the cosmetic effect of acetone extract of *A. sinicus* Linne seed, the inhibitory activity of the tyrosinase, elastase and collagenase and cytotoxicity were investigated *in vitro*. In addition, pigment, coarseness, elasticity, and moisture index for clinical trial using cream pack containing acetone extract of *A. sinicus* Linne seed were tested.

MATERIALS AND METHODS

1. Reagents and Materials

All chemicals were purchased from Sigma (USA). B16F10 mouse melanoma cells from the Korean Cell Line Bank were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin at 37 °C in a humidified incubator with 5% CO_2. *A. sinicus* Linne seed was obtained from Kwang ju (Korea). Extraction by 250 g of sample and 500 ml of acetone in soxhlet apparatus at room temperature for 3 to 6 hr was carried out. After standing overnight at 4 °C, the mixture was centrifuged, and the supernatants were evaporated. The

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extract was used as the *A. sinicus* Linne seed extract. The dried extracts were used directly for analyses and stored at 4 °C for further use.

2. Melanin Concentration

Melanoma B16 cells were stimulated with a melanocyte stimulating hormone and pretreated with various concentrations of *A. sinicus* Linne seed extract for 72 hr. After washing with 1% potassium phosphate buffered saline (PBS), cell pellets were dissolved in 100 μl of NaOH (1 N) at 80 °C and centrifuged for 10 min at 10,000 ×*g*. The optical density of each supernatant was measured at 450 nm.

3. Inhibitory Activity of the Tyrosinase

Tyrosinase assay was used to measure tyrosinase inhibition effect of the extract on both L-tyrosine and dihydroxy phenylalanine (DOPA). Briefly, normal human melanocyte cells were cultured in 24-well plates. After being treated with an individual herbal preparation for 24 h, cells were washed with 1% PBS and lysed with PBS (pH 6.8) containing 1% Triton X-100. Then, cells were disrupted by freezing and thawing, and lysates were clarified by centrifugation at 10,000 ×*g* for 10 min. Each well of a 96-well plate contained 40 μg protein, 2.5 mM-DOPA, and 0.1 M PBS (pH 6.8). After incubation at 37 °C for 1 h, the absorbance was measured at 450 nm using an ELISA (enzyme-linked immunosorbent assay, Synergy HT, BIOTEK, USA) reader. Tyrosinase inhibition was calculated with the following formula:

$$\text{Tyrosinase inhibition (\%)} = [1 - (\text{OD of sample}/\text{OD of control})] \times 100$$

4. Inhibitory Activity of Elastase

An aliquot of 20 μL of Human leukocyte elastase solution containing sodium acetate buffer solution (50 mM, pH 5.3) and 25-400 μg/mL of sample were reacted in 48-well plates and 200 μL of p-nitroanilide (400 μM) was added. After reaction at 37 °C for 20 min, an aliquot of 120 μL of reaction was added into 96 well plates. The absorbance was measured at 410 nm using an ELISA reader. Elastase inhibition was calculated with the following formula:

$$\text{Elastase inhibition (\%)} = [1 - (\text{OD of sample}/\text{OD of control})] \times 100$$

5. Inhibitory Activity of Collagenase

An aliquot of 300 μL of collagen solution (0.25 mg/mL), 600 μL of sample, and 600 μL of collagenase solution (0.5 unit) were mixed with 1,500 μL of PBS (pH 6.0). After pre-incubation for 20 min in a dark room, the absorbance was measured at 280 nm and 200 nm with a fluorescence spectrophotometer (F-4500, Hitachi, Japan). Collagenase inhibition was calculated with the following formula:

$$\text{Collagenase inhibition (\%)} = [1 - (\text{OD of sample}/\text{OD of control})] \times 100$$

6. Cell Viability

Cell viability for safety test of *A. sinicus* Linne seed extract was determined by using the MTT (3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl-2H-tetrazolium bromide) assay. For experiments, cells were plated in 24-well plates at 1×10^5 cells/well. After 24 h, the test sample was added to each well and incubated for 24 h. Cell survival was determined in a colorimetric assay using mitochondrial dehydrogenase activity in active mitochondria to form purple formazan.

7. Clinical Trial

An aliquot of 86.4% deionized water, 13% polyvinyl alcohol, 0.4% Tween 80, and 0.2% acetone extract of *A. sinicus* Linne seed was added into 300 ml of flask for cream preparation and mixed for 2 hr in room temperature. After 7 days of storage, it was used for clinical trial. The pigment, elasticity, and moisture indexes were measured by Aramo TS Diagnosis System (Aram HUVIS Co, Korea) after 8 weeks of clinical trial (women 24 people; range of 20 to 60 ages) on the cheek.

8. Statistical Analysis

All the tests were conducted in triplicate and data were averaged. Standard deviations were also calculated. Student's t-test was used to evaluate significant differences ($P<0.05$) between the means of each sample.

RESULTS AND DISCUSSION

Many researchers have studied the synthetic cosmetic additives [9,10]. However, the synthetic agents are being restricted because of skin inflammation. Therefore, the development of more effective cosmetic material of natural origins is desired. Recently, we investigated various extracts of *A. sinicus* Linne seed on antioxidant activity including DPPH radical scavenging activity, hydroxyl radical scavenging activity, superoxide radical scavenging activity, reducing power, chelating ability on ferrous, and xanthine oxidase activity were researched *in vitro*. In addition, antioxidant enzymes including, catalase, superoxide dismutase, and glutathione peroxidase activity were also studied in rats. Among various extracts, acetone extract showed the maximum antioxidant activity *in vitro* and *in vivo* (data not shown). Therefore, the acetone extract of *A. sinicus* Linne seed might be a strong candidate for cosmetic application and products. In the present study, in order to study the acetone extract effect of *A. sinicus* Linne seed on the skin bioactive effect, cytotoxicity and the inhibitory activity of the tyrosinase, elastase and collagenase were investigated. In addition, a clinical trial of acetone extract of *A. sinicus* Linne seed was carried out.

1. UV Absorption of Acetone Extract of *A. sinicus* Linne Seed

Solar ultra-violet (UV) light consists of electromagnetic radiation with wavelengths ranging from 100 to 400 nm that are conventionally divided in to three energy bands: UVA (320-400 nm), UVB (280-320 nm), and UVC (100-280 nm). Terrestrial UV light is only composed of UVA and UVB, since UVC is absorbed by stratospheric ozone in the earth's atimophere. It is already well known that UVB is the principal erythematous radiation in terrestrial sunlight and implicated in structure and cellular skin damage, such as actinic keratosis, telangiectasis, and skin cancer. In contrast, UVA radiation has been considered to be relatively harmless and only responsible for minimal skin damage [11]. Therefore, the UV absorption by cosmetic material is very important for skin protection. To measure the UV absorption ability of acetone extract of *A. sinicus* Linne seed, scanning using 100 μg/m of extract was carried out in range of 200 to 700 nm. The results are shown in Fig. 1. The absorbance of ascorbic acid in the range of 200 to 250 nm ranged of 0.25 to 0.6 and was zero at 290 nm. On the other hand, in the case of acetone extract of *A. sinicus* Linne seed, when the wavelength was increased from 200 to 260 nm, the absorbance was sharply decreased from 2.73 to 0.42 and the absorbance in the range of 260 to 400 nm ranged of 0.15 to 0.42.

2. Effect of Acetone Extract Concentrations on Melanin Concentration and Inhibitory Activity of Tyrosinase

Melanin biosynthesis can be inhibited by avoiding UV exposure, by inhibition of melanocyte metabolism and proliferation, by inhi-

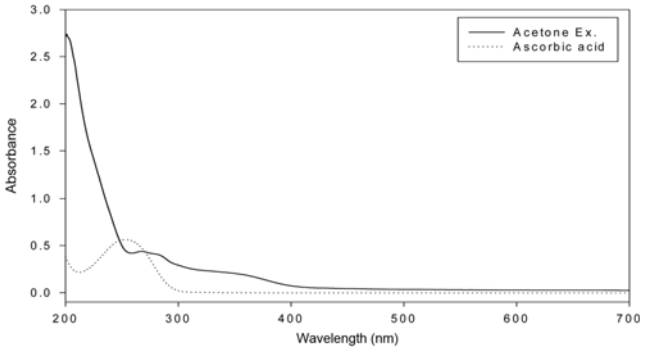

Fig. 1. Absorption spectra of acetone extract of *A. sinicus* Linne seed.

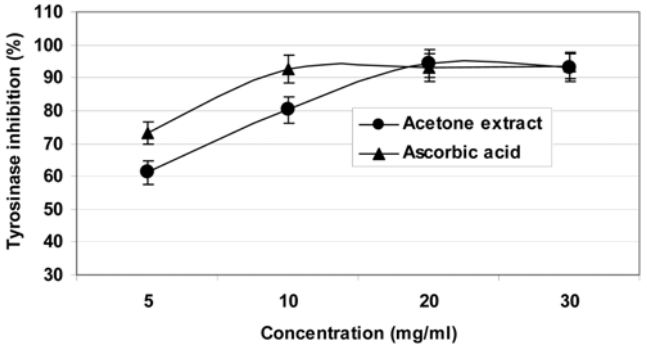

Fig. 3. Effect of acetone extract concentrations on the inhibitory activity of tyrosinase.

bition of tyrosinase, or by removal of melanin by corneal ablation. Apart from avoiding UV exposure, application of tyrosinase inhibitors may be the least invasive procedure for maintaining skin whiteness; such agents are increasingly used in cosmetic products [12]. Therefore, melanin formation is the most important determinant of mammalian skin color. Melanin is synthesized in a multi-step biochemical pathway that operates within a specialized intracellular organelle, the melanosome. In melanogenesis, the proximal pathway consists of the enzymatic oxidation of tyrosine or l-DOPA to its corresponding o-dopaquinone catalyzed by tyrosinase. After multibiosynthesis steps, further polymerization yields melanin [13]. Tyrosinase inhibitors are important constituents of cosmetics and skin-lightening agents. Tyrosinase, a copper-containing monooxy genase, is a key enzyme that catalyzes melanin synthesis in melanocytes. It catalyzes two major reactions, including hydroxylation of tyrosine and oxidation of the o-diphenol product, l-dopa. Dopa oxidation produces a highly reactive intermediate that is further oxidized to form melanin by a free radical-coupling pathway [14]. Fig. 2 is effect of acetone extract concentrations of *A. sinicus* Linne seed on melanin concentration in melanoma cell. When the acetone extract concentration of *A. sinicus* Linne seed was increased from 0 to 20 mg/mL, the melanin concentration was decreased from 100 to 62.1%. On the other hand, in the case of above 30 mg/mL, it did not decrease. This result indicates that the inhibition of melanin synthesis was affected by acetone extract concentration of *A. sinicus* Linne seed. Fig. 3 is the effect of acetone extract concentrations of *A. sinicus* Linne seed on the inhibitory activity of tyrosinase. When the acetone extract concentration of *A. sinicus* Linne seed was increased from 5 to 20 mg/mL, the inhibitory activity of tyrosinase was sharply increased from 61.3 to 93.8%. However, above 30 mg/mL, it did not increase. In comparison, ascorbic acid, a naturally occurring cosmetic vehicle and whitening agent with tyrosinase inhibitory activity, has 94.2% at 10 mg/mL. These results suggest that acetone extract of *A. sinicus* Linne seed exhibited potent inhibition of melanin synthesis which may be attributed to the antioxidation and competitive inhibition of tyrosinase. From Figs. 2 and 3, the whitening effects of acetone extract of *A. sinicus* Linne seed were excellent. Therefore, it is should be considered as a promising candidate for novel whitening agents.

3. Effect of Acetone Extract Concentrations on the Inhibitory Activity of Elastase and Collagenase

UV irradiation from sun produces free radicals and related reactive oxygen species (ROS) in human skin. These injure the DNA and extracellular matrix (ECM) in dermis of human skin. UV irradiation has been shown to stimulate fibroblast which secretes matrix metalloproteinases (MMPs) by cytokines. MMPs constitute more than 20 proteinase, and can degrade most components of ECM such as collagen, laminins, and elastins. Since collagen fibrils with elastin are responsible for the strength and resiliency of skin, their degradation causes wrinkles and skin aging [15,16]. To investigate the anti-wrinkle effect of acetone extract of *A. sinicus* Linne seed, the inhibition effects of elastase and collagenase were measured and compared with quercetin as a standard. The inhibition effects of elastase are shown in Fig. 4. The inhibition effects of elastase were

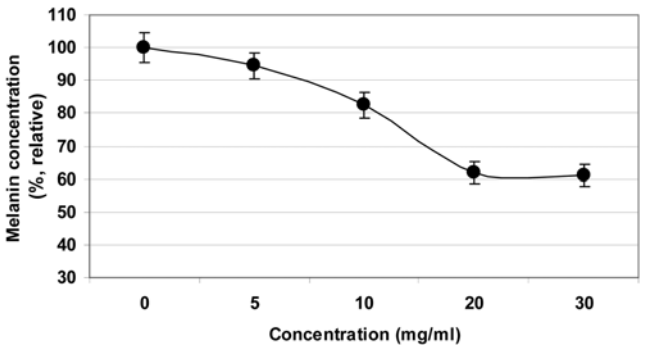

Fig. 2. Effect of acetone extract concentrations on melanin concentration in melanoma cell.

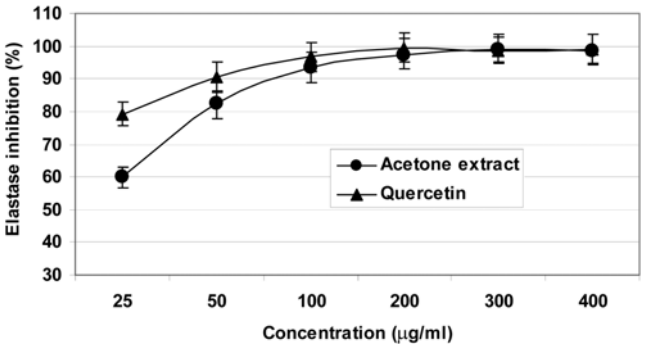

Fig. 4. Effect of acetone extract concentrations on the inhibitory activity of elastase.

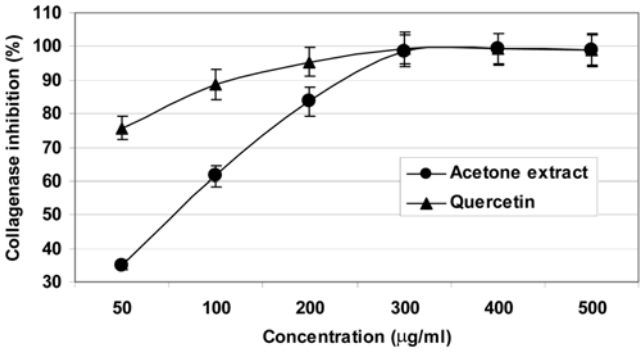

Fig. 5. Effect of acetone extract concentrations on the inhibitory activity of collagenase.

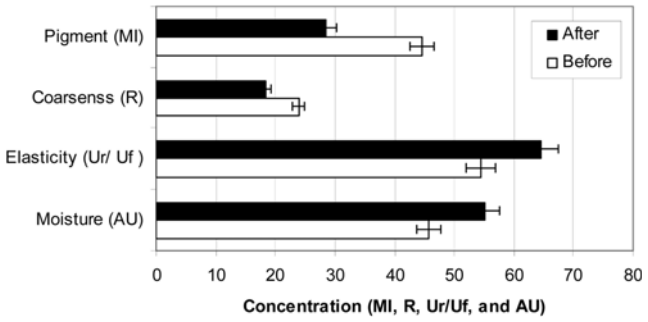

Fig. 7. Effect of acetone extract on the pigment, coarsenss, elasticity, and moisture effect.

increased with the extract concentration up to 200 μg/mL. Especially, when the acetone extract concentration of *A. sinicus* Linne seed was increased from 25 to 200 μg/mL, it increased from 60.2 to 97.5%. However, in the case of above 300 μg/mL, the inhibition effects of elastase did not increase. On the other hand, when the quercetin concentration as a standard was increased from 25 to 200 μg/mL, it increased from 79.7 to 99.0%. However, in the case of above 300 μg/mL, it was similar to that of acetone extract of *A. sinicus* Linne seed. These results showed that the inhibition effects of elastase were strongly affected by the acetone extract concentration of *A. sinicus* Linne seed. Fig. 5 is the inhibitory activity of collagenase of acetone extract of *A. sinicus* Linne seed. The inhibition effects of collagenase were increased with the extract concentration up to 300 μg/mL. Especially, when the acetone extract concentration of *A. sinicus* Linne seed was increased from 50 to 300 μg/mL, it increased from 35.0 to 99.0%. However, in the case of above 400 μg/mL, it did not increase and ranged from 99.0 to 99.3%. On the other hand, when the quercetin concentration was increased from 50 to 300 μg/mL, it increased from 76.5 to 99.2%. However, above 400 μg/mL, it was similar to that of acetone extract of *A. sinicus* Linne seed. These results showed that the effects of collagenase were affected by the acetone extract concentration of *A. sinicus* Linne seed. From Figs. 4 and 5, the anti-wrinkle effects of acetone extract of *A. sinicus* Linne seed were excellent.

4. Effect of Acetone Extract Concentrations on Cell Viability

To investigate the safety of acetone extract of *A. sinicus* Linne seed, cytotoxicity tests using MTT assay for cell viabilities of Raw 264.7 were carried out.

Cell viabilities of acetone extract of *A. sinicus* Linne seed are shown in Fig. 6. When the acetone extract concentration of *A. sinicus* Linne seed was increased from 0 to 10 mg/mL, cell viabilities of Raw 264.7 did not appear to have any significant direct cytotoxic effect. However, when the extract concentration was increased from 20 to 30 mg/mL, it was increased from 101.3 to 107. 5% and in the range of 40 to 60 mg/mL, it was ranged of 96.2 to 98.7%. Thus, the acetone extract of *A. sinicus* Linne seed were relatively safe as cosmetic material.

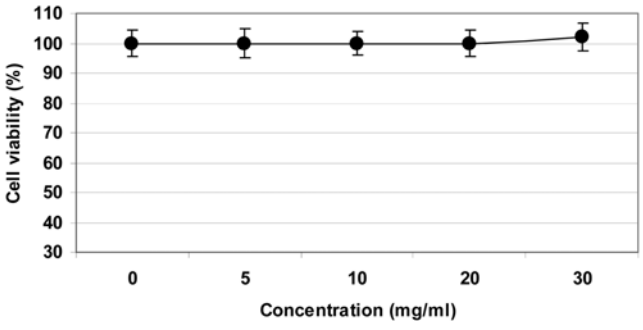

Fig. 6. Cell viability of acetone extract of *A. sinicus* Linne seed.

5. Effect of Acetone Extract on Clinical Efficacy

To clinical efficacy of cream containing acetone extract of *A. sinicus* Linne seed, pigment, coarsenss, elasticity, and moisture index were investigated. The results are shown in Fig. 7. The pigment index was 28.56 MI after clinical trial, which was about 62.7% compared to that of pre-clinical trial. The coarseness index was 18.45R-value after clinical trial, which was about 77.1% compared to that of pre-clinical trial. The elasticity index was 64.5Ur/Uf after clinical trial, which was about a 25.5% increase compared to that of pre-clinical trial. In the case of moisture index, it was 55.2AU after clinical trial, which was about 21.0% of compared to that of pre-clinical trial. These results demonstrated that pigment, coarseness, elasticity, and moisture index were strongly affected by the acetone extract of *A. sinicus* Linne seed.

CONCLUSION

For the possibility of a functional cosmetic agent using acetone extract of *A. sinicus* Linne seed, the effects of whitening, wrinkle, and safety were investigated. The strong absorbances of acetone extract of *A. sinicus* Linne seed were shown in UV A and B as well as UV C, compared to vitamin C. The maximum inhibitory activity of tyrosinase was obtained at 93.8% at 20 mg/mL. The maximum inhibition effects of elastase at 200 μg/ml and collagenase at 300 μg/mL were obtained, 97.5 and 99.0%, respectively. The indexes of pigment and coarseness after 8 weeks of clinical trial were about 62.7 and 77.1%, respectively, compared to that of pre-clinical trial. The indexes of elasticity and moisture after 8 weeks of clinical trial were about 25.5 and 21.0% of increase, respectively, compared to that of pre-clinical trial. From these results, we found that the acetone extract of *A. sinicus* Linne seed exhibited effects of whitening, wrinkling, and safety. Therefore, the acetone extract of *A. sinicus* Linne seed may be practically applicable as a functional cosmetic agent. However, further work is needed to identify the active mechanisms and identify more bioactive constituents in the various extracts of

A. sinicus Linne seed.

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