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Original Research Article

A Study on Anti-oxidative Activity of the *Lithospermum Erythrorhizon* Extracts for Application as a Cosmetic Ingredient

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Abstract - This study has assessed the anti-oxidative activities and cytotoxic effects of *Lithospermum erythrorhizon* ethanol extract and measured the effects of tyrosinase inhibition activities with a goal of estimating the usage of the medicinal plant as an ingredient of cosmetics. First, to perform a basic test on the extract, pH and UV-spectrum were measured. According to the measurement, the extract had control functions at pH 5.5, and maximum absorbance occurred at 530nm. In particular, DPPH (1-1-diphenyl-2-picryl-hydrazyl)-inhibiting activity (IC50) and polyphenol content were 149.81 μg/mL and 51.28± 2.52 mg/mL respectively. In addition, as extract concentration increased, tyrosinase inhibition activities improved as well. In raw 264.7 cell-based MTT assay, cell survival rates were 98% at 1000 ppm and 153% at 100 ppm. Therefore, it's been confirmed that there is almost no cytotoxin. According to the test results above, it appears that the *Lithospermum erythrorhizon* ethanol extract would be effective in anti-oxidation and application as a cosmetic ingredient.

Key words - *Lithospermum erythrorhizon*, Tyrosinase, Cytotoxin, Anti-oxidation

Introduction

These days, with the increased percentage of aged in the population, in recognition that living quality is getting higher, studies on biological rhythms, the treatment of diseases, and preventing aging have been active (Choi *et al*., 2010). Previous studies on natural substances from various plants are as follows: the anti-oxidation and anti-inflammation properties of peach blossoms (Lee and Ahn, 2010), the antibiosis of gardenia on aerobic bacteria (Ryu and Jo, 2004), anti-oxidation and anti-inflammation properties of *phellinus linteus* (Cheon *et al*., 2006), white mulberry extracts (Cho *et al.,,* 2006), anti-oxidation and anti-inflammation effects of wild peach trees (Cha and Lee, 2004), bioactivity of *cornus kousa* (Kim *et al.,* 2008), and the anti-oxidation properties of *Platyphyllum* extracts (Chang *et al*., 2011). With such trends and the need to be safe from chemical stimuli, we have a need to develop natural cosmetics and new products have been presented through increased interest in and demand for them (Sim *et al.,* 2008). The skin which is in direct contact with cosmetics is roughly divided into the epidermis and the dermis. The epidermis protects our skin from physical and chemical stimuli and its pH is an important element in its chemical protection mechanism. Healthy skin has a pH of 5.5 and it is weakly acidic, with the exception of the armpit and the scalp. Such weak acidity causes the skin to change to being alkaline through washing or external stimuli and to lose its chemical protection ability (Lee *et al.* 2004). Therefore, most basic cosmetics have the ability adjust and hold the proper pH, and to help the skin to optimally maintain a weak acidity. In particular, weakly acidic cosmetics have this function (Ha, 1999). Cosmetics are restricted by safety, stability, and usability. In order to see the possibility of natural substances or extracts used as ingredients in cosmetics, their bioactivity, safety, and functionality should be examined. Evaluating their anti-oxidation ability, whitening, and their influences on cytotoxicity is considered basic to predict the possibility of their use as ingredients in cosmetics.

Lithospermum erythrorhizon a plant of the Boraginaceae family with red-violet roots, is called Jicho, Jacho, Jageun, Jadan and grows perennially in the mountains of China and Japan as well as Korea (Cho *et al.,* 1999). It has been used as

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a medicinal and dyeing plant in Korea and Japan. Medicinal uses are to promote blood circulation, alleviate fever, treat eczema, and urinary tract infections (Lee *et al.,* 1998). The plants outer skin of its root is a red-violet color and contains shikonin, acetylshikonin, and isobutylshikonin which are derivatives of naphthoquinone (Ju *et al.,* 2010), as well as allantoin, cyanoglucoside, fumaric acid, and succinic anhydride as naphthoquinone coloring materials (Yun *et al.,* 1999). Also, studies on the effects of shikonin have been made focusing on its control of oxidation and reduction reactions and the promotion of recovery in wounds and burns, atopy dermatitis (Ju *et al.,* 2010), and anti-bacterial effects (Bae, 2004). This study aims to look into the possibility of *Lithospermum Erythrorhizon* as an ingredient for cosmetics by exploring its properties of pH, anti-oxidation, whitening, and cytotoxicity.

Materials and Methods

Specimen production

The *Lithospermum erythrorhizon* used in the study was purchased from Yeongduk, Gyeongsangbukdo Province, washed in running water, frozen rapidly to -80℃, kept frozen for 24 hours, freeze dried, and then ground. To observe the properties of the powder, it was retted with different solvents and its pH was measured. Also its absorption at each wavelength was observed. Fluids extracted after the ground powder was soaked in 70% ethanol at room temperature for 24 hours were filtered with a filtering bed (Whatman No.2) and the filtered fluid was concentrated in a vacuum evaporator and frozen. The powder extract concentrate was obtained using 100 mg/ml (DMSO:Ethanol=1:1). The extract was filtered with the use of a 0.2 μm filtering membrane(Fig 1).

Reagents and equipment

The extract solvents for the pH test were Grade 1 reagents (Duksan Pure Chemical Co., Ltd., Ansan, Korea) N-hexane, chloroform, ethyl acetate, butanol, ethanol, D.W (Distilled water), dimethyl sulfoxide (DMSO). These were selected according to their degrees of polarity. The reagent used for anti-oxidation was 1-1-diphenyl-2-picryl-hydrazyl (DPPH), and the enzyme used to inhibit tyrosinase activity was mushroom tyrosinase. MTT formazan for the cytotoxicity test was purchased from Sigma (Sigma-Aldrich Chemical Co. St.Louis, MO, USA). Also used was a rotary evaporator(Rotavapor-R, Buchi Labortechnik, Flawil, Switzerland), a color difference meter (Minolta CR-300, CT-310, Japan), a pH meter (InoLab pH 730, Germany), a spectrophotometer (Shimadzu, JP/UV-160A), and a cell incubator (MCO-15AC, Sanyo electric, Japan), ELISA Reader (BioTek, USA).

Measuring the pH with different solvents

Lithospermum erythrorhizon was frozen and dried to make a 5% sample and its pH was measured with a pH meter using a powdered sample. The glass electrode was already immersed in distilled water and the detector was washed with distilled water and the measurement was made at 25℃.

UV-spectrum measurement

The measurement values of pigments the sample held were presented in a Lab Color Space according to the standards of the Commission Internationale de I'Eclairage (CIE). First, 1g

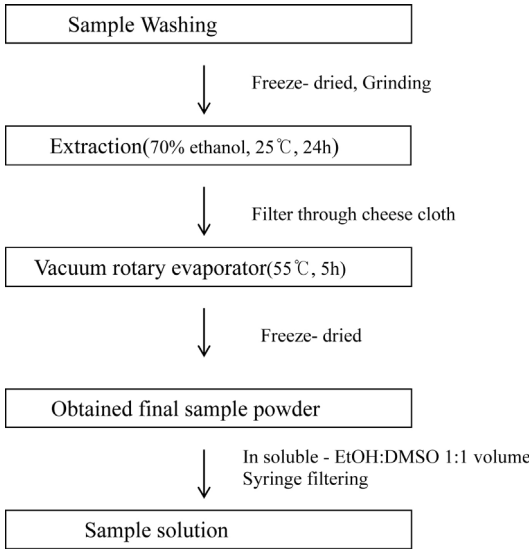

Fig. 1. The procedure for extraction from *Lithospermum erythrorhizon.*

of the specimen was frozen, dried, and ground. It was spun in a centrifuge after it was dissolved in 20 mL of each solvent to extract the supernatant, and after a certain amount of time it was absorbed into a Whatman paper, the values of L, a, and b were measured with the use of a color meter (Minolta CR-300, CT-310). For UV-spectrum measurement (Shimadzu, JP/UV-160A), 1 mL of supernatant extract was used and the absorption was measured between 200 and 700 nm.

Cell culture

For the cytotoxicity test, RAW264.7 cells, a macrophage of a mouse, were purchased from the Korea Cell Bank and for a cell culture, DMEM growth medium containing 10% FBS and 1% penicillin-streptomycin was used. The cells were cultured in CO_2 incubators (37°C, 5% CO_2 incubator).

Electron donating ability

The electron donating ability was measured through the new Blois (1958) method. After 500μL of fluid sample of the different concentrations was mixed with 2 mL of DPPH in which 1,1-diphenyl-2 picrylhydrazyl (DPPH) was dissolved in ethanol with a concentration of 0.15mM, it was vortexed for 10 seconds and reacted for 30 minutes at 25℃ and its absorption was measured at 517 nm. For EDA, the difference between absorptions of the test group and the control group was presented as a percentage. The IC_{50} value was used with concentrations which inhibit 50% of the oxidation from an EDA change curve according to the concentrations of the specimen. For a control group ascorbic acid was used.

Total polyphenol compound

The Total polyphenol amount measured through colorimetry with the Folin-Denis method (Swain *et al.,* 1959). After the same amount of Folin-Denis solution was added to 1 mL of specimen with different concentrations for 3 minutes, 1mL of 10% Na_2CO_3 was added for 1 hour in a dark room and the absorption was measured at 700 nm. Calibration curves were shown with gallic acid as a standard material.

DOPA autoxidation inhibition assay (*in vitro*)

To measure tyrosinase inhibitory activity, DOPA oxidase activity was measured with the use of an applied method developed by Moon *et al*. (2010). For DOPA oxidase activity, with L-DOPA as a substrate, absorption of dopachrome produced by tyrosinase was measured at 490 nm. For the reactor, 120 μL of L-DOPA (a solution dissolved in 8.3 mM, 67 mM phosphate buffer [pH6.8]) as a substrate and 40 μL of the specimen solution were placed into the 96-well plate. Then, 40 μL of tyrosinase (125 U/mL) was added to it. After it was reacted at 37℃ for 30 minutes, the activity was measured with a use of an ELISA reader at 490 nm. For the control group, ascorbic acid was used to obtain an activity inhibitory rate.

MTT assay

To measure the cell survival rate, the MTT method was used. It measured the changes in color of yellow tetrazolium as it changed into purple fomazan by mitochondria enzyme of cells. 1×10^5 cells/mL of RAW 264.7 cells were divided into 100 μL and each of them was transplanted into a 96well tissue culture plate and cultured at 37℃ in a 5% CO_2 incubator for 24 hours. To the cells cultured, the specimen solution of different concentrations was added and cultured for 18 hours, and then 50 μL of 5 mg/mL MTT solution which was dissolved in a phosphate buffered solution (PBS) that was put into each well and cultured for 4 hours. After the cultures were completed, the supernatant was removed and 100 μL of DMSO was put into each well and fomazan was dissolved and the absorption was measured at 570 nm. Without the specimen solution added, the growth medium was added and the cell survival rate was measured based on the absorption of the control group.

Results and Discussion

Measuring pH according to solvents

To speculate on the possibility of the subject extract to be

Table 1. pH measurement of *Lithospermum erythrorhizon*

Solvent	Solvent pH	Extract solution pH
Hexane	6.4 ±0.2**	5.8 ±0.3**
Chloroform	3.2 ±0.4**	4.4 ±0.9**
E.Acetate	2.6 ±0.3**	3.1 ±0.6*
Butanol	5.1 ±0.3**	4.5 ±0.5
Ethanol	5.3 ±0.2	5.8 ±0.4
D.W	7.0 ±0.1**	6.5 ±0.5**
DMSO	9.5 ±0.2**	8.4 ±0.2**

[z]All values are mean ± SD of triplicate determinations.
** and *: Significant at p<0.001 and p<0.005.

Table 2. L-a-b value of *Lithospermum erthrohizon*

	L	a	b
Hexane	61.67±0.649	46.46±1.785**	15.47±0.962**
Chloroform	71.31±0.264**	31.54±1.545**	7.9±0.648**
E.Acetate	64.39±0.271**	44.17±0.120**	14.21±0.066**
Butanol	66.69±0.327**	38.74±1.010**	10.90±0.637**
Ethanol	76.29±0.302**	24.32±1.231**	3.77±0.459
DMSO	34.15±0.106**	13.01±1.846*	4.52±0.259**
Water	89.92±0.914	3.20±1.065*	7.97±0.191**

[z]All values are mean ± SD of triplicate determinations.
[y]L: lightness, a: redness, b: yellowness.
** and *: Significant at p<0.001 and p<0.005.

used as a cosmetic ingredient, this study measured the pH. The changes in the pH depending on the solvents when the extract was dissolved are presented in Table 1. The pH for hexane (6.4±0.2), DMSO (9.5±0.2) and D.W (7±0.1) was higher than the ideal acidity of skin, a pH of 5.5, but after the specimen was dissolved, the values lowered. The alkali grew weaker and the pH approached 5.5. The results were as follows: hexane (5.8±0.3), DMSO (8.4±0.2), and D.W (6.5±0.1). For Chloroform (3.2±0.4), ethyl acetate (2.6±0.3) and ethanol (5.3±0.2), after the specimen was dissolved, strong acidity changed into weak acidity as follows: chloroform (4.4±0.9), ethyl acetate (3.1±0.6) and ethanol (5.8±0.4). Thus, it was found that solvents with different polarities have the ability to control acidity centering on a pH of 5.5.

UV-spectrum measurement

In the lab values of the Commission Internationale de I'Eclairage : CIE, L indicates luminosity, a indicates colors from red to green, and b indicates those from yellow to blue. In each solvent, the extract was colored red in an acid solvent such as chloroform or ethyl acetate, dark purple in higher pH solvents such as hexane, butanol, and ethanol, and purple in an alkali such as DMSO, and a L*a*b* value based on the colorimetry is presented in Table 2.

The UV-spectrum of the extract in different solvents was measured between 200 nm and 700 nm. It was discovered that hexane, chloroform, ethyl acetate, butanol and ethanol had similar absorption zones with the largest values at 520 nm and

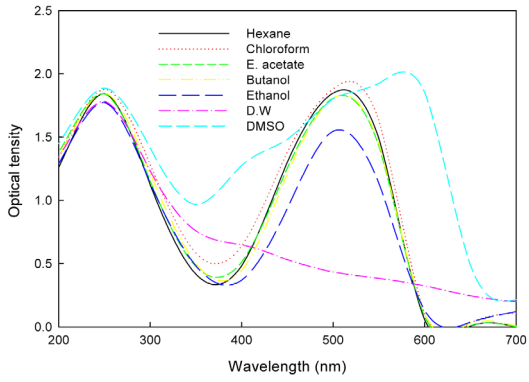

Fig. 2. UV-spectrum value of *Lithospermum erythrorhizon.*

530 nm, and DMSO had a wider absorption zone. In distilled water, the absorption for the visible light zone was not significant, but it is suggested that this is because the pigment of the extract is not water-soluble. UV-spectrum measurements are presented in Fig. 2.

Electron donating ability

In measuring the EDA, the degrees to which electrons are donated to active radicals and how strongle oxidation is inhibited are measured. Anti-oxidation is measured through the degree the dark purple color of DPPH is washed out by the polyhydroxyl aromatic compound, aromatic amine, and ascorbic acid. Electron donating ability effects in measuring the anti-oxidation activity of the 70% ethanol extract were as follows: 34.9%±1.11 at 62.5 ppm and 74.47%±0.73 at 1000 ppm. The scavenging effects were higher in comparison at

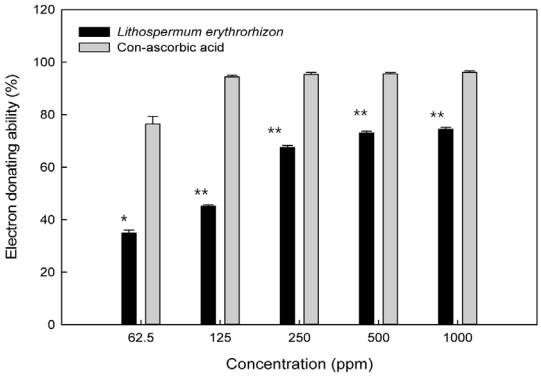

Fig. 3. Electron donating ability of the ethanol extracts from *Lithospermum erythrorhizon*.
The bars represent the standard deviation. Asterisk indicates a significant difference at **p<0.001, *p<0.005 level.

Table3. Antioxidant activity, phenolics contents of the *Lithospermum erythrorhizon* studied

	Total polyphenol(mg/g GAE[x])	DPPH (IC_{50})(μg/ml)
Sample[y]	51.284±2.552	149.818

[z]All values are mean ± SD of triplicate determinations.
[y]Sample: experiment extract obtained 70% ethanol condition.
[x]GAE: gallic acid equivalent.

76.48%±2.85 and 96.14%±0.58 in ascorbic acid, the control group (Fig. 3).

As one of the methods to evaluate anti-oxidation in natural substances, for an IC_{50} value for which a specimen concentration needed to scavenge 50% of the DPPH radical specimen, the concentration was 0.015% and the concentration was 149.8 μg/ml. Previous studies reported that the IC_{50} value of ethyl acetate fraction was 122.95 μg/ml, which was the highest value for the extract fractions for *Lithospermum erythrorhizon* (Kim *et al*., 2010) and the 70% ethanol extract in this study showed similar results. Also, as previous studies reported that anti-oxidation of EDA had correlations with the total phenolic compounds, EDA was compared with the amount of phenolic compounds and the results are presented in Table 3. Based on a report (Tobin and Thody, 1994) that removal of reactive oxygen is effective for inhibition of pigmentation, the high reactive oxygen scavenging effects showed the possibility that the subject extract to be used as a material for whitening cosmetics.

Total polyphenol compound

A polyphenol compound, as a representative anti-oxidative material, has a phenolic hydroxyl which can be connected with protein and macromolecules, it shows different forms of bioactivity such as anti-oxidation and anti-cancer effects (Jung *et al*., 2004). The total amount of polyphenol compound measured with gallic acid as a standard material was 51.28± 2.55 (mg/g) and Table 3 shows its relationships with the EDA. The relationship between the wave absorption of natural pigments extracted from natural material and other ingredients were examined through a UV/VIS spectrum measurement. The ethanol extract of *Lithospermum erythrorhizon* varies in color from red to purple depending on the solvents. Compounds with such a color must be conjugations of long-chains, have polycyclic aromatic chromophores or benzenoids with conjugating substituents, and as the polarity of solvents increases, they were observed at 400~500 nm UV Spectra in the same area where compounds such as flavones and flavonols are observed. As in Fig. 2, as all the maximum absorption frequencies ranged from 270 to 280 nm and from 525 to 530 nm, at the same wavelength as napthoquinone pigment containing shikonin, a high absorption appeared.

DOPA autoxidation inhibition assay (*in vitro*)

When skin is exposed to ultraviolet rays, Tyrosin in pigmentation cells, which exists in the basal layer of skin, produces melanin through enzyme action of tyrosinase. Melanin which is produced excessively at this time causes pigmentary deposits such as melasma or freckles. It has been reported that effective materials which inhibit tyrosinase activity are ascorbic acid, arbutin, kojic acid, azelaic acid, and tropolone (Jung *et al.,* 1995). Recently, experiments using extraction to search for effective naturally sourced materials which will inhibit melanin synthesis have increased. The values for tyrosinase activity inhibition within pigment cells are presented in Fig. 4.

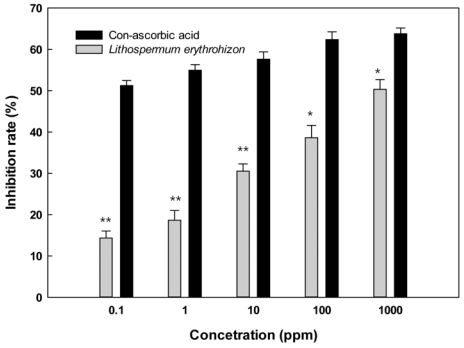

Fig. 4. DOPA autoxidation inhibition rate of the crude ethanol extracts from *Lithospermum erythrorhizon.* The bars represent the standard deviation. Asterisk indicates a significant difference at **p<0.01, *p<0.05 level.

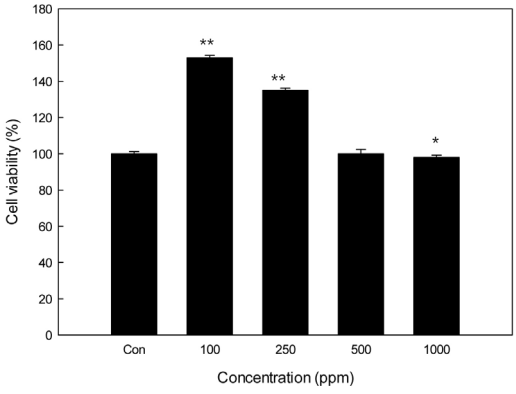

Fig. 5. Cytoxicity of the crude ethanol extracts from *Lithospermum erythrorhizon*.
The bars represent the standard deviation. Asterisk indicates a significant difference at **p<0.001, *p<0.005 level.

Ascorbic acid of the control group showed more than a 50% inhibitory activity both at 0.1 ppm, the lowest concentration and 1000 ppm, the highest concentration. The extracts of *Lithospermum erythrorhizon* had a 16% inhibitory activity at 0.1 ppm, but had a 51% inhibitory activity at 1000 ppm. At 10 ppm, a 41% inhibitory activity appeared and when it was compared with the 30% shown by angelica anchangelica (Son *et al*., 2011) and the 31.8% of *kousa* at the same concentration, it was discovered that there is a possibility that this material can be applied for whitening cosmetics as a tyrosinase activity inhibitory ingredient. Such results showed the correlations between anti-oxidation and whitening effects in the same context as discussion of the possibility involving EDA measurement (Tobin and Thody, 1994).

Measuring cytotoxicity

The results on the survival rates discovered through the MTT assay are presented in Fig. 5. The extracts of *Lithospermum erythrorhizon* were treated with a macrophage (Raw 264.7) at 100, 250, 500, and 1000 ppm, the cell survival rates were 153% at 100 ppm, 135% at 250 ppm, 100% at 500 ppm, and 98% at 1000 ppm. Proliferation of 150% cell growth occurred at 100 ppm where the extracts were added and at the concentrations of 500 ppm and 1000 ppm, cell growth slowed down with similar survival rates to that of the control group, but as the survival rate did not significantly slow down in comparison with that of the control group, it was suggested that it was not affected by the toxicity of the specimen.

This study measured the pH of *Lithospermum erythrorhizon* as well as the UV-spectrum, anti-oxidation ability, whitening, and cytotoxicity to discover the possibility of using the ethanol extract as an ingredient in cosmetics. As a result of the test, the following results were discovered: This material had the ability to control strongly acid and alkaline solvents centering on a pH of 5.5, the most ideal pH for skin. The maximum absorption peak was observed between 270-280nm and at 530nm in the UV-spectrum, and the total amount of polyphenol compound was 51.28±2.55 (mg/g). Also, as the DPPH scavenging effects were 34.9±1.11 at 62.5 ppm, in low activity, they grew greater according to the concentration of the specimen, and at 1000 ppm, the activity effects was 74.47%±0.73. This was 80% of that for ascorbic acid at the same concentration for an IC_{50} value needed to scavenge DPPH radicals, the concentration of the specimen was 0.015% and the concentration was 149.8 μg/ml.

The tyrosinase activity inhibition value was 33% at 10 ppm and 51% at 1000 ppm. In considering that ascorbic acid, the control group, was 58% at the same concentration, it showed high inhibitory activity. As in previous studies, as the reactive oxygen scavenging rate of EDA is higher, correlations with the whitening effects to inhibit melanin synthesis were signi-

ficant. In the cytotoxicity test, the cell survival rate at 1000 ppm was above 98 %, which means there is no cytotoxicity.

To sum up the results above, the ethanol extract of *Lithospermum erythrorhizon* had high anti-oxidation ability and whitening effects, and has a possibility that this material can be applied as an ingredient for whitening cosmetics using natural materials as it is not involved in cytotoxicity.

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