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Original article

Antioxidant effects and anti-aging characteristics of *Leonurus japonicus* H. ethanol extracts

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Summary. *Leonurus japonicus* H. is a biennial wild plant that grows naturally in Asian countries such as Korea, China and Japan and belongs to Labiatae and has been used in lowering blood pressure, promoting urination, as a pain-killer, sedation and in promoting menstruation. In this study, Leonuri herba's antioxidant function, improvement were investigated. *L. japonicus* H. extract was fractioned into Hexane, Ethyl Acetate, Water, H_2O, 30% EtOH, 60% EtOH and 100% EtOH. The investigator carried out an experiment of confirming the capability of superoxide erasure by using the DPPH technique of antioxidant experiment and Xanthine oxidase hypoxanthine and measured the activation of antioxidant with ABTS technique. This Study showed that the 30% EtOH fraction was highest in antioxidant effect. Collagense synthesis was significantly increased in the experiment of anti-wrinkle effcet. All the *L. japonicus* H. extracts inhibited the generation of H_2O_2 in a dose dependent manner. Based on the above study findings, the anti-aging effect of 30% EtOH fraction of *L. japonicus* H. was most excellent. In conclusion, the rutin and adenosine of *L. japonicus* H. extract had a strong antioxidant function. If it is used in cosmetics, a variety of natural functional cosmetics, such as excellent natural moisturizers, antioxidant agents and anti-aging agent, can be developed.

Key words: *Leonurus japonicus* H., cosmetics, natural moisturizers, antioxidant agent, anti-aging agent

Introduction

Environmental pollution due to rapid industrialization and living amidst a highly competitive society force modern people to long for a more leisurely and monotonous life of naturalism and this desire has driven the well-being culture in which consumers could enhance the richness of their lives by obtaining peace of mind rather than caring for their appearance deeply entrenched in society and brought about many changes throughout the society. As seeking naturalism, the well-being culture has promoted the development of cosmetics using various natural ingredients, and therefore, the golden age of Korean herbal medicine cosmetics has come.

At present, the cosmetics industry accounts for 1 percent of Korea's gross natural product and has been settled as daily necessity, while coming into the spotlight as a promising industry. By virtue of the advancement of science, various fine ingredients and resources have been developed which have greater effects on the skin, thereby the development of functional cosmetics with such efficacy as anti-wrinkle effect and whitening has been extended (1).

Increase of various consumer classes, development of medical technology and beauty treatment,

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and concerns of those who want to have beautiful and healthy skin have encouraged the development of such cosmetics as related to anti-aging including anti-wrinkle effect so as to be focused by the business world.

For the research trend of functional cosmetics, oxidation reaction of cell membrane lipid, one of the causes of promotion of wrinkle formation, arouses cell dysfunction or necrosis. Reactive oxygen species (ROS) which increases in generation abnormally by in vivo and in vitro stimulus is classified into free radical (2). ROS also increases in generation even in normal cells by subcellular metabolism and inflammatory reactions such as prostaglandin biogenesis which is removed by self-protection mechanism of enzymes such as superoxide dimutase (SOD) and catalase (CAT). However, because of external factors such as ultraviolet, pollutant and abused chemicals, internal factors such as inflammatory reactions and stress, and abnormal metabolism, ROS of superoxide radical (O_2^-), hydroxyl radical (OH), hydrogen peroxide (H_2O_2), nitric oxide (NO), and peroxinitrite ($ONOO^-$) and transition metal ion such as iron and copper are generated excessively (3,4). Since they have unpaired one-electron or show a strong reactivity by taking ionic character, they damage most of cell organs including cell membrane lipid, DNA, functional protein, and mitochondria, and ultimately degrade self resilience (5-6). Therefore, the purpose of antioxidants is to prevent oxidation of subcellular organs by ROS and free radical (7).

For representative ingredients relative to anti-wrinkle effect, retinol was the first-generation ingredient for anti-wrinkling. It was followed by retinol derivatives which stabilize retinol and became popular as the second-generation ingredient. Since 2000, ingredients have varied in type and function and Angelica extracted from natural substances, Adeonsine, NAG (N-Acetyl Glucosamine), Kinetine, a kind of phytohormone which combines Ursolic acid contained in plants such as apple, pear and rosemary and herb, Coenzyme Q-10, and enzyme extracts attracted popularity as the third-generation ingredients. Recently, various ingredients have been developed such as EGF, RG II, Peptide, and snail secretion.

Typical antioxidative agents for cosmetics include vitamin A, C, and E (8). In particular, carotenoid has received attention as a new anti-wrinkle agent as various mechanisms of carotenoid have been revealed. Among carotenoid, α- and β-carotene which exist abundantly in green and yellow vegetables and fruits, γ-carotene contained in carrot and apricot, lycopene contained in tomato, lutein in green vegetables such as spinach and kale, fucoxanthine contained in brown algae such as sea mustard and kelp, zeaxanthine in mango and egg yolk, capsaicin in pepper, astaxanthin, a red pigment found in crustacean such as shrimp and crab, catechin in gree tea, isoflavone such as flavonoid, genistein, coumestrol of curcurmin in curry, and polyphenol of resvertrol in red wine are typical antioxidative agents existing in nature (9-11).

These antioxidative agents are used variously for anti-inflammatory drugs, antibiotics, immunity improvement, and whitening agents as well as anti-wrinkling agents through various mechanisms in cosmetics (12-13). In particular, these antioxidative agents are in the spotlight as new anti-wrinkling agents as the mechanism which effectively restraints the activation and revelation of MMPs, skin collagen breakdown enzyme were found (14).

Leonurus japonicus H. is a plant of Labiatae and known to be 200 genus and 3,200 species in the world. In Korea, there are 25 genus 55 species of *L. japonicus* H. The whole plant is covered by lines with unique scent (15). The flowers bloom July to September and are light reddish violet. When the flowers bloom, the top is termed *Leonuri Herba.* Since *L. japonicas* H. has beautiful flowers and scent, many kind of them are grown as ornamental plants or used as the source of favorite food and flavorings (16). Because the natural oil contained abundantly in *L. japonicus* H. has pharmacological effects such as immunity improvement, anticancer and anti-aging effect and antioxidative activity, many studies in the field of medicine, food and cosmetics have actively progressed (17).

Figure 1. Photograph of *Leonurus japonicus H.*

Although there have been many studies on pharmacological activities of *L. japonicus* H., few studies on functional cosmetics and skin anti-aging related to the plant are present. This experiment carried out an in vitro activities using NIH-3T3 fibroblast cells to determined if *L. japonicus* H. extracts can be used for anti-wrinkle functional cosmetics, which has the purpose to develop anti-wrinkle functional cosmetics using the existing herbs.

Materials and methods

Materials

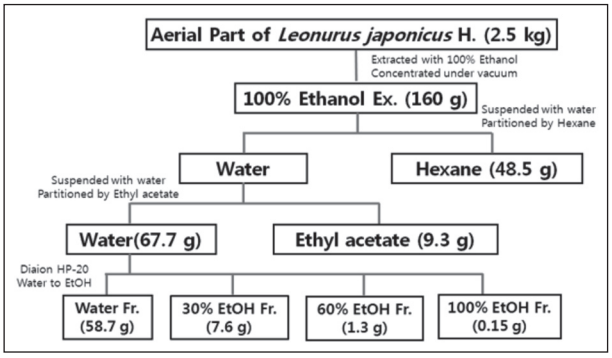
For *L. japonicus* to be used in this experiment, we bought the plants grown in Cheongsong, Gyeongsangbukd at the Gyeongdong Yangnyung market, Seoul, in August 2010 and put them under a botanical assessment by the Medicine Resource Botanical Class of Jungang University, then took 2.5 kg out of them for our ingredients.

Cell culture

NIH-3T3 cells were cultured at a 5% CO_2culture medium containing the Dulbecco's modifide Eagle's medium (DMEM, Invitrogen Co. Carlsbad, CA) solution which includes the 10% fetal bovine serum (FBS, Invitrogen Co. Carlsbad, CA) and the penicillin/ streptomycin (100 IU/50 μg/ml, Invitrogen Co. Carlsbad, CA) at 37°C.

Extract and fraction

2.5 kg of dried *L. japonicus* were pulverized and put to 18L of 100% EtOH. They were extracted for 24 hours and filtered. 18L of 100% EtOH was put to the residue and the abovementioned process was repeated five times. Extraction liquid was collected and subject to decompressive concentration. EtOH extracts was made to suspension with distilled water and hexane was added for shaking repeated extraction. Fractioned from a graduation hopper to obtain the hexane layer and the water layer separately. They were subject to decompressive concentration to get hexane extracts and ethly acetate extracts was also obtained by the same method. The remainder of water layer was concentrated to obtain water extracts which was fractioned into H2O, 30%, 60%, 100% EtOH fraction through Gels for Column Chromatography (Diaion HP-20, Nippon Rensui Co., Tokyo, Jjapan) at 20°C (Scheme 1).



Scheme 1. Extraction and Isolation of the Constituents from *Leonuri Herba*

Thin layer chromatography test for fractions

In order to determine whether rutin and adenosine are contained, 0.1g of the fraction powder of *L. japonicus* was weighted and put to 1ml of 70% MeOH and dissolved to make a test liquid. 20μl of the test liquid was dropped on a thin-layer board which is made of silica gel for thin-layer chromatography (TLC) and developed with a development solvent consisting of chloroform: methanol : water = 70: 30: 4. After development by 10cm, each of the thin-layer boards was dried in the wind and the result is as shown in Fig. 2 at UV 254nm. Another thin-layer board on which 10% sulfuric acid test solution was applied and temporarily heated at 105°C is shown in Fig. 3. In the next figure, rutin, adenosine, H_2O, 30% EtOH, 60% EtOH, 100% EtOH, water layer, ethly acetate layer, and hexane layer are sequenced from the left. From the TLC result, compared with samples at 254 nm, rutin and adenosine were clearly shown at 30% EtOH, in comparison with other extracts. Analysis of rutin, leonurin, and adenosine contents through HPLC (Waters 600, Waters Co., Milford, MA). 1mg of 30% EtOH from which rutin was clearly observed through TLC was taken and put to 10 ml of 70% ethanol and subject to a 2-hour ultrasonic extraction and filtered. The residue after supernatant was taken was put to 10 ml of 70% ethanol and the same process was repeated twice. All the remaining liquid was collected and subject to decompressive concentration and dissolved in 5 ml of

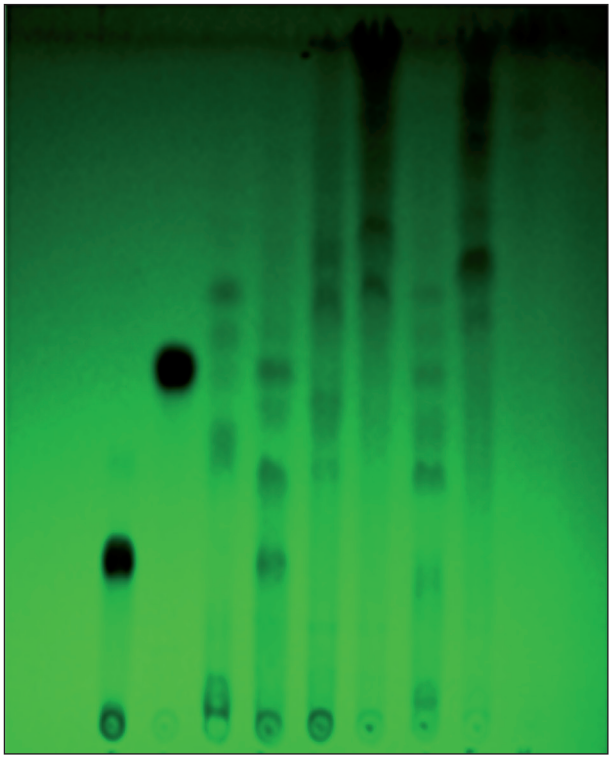


Figure 2. TLC of Extracts at UV-lamp 254 μ

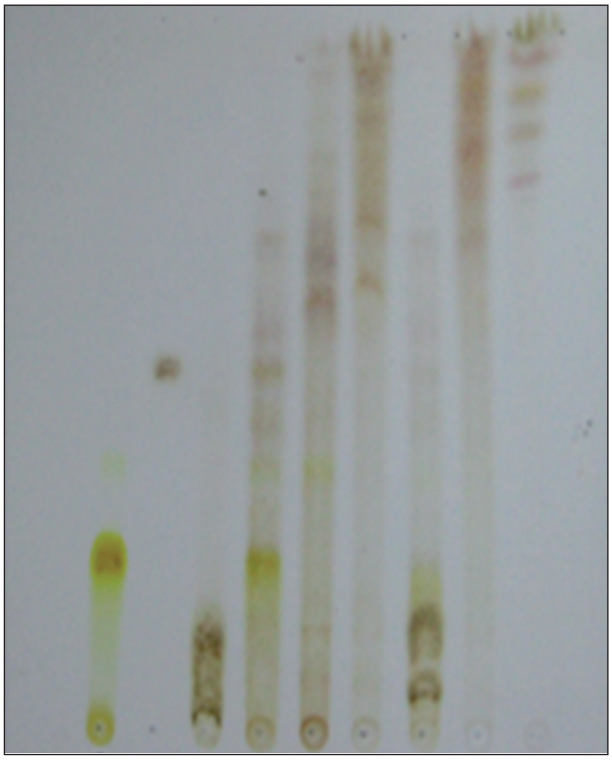
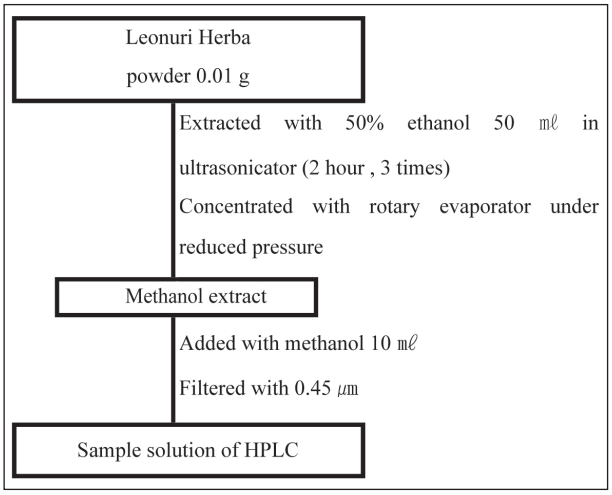


Figure 3. TLC of Extracts in 10%-H_2SO_4

methanol and passed through a membrane filter (0.45 μm). Among them, 10 μl of liquid was taken to use as the test liquid for each specimen (Scheme 2).

For analysis instrument, the HPLC analysis equipment of Waters was used. For analysis conditions, AKZO NOBEL. KR100-5C18 (4.6×250 mm) was used for column. For moving phase, methanol and tertiary distilled water were used in the proportion of 20: 80, and measured at UV 254 nm, while keeping the velocity of moving fluid at 1 ml/min (Table 1). For a standard solution, 1mg of rutin, leonurin and adenosine were dissolved in 1ml of water and passed through a membrane filter (0.45 ml). Then 10 μl was taken out of it to use as a test liquid (Fig. 4, 5).



Scheme 2. Procedure for HPLC analysis of *Leonuri herba*

Table 1. HPLC analytical condition-1

Detector	UV (280 nm)		
Column	AKZO NOBEL. KR100-5C-18 (4.6x250 mm)		
Mobile phase	A: Water/Methanol = 95/5 in TFA 0.1%		
	B: Methanol/Water = 5/95 in TFA 0.1%		
	Time (min)	A	B
	0	75	10
	10	45	20
	20	20	40
	30	0	60
Flow rate	1 ml/min		
Injection	10 μl		

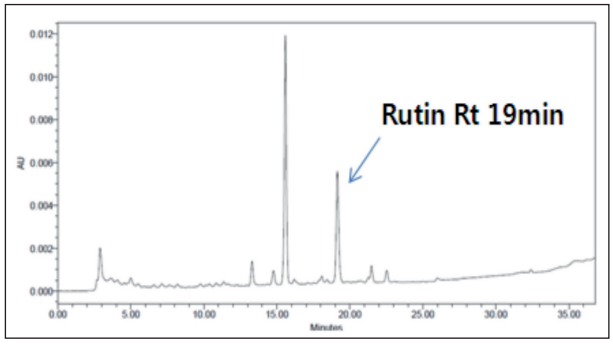


Figure 4. HPLC pattern of Rutin and Leonurin from 30% EtOH

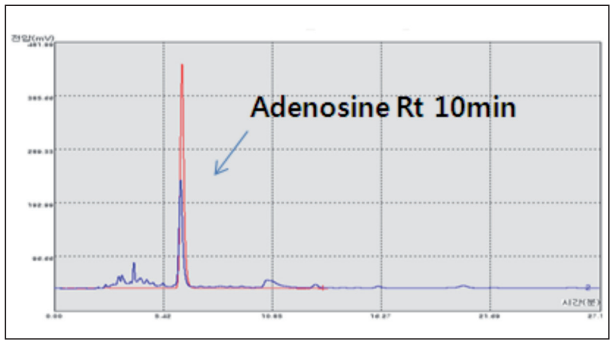


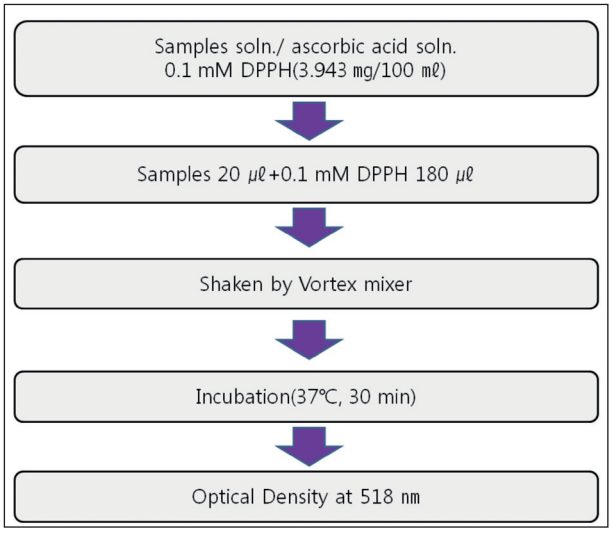
Figure 5. HPLC pattern of Adenosine and 30% EtOH

Measurement of antioxidative activities using DPPH

DPPH (1,1-diphenyl-2-picrylhydrazyl, Sigma-Aldrich Co., St. Louis, MO) is a very stable free radical. It is a dark violet compound which shows optical absorption at 518 nm. As it is bleached to yellow quantitatively by antioxidant of radical scavenging activity and easy to measure antioxidative activity, it has widely been used to search antioxiant. After each sample was prepared by concentration, 180 μl of 0.1 mM DPPH solution was added to 20 μl of each sample and subject to shaking for 10 seconds using a vortex mixer and cultured for 30 minutes at 37°C. Then absorbance was measured at 518 nm using spectrophotometer. For positive contrast agent, L-ascorbic acid (Sigma-Aldrich Co., St. Louis, MO) was prepared by concentration. To measure the antiaxidation effect of sample, antioxidative activities were displayed as radical scavenging activity of the degree of controlling oxidation and IC_{50}, the amount of samples necessary for radical scavenging activity to be 50%, was measured to compare and examine antioxidative activites of sample. Each fraction was divided into 5 types of concentration of 10, 50, 100, 500, and 1000 ppm (99.5% ethanol) and 180 μl was added to 0.1 mM DPPH solution (99.5% ethanol) to 20 μl of the solution (99.5% ethanol of control solution). After a 10-min shaking with vortex mixer, incubation was performed for 30 minutes at 37°C. Then, absorbance was measured at 518 μm using a spectrophotometer. As positive contrast agent, L-ascorbic acid was selected into 5 types of concentration of 10, 50, 100, 500, and 1000 ppm (99.5% ethanol). Antioxidative activites of each sample were indicated as DPPH radicaal scavenging activity (%) and IC_{50} values (concentration necessary to hold down 50% of DPPH radical formation), and inhibition concentration (50%) (18) (Scheme 3).

Measurement of superoxide antioxidative activities using hypoxanthine/xanthine oxidase system

Xanthine oxidase (Sigma-Aldrich Co., St. Louis, MO) involved in the generation of uric acid in human body generates uric acid with hypoxanthine and xanthine as matrix. However, superoxide is generated in this process, which gives oxidative damage to human body. Antioxidative effect can be weighed up by reproducing such an in vivo process at in vitro and check-



Scheme 3. Measurement of radical scavenging activity

$$\text{Inhibition rate (\%)} = \frac{\text{Control O.D.} - \text{Sample O.D.}}{\text{Control O.D.}} \times 100$$

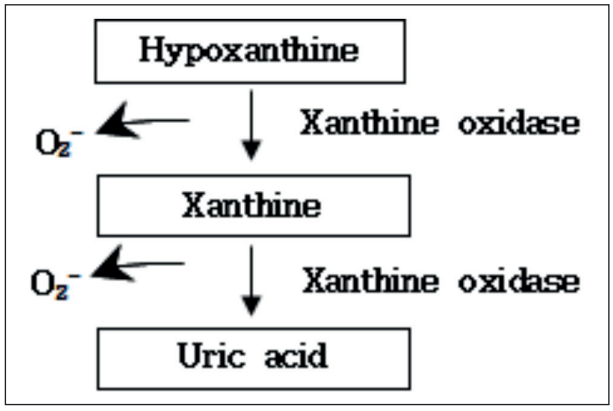
Sample O.D. : Absorbance of the test liquid with addition of sample
Control O.D. : Absorbance of the test liquid with addition of ethanol instead of sample

ing how much superoxide could be scavenged by target herb (Scheme 4).

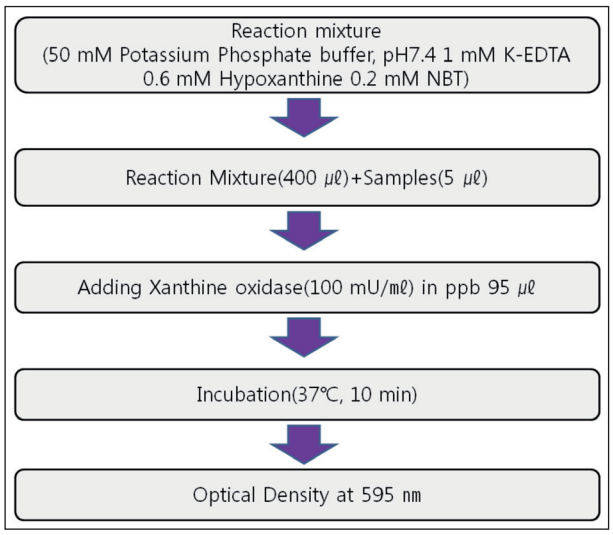
First, reaction mixture was made of 50 mM potassium phosphate buffer (PPB), pH 7.4, 1 mM EDTA, 0.6 mM hypoxanthine, 0.2 mM NBT. They were subfractioned into tubes by 400 μl and stock solution was made by setting proper concentration for samples and 5 μl of them were added. Xanthine oxidase was diluted to be 100 mU/ml of concentration and 100 μl of them was added (so that the final concentration of xanthine oxidase to be 20 mU/ml). Incubation was carried out for 10 minutes at 37°C and absorbance was measured at 595 nm. Check whether hypoxanthiine/xanthine oxidase system was activated using allopurinol known to be non-competitive antagonic agent of xanthine oxidase. The evaluation of them is expressed in superoxide scavenging activity relatively against control (Scheme 5).

Assay of antioxidative activity using ABTS

ABTS (2,2'-Azinobis(3-ethylbenzothizoline-6-sulfonic Acid Ammonium Salt, Sigma-Aldrich Co., St. Louis, MO) assay is used to evaluate antioxidative activity of sample using $ABTS^{\cdot+}$ cation radical scavanging activity generated from reaction between ABTS and potassium persulfate according to the method by Arnap et al (19). First, mix 7 mM ABTS and 2.6 mM potassium sulfate solution and leave them for 12 hours. Using methanol, dilute the stock solution so that absorbance at 732 nm is about 1.2. Then, 950 μl of diluted $ABTS^{\cdot+}$ cation radical solution was added to stock solution 50 μl of the sample prepared by concentration and absorbance was measured at 732 nm using a spectrophotometer. For positive contrast agent, Trolox was selected for comparison. Antioxidative activity of each sample was indicated in inhibition rate (%) for $ABTS^{\cdot+}$ cation radical oxidation and IC_{50} value (concentration necessary for inhibiting 50% of $ABTS^{\cdot+}$ cation radical formation (Scheme 6).



Scheme 4. Procedure on inducing superoxide



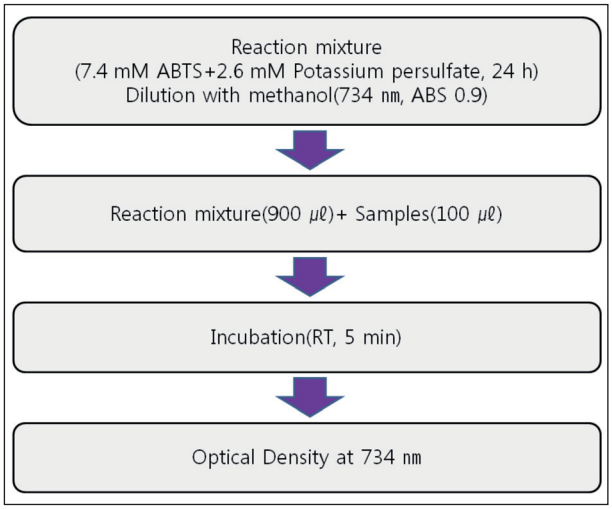
Scheme 5. Measurement of superoxide scavenging activity

$$\text{Inhibition rate}(\%) = \left(1 - \frac{C-D}{A-B}\right) \times 100$$

A: Absorbance at 292 nm without test sample after incubation
B: Absorbance at 292 nm without test sample before incubation
C: Absorbance at 292 nm with test sample after incubation
D: Absorbance at 292 nm with test sample before incubation

Collagen-like polymer dose at NIH-3T3 fibroblast cells

For dose of collagen-like polymer was, the method of Yin et al (20) was used as amended. NIH-3T3 cells were sufficiently cultured at the DMEM containing 10% FBS and fractioned into 6 well plates by 10^5 cells/3 ml. After 24 hours, it was cultured for 48 hours to handle *L. japonicus* extracts. Supernatant was removed and the remaining was transferred to an eppendorf tube by separating cells using a scraper. The cells were put into the Buffer A (20 mM Tris HCl, pH 7.5 + 5 mM CaC_2) and cell debris was removed by centrifugation (12,000 rpm, 20 min). Crude cell lysate was subject to a 10-minute heat treatment at 80°Cand deposited protein was removed by centrifugation (12,000 rpm, 20 min). Collagen-like polymer (CLP) is remained as being dissolved in supernatant. 4 tubes per sample were prepared and operated as below and cultured for 1 hour at 37°C. 1ml of the ninhydrin reagent (Buffer B : Solution A =



Scheme 6. Measurement of radical scavenging activity

$$\text{Inhibition rate (\%)} = \frac{\text{Control O.D.} - \text{Sample O.D.}}{\text{Control O.D.}} \times 100$$

Sample O.D. : Absorbance of the test liquid with addition of sample
Control O.D. : Absorbance of the test liquid with addition of ethanol instead of sample

1: 1; Buffer B: 0.5 M acetic acid + 0.1 M citric acid, pH 5.0; Solution A: 200 mg ninhydrin + 20 mg $SnCl_2$ in 10 ml of methylcellosolve) prepared just before was added to each tube and cultured for 20 minutes at the boiling water bath. It was moved to an ice-cold water bath and rapidly chilled to stop reaction and diluted in five times using a dilution solution (n-propanol : water = 1 : 1, v/v) and absorbance was measured at 570 nm. The amount isolated by collagenase type IA was considered as collagen-like polymer (CLP) (Scheme 7).

(1) Blank 1: 0.1 ml of buffer A + 0.01 ml of buffer A

(2) Blank 2: 0.1 ml of sample + 0.01 ml of buffer A

(3) Blank 3: 0.1 ml of buffer A + 0.01 ml of 300 U/ml collagenase

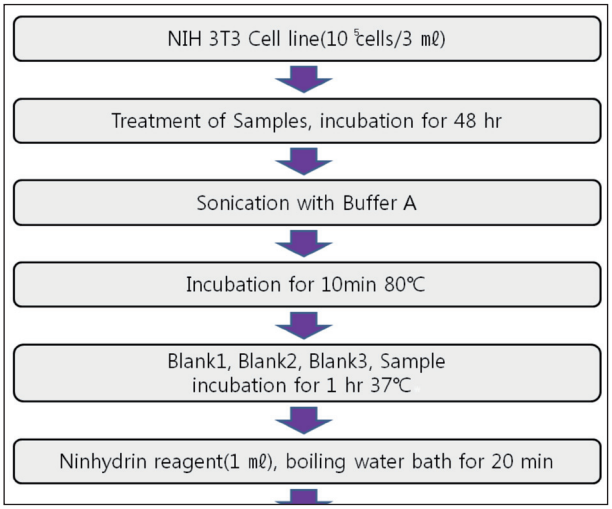
(4) Sample : 0.1 ml of sample + 0.01 ml of 300 U/ml collagenase

Absorbance increase caused by the liberated amino acid (ΔA)

ΔA = (sample - blank 3) - (blank 2 - blank 1)

Measurement of ROS in cells using DCF/DA

To measure oxygen radical reactive oxygen species (ROS) generated in NIH-3T3 cells, 2',7'-dichlo-



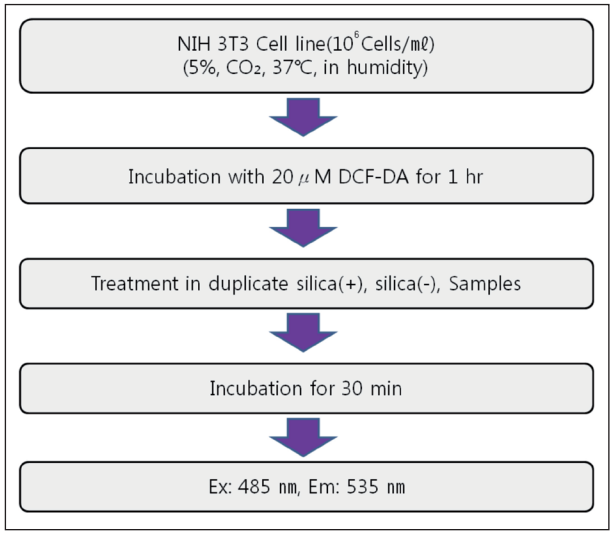
Scheme 7. Measurement of collagen-like polymer in NIH 3T3 cells

rofluorescin diacetate (DCF-DA) were used. Cells were cultured in a 5% CO_2 culture medium consisting of the dulbecco's modified eagle's medium (DMEM) solution which contains NIH-3T3 cells, 10% fatal bovine serum (FBS), and penicillin (100 IU/ml)/streptomycin (50 μg/ml) and is maintained at 37°C. DCF-DA is put into the cells and oxidized by oxygen radical and subject to deacetylation and converted into fluorescent material (DCF). NIH-3T3 cells were suspended in 15 ml of krebs buffer solution (NaCl 137 mM, KCl 2.7 mM, Na_2HPO_4 0.4 mM, $MgCl_2$ 0.5 mM, HEPES [pH 7.4] 10 mM, $CaCl_2$ 1.8 mM, glucose 5 mM) with addition of 20 μM DCF-DA and cultured in a place where light is blocked out for 1 hour. They were washed once with the Krebs solution free of DCF-DA and fractioned into 10^5 cells/ml and subject to pre-treatment of *L. japonicus* extracts and induced to produce H_2O_2 for 30 minutes with addition of 1 mg/ml of silica. After centrifugation, cell pellets were dispersed in 200 μl of the Krebs solution and fluorescence (Ex: 485 nm; Em; 535 nm) was measured (21) (Scheme 8).

Results

Antioxidative activity measurement using DPPH

As a result of the experiment by preparing each of the fractions of EtOH extracts of *L. japonicus* by con-



Scheme 8. Measurement of reactive oxygen species(ROS) scavenging activity

centration (1000 10 ppm), radical scavenging activity showed activity in some fractions and increased depending upon concentration in order of 30% EtOH> 60% EtOH> Ethly acetate> 100% EtOH> Water> H_2O> Hexane. In particular, 30% of fractions showed relatively high radical scavenging activity as IC_{50}= 61.9 μg/ml (Fig. 6, 7).

Superoxide antioxidative activity measurement using hypoxanthine/xanthine oxidase system

The fractions of hexane, ethyl acetate, and water obtained by solvent fraction of 100% ethanol extracts of *L. japonicus* were prepared by concentration (10 200 μg/ml) and checked for reaction using allopurinol, a xanthine oxidase inhibitor. The result showed that superoxide scavenging activity of 30% EtOH was good and increased depending upon concentration in order of 30% EtOH> 60% EtOH> Ethly acetate> H_2O> Water> 100% EtOH> Hexane. Among them, EtOH showed the best antioxidative activity of IC_{50}=109.9 μg/ml (Fig. 8, 9).

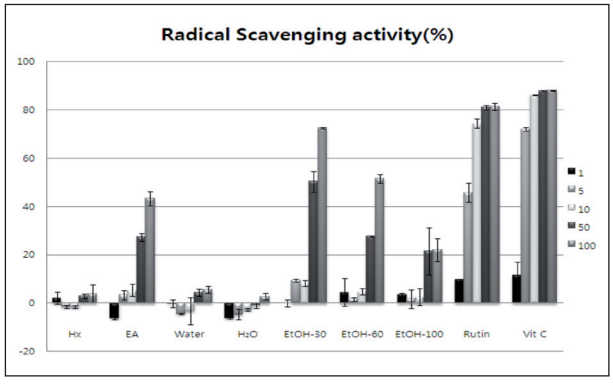


Figure 6. The Radical Scavenging Activities of *L. japonicus* H. active fraction

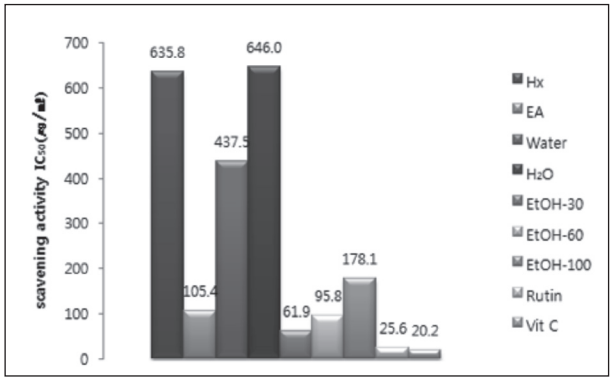


Figure 7. IC_{50} of *L. japonicus* H. active fraction in radical scavenging activity

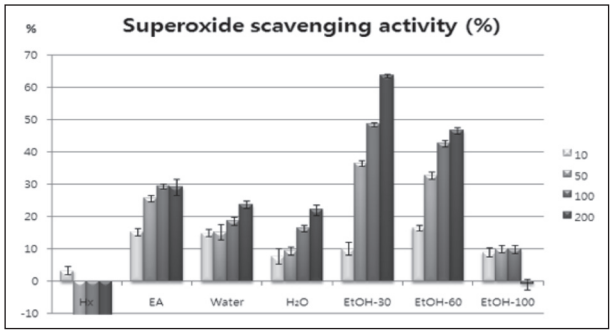


Figure 8. Superoxide scavenging activity of *L. japonicus* H. active fraction

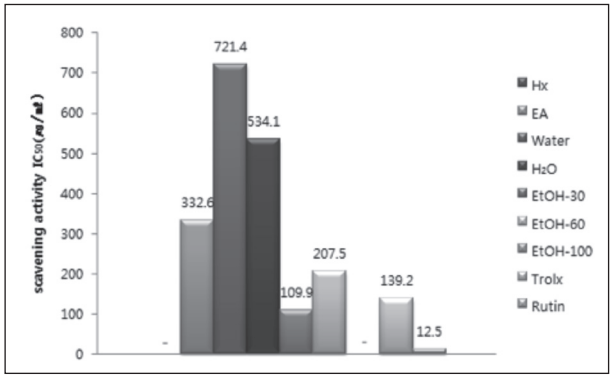


Figure 9. IC_{50} of *L. japonicus* H. active fraction in radical scavenging activity

Antioxidation effect using ABTS

The fractions of hexane, ethyl acetate and water obtained by solvent fraction of 100% ethanol extracts of *L. japonicus* were prepared by concentration (1 100 μg/ml). The result showed that radical scavenging activities of the fractions of 30% EtOH and 60% EtOH were good and increased depending upon concentration in order of the fractions of 30% EtOH> 60% EtOH> Ethly acetate> 100% EtOH> Water> Hexane, with no H_2O values. Among them, 30% the EtOH fraction was IC_{50} 16.1 μg/ml (IC_{50} 16.3 μg/ml of rutin), showing similar antioxidative activity to that of rutin (Fig. 10, 11).

Effect of L. japonicus extract on collagen combination in NIH-3T3 fibroblast cell

Collagen, one of the major components of extracellular matrix, is the main matrix protein generated from fibroblast of skin. The main functions of collagen are known to be mechanical strength of the skin, resistance of connective tissue and coherence of tissue, maintenance of cell adhesion, cell division and induction of division. It is known that collagen decreases by age and optical aging due to ultraviolet irradiation, which is closely related to wrinkle formation of the skin (22-24). This experiment reacted digested amino acid by collagenase with ninhydrin and measured indirectly collagen-like polymer. *L. japonicus* extract fractions were treated with NIH-3T3 cells by concentration and collagen was quantified after 48 hours. As a result, although NI was not significant, the amount of collagen was increased to 113.1%, 126.4%, and 133.6%, respectively at 30% EtOH NI liquid @ ym, 10, 100 μg/ml (based on control 100%) (Fig. 12).

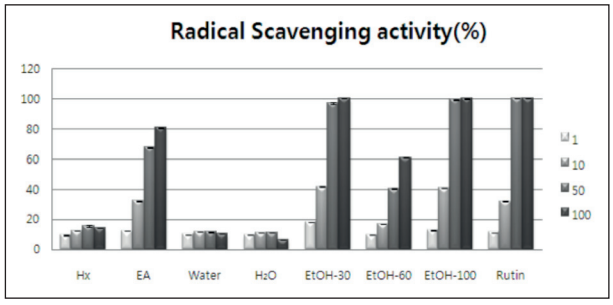


Figure 10. Superoxide scavenging activity of *L. japonicus* H. active fraction

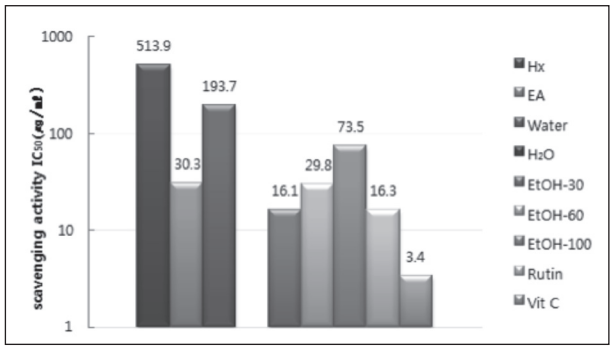


Figure 11. IC_{50} of *L. japonicus* H. active fraction in radical scavenging activity

Scavenging activities of reactive oxygen species (ROS) in NIH-3T3 cells

Cells using oxygen endlessly produce ROS. Among ROS, superoxide anion is generated first and converted to hydrogen peroxide by superoxide dimutase, a removing enzyme. Hydrogen peroxide is converted into harmless water and oxygen by intracellular catalase or peroxidase. However, in this process, peoxynitrite, lipid hyeroxide or hydroperoxid is sometimes produced in cells (25). *L. japonicus* extract itself shows strong antioxidative activity in a test tube, and since the 30% fraction of collagen-like polymer increased the amount of collagen in this experiment, superoxide anion, hydrogen peroxide, and hydroxyl radical which are produced in cells from the 30% EtOH fraction were measured using DCF-DA fluorescence. Silica is known to produce reactive oxygen species even in fibroblast. 1 mg/ml of silica was used to check whether *L. japonicus* extract has an effect to activate ROS scavenging in cells. The 30% EtOH fraction measuring the production of H_2O_2 in cells using DCF-DA, it was found that the production

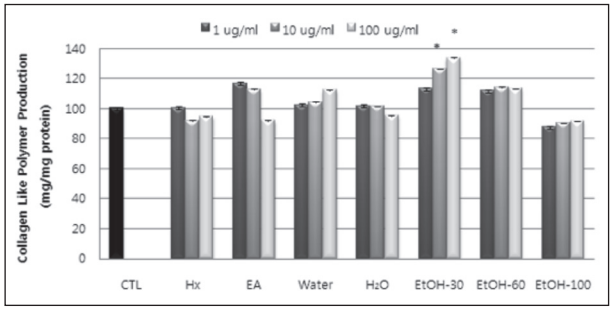


Figure 12. Effect of *L. japonicus* H. on collagen-like polymer production in NIH-3T3 cells.

of 1, 10, and 100 ug/ml of H_2O_2 were inhibited depending upon concentration of 418%, 390%, and 312%, respectively (Fig. 13).

Discussion

For physiological activity of *Leounri herba*, it was used for treatment of inflammatory disease as the Korean folk remedy (26), has antibacterial function (27), and includes various activity component such as alkaliod, flavonoid, diterpenes compound and fatty acid. Recent studies showed that these components have a beneficial effect on the treatment of ischemic stroke through protection and mechanism for coronary artery disease, cerebral infarction and antioxidation, anti-coagulation, anti-cell death (28, 29).

Various studies have reported that antioxidative activity has penol compound action and increases in proportionate to the content of phenol compound. In particular, the DPPH analysis showed the total penol content and good correlation of measuring equipment, indicating that labiatae plants are useful to prevent or treat oxidation stress diseases and can be used as a good potential source of natural antioxidant (30). One study showed that fluid extract of *L. japonicus* H. has higher antioxidative activity than that of ginko and hawthorne (31). On the contrary, Masteikova R et al (32) showed that it is the rutin of *L. japonicus* H. that most contributes to antioxidative action in high concentration of tincture in the fruites of hawthorne and *Leounri herba.*

One study reported that *L. japonicus* H. is effective in reinforcing antibiosis activity in 70% EtOH with strong antioxidative effect (34).

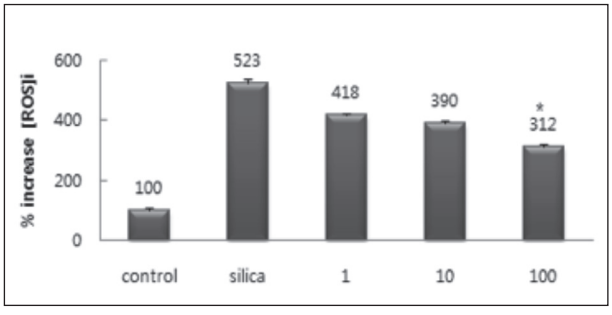


Figure 13. Inhibitory activity of *L. japonicus* H. on silica induced intracellular reactive oxygen species (ROS) production in NIH-3T3 cells.

In this study, 30% fraction is IC_{50}= 61.9 μg/ml and showed relatively high radical scavenging activity. Optical protection and exposure efficiency was evaluated in relation to single plant extract administration and concentration of human organism. The effect was decreased in order of L. > Hypericum > Aralia > Schizandra > Echinopanax > Eleutherococcus > Valeriana > Panax ginseng. Superoxide radical did not greatly contribute to the formation of chemical luminous substance (35).

Antioxidative effect of *L. japonicus* H. preserves selectively the activity of superoxide dismutase and glutation per oxidase and in particular, in the acute period of acute MI, it inhibits the formation of malon dialdehide, under oxidation stress conditions. The effect to inhibit the formation of reactive oxygen plays an important role in protecting endogenetic antioxidative system from oxidative stress. It also restraints production of mitochondria active oxygen and biogenesis of adenosin triphosphate (36-37).

With the action of leonurine on the cardiac muscle of low oxygen newborn mouse by induction of superoxide dismutase and catalase, the cardiac mechanism of infarction mice heart is related to antioxidation and anti-cell death. In addition, the level of revelation of antioxidant enzyme MN-SOD increased by 1.23 times (P <0.05), and this discovery increases SOD activity and countered the reduction lipid peroxidation as best it could (38). Through anxioxidation and anti-cell death effect and leonurine, it was shown that leonurine damping heart damage during the MI can be a useful assist cardiac protective agent (39).

In this study, superoxide scavenging activity increased depending up concentration in order of 30% EtOH> 60% EtOH> Ethly acetate> H_2O> Water> 100% EtOH> Hexane, and among them 30% EtOH was IC_{50} 109.9 μg/ml, showing the best antioxidative activity. ABTS radical scavenging activity is also frequently used in screening antioxidative activity. As a result of ABTS analysis, high antioxidative activity was found in ginko fluid extract comparing with that of *L. japonicus* H. and hawthorne (31). Zhu YZ et al (40) explained various antioxidative properties of CHM (Chinese Herbal Medicine) using in vitro antioxidative assay using diphenyl-L-picrylhydrazyl. Among them, 30% EtOH fraction was IC_{50}=16.1 μg/

ml(Rutin IC_{50}=16.3 μg/ml), showing antioxidative activity similar with ruin. The death of cells of all tumor cell in testing could be induced.[41] It was found that antiproliferative effect depends upon administration and time and mitochondria is involved in cell death induced by left extract. Cell surface area and protein/DNA ratio were detected as index for obese of cardiac muscle cell (42). Alkaliod found in *L. japonicus* H. showed biological effect such as antioxidation, anti-coagulation, anti-cell death and protection from ischaemic heart diseases and decreased reactive oxygen ROS level at isolated mitochondria from ischaemic outer cover (43). ROS is also increased in production by metabolism of cell organelle such as mitochondria and micromsome even in normal cells and inflammatory reaction such as prostaglandin biogenesis. This is removed by enzyme self protective mechanism such as superoxide dimutase (SOD) and catalase (CAT) (3-4). It was found to inhibit cardiac muscle fibrosis (44). Various concentrations of stachydrine involvement (10^{-6} - 10^{-4} mol / L) showed better anti-obeses effect and cardiac muscle cell was decreased (P <0.05) (42). Leonurine was found to decrease generation of intracellular reactive oxygen species (ROS) in H_2O_2 activated cells. In addition, H9C2 cells motivated by H has the potential of loss of DNA, cell death body formation, release of cytochrome c, mitochondria of mitochondria membrane. Through the blocking of the activation of JNK 1/2 and related mitochondria modulated impaired functioning it was shown that the H9C2 cells could be protected from cell death H_2O_2 (45). It was found the 30% EtOH fraction measuring the production of H_2O_2 in cells using DCF-DA, it was found that the production of 1, 10, and 100 ug/ml of H_2O_2 were inhibited depending upon concentration of 418%, 390%, and 312%, respectively.

Conclusions

L. japonicus H. is a herb and known to have various activities such as antioxidation, antiinflammatory effect, whitening and collagen combination through activity expreiments. It is commonly found around us. This study carried out an activity experiment on the effect of *L. japonicus* H. of these advantages on the skin for development of cosmetics ingredients and can be concluded as follows:

First, *L. japonicus* H. was subject to concentration, filtering and extracted with 100% EtOH and fractioned into Hexane, Ethyl Acetate, Water. Then the water layer of high content of rutin and adenosine was fractioned into H2O, 30% EtOH, 60% EtOH, 100% EtOH using Diaion HP-20 through TLC. Among them, the 30% fraction with high content of rutin was used as the ingredient of functional cosmetics.. For antioxidative activity measurement using DPPH at an in vitro activity experiment using an active fraction, the 30% EtOH showed the highest anti-oxidation activity in order of 0% EtOH> 60% EtOH> EA> 100% EtOH> Water> Hx> H2O and showed relatively high radical scavenging activity with IC_{50} =61.9 μg/ml. In the experiment to check superoxide scavenging activity using xanthine oxidant/ hypoxanthine, 30% EtOH was IC_{50}=109.9 μg/ml, showing the best antioxidative activity in order of 30% EtOH> 60% EtOH> Ethyl Acetate> 100% EtOH> Water> H2O> Hexane. In the ABTS antioxidative activity, the 30% EtOH fraction was IC_{50}=16.1 μg/ml (for Rutin, IC_{50}=16.3 μg/ml) and showed antioxidative activity similar to rutin. It is an active test Collagena. In the antioxidative activity, in NIH-3T3 fibroblast, the 30% EtOH significantly increased Collagen-like Polymer compound and ROS depend upon concentration and inhibition of the production of H_2O_2. From the abovementioned results, it was found that the 30% EtOH of *L. japonicus* H. has the best antioxidative activity. As mentioned above, the extract of *L. japonicus* H. found around us is considered to be far better in terms of efficacy compared with the existing functional cosmetics. Various developments of functional cosmetics using *L. japonicus* H. are expected.

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