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멸종위기 식물 날개하늘나리의 항산화 및 항암활성

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Characterization of Antioxidant Activity and Anticancer Effect of an Endangered Plant, *Lilium dauricum* Ker-Gawl

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ABSTRACT

Background: *Lilium dauricum* Ker-Gawl is an endangered plant, and it is highly valued as a medicinal plant owing to its excellent functionality.
Methods and Results: *L. dauricum* leaf extract was shown to have high antioxidant activity and an inhibition concentration of 50% (IC_{50})value of 71.8 ± 0.13 ㎍/㎖ was shown for 2,2-diphenyl-1-picrylhydrazyl radical scavenging activity. Its total phenol and flavonoid contents were 74.76 ± 0.24 ㎎·gallic acid equivalent (GAE)/g and 31.44 ± 0.27 ㎎·quercetin equivalent (QE)/g, respectively. Nitric oxide (NO) production was measured after oxidative stress was induced in lipopolysaccharide-stimulated RAW 264.7 cells, and 200 ㎍/㎖ of leaf extract effectively inhibited NO production at a rate of 17.84 ± 0.18%. Gastric cancer cells (AGS) and lung cancer cells (A549) were treated with 200 ㎍/㎖ of bulb extract, the inhibition rate for gastric cancer cell growth was 48.12 ± 2.60% whereas the inhibition rate for lung cancer cell growth was 55.83 ± 4.08%.
Conclusions: These results provide basic research data that can be used to identify the active constituents present in *L. dauricum* leaf and bulb extracts for the development of anticancer drugs, antioxidants, and functional products.

Key Words: Lilium dauricum Ker-Gawl, Anticancer Effect, Antioxidant Activity, Nitric Oxide Production, Total Flavonoid Contents, Total Phenol Contents

INTRODUCTION

Exposure to various substances and environmental pollutants such as smoking, alcohol, and radiation have been exacerbated by industrialization. These environmental triggers generate unstable and highly reactive oxygen species (ROS) in the human body, which easily combine with various substances to synthesize oxidation products. These oxidized substances cause irreversible cell and tissue damage and promote toxicity and cancer cell formation (Lee *et al.*, 2006).

Although the body maintains an antioxidant system that responds to ROS, a prolonged state of oxidative stress that exceeds the defense of this system can promote aging and chronic degenerative diseases such as cardiovascular disease, diabetes, cancer, and neurological diseases (Lee *et al.*, 2016).

Therefore, to suppress the generation of free radicals in the living body and to lower their prevalence, the frequency of supplementary antioxidant intake has been increasing. Although

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there are synthetic antioxidants such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BTT), they cause side effects when consumed above threshold levels. As a result, there has been increasing interest in the development of safe alternatives such as natural antioxidants derived from plant or food materials (Kumar *et al.*, 2008; Ku and Kang, 2010).

Recent studies have been conducted to improve or treat various human diseases by identifying species with excellent antioxidant effects from edible or medicinal plants (Geronikaki and Gavalas, 2006). Natural antioxidant compounds are mainly polyphenols such as flavonoids, tannins, anthocyanins, carotenoids, and organic acids (Choi *et al.*, 2015).

The safety of medicinal plants such as *Astragalus propinguus*, *Simplicillium chinense*, *Lycium chinense*, *Glycyrrhiza uralensis*, *Chrysanthemum morifolium*, *Angelica gigas*, *Plantago ovate*, and *Cnidium officinale* was also guaranteed, and studies on their antioxidant properties were conducted (Park, 2002; Kim *et al.*, 2005; Park *et al.*, 2005; Seo *et al.*, 2008; Lee *et al.*, 2011; Park *et al.*, 2011).

Most of these antioxidant components are phenolic compounds of the flavonoid family. Representative anticancer substances derived from medicinal plants with antioxidant activity are taxol and vinblastine. Anticancer drugs obtained from these plants are reportedly more effective than synthetic anticancer drugs (Lee *et al.*, 2016).

Clinical results showed that existing synthetic chemical products act on normal cells or tissues more than cancer cells, which may cause secondary infections such as the weakening of resistance due to the destruction of bone marrow cells and lymphocytes.

L. dauricum Ker-Gawl grows 1,400 m above sea level in the high mountainous regions of Gangwon-do, Jeollabuk-do, and Gyeongsangbuk-do, Korea. It grows to 1.5 m in height and has narrow wings on the stem. Underground scales were white, round, and 3 ㎝ - 5 ㎝ in diameter. The lanceolate leaves are 5 ㎝ - 12 ㎝ long and run alternately without petioles, and three to five veins have fine processes along the edges. It has upward-facing flowers from July to August, and the yellow-red flowers run upward in an umbel at the end of the main stem. Inflorescences are 7 ㎝ - 8 ㎝ long, six in number, broadly lanceolate, spread obliquely, and the tip is slightly bent back. Purple spots appeared around the inside of the flowers. The fruit has a narrow, obovate capsule. *L. dauricum* grows only in high mountains, and because of its beautiful flower shape and color, it has a very high ornamental value and is of great value as a genetic resource for cultivating new varieties.

However, it is currently considered an endangered species on the Korean Red List and is in danger of extinction owing to the destruction of its native habitat and indiscriminate harvesting for ornamental purposes (Kim, 2020).

Research into potential uses and functions has been limited this study, extracts were prepared for each tissue part to examine the antioxidant activity by measuring total phenol and flavonoid contents, and 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging ability. The potential of *L. dauricum* as a new functional food material was also assessed. In addition, we aimed to demonstrate the inhibitory effect of *L. dauricum* extract on the proliferation of human cancer cells.

MATERIALS AND METHODS

1. Plant material

To analyze biological activity, *L. dauricum* was acclimatized to aseptic culture using plant cell tissue culture technology. As for the acclimatization method, aseptic plants planted in the medium were adapted to the soil for gardening after removing the medium. After 12 weeks of acclimatization, the plants were separated into bulbs, stems, leaves, petals and pistil and stamens and used for extraction and concentration experiments (Fig. 1).

2. Extraction and concentration

The *L. dauricum* used in this experiment was washed cleanly, dried in the shade, and then ground with blender and used as the solvent, and 70% EtOH was used. Phenolic compounds were extracted using maceration technique with modification.

Each sample (10 g rhizome, 10 g stem, 10 g leaf, 5 g flower, 2 g pistil and stamens) added 20 times the solvent was extracted after sonication for 30 minutes. The extraction process was repeated 3 times, and after extraction, it was concentrated under reduced pressure (EYELA N-1000, Tokyo Rikakikai Co., Ltd., Tokyo, Japan) and used in the experiment.

3. Antioxidant activity assay by DPPH free radical scavenging

The DPPH radical scavenging activity assay of the extracts from each part of *L. dauricum* was performed by partially modifying the method described by Blois (1958).

After mixing 0.1 ㎖ of the sample with 0.1 ㎖ of 0.15 mM DPPH and reacting at room temperature for 30 min, absorbance

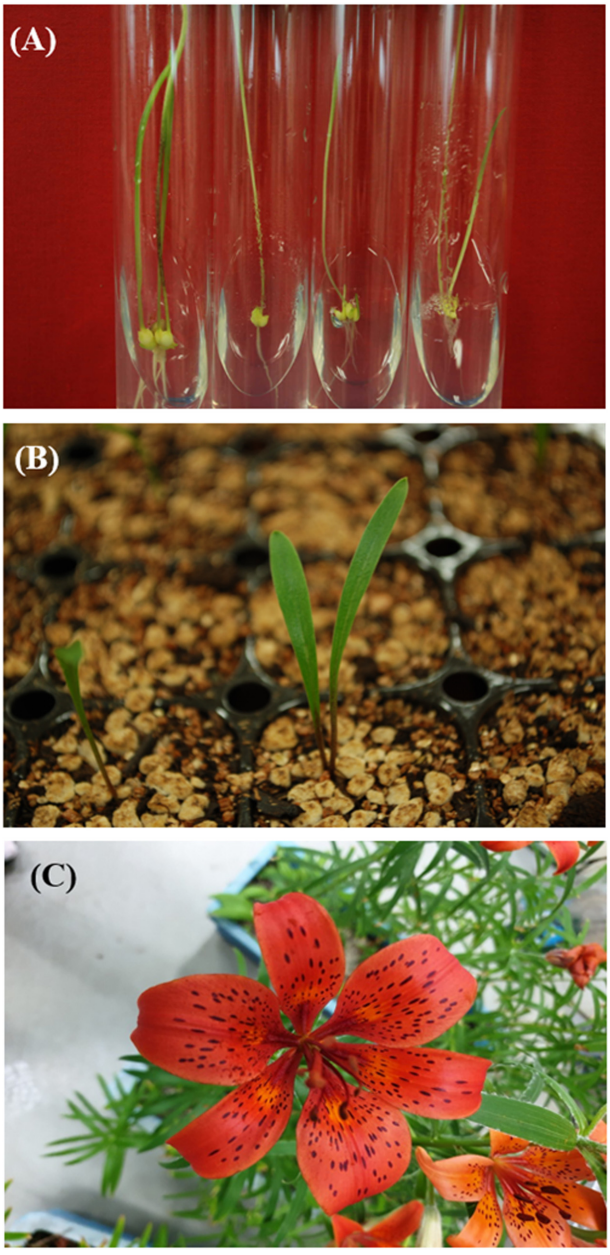

Fig. 1. Production of regenerated plants using plant tissue culture technology in *L. dauricum*. (A); plant tissue culture processing, (B); acclimatization processing of regenerated plant, (C); flowering of regenerated plants.

was measured at 517 ㎚ with a UV/VIS spectrophotometer (V530, Jasco Co., Tokyo, Japan) to test the activity.

Ascorbic acid was used as a positive control, and the DPPH radical scavenging ability according to the concentration change was expressed as inhibitory concentration (IC_{50}) and obtained as a slope to measure the sample concentration at which the scavenging ability was 50%.

4. Determination of total phenol and flavonoid contents

The total phenol content was measured by modifying the method of Folin-Ciocalteu using the general oxidation-reduction properties of phenolic substances (Singleton and Rossi, 1965).

After mixing 0.1 ㎖ of the sample with 0.05 ㎖ of Folin-Ciocalteu reagent, 0.3 ㎖ of 20% sodium carbonate was added. The solution was then stabilized at 20℃ for 15 min, and the absorbance was measured at 725 ㎚ (Multiskan FC Microplate Photometer, Thermo Fisher Scientific Inc., Waltham, MA, USA) by adding 1 ㎖ of distilled water.

A standard calibration curve was prepared by treating gallic acid used as a standard at a concentration of 0 ㎎/㎖ - 100 ㎎/㎖, and the phenolic compounds of the sample were quantified and expressed as gallic acid equivalent (GAE).

Total flavonoid content was measured by partially modifying the method described by Moreno *et al.* (2000). Solutions of 0.1 ㎖ of a sample diluted with 80% ethanol, 0.02 ㎖ of 1 M potassium acetate, 0.02 ㎖ of 10% aluminum chloride, and 0.86 ㎖ of 80% ethanol were combined, reacted at room temperature for 40 min, and absorbance was measured at 415 ㎚ (Multiskan FC Microplate Photometer, Thermo Fisher Scientific Inc., Waltham, MA, USA). Total flavonoid content was quantified using a quercetin calibration curve and expressed as quercetin equivalents (QE).

5. Cytotoxicity measurement

The cytotoxicity of the sample was measured by the 3-(4,5-dimethy-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) method, which was partially modified from the method of Mosmann *et al.* (1983).

RAW264.7 cells were seeded in a 96-well plate at 1×10^5 cells/well and cultured in a CO_2 incubator for 24 h. After adding samples and treatment with LPS at a concentration of 4 ㎎/ℓ, the solution was incubated for an additional 24 h. MTT dissolved in phosphate-buffered saline (PBS) was added at a concentration of 0.5 ㎎/㎖, the supernatant was removed, and absorbance was measured at 540 ㎚ by treatment with 100 ㎕ of dimethyl sulfoxide.

6. Measurement of nitric oxide (NO) production rate

NO, an ROS, was measured using Griess reagent. RAW264.7 cells were seeded in a 96-well plate at 1×10^5 cells/well and cultured in a CO_2 incubator for 24 h. The lipopolysaccharide (LPS) and samples were added and cultured for an additional 24 h. Then, 50 ㎕ of the supernatant was mixed with 50 ㎕ of

Griess reagent (A reagent: 1% sulfanilamide, B reagent: 0.1% N-(1-naphthyl)-ethylenediamine dihydrochloride dissolved in 5% phosphoric acid), and absorbance was measured at 540 ㎚.

7. Measurement of anti-cancer activity

All cells used in the study were purchased from the Korea Cell Line Bank (Seoul, Korea).

For the anti-cancer activity assay, all cells related to cancer were cultured for 24 h in a CO_2 incubator using a Dulbecco's Modified Essential Medium (DMEM) medium supplemented with 10% fetal bovine serum (FBS) and 1% antibiotic (penicillin, 100 U/㎖). Normal cells (HEK 293) and human cancer cells including cervical cancer cells (HeLa), lung cancer cells (A549), gastric cancer cells (AGS), prostate cancer cells (PC-3) were used in anticancer experiments.

In the DMEM medium supplemented with 10% FBS and 1% antibiotics (penicillin, 100 U/㎖), HeLa and HEK 293 were cultured. AGS, PC-3, and A549 were cultured in a CO_2 incubator using Roswell Park Memorial Institute Medium (RPMI) medium supplemented with 10% FBS and 1% antibiotic (penicillin, 100 U/㎖).

8. Data analysis

All data were represented as mean ± standard deviation by performing at least three repetitions, and the software used for statistical analysis was IBM SPSS Statistics V26 program (SPSS Inc., Chicago, IL, USA). Variation analysis was analyzed for statistical significance at the 5% level using Duncan's Multiple Range Test (DMRT, $p < 0.05$).

RESULTS

1. Antioxidant activity of the leaf tissue

The antioxidant activity was measured using the DPPH scavenging method by extracting bulb, stems, leaves, petals and pistil and stamens for each part. The antioxidant activity of the leaf extract was 71.8 ± 0.13 ㎍/㎖, indicating the highest antioxidant activity (Table 1).

The lowest radical scavenging ability was 263.29 ± 5.67 ㎍/㎖ in a pistil and stamens extract. The total phenol content of each extract was measured and ranked in descending order: Leaf (74.76 ± 0.24 ㎎·GAE/g); flowers (68.46 ± 0.53 ㎎·GAE/g); pistil and stamens (53.46 ± 0.59 ㎎·GAE/g); stem (32.2 ± 0.24 ㎎·GAE/g); and rhizome (27.32 ± 0.44 ㎎·GAE/g).

The total flavonoid content also showed the highest value in the leaf extract at 31.44 ± 0.27 ㎎·QE/g. The correlation between the DPPH scavenging ability, total phenol content, and total flavonoid content was shown, suggesting that the overall antioxidant activity of the leaf was high.

Table 1. The total phenolic, flavonoid contents and DPPH radical scavenging activity of extracts from different parts in *L. dauricum*.

Extraction	DPPH radical scavening activity IC_{50} (㎍/㎖)	TPC[1] (㎎·GAE[3]/g)	TFC[2] (㎎·QE[4]/g)
Bulb	432.38±0.76[d]	27.32±0.44[e]	1.50±0.13[e]
Stem	313.22±0.58[c]	32.20±0.25[d]	5.23±0.40[d]
Leaf	71.8±0.13[a]	74.76±0.24[a]	31.44±0.27[a]
Petal	82.68±0.77[a]	68.46±0.59[b]	6.66±0.08[c]
Pistil and Stamens	263.29±5.67[b]	53.46±0.47[c]	8.15±0.29[b]
Ascorbic acid	3.78±0.41	-	-

[1]TPC; total phenolic content, [2]TFC; total flavonoid content, [3]GAE; gallic acid equivalent, and [4]QE; quercetin equivalent. Values represent means of data obtained from three independent experiments ($p < 0.05$). *Values with different letters within a column are significantly different by Duncan's Multiple Range Test (DMRT, $p < 0.05$).

2. Anti-inflammatory activity of the leaf tissue

RAW264.7 cells were treated with extracts of each part of *L. dauricum* at various concentrations (10, 50, 100, and 200 ㎍/㎖), and cell viability was investigated.

No toxicity was observed at concentrations below 200 ㎍/㎖ (Fig. 2A). Accordingly, the extracts for each part of *L. dauricum* were treated at a concentration of 200 ㎍/㎖ or less, and LPS stimulation was applied to measure the production of NO, which is an inflammatory sub-stance. NO production decreased as the concentration increased in the treatment group, except for the flower extract (Fig. 2B).

At 200 ㎍/㎖ of the leaf extract, each showed an NO production rate of 17.84 ± 0.18%. As a result of statistically significant by tissue parts, the leaf extract of *L. dauricum* was confirmed as a potential biomaterial for inflammation drug development.

3. Anticancer activity of the bulb extract

To check the presence or absence of anticancer activity of extracts from each tissue part of *L. dauricum*, the anticancer activity was tested using gastric cancer cells (AGS), lung

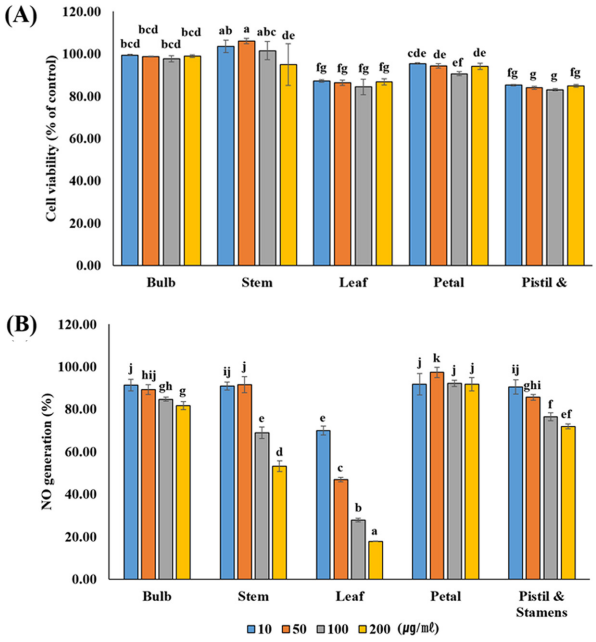

Fig. 2. Cell viability (A) by MTT assay and NO production (B) in LPS-induced Raw 264.7 cell with extractions obtained from various tissue parts of *L. dauricum*. This value was performed to triplicate and represented as means ± standard deviation. Statistical significance was indicated by different letters at the 5% level by Duncan's Multiple Range Test (DMRT) analysis ($p < 0.05$).

cancer cells (A549), prostate cancer cells (PC-3), and cervical cancer cells (HeLa).

The cell viability of extracts of various concentrations of *L. dauricum* was measured using HEK 293 cells. Most showed a survival rate of more than 90% and showed no toxicity, however, to have less effect on cells in relation to cell osmotic pressure, the anticancer activity test was conducted at 200 ㎍/㎖ or less for each extract (Fig. 3A).

When the bulb extract, which showed low antioxidant activity, was treated with different concentrations (50, 100, and 200 ㎍/㎖) of HeLa cells, the inhibition rates of cancer cell growth were statistically significant, 59.53%, 46.09%, and 37.28%, respectively. (Fig. 3B).

In addition, when AGS cells were treated with bulb extract at a concentration of 200 ㎍/㎖, the survival rate was 48.12%, indicating a high cancer cell growth inhibition rate (Fig. 3C), and A549 cells were also treated with the bulb extract of a concentration of 200 ㎍/㎖. The mean survival rate was 55.83%, and cancer cell growth was inhibited (Fig. 3D). The inhibition rate for cancer cell growth of AGS and A549 was the highest in the bulb extract, which was statistically

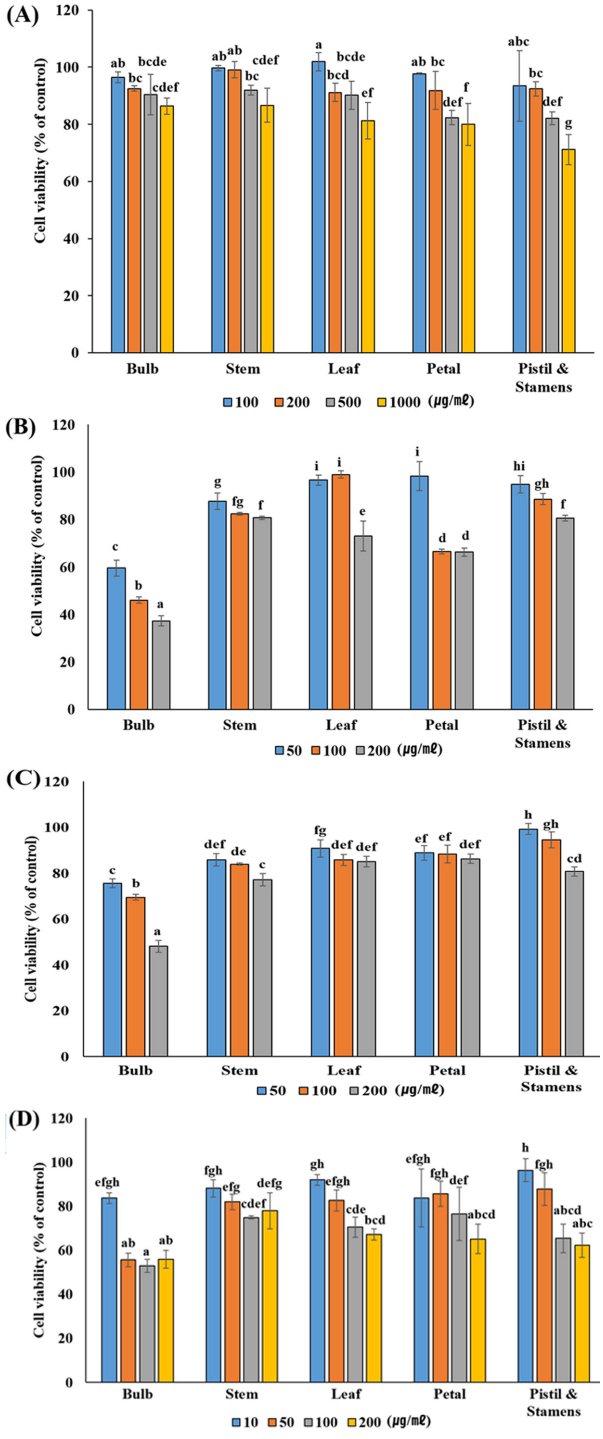

Fig. 3. Cell viability by MTT assay in HEK293 cell (A) and inhibitory effect of extractions obtained from various tissue parts of *L. dauricum* on proliferation of HeLa (B: Cervical cancer), AGS (C: Gastric cancer), A549 (D: Lung cancer) and PC-3 (E: Prostate cancer) cells. This value was performed to triplicate and represented as means ± standard deviation. Statistical significance was indicated by different letters at the 5% level by Duncan's Multiple Range Test (DMRT) analysis ($p < 0.05$).

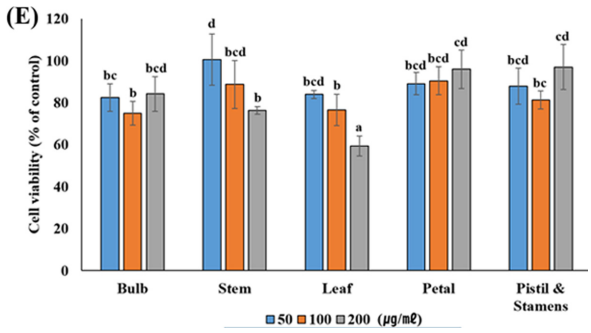

Fig. 3. Continued.

significant. The PC-3 cells showed the highest rate of 59.32% in anticancer activity when 200 ㎍/㎖ of *L. dauricum* leaf extract was treated (Fig. 3E).

DISCUSSION

Oxidative stress is known to cause various diseases by causing an imbalance between pro-oxidants and antioxidants (Bengmark *et al*., 2009). For example, in a patient suffering from chronic cardiovascular disease, a large amount of free radicals is produced and cause fatal damage to cells or tissues (Lee *et al*., 2003; Geronikaki and Gavalas, 2006; Kondo *et al*., 2009).

Therefore, to prevent or treat chronic and inflammatory diseases, the activity of antioxidant enzymes such as SOD (superoxide dismutase), catalase, and glutathione peroxidase as well as external natural substances such as vitamins C and E, selenium, polyphenols, and flavonoids must be maintained. Recently, studies on the discovery of antioxidants derived from natural products that can effectively remove active oxygen, such as superoxide anions (O_2^-), H_2O_2, $ONOO^-$ and NO, have been conducted (Kumar *et al*., 2008).

The main goal of this study was to evaluate the antioxidant potential and anticancer activity of *L. dauricum*. However, the total antioxidant capacity of individual plants was affected by various phytochemicals in plant tissues or cells, and this potential could not be accurately assessed using a single procedure (Chu *et al*., 2000).

Therefore, we determined the overall antioxidant capacity of *L. dauricum* using various methods including a DPPH assay, total phenol content, and total flavonoid content to standardize the activity characteristics of each tissue extract.

In this experiment, the antioxidant capacity of the leaf tissue extract of *L. dauricum* was found to be much higher than that of the other tissue parts, including bulb, stem, petal and pistil and stamens. The total flavonoid content in the leaf extract was 31.44 ± 0.27 ㎎·QE/g, which was 4 - 5 times higher than that of other tissue extracts. The leaf extract also showed the highest total phenol content (74.76 ± 0.24 ㎎·GAE/g) and the highest antioxidant activity as determined using the DPPH method.

A study by Mok *et al.* (2011) showed that the total polyphenol and flavonoid contents were found to be the highest in the leaf and flower tissue extracts, showing a similar trend to our results. Polyphenols and flavonoids are widely distributed throughout the plant kingdom. Polyphenol compounds have two or more hydroxyl groups, and flavonoid compounds are aromatic compounds with a basic skeleton of C6-C3-C6, which easily bind to proteins (Yu *et al*., 2006).

Flavonoids with flavones as a basic structure are abundantly present in the flowers, stems, and fruits of plants and reportedly have antioxidant, anticancer, and anti-inflammatory effects (Vijaya *et al*., 1995). As described above, this study shows that the leaf-derived antioxidant activity of *L. dauricum* is excellent because it contains flavonoids and phenols.

Most human diseases involve a degree of inflammation; therefore, drugs with anti-inflammatory effects are in constant development. These anti-inflammatory drugs are generally based on synthetic materials; however, natural plant materials may minimize side effects and provide alternative treatments.

This study determined that for *L. dauricum*, the leaf tissue extract had the highest anti-inflammatory effect compared to tissue extracts from the other plant parts, including bulb, stems, petal, pistils, and stamens. In a study of the anti-inflammatory properties of *C. japonicum*, the leaf extract, which had the best antioxidant activity, was reported to significantly inhibit NO production by effectively inhibiting the expression of iNOS and COX-2 in LPS-stimulated RAW264.7 macrophages (Mok *et al*., 2011). Another study examined the anti-inflammatory effects of *T. kirilowii* and showed that the NO production inhibitory ability was ranked in the order of seed, fruit, and flower extracts (Park and Kang, 2016).

In the present study of *L. dauricum*, the leaf extract had high antioxidant activity and a high anti-inflammatory effect. Similar results were reported in studies of *C. japonicum*. The exact relationship between antioxidant activity and anti-inflammatory effect may vary depending on the plant species and the parts these tissue extracts are obtained.

Medicinal plants have been used for centuries because of

their proven therapeutic effect on anticancer activity. As many compounds isolated from plants exist in a mixed form, the anticancer activity of plant extracts has been extensively studied (Liu, 2003; Karna *et al.*, 2012).

Studying secondary metabolites in plant component mixtures is important in determining the biological effects of medicinal plants. The effect of the tissue extract varies depending on the plant part (Reichelt *et al.*, 2002; Chen *et al.*, 2003). This study demonstrated that the anticancer activity of the tissue extracts for each part of *L. dauricum* was different.

The cancer cell suppression activity of bulb extract against cervical, gastric, and lung cancer cells was found to have a significant effect compared to other tissue extracts. However, the anticancer activity of the leaf tissue extract of *L. dauricum* was found to have the highest efficacy in the inhibition of prostate cancer cells.

For *Trapa japonica*, the cancer cell proliferation inhibitory activity of the pericarp extract was more effective than that of the seed extract, which was assessed using a total of 7 cancer cell lines, including A549, AGS, HeLa, PC-3, HCT-116, HT29, SW620 (Han *et al.*, 2016).

Lee *et al.* (2016) studied the inhibitory effects of *E. japonica* seed, flesh, and leaf ethanol extracts on liver cancer cells (H460), gastric cancer cells (AGS), and lung cancer cells (A549) and showed that seed extracts yielded the optimal results.

Many anticancer studies have tested whole plant extracts instead of isolated plant components and compounds. There are currently only a few studies on the anticancer mechanisms of the components of *L. dauricum*. Therefore, additional in-depth research on the development of natural materials from endangered plants is required. Tissue extracts from the leaves and bulbs of *L. dauricum* show excellent biological activity and should be used as a starting point for the development of pharmaceutical materials related to antioxidant and cancer cell inhibition agents and functional foods.

Limited research on the physiological activity mechanism of *L. dauricum* has been previously reported, despite the high scarcity value of this endangered plant. This study determined the antioxidant activities of tissue in separate plant parts of *L. dauricum* and examined whether the highest antioxidant activity was also related to the highest anti- inflammatory and anticancer activities.

For example, the leaf extract, which had high antioxidant activity, also showed good anti-inflammatory activity with high total phenol and flavonoid contents. The leaf extract also showed good cancer cell suppression activity in prostate cancer cells. Bulb tissue extract displayed significant inhibitory activity against cervical cancer, gastric cancer, and lung cancer. Each separate extract corresponding to a separate part of *L. dauricum* may express the mechanism for anticancer activity differently.

This shows considerable potential for developing future anticancer and anti-inflammatory treatments related to active oxygen scavenging ability.

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