

Effect of Extraction Ethanol Concentration on Antioxidant and Anti-Inflammatory Activity of 30-Year-Old and 120-Year-Old Dangyuja (*Citrus maxima* (Burm.) Merr.)

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Dangyuja (*Citrus maxima* (Burm.) Merr.) is a native fruit of great economic importance in Jeju island in Korea. To provide experimental evidence for the antioxidant and anti-inflammation properties on extraction ethanol concentration of Dangyuja, 2 cultivars, including 30-year-old and 120-year-old were evaluated. 30-year-old Dangyuja 50%, 70% ethanol extracts had the highest polyphenol and flavonoid content, and the strongest 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical scavenging activity. To investigate the anti-inflammatory activity of Dangyuja ethanol extracts, we used BV-2 microglia cells and induced inflammation using lipopolysaccharide (LPS). Then, we measured levels of inflammatory mediators as nitric oxide (NO). Among the 6 extracts, 30-year-old Dangyuja 50% ethanol extracts show the highest anti-inflammatory activity. The results suggest that 30-year-old Dangyuja 50% ethanol extracts provides significant health benefits and may be used for developing new functional materials.

Key Words: *Citrus maxima* (Burm.) Merr., Extraction condition, ABTS, Polyphenol, NO

Dangyuja (*Citrus maxima* (Burm.) Merr.), a traditional citrus in Jeju Island, is called Jeju 'Daengyuji', and is one of native Citrus species (Song et al., 1997). Peel of Dangyuja are known to be rich sources of polyphenolic compounds, particularly flavonoids such as naringin, hesperidin, and neohesperidin (Kim et al., 2007; Lee et al., 2006). Flavonoids in Dangyuja have been attracting interest because of their significant bioactivities. The health benefits of Dangyuja, including anticancer, antiviral, antioxidant, and anti-inflammatory, have been reported (Kim et al., 2007; Lee et al., 2006; Lim et al., 2006; Lim et al., 2009).

There are many techniques to recover active component

from plants, such as ultrasound-assisted extraction, maceration, supercritical fluid extraction, subcritical water extraction, and Soxhlet extraction. However, active component extraction yield and bioactive not only depend on the extraction method but also on the solvent used for extraction. Polar solvents are frequently used for recovering polyphenols from plants (Turkmen et al., 2006). Especially, ethanol has been known as a good solvent for polyphenol extraction and is safe for human consumption (Dai and Mumper, 2010).

The objective of this study was to investigate the effects of ethanol concentration on the extraction of polyphenol and flavonoid from Dangyuja and investigate the antioxidant

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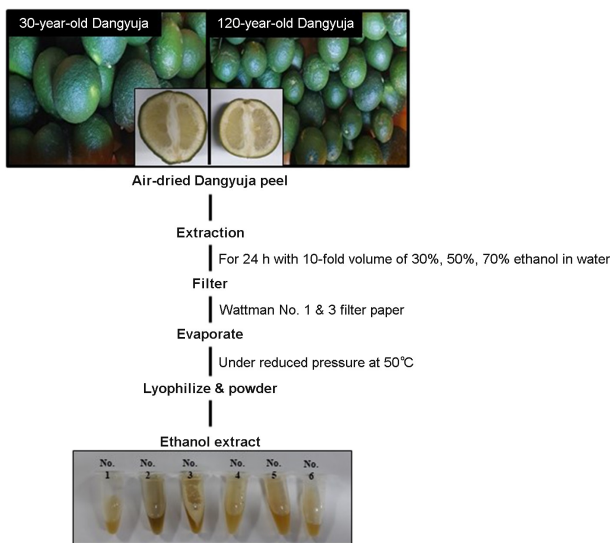


Fig. 1. Schematic diagram for preparation of Dangyuja peel ethanol extracts. No. 1; 30-year-old Dangyuja peel 30% EtOH extract, No. 2; 30-year-old Dangyuja peel 50% EtOH extract, No. 3; 30-year-old Dangyuja peel 70% EtOH extract, No. 4; 120-year-old Dangyuja peel 30% EtOH extract, No. 5; 120-year-old Dangyuja peel 50% EtOH extract, No. 6; 120-year-old Dangyuja peel 70% EtOH extract.

and anti-inflammatory activity of the extracts by *in vitro* methods.

The Dangyuja 2 cultivars, including 30-year-old and 120-year-old used in this study was bought at a Jeju Island. First, Dangyuja peel was separated, air dried at room temperature (RT). It was pulverized using a milling machine and extracted with 30%, 50%, 70% ethanol by stirring for 24 h at RT. The extract was filtered, concentrated with a vacuum rotary evaporator under reduced pressure, and lyophilized (Fig. 1). The yield of Dangyuja extract was calculated by the dry weight ratio of the extract. Yields of 30%, 50%, and 70% ethanol extracts of 30-year-old Dangyuja peel were 22.5%, 25.3%, and 28.1%, respectively. Yields of 30%, 50%, and 70% ethanol extracts from 120-year-old Dangyuja peel were 20.3%, 22.6%, and 27.2%, respectively. It was confirmed that the higher the concentration of ethanol, the higher the extraction yield.

To investigate anti-oxidative activities was confirmed using total polyphenol and flavonoid contents, and ABTS radical scavenging effect. The total polyphenol content was measured by modifying the Folin-Denis (Folin and Denis,

1912) method. After diluting the Dangyuja ethanol extracts to various concentrations, the diluted samples 60 μ L were dispensed in 96 well-plates. After adding 2-fold diluted Folin reagent and reacting for 3 min, the same amount of 10% sodium carbonate (Na_2CO_3) was added and reacted for 1 h to measure absorbance at 700 nm. The total polyphenol content was calculated based on a standard curve obtain with gallic acid (Sigma Chemical Co., St. Louis, Mo. USA). Total flavonoid content was measured by the method of Nieva Moreno et al. (2000). After diluting the Dangyuja ethanol extracts (100 μ L) was mixed with 860 μ L of 80% ethanol, adding 20 μ L of 10% aluminum nitrate and 1 M potassium acetate. The absorbance was then measured at 415 nm after incubation for 40 min. A standard calibration curve was prepared using Quercetin (Sigma Chemical Co.). The ABTS radical scavenging activity measurement was performed by previously described method (Re et al., 1999). To ABTS radical formation, 14 mM 2,2-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS, Sigma Chemical Co., USA) and 4.9 mM potassium persulfate were mixed at room temperature for 24 h, it was diluted with phosphate buffer saline (PBS, pH 7.4) so that the absorbance value at 732 nm was 0.70 (\pm 0.02). After adding 20 μ L of sample to 180 μ L of the diluted solution, it was allowed to stand for exactly 1 minute, and absorbance was measured at 732 nm. All experiments were repeated three times.

To measure the anti-inflammatory activity of the Dangyuja ethanol extracts, we used BV-2 microglia cells and induced inflammation using LPS (Sigma Chemical Co.). BV-2 cells were cultured in RPME1640 (Gibco BRL, NY, USA) supplemented with 10% fetal bovine serum (FBS; Gibco BRL) and 1% antibiotic-antimycotic (Gibco BRL) at 37°C in a humidified incubator with 5% CO_2 . To measure cell viability against Dangyuja ethanol extracts, it was measured by a 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT) assay. Cells (1×10^5 cell / mL) were dispensed in 100 μ L in 96 well-plates and cultured in a CO_2 incubator for more than 12 h, and then followed by incubation for 24 h by treating 100 μ L of fresh medium and Dangyuja ethanol extracts (250 μ g/mL). After 24 h, 10 μ L of MTT (2.5 mg/mL) was added, followed by incubation for 4 h. And then, the culture medium was removed, and 100 μ L of dimethyl-

sulfoxide (DMSO) was added to dissolve the generated formazone crystals, and measured at 540 nm using a microplate reader. Cell viability was expressed as a percentage (%) compared to the control. The inhibitory effect of nitric oxide (NO) was measured by reacting the NO_2^- form present in the cell culture with Griess Reagent using the method of Green et al. (1982). BV-2 cells (1×10^5 cell / mL) were cultured in 96-well plates overnight. The cells were treated with Dangyuja ethanol extracts (250 $\mu\text{g}/\text{mL}$) and 100 $\mu\text{g}/\text{mL}$ of lipopolysaccharide (LPS) for 24 h. And then, 100 μL of cell culture solution and 100 μL of Griess reagent (Sigma Chemical Co.) were mixed and reacted in 96 well-plates for 10 min, and then measured at 540 nm using a microplate reader.

All statistical analyses were performed using SPSS version 12.0 for Windows (SPSS, Chicago, IL, USA). Values were expressed as mean \pm standard error of the mean (SEM) for $N=3$. Data were analysis using one-way analysis of variance (ANOVA) and Duncan's multiple-range tests with $P < 0.05$ were considered significant.

To determine the antioxidant activity of Dangyuja peel extracted by 30%, 50%, 70% ethanol concentration, we first measured polyphenol and flavonoid content. Phenolic compounds have long been known to act on various biologically active, and many studies have been conducted (Ho et al., 2018). Total polyphenol content values were obtained from the calibration curve using gallic acid (Fig. 2A). The total polyphenol content values of the extracts range from 51.54 mg/g to 71.96 mg/g and they decrease in the following order: No. 2 (71.96 mg/g) > No. 3 (71.54 mg/g) > No. 6 (66.23 mg/g) > No. 5 (65.33 mg/g) > No. 4 (61.48 mg/g) > No. 1 (51.54 mg/g). The total flavonoid content of the extracts was reported in Fig. 2B. Total flavonoid content was calculated using quercetin. The total flavonoid content values of the extracts range from 37.32 mg/g to 66.74 mg/g and they decrease in the following order: No. 3 (66.74 mg/g) > No. 2 (65.24 mg/g) > No. 6 (53.67 mg/g) > No. 5 (52.12 mg/g) > No. 4 (50.24 mg/g) > No. 1 (37.32 mg/g). Based on the results of total polyphenol and flavonoid content, the 30-year-old Dangyuja peel is higher than 120-year-old Dangyuja peel, and the best extracting concentration of ethanol was 50% to 70%. 30-year-old Dangyuja peel 50% ethanol extract

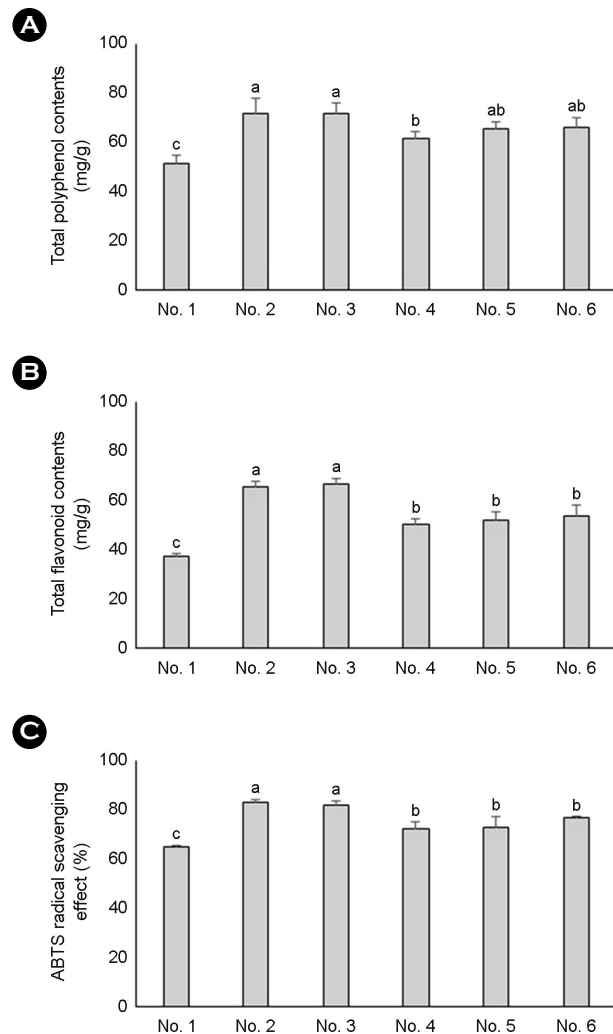


Fig. 2. Antioxidant activity of Dangyuja peel ethanol extracts. (A) Total polyphenol contents; (B) Total flavonoid contents; (C) ABTS radical scavenging effect. ABTS radical treatment extract concentration is 100 $\mu\text{g}/\text{mL}$. Data were analysis using one-way analysis of variance (ANOVA) and Duncan's multiple-range tests with $P < 0.05$ were considered significant.

also showed the highest ABTS radical scavenging activities (Fig. 2C).

We measured cell viability of BV-2 cells to investigate the cell cytotoxicity of Dangyuja peel extract. Although the survival rate of 90% or more was showed in almost all extracts, the 120-year-old Dangyuja peel 70% ethanol extract showed cytotoxicity with a survival rate of 65.32% (Fig. 3A). To determine the inflammatory activity of Dangyuja peel extract, we measured major inflammatory mediators, nitric oxide (NO). In LPS-induced BV-2 cells, 120-year-old

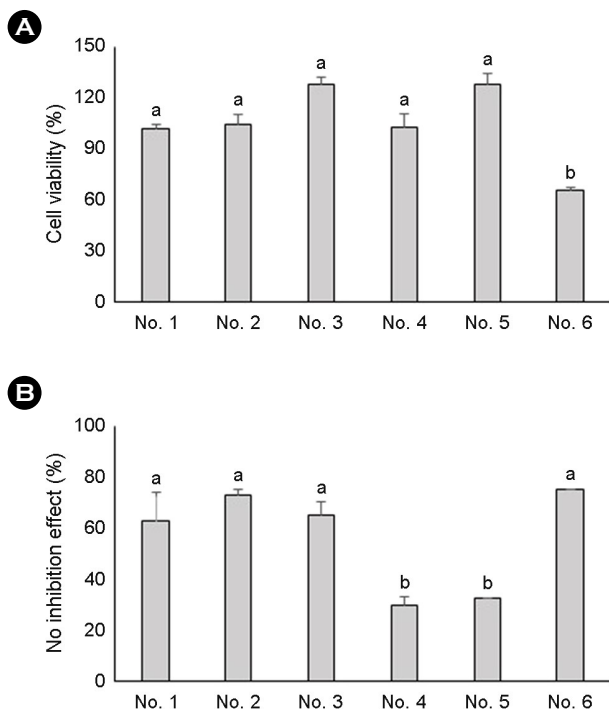


Fig. 3. Cell viability and anti-inflammatory activity of Dangyuja peel ethanol extracts in LPS-induced BV-2 cells. (A) Cell viability was measured using an MTT assay; (B) NO inhibition was represented as % against control: Inhibition effect (%) = $\{(\text{Control} - \text{Sample}) / \text{Control}\} \times 100$. The concentration of the extracts is 250 $\mu\text{g/mL}$. Data were analysis using one-way analysis of variance (ANOVA) and Duncan's multiple-range tests with $P < 0.05$ were considered significant.

Dangyuja peel 70% ethanol extract showed the best NO inhibition effect, but it is thought to be a result of cytotoxicity. In the extracts except 120-year-old Dangyuja peel 70% ethanol extract, 30-year-old Dangyuja peel 50% ethanol extract showed the best NO inhibitory ability (Fig. 3B).

In conclusion, it is clear that 30-year-old Dangyuja peel 50% ethanol extract gave the highest antioxidant and anti-inflammatory in all *in vitro* assays studied. Therefore, 30-year-old Dangyuja 50% ethanol extracts provides significant health benefits and may be used for developing new functional materials. Also, further research is required that on the separation and purification of active compounds with antioxidant and anti-inflammatory effects are expected.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication this articles.

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