



섬개야광나무의 부위별 메탄올 추출물의 항산화 및 항염 활성 비교

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Comparison of Anti-oxidant and Anti-inflammatory Activities of Methanolic Extracts Obtained from Different Parts of *Cotoneaster wilsonii* Nakai

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ABSTRACT

Background: The Ministry of Environment, Korea recognizes *Cotoneaster wilsonii* Nakai as a second-class endangered plant. It is a native species that grows in Ulleung-do, Korea. To our knowledge, the bioactivity of this plant has not yet been reported. Therefore, in this study, we have reported the bioactivity of *C. wilsonii* Nakai.

Methods and Results: The anti-oxidant activities of *C. wilsonii* methanolic extract were investigated *in vitro*. The anti-oxidant activity was evaluated using the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical assay, and the total phenolic and flavonoid content were measured. The leaf methanolic extract had the highest DPPH radical scavenging activity (IC₅₀; 15.74 µg/ml), and it also had the highest total phenolic and flavonoid content (220.95 mg·GAE/g, and 36.46 mg·QE/g extract respectively). Through high performance liquid chromatography (HPLC) analysis, chlorogenic acid was found to be the predominant among all phenolic compounds, showing a concentration of 84.24 mg/g extract. More than 60% decrease in nitric oxide (NO) production was found in the leaf methanolic extract.

Conclusions: To the best of our knowledge, this is the first report of the bioactivities of *C. wilsonii*. The results demonstrate that the leaf methanolic extract of *C. wilsonii* shows potent anti-oxidant, and anti-inflammatory activities.

Key Words: *Cotoneaster wilsonii* Nakai, Anti-oxidant, Anti-inflammatory, Caffeic Acid, Chlorogenic acid, Ferulic Acid, Gallic Acid, High Performance Liquid Chromatography, Syringic Acid

INTRODUCTION

Recently, there has been a lot of attention for the studies of reactive oxygen species (ROS). ROS cause aging and disease by breaking the balance with the anti-oxidant defense system in the human body (Nordberg *et al.*, 2001; Duan *et al.*, 2015; Yi, 2017). Research on natural anti-oxidants is actively conducted as medicinal plants used for treatment and prevention to remove free radicals (Bauer, 2000). ROS can be

inhibited by phenolic and flavonoid compounds. Anti-oxidants materials can be used to prevent adult diseases and contribute to the delay and prevention of aging.

Currently, synthetic anti-oxidants with good effects such as butylated hydroxyanisole (BHA) and butylated hydroxy toluene (BHT) have been developed and used. However, problems have been reported that they are carcinogenic, toxic, disrupting reserved nutrient consumption and cell metabolism and breathing. Therefore, it is necessary to develop a natural

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Received 2019 April 16 / 1st Revised 2019 May 8 / 2nd Revised 2019 June 2 / 3rd Revised 2019 June 9 / Accepted 2019 June 9

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antioxidant in plant origin that has a high anti-oxidant effect, safe and economical (Omaye, 1997).

Researchers suspect that the seeds of *Cotoneaster multiflorus* floating in the sea or carried by birds or winds may have adapted to the unusual environmental conditions of Ulleung-do and have been differentiated into *C. wilsonii* Nakai. The height of *C. wilsonii* Nakai is 1 to 4 meters tall. The leaves are irregular shape, oval or obovate, with narrow ends. Flowers bloom from May to June and the color is white. The sepals are white, furry at the end, and two petals are longer than stamen and have a style. It bears a fruit in September. It is red and egg-shaped (Kim *et al.*, 2002).

Currently, the study of the *C. wilsonii* Nakai has been conducted up to plant characteristics, genetic diversity conservational status (Kim *et al.*, 2002), a paper comparing flavonoids of East Asian *Cotoneaster* and *C. wilsonii* Nakai leaves (Chang and Jeon, 2003). Germination and other characteristics of seed (Lim *et al.*, 2013) and so on. No studies on bioactivity have been conducted.

The main aims of this study was to provide scientific basis for the possibility of development as a natural product material by evaluating the effects of anti-oxidant, anti-inflammatory activities.

MATERIALS AND METHODS

1. Preparation of *C. wilsonii* Nakai extracts.

Methanolic extraction was provided by Gangwon Nature Environment Research park (Hongcheon, Korea) and used for this experiment. Dried leaves, stems, and fruits were extracted at room temperature by three times with 100% methanol. And, the methanolic extract was filtered and then evaporated using a vacuum rotary evaporator (N-1200A, Eyela, Tokyo, Japan) under reduced pressure at 40°C. It was stored at -70°C in deep freezer.

2. Chemicals and reagents.

1,1-diphenyl-2-picrylhydrazyl (DPPH), ascorbic acid, gallic acid, α -tocopherol, quercetin, trichloroacetic acid, aluminium chloride, 2,6-di-tert-butyl-4-methylphenol (BHT), 3-tert-butyl-4-hydroxyanisole (BHA) used Sigma-Aldrich (St. Louis, MO, USA) products to measure anti-oxidant activities, and all other reagents were purchased and used with premium products from Deajung (Siheung, Korea) and Junsei (Tokyo, Japan). HPLC grade water and acetonitrile was purchased from

Avantor (Delaware, PA, USA).

Macrophages (RAW264.7) used in the experiment was obtained from Korean Cell Line Bank (Seoul, Korea). Phosphate buffered saline (PBS), 1-(4,5-dimethylthiazol-2-yl)-3,5-diphenyl-formazan (MTT), lipopolysaccharides (LPS) used Sigma-Aldrich (St. Louis, MO, USA) products. Dulbecco's modified eagle's medium (DMEM), fetal bovine serum (FBS) and penicillin were purchased from biowest (Maine-et-Loire, France).

3. Determination of anti-oxidant activity.

1) DPPH radical scavenging activity assay

DPPH radical scavenging activity was evaluated using the modifying method of Blois (1958). Zero point one ml of the different concentrations extracts added with the 0.1 ml of 0.15 mM 1,1-Diphenyl-2-picrylhydrazyl (DPPH). And incubated for 30 min at room temperature in dark. This was detected at 517 nm using a UV/VIS spectrophotometer (V530, Jasco Co., Tokyo, Japan). BHA, BHT, α -tocopherol and ascorbic acid were used as positive controls. The concentration of the samples at the time of 50% inhibition of DPPH was represented by IC₅₀.

DPPH radical scavenging activity (%)

$$= \left(1 - \frac{\text{absorbance value of sample}}{\text{absorbance value of control}} \right) \times 100$$

2) Reducing power assay

Reducing power of *C. wilsonii* Nakai was determined according to modifying the method by Oyaizu (1986). Zero point one ml of the diluted extracts using distilled water was added with 0.1 ml of 0.2 M sodium phosphate buffer (pH 6.6), 0.1 ml of 1% potassium ferricyanide, 0.1 ml of 10% trichloroacetic acid and 0.4 ml of distilled water. Added 0.05 ml ferric chloride and then directly measured it using a UV/VIS spectrophotometer (V530, Jasco Co., Tokyo, Japan) on 700 nm.

4. Determination of total phenolic and total flavonoid contents.

Total phenolic content of *C. wilsonii* Nakai was determined according to modifying the Folin-Ciocalteu method of by Singleton and Rossi (1965). Zero point one ml of the extracts was added to 0.05 ml of Folin-Ciocalteu reagent and incubated for 3 min at room temperature. Then added the 0.3 ml of 20% sodium carbonate. And after 15 min, added the 1 ml of distilled water. The mixture was detected at 725 nm using a

UV/VIS spectrophotometer (V530, Jasco Co., Tokyo, Japan). The phenolic content was calculated using the linear equation based on the gallic acid calibration curve. And the results were expressed as the gallic acid equivalence per gram of extract (mg·GAE/g).

Total flavonoid content of *C. wilsonii* Nakai. was determined according to modifying the method of by Nieva Moreno *et al.* (2000). Zero point one ml of the diluted extracts using 80% EtOH was added with 0.02 ml of 10% aluminum nitrate, 0.02 ml of 1 M potassium acetate and 0.86 ml 80% EtOH. After 40 min at room temperature, the mixture was detected at 415 nm using a UV/VIS spectrophotometer (V530, Jasco Co., Tokyo, Japan). The flavonoid content was calculated using the linear equation based on the quercetin calibration curve. And the results were expressed as the quercetin equivalence per gram of extract (mg·QE/g).

5. MTT assay.

Cytotoxicity of the *C. wilsonii* Nakai to RAW264.7 was determined according to modifying the MTT method of Mosmann (1983). RAW264.7 macrophage cells were seeded in 96-well culture plate at 1×10^4 cells/well and incubated for 24 hr. And then, all medium was replaced 0.1 ml of different concentrations of the samples (0 - 200 $\mu\text{g/ml}$) and incubated for 24 hr. And the changed 0.1 ml of 500 $\mu\text{g/ml}$ of MTT. After 4 hr, 0.1 ml of dimethyl sulfoxide (DMSO) was treated. After the treatment, it was detected at 540 nm using a ELISA (Model 680, Bio-Rad, Hercules, CA, USA) microplate absorbance reader.

6. Determination of NO production of RAW264.7 macrophages.

NO production of *C. wilsonii* Nakai was determined according to modifying the Griess reagent system of by Green and Kroemer (1982). RAW264.7 macrophage cells were seeded in 96-well culture plate at 1×10^5 cells/well and incubated for 24 hr. Medium was replaced different concentrations of the samples (0 - 200 $\mu\text{g/ml}$). After 30 min of the incubation, 2 $\mu\text{g/ml}$ of LPS was added and incubated for 24 hr. After the treatment, 0.05 ml supernatant mixed with 0.05 ml Griess reagent (A reagent; 1% sulfanilamide, B reagent; 0.1% n-(1-naphthyl)-ethylenediamine dihydrochloride melted in 5% phosphoric acid) was detected at 540 nm using a ELISA (Model 680, Bio-Rad, Hercules, CA, USA) microplate absorbance reader.

7. Determination of phenolic derivatives content in *C. wilsonii* Nakai.

Six phenolic derivatives were analyzed simultaneously using HPLC. Six phenolic derivatives were gallic acid, chlorogenic acid, caffeic acid, syringic acid, coumaric acid and ferulic acid. The HPLC analysis was analyzed using e2695 system of the Waters (Milford, MA, USA). The detector was 2489 UV-Vis detector of the Waters (Milford, MA, USA). The analytical column was a CAPCELL PAK C18 (5 μm , 4.6 mm \times 250 mm) of the Shiseido (Tokyo, Japan). The mobile phase was a mixture of 0.01% phosphoric acid in water and acetonitrile. The flow rate of mobile phase was 1.0 ml/min. The wavelength of UV detector was set at 272 nm.

8. Statistical analysis method.

Each data is means \pm standard derivation (SD) from triplicate samples of three independent experiments. Overall differences among the treatment groups were determined using One-way analysis of variance (ANOVA) by IBM SPSS Statistic 24 Software (Armonk, NY, USA). $p < 0.05$ is regarded as significance.

RESULTS

1. Anti-oxidant activity of *C. wilsonii* Nakai.

1) DPPH radical scavenging activity

The results of the DPPH radical scavenging activity, the IC₅₀ values of the stem methanolic extract was $22.00 \pm 0.14 \mu\text{g/ml}$, the leaf methanolic extract was $15.74 \pm 0.16 \mu\text{g/ml}$ and the fruit methanolic extract was $80.58 \pm 0.84 \mu\text{g/ml}$ (Table 1). The

Table 1. DPPH radical scavenging activity of extractions from *C. wilsonii* Nakai.

Sample	IC ₅₀ ¹⁾
Stem	$22.00 \pm 0.14^{\text{b*}}$
Leaf	$15.74 \pm 0.16^{\text{a}}$
Fruit	$80.58 \pm 0.84^{\text{c}}$
Ascorbic acid	2.87 ± 0.01
BHA	7.29 ± 0.06
α -Tocopherol	12.33 ± 0.14
BHT	147.56 ± 0.84

¹⁾Concentration causing 50% inhibition of DPPH at 30 min after starting the reaction [IC₅₀ ($\mu\text{g/ml}$)]. *Each Data is means \pm standard deviation of three replicate tests. The all data were statistically analyzed used by One-way analysis of variance (ANOVA). And differences were assessed that significant at 5% level of probability ($p < 0.05$).

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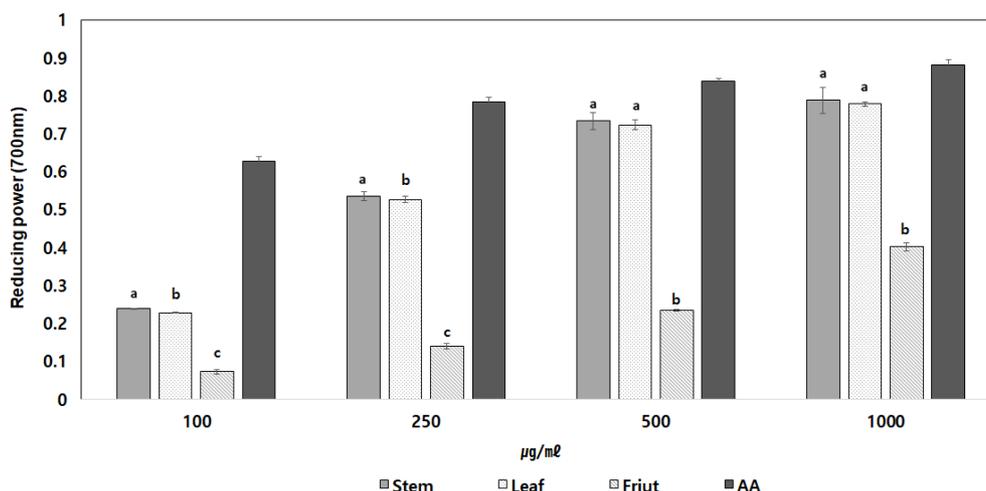


Fig. 1. Reducing power of extractions from *C. wilsonii* Nakai. AA; Ascorbic acid. Each Data is means \pm standard deviation of three replicate tests. The all data were statistically analyzed used by One-way analysis of variance (ANOVA). And differences were assessed that significant at 5% level of probability ($p < 0.05$).

leaf methanolic extract showed the highest anti-oxidant effect and showed higher activity than BHT ($147.56 \mu\text{g/ml}$) used as an antioxidant and showed activity similar to α -tocopherol ($12.33 \mu\text{g/ml}$). The availability of *C. wilsonii* Nakai extract as antioxidant was confirmed.

2) Reducing power assay of *C. wilsonii* Nakai

The different part extracts of *C. wilsonii* Nakai were treated with 100, 250, 500, and 1,000 $\mu\text{g/ml}$. Respectively, the reducing power was increased as the concentration increased. The leaf methanolic extract and stem methanolic extract showed the high reducing power effect. Effect was found similar to the ascorbic acid used as positive control when treated at 1,000 $\mu\text{g/ml}$ concentrations (Fig. 1).

This supports the correlation between anti-oxidant activity and reducing power reported in many studies, as well as results such as Kim *et al.* (2013) similar to DPPH inhibition assay.

2. Total phenolic and total flavonoid content of *C. wilsonii* Nakai.

The total phenolic content of extracts from different parts in *C. wilsonii* Nakai was high in order of leaf methanolic extract ($220.95 \pm 0.50 \text{ mg}\cdot\text{GAE/g}$) > stem methanolic extract ($214.65 \pm 2.43 \text{ mg}\cdot\text{GAE/g}$) > fruit methanolic extract ($60.71 \pm 2.03 \text{ mg}\cdot\text{GAE/g}$). And the total flavonoid content was also the highest in leaf methanolic extract with $36.46 \pm 1.89 \text{ mg}\cdot\text{QE/g}$ (Table 2). This supports the results that total phenolic and total flavonoid content was involved in anti-oxidant activity reported

Table 2. The total phenolic and flavonoid contents of extractions from *C. wilsonii* Nakai.

Sample	Total phenolic ¹⁾	Total flavonoid ²⁾
Stem	214.65 ± 2.43^b	6.09 ± 0.71^b
Leaf	220.95 ± 0.50^a	36.46 ± 1.89^a
Fruit	60.71 ± 2.03^c	0.23 ± 0.20^c

¹⁾Gallic acid equivalent (mg·GAE/g), ²⁾Quercetin equivalent (mg·QE/g). Each value is means \pm standard derivation of three replicate tests. *Each Data is means \pm standard deviation of three replicate tests. The all data were statistically analyzed used by One-way analysis of variance (ANOVA). And differences were assessed that significant at 5% level of probability ($p < 0.05$).

by Rice-Evans *et al.* (1996) and Zou *et al.* (2012), along with DPPH inhibition assay results, and indicates the high anti-oxidant effects of the *C. wilsonii* Nakai extract.

3. Effect of *C. wilsonii* Nakai on NO production in LPS-stimulated RAW264.7 macrophages.

An experiment was conducted using the macrophage RAW264.7 to determine the anti-inflammatory activity of the *C. wilsonii* Nakai extract. For the determination of cytotoxicity, various concentrations of extracts (10, 50, 100, 200 $\mu\text{g/ml}$) were treated. Stem methanolic extract showed a survival rate of less than 20% at 50 $\mu\text{g/ml}$, while leaf methanolic extract produced a survival rate of 67% at 200 $\mu\text{g/ml}$, and fruit methanolic extract produced a survival rate of 43% (Fig. 2).

RAW264.7 cells were treated with extracts at various concentrations (10, 50, 100, 200 $\mu\text{g/ml}$) and NO production

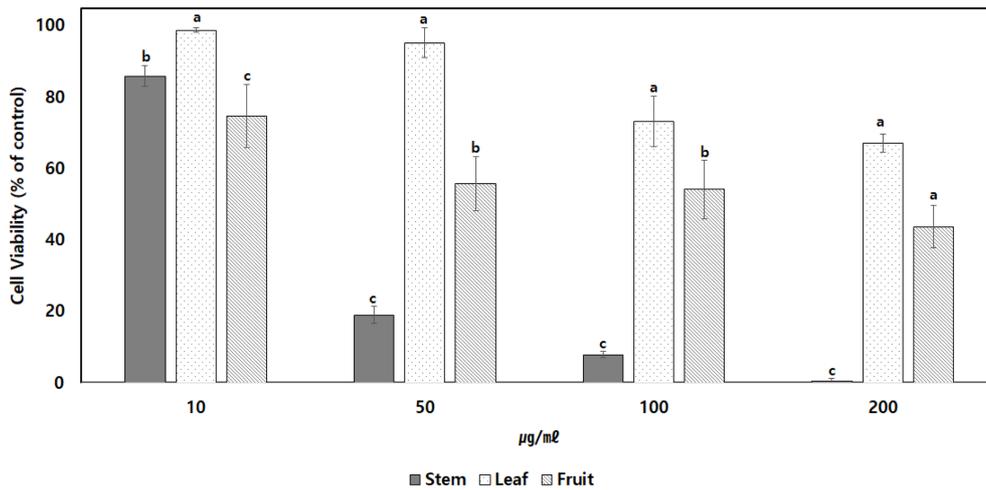


Fig. 2. Inhibitory effect of extract from *C. wilsonii* Nakai on cytotoxicity of RAW264.7 cell. Each Data is means \pm standard deviation of three replicate tests. The all data were statistically analyzed used by One-way analysis of variance (ANOVA). And differences were assessed that significant at 5% level of probability ($p < 0.05$).

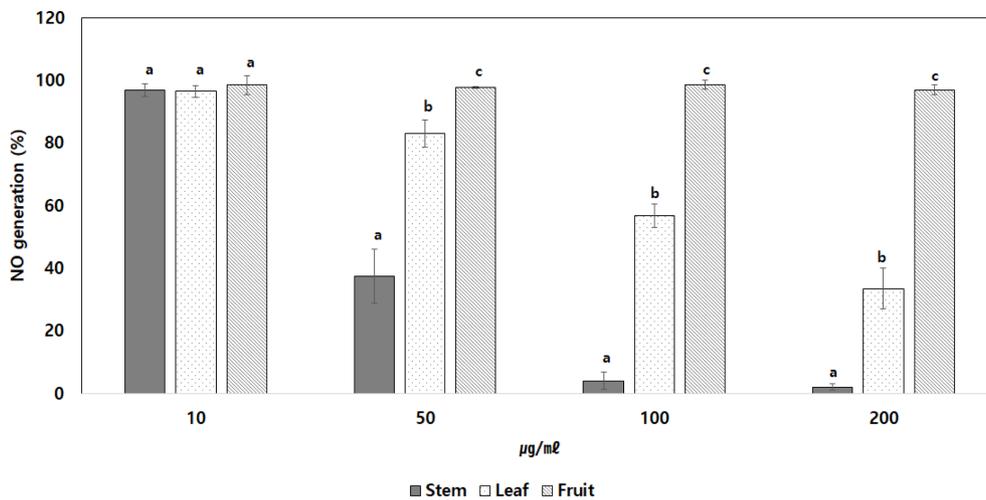


Fig. 3. The effect of *C. wilsonii* Nakai on LPS-induced NO production in RAW264.7 cell. Each Data is means \pm standard deviation of three replicate tests. The all data were statistically analyzed used by One-way analysis of variance (ANOVA). And differences were assessed that significant at 5% level of probability ($p < 0.05$).

was measured by stimulating LPS, an inflammatory substance. As a result, NO production was reduced in proportion to the concentration in leaf methanolic extract (Fig. 3). Stem methanolic extract was unable to confirm its effect due to cytotoxicity. RAW264.7 effectively inhibited NO production within cells, confirming that the *C. wilsonii* Nakai leaf methanolic extract is available as a natural substance to anti-inflammatory.

4. Phenolic derivatives contents of the *C. wilsonii* Nakai using HPLC.

It was estimated that the anti-oxidant and anti-inflammatory

effect's substance in *C. wilsonii* Nakai extract was phenolic derivatives. Therefore, phenolic derivatives were analyzed using HPLC. In Fig. 4, six phenolic derivatives and extracts by different parts of the *C. wilsonii* Nakai were shown the chromatogram by HPLC. Five phenolic derivatives were detected in leaf methanolic extract, three phenolic derivatives detected in stem methanolic extract and two phenolic derivatives detected in fruit methanolic extract.

The calibration curve and limit of detection (LOD), limit of quantitation (LOQ) for each standard for quantifying the content of detected compounds are summarized in Table 3. The calibration curve and correlation coefficient (R^2) were

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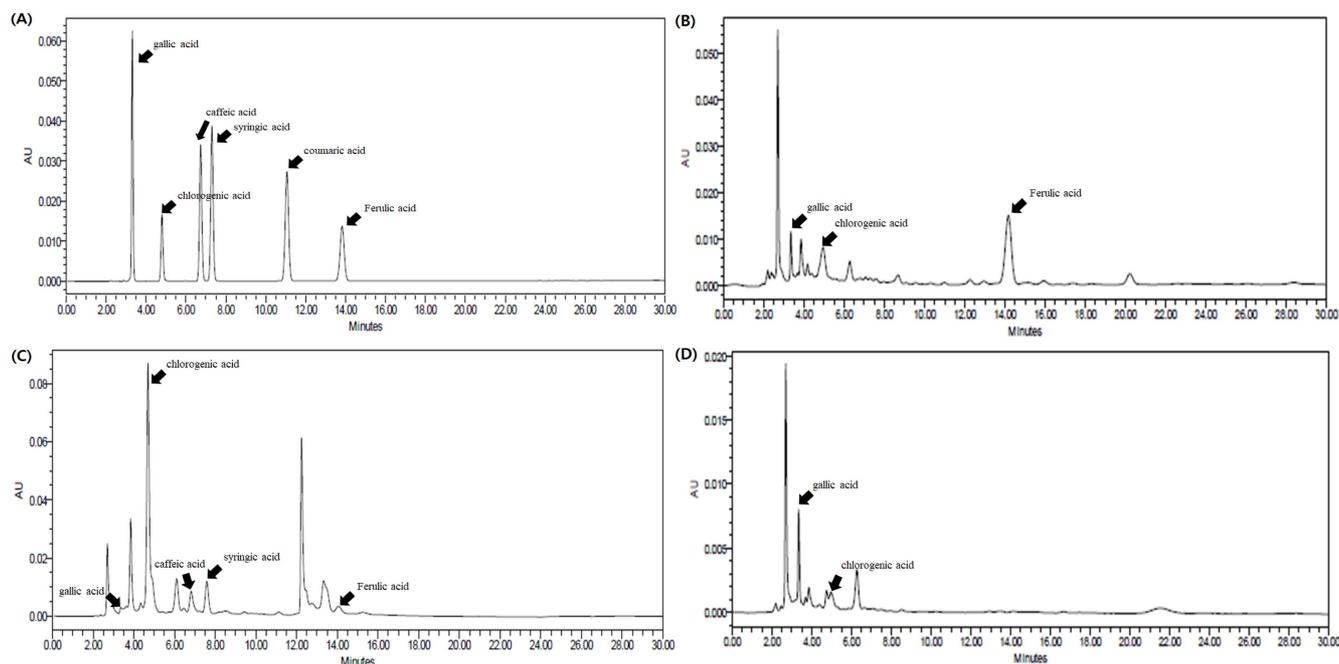


Fig. 4. HPLC chromatograms of standard compounds and methanolic extracts from *C. wilsonii* Nakai. (A); standard compounds, (B); stem methanolic extract, (C); leaf methanolic extract, (D); fruit methanolic extract.

Table 3. Calibration curve of standard and limit of detection (LOD) and, limit of quantitation (LOQ).

Compounds	Calibration curve	Correlation coefficient (R ²)	LOD ¹⁾ (μg/ml)	LOQ ²⁾ (μg/ml)
Gallic acid	Y = 2.85e+004X + 9.62e+004	0.996555	0.04	0.16
Chlorogenic acid	Y = 1.21e+004X + 4.53e+003	0.999867	0.05	0.16
Caffeic acid	Y = 2.84e+004X + 1.80e+004	0.999793	0.02	0.06
Syringic acid	Y = 3.49e+004X - 5.52e+003	0.999839	0.02	0.05
Coumaric acid	Y = 3.36e+004X + 3.30e+003	0.999871	0.02	0.08
Ferulic acid	Y = 2.06e+004X + 3.55e+003	0.999872	0.04	0.16

¹⁾LOD; Limit of detection, ²⁾LOQ; Limit of quantitation. The standard concentration were 10, 25, 50 and 100 μg/ml.

Table 4. The difference in the content of phenolic acid composition of the *C. wilsonii* Nakai. (mg/g·Ext.)

Compounds	Stem	Leaf	Fruit
Gallic acid	1.34±0.10 ^a	1.19±0.02 ^a	2.07±0.06 ^a
Chlorogenic acid	8.61±2.52 ^b	84.24±0.64 ^a	4.05±0.08 ^b
Caffeic acid	ND	1.67±1.44 ^a	ND
Syringic acid	ND	1.99±0.01 ^a	ND
p-Coumaric acid	ND	ND	ND
Ferulic acid	15.46±1.23 ^a	1.71±0.04 ^b	ND

ND; Not detected. *Each Data is means ± standard deviation of three replicate tests. The all data were statistically analyzed used by One-way analysis of variance (ANOVA). And differences were assessed that significant at 5% level of probability (p < 0.05).

calculated using four concentrations (10, 25, 50 and 100 μg/ml). LOD, LOQ was obtained by signal-to-noise (S/N). LOD was calculated as the S/N value of 3 : 1. And. LOQ was

calculated as the S/N value of 10 : 1. The quantification results are shown in Table 4. This similar to the previous experiment of total phenolic content, the most contents in leaf methanolic

extract was detected. Similar to the total phenolic content experiment that previously tested, the largest contents was detected in leaf methanolic extract, and in order to high chlorogenic acid (84.24 ± 0.64 mg/g·Ext.) > syringic acid (1.99 ± 0.01 mg/g Ext.) > ferulic acid (1.71 ± 0.04 mg/g·Ext.) > caffeic acid (1.67 ± 1.44 mg/g·Ext.) > gallic acid (1.19 ± 0.02 mg/g·Ext.).

This results supports that the total phenolic content is involved in anti-oxidant activity reported by Rice-Evans *et al.* (1996) and is involved in anti-inflammatory by Elizabeth *et al.* (2005).

DISCUSSION

DPPH radical inhibition assay, total phenolic content and total flavonoid content, reducing power assay, HPLC qualitative and quantitative analysis were conducted to review the availability of natural antioxidants through anti-oxidant activity effect about *C. wilsonii* Nakai. According to the results, high anti-oxidant activity was found in leaf methanolic extract.

The IC₅₀ value of 15.74 ± 0.16 µg/ml in DPPH radical inhibition assay showed higher activity than the BHT used as an anti-oxidant and similar activity to α-tocopherol. Total phenolic and flavonoid content were 220.95 ± 0.50 mg·GAE/g, 36.46 ± 1.89 mg·QE/g, HPLC analysis showed that chlorogenic acid was the highest with 84.24 ± 0.64 mg/g·Ext. The anti-oxidant activity in *C. wilsonii* Nakai has not been reported yet, and the anti-oxidant effect is excellent.

When compared with the *C. multilorus* fruit which is believed to have been transferred and separated into *C. wilsonii* Nakai on Ulleung-do, It is reported that DPPH radical inhibition assay IC₅₀ could not be found in the ethanol-water (7 : 3, v/v) extract of *C. multilorus* fruit in a scientific paper (Liu *et al.*, 2018). Thus, the extracts of the *C. wilsonii* Nakai fruit studied in this paper showed a higher anti-oxidant effect. And *C. integrerimus* (Uysal *et al.*, 2016), which a plant of the same genus *Cotoneaster*, has been reported that the phenolic content was 115.15 mg·GAE/g and flavonoid content was 16.29 mg/g in twing methanolic extract. Therefore, it was confirmed that the phenol content (214.65 mg·GAE/g) is higher in the stem methanolic extract of the *C. wilsonii* Nakai.

Inflammation is the cause of chronic diseases such as obesity, cardiovascular disease, metabolic syndrome and dementia (Chung *et al.*, 2009). Measurement of NO generation, an inflammatory substance, showed that the amount of NO

produced in RAW264.7 cells was dependently reduced on concentration compared to negative control in leaf methanolic extract. In particular, NO production was reduced by 67% at 200 µg/ml.

This result is attributed that chlorogenic acid is high in the leaf methanolic extract (84.24 ± 0.64 mg/g·Ext.). This supports the results that chlorogenic acid is involved in anti-inflammatory by Hwang *et al.* (2014). For stem methanolic extract, RAW264.7 cells were found to be toxic (Fig. 2) and fruit methanolic extract were found to be ineffective in the NO generation (Fig. 3).

This is consistent with the results that inflammatory reactions are effectively inhibited if oxidative stress is blocked by an anti-oxidant reported by several researchers (Kim *et al.*, 2011; Kim *et al.*, 2015). Therefore, the anti-inflammatory effect of the *C. wilsonii* Nakai is thought to be due to the strong anti-oxidant power of the *C. wilsonii* Nakai as aforementioned.

The results of this study are the first report of anti-oxidant effect in the *C. wilsonii* Nakai. Comparing with the same *Cotoneaster* plants, it has been found that the high anti-oxidant effect. The leaf extract of the *C. wilsonii* Nakai is thought to be available as a natural antioxidant. Further research on bioactivities is required for the possibility of development as a natural material of the *C. wilsonii* Nakai.

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