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Radical Scavenging Activity and Content of Cynarin (1,3-dicaffeoylquinic acid) in Artichoke (*Cynara scolymus* L.)

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The contents of total phenol and total flavonoid of artichoke (*Cynara scolymus* L.) were measured. The antioxidant activity of the artichoke was evaluated based on its potential as a scavenging the ABTS radical. These results showed the antioxidant activity of artichoke has a close relationship with the total flavonoid content. The compound showing antioxidant activity was isolated from the artichoke by repeated column chromatography and recrystallization. Based on the spectrometric studies, the compound was identified as 1,3-dicaffeoylquinic acid, known as cynarin. The content of cynarin from heads and leafs of the artichoke determined by C_{18} reversed phase HPLC (high-performance liquid chromatography) coupled with photodiode array detector was 10.15 and 0.67 mg/g, respectively. This compound showed potent antioxidant activities against DPPH and ABTS radicals (EC_{50} = 14.09 and 28.85 μM, respectively).

Key words: *antioxidant activity, artichoke,* Cynara scolymus *L., cynarin, total flavonoid, total phenol*

Artichokes (*Cynara scolymus* L.) are widely cultivated in Europe and the United States of America. The artichoke head, an immature flower, constitutes the edible part of this vegetable. Artichokes is not only known for its pleasant bitter taste, but is also used as a widespread herbal drug. Extensive studies on the chemical components of the artichoke have revealed it to be a rich source of the polyphenol compounds, with mono- and dicaffeoylquinic acids and flavonoids as the major chemical components [Nichiforescu, 1970; Adzet *et al.*, 1985; Dranik *et al.*, 1991; Wagenbreth, 1996]. Artichoke has been cultivated in Jeju since 2004. With the increasing demands for only artichoke heads by the hotels for luxurious cooking, it, therefore, became necessary to analyze the functional materials in other parts of the artichokes for the processing industry. Two major compounds in the artichoke are the salts of chlorogenic acid and cynarin (1,3-dicaffeoylquinic acid), phenolic compounds that are derivatives of caffeic acid. Extracts containing cynarin have been found to be effective on the treatments of hepatobiliary diseases, hyperlipidaemia and cholesterol metabolism [De Malach *et al.*, 1976; Foury, 1977; Zaki *et al.*, 1991; Schrader, 1994; Miguel *et al.*, 1997; Garcia *et al.*, 1998; Kocer and Eser, 1999; Garcia *et al.*, 1999].

In this study, the contents of the functional materials such as total phenols and flavonoids from the head and leaf of the artichoke were measured, and their antioxidant activities were determined at varying concentrations of the methanol for commercial use of the artichokes. One compound was isolated from the artichoke, and its structure was determined by spectroscopic methods, analyzed by C_{18} reverse phase HPLC, and evaluated for radical scavenging activities against DPPH and ABTS. The antioxidant activity of artichokes has been reported by Gebhardt (1997). However, the contents of the functional materials at varying concentrations of the methanol and the antioxidant activity of the cynarin against ABTS have never been reported.

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Abbreviations: ABTS, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid); DPPH, 1,1-Diphenyl-2-picrylhydrazyl.

Materials and Methods

Plant material. The cultivated artichoke was collected at Jeju island (Korea) in June, 2006. Extra pure grade solvents were purchased from Duksan Pure Chemicals (Ansan, Korea).

Reagents. Rutin, ABTS, DPPH, sodium persulfate, butylated hydroxyl anisol (BHA), and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). HPLC-grade water and acetonitrile were obtained from EMD Chemicals (Darmstadt, Germany).

Instruments. IR spectra were recorded on a Bruker Vertex 70 infrared Fourier transform spectrophotometer (KBr) and UV spectra were measured on a Varian Cary100 spectrophotometer. ^{1}H-NMR and ^{13}C-NMR at 400 and 100 MHz, respectively, were obtained on a Bruker AM 400 spectrometer in CD_3OD. EIMS was obtained on a JEOLJMS-700 mass spectrometer. TLC was conducted on precoated Kieselgel $60F_{254}$ plates (Art. 5715; Merck) and the spots were detected either by examining the plates under a UV lamp or treating the plates with a 10% ethanolic solution of phosphomolybdic acid (Wako Pure Chemical Industries) followed by heating at 110°C. HPLC was performed using a Waters 2695 Alliance Separations Module, Waters 2996 Photodiode Array Detector, Waters Micromass Quattro Micro API Mass Spectrometer (Waters, USA), and Bondapak™ C_{18} column (10 μM, 3.9 × 300 mm, Waters, Ireland)

Determination of the total phenol contents. The total phenol contents of artichoke were measured by the Prussian blue assay of Price and Buttler (1977). Dried artichoke head and leaf were extracted with different concentrations of methanol at 37°C for 12 h. The artichoke head and leaf extracts were diluted with 2 mL methanol, followed by the addition of 200 μL of 50 mM $FeCl_3$ in 0.1 M HCl plus 200 μL of 8 mM $K_3Fe(CN)_6$ to the sample at room temperature for 5 min. The total phenol contents were determined by measuring the absorbance at 670 nm on a spectrophotometer (Varian, Cary100, Australia). A calibration curve was constructed with different concentrations of chlorogenic acid as a standard. The total phenol contents were determined as mg of chlorogenic acid equivalents (mg CGA/g extract).

Determination of the total flavonoid contents. The total content of flavonoid of artichoke extract was determined following the modified procedures described by Davis [Jo and Jung, 2000]. Dried artichoke head and leaf were extracted with the different concentrations of methanol at 37°C for 12 h. Each one milliliter of artichoke head and leaf extracts were mixed with 10 mL of diethylene-glycol and 100 μL of 1 N NaOH at 37°C for 1 h. The total flavonoid contents were determined as mg of rutin equivalents (mg rutin/g extract) by measuring the absorbance at 420 nm in spectrophotometer with rutin as standard.

Extraction and isolation. The head of artichoke (1.0 kg) was air-dried, chopped and extracted three times with 80% MeOH (3 × 7 L) for 14 days at room temperature. The combined extracts were evaporated to dryness under reduced pressure at below 40°C. After filtration and concentration, the resulting extract (35 g) was suspended in H_2O (2 × 300 mL). The dilution with H_2O was partitioned with the organic solvents ($CHCl_3$, *n*-BuOH) of different polarities to afford soluble-$CHCl_3$ (7 g), soluble-*n*-BuOH (12 g), and soluble-H_2O (10 g) extracts. The *n*-BuOH extracts were chromatographed over silica gel using *n*-hexane: Acetone and $CHCl_3$: MeOH gradient to give 15 fractions (P1-P15). The eighth fraction (P8) (350 mg) was subjected to the silica gel column chromatography with *n*-hexane: Acetone (10 : 1 → 1 : 20) to afford 24 subfractions. Subfractions 10-15 were subjected to silica gel chromatography ($CHCl_3$: MeOH = 25 : 1 → 5: 1) and purified by recrystallization with $CHCl_3$-MeOH mixture to yield 1,3-dicaffeoylquinic acid (**1**) (52 mg).

HPLC apparatus and measurements. The head and leaf of artichoke (1.0 g) were extracted with 20 mL MeOH overnight in a vortex mixer at room temperature to form the final extract, which was and then centrifuged. The extracts used for the HPLC analysis were passed through a 0.45 μm filter (Advantec MFS, Inc. CA, USA) before injection into a reverse phase Bondapak™ C_{18}. Subsequently, 20 μL portion of these solutions was injected into the HPLC system (Waters 2695 Alliance). The mobile phase was water containing 0.1% formic acid (A) and acetonitrile (B). The linear gradients of A-B were: 0 min 80 : 20, 33 min 0 : 100, 40 min 80 : 20 (v/v). The flow rate was adjusted to 1.0 mL/min, and the wavelength of detection was set at 330 nm with the temperature held constant at 30°C.

Standard solution and calibration curves. An external standard method was utilized for the quantification. About 5-10 mg of a standard was dissolved in a 10-mL volumetric flask with MeOH to obtain the stock solution and stored in a freezer. The working standard solutions were diluted to a series of concentrations with MeOH. The mean areas generated from the standard solutions were plotted against the concentration to establish calibration equations.

Measurement of scavenging activity of the DPPH radical. The method described by Hatano *et al.* (1988) was used for determining the scavenging activity of artichoke extracts on DPPH radicals. Absorbance of the remaining DPPH radical was measured at 517 nm for 35 min against a blank of pure methanol including only

DPPH radical using the UV-visible spectrophotometer at room temperature. The DPPH radical scavenging capacity was calculated as the difference in the absorbance with tested compounds and expressed as percent remaining of the DPPH radical. Inhibition of the free radical DPPH in percent (%) was calculated as follows:

$$\text{Antioxidant activity of DPPH (\%)} = (A_{blank} - A_{sample}/A_{blank}) \times 100$$

Measurement of Trolox equivalent antioxidant capacity (TEAC). TEAC assay was based on the relative ability of antioxidants to scavenge the radical cation $ABTS^{\cdot+}$ in comparison to a standard (Trolox) [Re *et al*, 1999; Choi *et al*, 2005]. The radical cation was prepared by mixing 7 mM ABTS stock solution with 2.45 mM potassium persulfate. The reaction mixture was maintained for 4-8 h until the mixing was completed and the absorbance become stable. $ABTS^{\cdot+}$ solution was diluted with ethanol and the absorbance was read at 734 nm. For the photometric assay, 0.9 mL $ABTS^{\cdot+}$ solution and 0.1 mL of the test compound were mixed for 1 min and the absorbance was measured immediately after 1 min at 734 nm. Antioxidant activity of each compound was calculated by determining the decrease in absorbance at different concentrations using the following equation: $E = [A_{blank} - A_{sample}/A_{blank}] \times 100$, where A_{sample} and A_{blank} were the absorbance of the samples with and without the test samples, respectively. Antioxidant activity was expressed as TEAC values.

Results and Discussion

Total phenol contents of artichoke extracts. The total phenol contents in the methanol extracts of the artichoke were expressed as mg/g of chlorogenic acid. A calibration curve was constructed using different concentrations of chlorogenic acid (400, 200, 100, 50, 25, 12.5, and 6.25 μg/mL) as standard. The linear regression equation of this curve and coefficient of determination (R^2) were calculated as $y = 0.0161x - 0.01104$, $R^2 = 0.9981$. These results showed that content of total phenols was higher at 30~60% methanol solvent in the head and at 70% methanol solvent in the leaf (Fig. 1).

Total flavonoid contents of artichoke extracts. Contents of total flavonoids in the head and the leaf of the artichoke were estimated following the modified procedures described by Davis [Jo and Jung, 2000] and expressed as mg/g of rutin. A calibration curve was constructed using different concentrations of rutin (400, 200, 100, 50, 25, 12.5, and 6.25 μg/mL) as the standard. The linear regression equation of this curve and the coefficient of determination (R^2) were calculated as $y = 0.0014x - 0.0126$, $R^2 = 0.9992$.

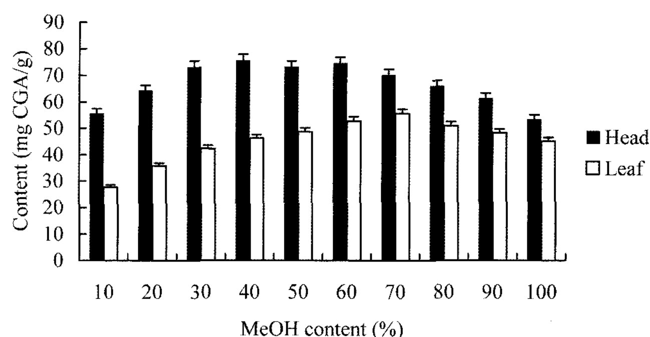

Fig. 1. Total phenol contents of artichoke extracts.

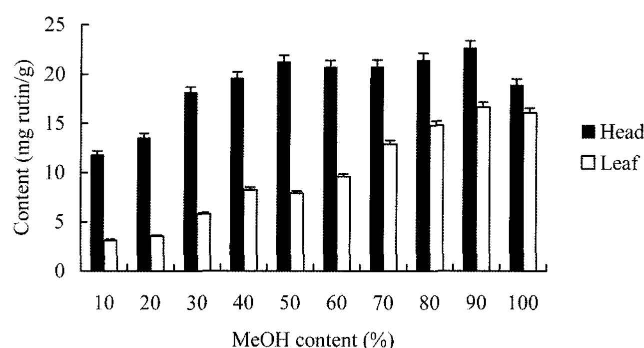

Fig. 2. Total flavonoid contents of artichoke extracts.

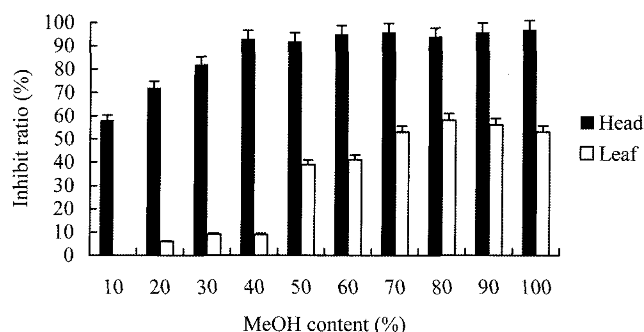

Fig. 3. ABTS radical scavenging activity of the artichoke extracts.

Results showed the contents of total flavonoids were highest at 80~90% methanol solvent in the head, and at 90% methanol solvent in the leaf (Fig. 2).

Radical ABTS scavenging activity. Dried artichoke head and leaf (1 g) were extracted in different concentrations of methanol solutions (100 mL) at 37°C for 12 h. Each 0.1mL of head and leaf extracts were added to 0.9 mL $ABTS^{\cdot+}$ solution at room temperature for 1 min and the absorbance was measured immediately after 1 min at 734 nm. Results showed the free radical scavenging activity of artichoke was highest at 40~100% methanol solvent in the head and at 70~80% ethanol solvent in the leaf, demonstrating that the antioxidant activity was closely related to the total flavonoid contents (Fig. 3).

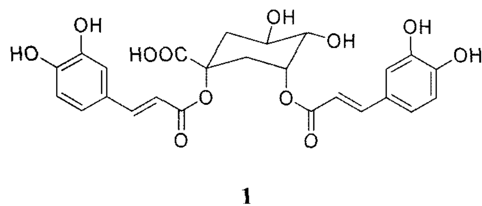

Fig. 4. Structure of the isolated compound from the head of artichoke.

The heads of artichoke were extracted with 80% MeOH. After filtration and concentration the resulting extracts were suspended in H_2O and successively partitioned with $CHCl_3$, *n*-BuOH, yielding $CHCl_3$-, *n*-BuOH-extractable residues. A compound was isolated through repeated chromatographic separation of the *n*-BuOH fractions. Structural identification of the compound was carried out through the interpretation of several spectral data, and comparison with the data described in the literature [Horman *et al*, 1984; Schram *et al*, 2004; Wang *et al*, 2003]. The isolated compound was readily identified as 1,3-dicaffeoylquinic acid (**1**) known as cynarin.

1,3-Dicaffeoylquinic acid (Cynarin): Yellow power. EIMS m/z 516; UV λ_{max} nm 330, 233 (MeOH); ^{1}H NMR (CD_3OD, 400 MHz) δ 7.57 (2H, d, J = 16.1 Hz), 7.05 (1H, d, J = 1.5 Hz), 7.04 (2H, d, J = 1.5 Hz), 6.95 (2H, m), 6.78 (1H, d, J = 1.5 Hz), 6.76 (1H, d, J = 1.5 Hz), 6.28 (1H, d, J = 16.0 Hz), 6.26 (1H, d, J = 16.0 Hz), 5.37 (1H, m), 4.29 (1H, m), 3.77 (1H, dd, J = 8.0, 3.4 Hz), 2.55 (1H, dd, J = 13.7, 3.9 Hz), 2.43 (2H, m), 2.06 (1H, dd, J = 13.7, 8.8 Hz); ^{13}C NMR (CD_3OD, 100 MHz) δ 168.6 (s), 168.0 (s), 168.0 (s), 149,6 (s), 149.6 (s), 147.5 (d), 147.3 (d), 146.8 (s), 146.8 (s), 127.8 (s), 127.8 (s), 123.1 (d), 123.0 (d), 116.5 (d), 116.5 (d), 115.2 (d), 115.1 (d), 115.1 (d), 115.0 (d), 72.7 (s), 71.5 (d), 71.5 (d), 69.3 (d), 36.8 (t), 35.6 (t)

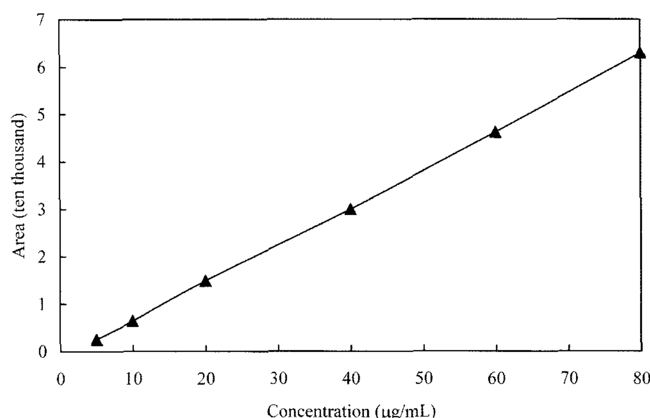

Fig. 5. Calibration curve of the isolated cynarin from artichoke.

Determination of cynarin. The isolated cynarin was investigated by quantitative analysis using HPLC, and identified by comparing its physical and spectroscopic data with those of the authentic standard. A calibration curve was constructed at different concentrations of cynarin (80, 60, 40, 20, 10, and 5 μg/mL) as a standard (Fig. 5). The linear regression equation of this curve and coefficient of determination (R^2) were calculated as $y = 796.6x - 1273.0$, $R^2 = 0.9996$. Results of the analysis revealed that artichoke contains 10.15 and 0.67 mg of cynarin in 1 g of the head and leaf, respectively (Fig. 6).

Radical scavenging activity of cynarin. The systems ABTS and DPPH are both commonly used to measure the total antioxidant status of various biological specimens because of their reproducibility and ease of quality control. Therefore, ABTS and DPPH radicals were

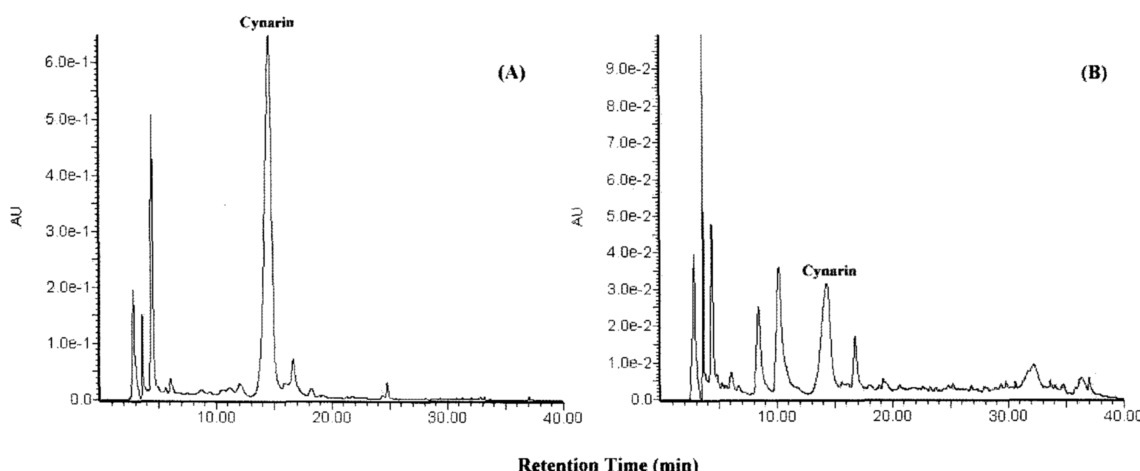

Fig. 6. Characteristic of HPLC chromatograms: (A) Profile of MeOH extract from head of artichoke; (B) Profile of MeOH extract from leaf of artichoke.

Table. 1. Antioxidant activity of the isolated cynarin from artichoke on DPPH and ABTS radicals

Compound	EC_{50} (μM)[1]	
	DPPH	ABTS
Cynarin	14.09 ± 0.92	28.85 ± 2.16
BHA/Trolox	33.4 ± 2.15 (BHA)	13.5 ± 2.25 (Trolox)

[1]Antioxidant activity was expressed as the mean of 50% effective concentration of triplicate determinations.

chosen to test the antioxidant activities of the isolated compound. For the measurement of the antioxidant activity, the UV/Vis spectrophotometry method was used to detect ABTS and DPPH radicals. Radical scavenging activity of cynarin showed suppressed absorbance of the ABTS and DPPH radicals with EC_{50} value of 28.85 and 14.09 μM, respectively (Table 1).

In conclusion, the total phenol and total flavonoid contents of the artichoke were measured. The antioxidant activity of artichoke was evaluated its potential for scavenging stable ABTS radical. A compound, identified as a cynarin, was isolated from the methanol extract of the artichoke and characterized using physical and spectroscopic data. The content of cynarin was determined by C_{18} reversed phase HPLC analysis. Cynarin showed strong antioxidant activities against DPPH and ABTS radicals.

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