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Cynarin-Rich Sunflower (*Helianthus annuus*) Sprouts Possess Both Antiglycative and Antioxidant Activities

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ABSTRACT: The present study examined the antiglycative and antioxidant properties of four edible sprouts popular in Chinese markets. In a protein-reducing sugar model, the sunflower sprout *Helianthus annuus* exhibited the strongest inhibitory effects against the formation of advanced glycation end products (AGEs). At a concentration of 1.0 mg/mL, its inhibitory rate achieved 83.29%, which is stronger than that of aminoguanidine (1 mM), a well-known synthetic antiglycative agent (with an inhibitory rate of 80.88%). The antioxidant capacity of *H. annuus* was also much stronger than other sprout samples in terms of free radical scavenging and reducing properties. An active ingredient contributing to the observed activities was identified as cynarin (1,5-dicaffeoylquinic acid). This is the first report of the novel function of cynarin to intervene against glycoxidation. Given the key roles of AGEs and oxidation in the pathogenesis of diabetes, the sunflower sprout *H. annuus* rich in cynarin may be regarded as a beneficial food choice for diabetic patients.

KEYWORDS: *Advanced glycation end products, antioxidant, sunflower sprout, cynarin*

INTRODUCTION

From very ancient times, *Huangdi Neijing* (also known as *Yellow Emperor's Inner Canon*), the earliest and most fundamental Chinese medicine text, has suggested that medicine and food could have shared sources. The concept of "Chinese dietary therapy" has been handed down by our ancestors, gaining more and more scientific support from past to present. The intake of natural foods has much fewer side effects than synthetic chemical drugs and is safe for human consumption, which may correct physiological malfunctions in people and help to prevent or alleviate a number of diseases, such as diabetes. As one of the most significant public health threats, diabetes can be found in almost every population in the world and is responsible for several long-term complications.[1] Asia is the major region of the increasing prevalence of diabetes, and the latest study in 2010 showed China has overtaken India as the new global epicenter of diabetes.[2] It is believed that, under hyperglycemic conditions, the formation and accumulation of advanced glycation end products (AGEs) is an important pathogenic factor responsible for diabetes. AGEs are generated from the non-enzymatic glycation between reducing sugars and amino groups of proteins.[3] They may form cross-links with proteins in the basement membrane of the extracellular matrix, resulting in structural modifications and functional impairments. Furthermore, AGEs may interact with their receptors (RAGE) to promote the intracellular signals disrupting cellular functions. For example, their interaction on macrophages induces oxidative stress and the activation of nuclear factor-κB (NF-κB), which in turn upregulates the expression of several genes relevant for inflammation, vasoconstriction, and coagulation.[4] In recent years, the research exploring the anti-AGE activities of natural foods has attracted much attention in China as well as some other Asian countries, and some very promising results have been obtained, e.g., from mung bean,[5] tomato paste,[6] guava,[7] and microalgae.[8,9] These findings, with a few exceptions, highly correlate with free radical scavenging activities, providing very good evidence for the management of diabetes by dietary therapy. Because very few of them focused on vegetable sprouts, in the present work, four sprouts were examined to fill this knowledge gap. These sprouts, namely, *Toona sinensis*, *Lathyrus palustris*, *Vigna angulariz*, and *Helianthus annuus*, are very popular in the Chinese market, especially for five-star hotels and famous restaurants.

MATERIALS AND METHODS

Reagents and Chemicals. All reagents were purchased from Sigma Chemical Co. (St. Louis, MO), unless otherwise stated. All analytical and high-performance liquid chromatography (HPLC)-grade solvents were obtained from BDH Laboratory Supplies (Pool, U.K.).

Preparation of Crude Extracts from Vegetable Sprouts. The fresh edible sprouts (*T. sinensis*, *L. palustris*, *V. angulariz*, and *H. annuus*) were purchased from a local retail vegetable market in Beijing, China. The fresh vegetable samples were dried immediately at 50 °C in the oven. The dried sprouts were ground with a mill beaker and extracted with 2 volumes of 80% aqueous methanol for 5 days at room temperature. The extracts were filtered through filter paper. The extraction was repeated 3 times, and the combined extracts were concentrated with a rotary evaporator at 50 °C under reduced

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pressure. The concentrated extract was subjected to D101 resin column chromatography (Mosu Co., Shanghai, China). A total of 5 bed volumes of deionized water were used to remove amino acids and simple sugars, followed by elution with 5 bed volumes of 95% ethanol. The ethanol eluent was evaporated to dryness under vacuum. The dried extracts were used in assays directly.

Bovine Serum Albumin (BSA)−Glucose Assay. The BSA−glucose assay was performed as we described previously.[10] BSA (50 mg/mL) was dissolved in phosphate buffer (0.2 M, pH 7.4) with glucose (0.8 M). NaN_3 (0.2 g/L) was added to the test solution to ensure an aseptic condition. Aminoguanidine (AG) solution (1 mM) was used as the positive control. All incubations were carried out at 37 °C for 7 days in the absence or presence of inhibitors. The measurement of the fluorescent intensity of AGEs was performed using a Hitachi F-2500 fluorescent spectrometer (Hitachi Corporation, Tokyo, Japan). The generation of AGEs was characterized by a typical fluorescence with excitation and emission maxima at 330 and 410 nm, respectively. The percent inhibition of AGE formation was calculated using the following equation: percent inhibition = [1 − (fluorescence of the solution with inhibitors/fluorescence of the solution without inhibitors)] × 100.

1,1-Diphenyl-2-picrylhydrazyl (DPPH) Radical Scavenging Assay. The DPPH radical scavenging assay was carried out as previously described, with slight modifications.[11] Briefly, 1 mL of freshly prepared DPPH (0.19 mM) was mixed with 200 μL of tested compounds in 95% ethanol. After 1 h of incubation at 25 °C with vigorous shaking, the solution was centrifuged and 1 mL of aliquot of supernatant was transferred to a 1.5 mL plastic cuvette. The absorbance was measured at 520 nm. The percentage of inhibition was calculated as follows: percent inhibition = [1 − (absorbance of the solution with inhibitors/absorbance of the solution without inhibitors)] × 100. Ascorbic acid (AA), butylated hydroxyanisole (BHA), and Trolox (TX) were used for comparison.

Iron(III) to Iron(II) Reducing Capacity Assay. The iron reducing capacity was assessed using a method as previously described.[12] The sample mixture (600 μL) consisting of 100 μL of tested compounds in 95% ethanol, 250 μL of potassium phosphate buffer (200 mM, pH 6.5), and 250 μL of $K_3Fe(CN)_6$ (30 mM) was incubated for 20 min at 50 °C. After cooling rapidly, 250 μL of trichloroacetic acid (TCA) (600 mM) was added and the mixture was shaken vigorously before being centrifuged at 10000*g* for 10 min. An aliquot of supernatant (100 μL) was placed in a 1.5 mL plastic cuvette and supplemented by 900 μL of osmosed water and 100 μL of $FeCl_3{\cdot}6H_2O$ (3.7 mM). After incubation at room temperature for 15 min, the absorbance was measured at 700 nm. The higher the absorbance of the sample mixture, the better the iron reducing capacity of the sample. AA, BHA, and TX were used for comparison.

***β*-Carotene Protection Assay.** The protective effect against oxidization of *β*-carotene was evaluated using a method as previously described, with slight modifications.[13] The emulsion, consisting of 1 mg of crystalline *β*-carotene, 20 μL of linoleic acid, and 200 μL of commercial surfactant Tween 40 (polyoxyethylene sorbitan monopalmitate), was prepared in 10 mL of reagent-grade chloroform. The solvent was evaporated at 40 °C using a rotary evaporator. The resulting viscous oil was immediately mixed with 100 mL of O_2-saturated osmosed water and vigorously shaken for 2 min. A total of 5 mL of each aqueous emulsion was transferred to test tubes containing 200 μL of tested compounds in 80% methanol. A zero-time reading (A_0 for the control solution and A_0* for the sample mixture) was taken at 470 nm for each reaction mixture immediately after the addition of emulsion to a sprout solution. The tubes were incubated at 50 °C and exposed to daylight in a rotary shaker. The absorbance of aliquots of the reaction mixture incubated for 60 min was monitored at 470 nm (A_{60} for the control solution and A_{60}* for the sample mixture). The protective effect was expressed as the percentage of *β*-carotene protection against bleaching, calculated as follows: percent protection = $[1 - (A_{60}^* - A_0^*)/(A_{60} - A_0)] \times 100$. BHA was used for comparison.

HPLC and Liquid Chromatography−Tandem Mass Spectrometry (LC−MS/MS) Analysis. Components of the *H. annuus* extract were analyzed using the HPLC system equipped with a Waters Alliance 2695 separation module and Waters 2996 diode array detectors. Separation was carried out with an Alltima C18 (5 μm, 250 × 4.6 mm) column. The mobile phase was composed of 0.1% formic acid water (solvent A) and acetonitrile (solvent B) of the following gradients: 0 min, 85% A/15% B; 30 min, 70% A/30% B; 40 min, 20% A/80% B; and 45 min, 10% A/90% B. The flow rate was set at 1.0 mL/min. The total running time was 50 min, and the post-running time was 10 min for equilibration of the column.

The sample was further analyzed on a LC−MS/MS instrument equipped with a separation model (Agilent 1200, Agilent Technologies, Santa Clara, CA) containing an electrospray ionization (ESI) source. The separation condition adopted was the same as mentioned in HPLC analysis, while separation was conducted on a YMC-pack Pro column (2.1 × 150 mm, 5 μm). The flow rate was set at 0.2 mL/min. The MS conditions were as follows: negative-ion mode; spray voltage, 4.5 kV; scan range, 100−800 Da; DP, 65 V. Independent data analysis (IDA) was used, and the enhanced product ions (EPIs) originated from the largest abundance m/z in enhanced mass scan (EMS) mode were recorded.

Statistical Analysis. Experimental results were obtained as the mean value ± standard deviation (SD) (n = 3). Statistical analyses were performed using the SPSS statistical package (SPSS, Inc., Chicago, IL). A paired sample t test was applied. The statistical significances were achieved when $p < 0.05$.

RESULTS AND DISCUSSION

The investigation of AGE inhibitors may provide a novel preventive/therapeutic approach for diabetes. Although a number of synthetic compounds, e.g., AG, have shown strong antiglycative capacities, their side effects and high toxicity are serious concerns for people, hindering the ultimate commercial application.[14] On the other hand, the benefits of developing natural anti-AGE agents from foodstuffs that are safe for human consumption without adverse effects have been highlighted.[15] In the present study, for the first time, the AGE inhibitory capacities of four vegetable sprouts popular in Chinese restaurants and hotels have been evaluated. The BSA−glucose system employed is commonly used in non-enzymatic glycation studies. Proteins can be modified when exposed to reducing sugars through the spontaneous glycation process, and the sugar-mediated fluorescence intensity, which is the characteristic of AGEs, is increasing during the incubation at 37 °C. The formation of AGEs can be monitored at excitation and emission of 330 and 410 nm, respectively.[16] As shown in Figure 1, the sprout samples exhibited very diverse effects against the formation of AGEs. At a concentration of 0.2 mg/mL, the extract of *H. annuus* showed the strongest inhibitory capacity (inhibitory rate of 37.23%, $p < 0.05$), followed by *T. sinensis* (21.62%) and *L. palustris* (2.74%), whereas the *V. angulariz* extract had no effects. It was observed that increasing the concentrations of sprout samples led to attenuated fluorescence intensity. At a concentration of 1.0 mg/mL, the AGE inhibitory rate of *H. annuus* achieved 83.29%, higher than that of AG (1 mM), a well-known synthetic AGE inhibitor employed as the positive control in the present study (inhibitory rate of 80.88%).

The formation of AGEs has tight association with oxidation. Under the presence of metals and O_2, Amadori products are modified to create protein dicarbonyl compounds, which subsequently participate in the AGE formation.[17] Alternatively, glucose may be auto-oxidized, converting itself to a dicarbonyl ketoaldehyde that facilitates the generation of AGEs and giving rise to superoxide radicals by reducing molecular oxygen. These superoxide radicals may be subsequently converted to highly

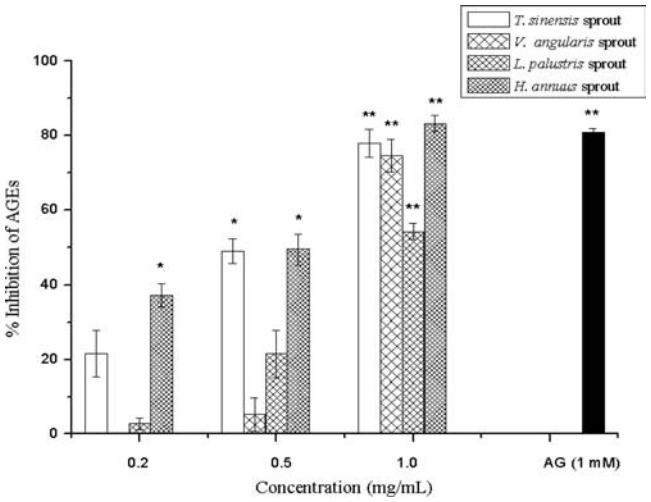

Figure 1. Inhibitory effects of sprout extracts (0.2−1.0 mg/mL) against the formation of AGEs. Each value represents the mean ± SD (n = 3). Significant differences were marked with ∗ (p < 0.05) or ∗∗ (p < 0.01) compared to the control solution.

reactive hydroxyl radicals via the Fenton reaction, inducing further oxidative protein degradation.[18] In fact, there is growing evidence that reactive oxygen species (ROS) is the trigger driving various biochemical pathways associated with diabetic complications, and diabetic patients are suffering from an increased risk of free-radical-mediated damage.[19] Therefore, natural products with both antioxidant and antiglycative properties have been proven to be more effective in the treatment or prevention of diabetes.[20,21] In the present work, several assays were introduced to evaluate the antioxidant activity of sprout extracts. In the DPPH radical scavenging assay, the addition of sprout extracts induced a decrease in absorbance at 520 nm, of which *H. annuus* exhibited the most potent scavenging activity (Figure 2A). The reductive power is another indicator of the antioxidant activity. The iron reducing assay employed in the present study is based on the reduction of the ferric (Fe^{3+}) form to the ferrous (Fe^{2+}) form. At low pH, the ferrous form reacts with $FeCl_3$, and the resulting Prussian-blue-colored complex can be spectrophotometrically monitored at 700 nm. Results showed that an addition of the *H. annuus* extract into the reaction mixture resulted in a markedly increased value, suggesting its significant iron-reducing potential. For other sprout samples, similar to DPPH results, dose-dependent antioxidant actions were observed (Figure 2B). The protection effects of sprout samples against β-carotene oxidization were also evaluated. Under the presence of light, air, heat, and linoleate-free radicals, the red−orange β-carotene may be oxidized, losing double bonds, and become colorless. Such β-carotene bleaching could be inhibited by antioxidant agents.[22] Results showed that the sprout extracts effectively diminished the oxidative degradation of β-carotene, while increasing the concentration was associated with higher absorbance values at 470 nm (Figure 2C). These data clearly showed that *H. annuus* had the highest antioxidative activities among the sprouts tested, be it free radical scavenging or reducing properties. The EC_{50} values of *H. annuus* and the other three spout samples were compared to some pure antioxidant chemicals commercially used in food, including AA, BHA, and TX. As shown in Table 1, although the antioxidant

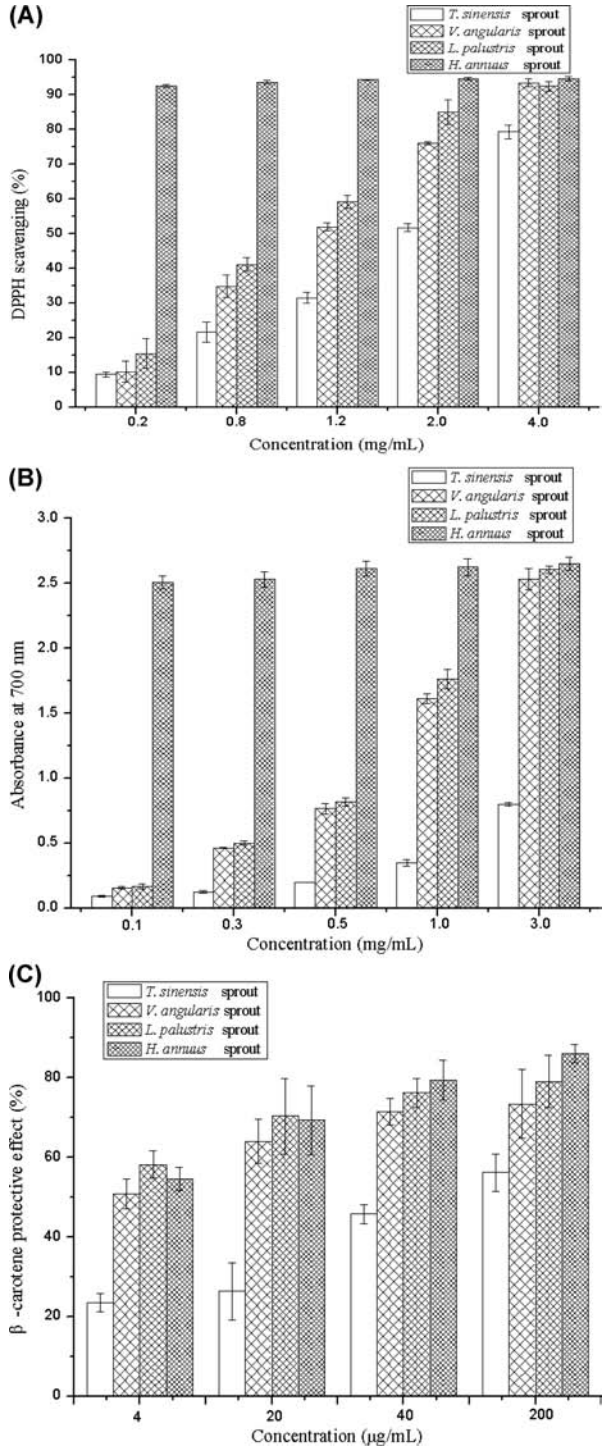

Figure 2. (A) Scavenge effects of sprout extracts (0.2−4.0 mg/mL) on the DPPH radical. Each value represents the mean ± SD (n = 3). (B) Iron reducing capacities of sprout extracts (0.1−3.0 mg/mL). Each value represents the mean ± SD (n = 3). (C) Protective effects of sprout extracts (4−200 μg/mL) against β-carotene bleaching. Each value represents the mean ± SD (n = 3).

capacity of *H. annuus* was weaker than them, it is still relatively strong compared to many other edible vegetables or even Chinese medicines.[21,23] These results, taken together, strongly supported the possibility of using natural antioxidants as glycoxidation inhibitors and suggested the potential benefits of *H. annuus* for diabetic patients.

Table 1. EC_{50} Values of Sprout Samples and Cynarin in the Antioxidant Assays Compared to Some Pure Antioxidant Chemicals Commercially Used in Food[a]

	EC_{50} value (μg/mL) (mean ± SD; n = 3)		
product	DPPH[b]	Fe^{III} [c]	β-carotene[d]
H. annuus	35.00 ± 3.82	18.00 ± 0.31	2.93 ± 0.23
L. palustris	870.00 ± 19.06	280.00 ± 18.71	2.56 ± 0.66
V. angulariz	1000.00 ± 154.64	300.00 ± 25.96	3.87 ± 0.86
T. sinensis	1600.00 ± 143.23	1800.00 ± 18.08	110.00 ± 4.67
AA	6.89 ± 1.06	3.34 ± 0.30	nt
BHA	6.31 ± 0.30	4.87 ± 0.20	0.91 ± 0.05
TX	11.51 ± 0.74	8.01 ± 0.72	nt
cynarin	10.17 ± 0.91	525.00 ± 12.50	42.15 ± 6.20

[a]AA, ascorbic acid; BHA, butylated hydroxyanisole; TX, trolox; nt, not tested. [b]EC_{50} value: effective concentration at which 50% of radicals are scavenged. [c]EC_{50} value: effective concentration at which the absorbance is 0.5. [d]EC_{50} value: effective concentration needed to prevent β-carotene bleached by 50%.

The chemical components in the extract of the *H. annuus* sprout were analyzed by HPLC. As shown in Figure 3A, the major peak, t_R of 23.2 min, was identified as cynarin (1,5-dicaffeoylquinic acid) by comparison to the authentic cyanrin purified by us before from artichoke. Its negative ESI−MS spectrum (Figure 3B), which showed a molecular ion peak at 515.0 and major fragment ion peaks at 353.2 and 191.3, further validates the structure of this compound. Cynarin is found in the leaves of artichoke (*Cynara scolymus* L.), an ancient herbal medicine. To the best of our knowledge, this is the first report of cynarin in *H. annuus* sprouts. Furthermore, its content was calculated to be over 8% (w/w), much higher than that in artichoke leaves, of which the total amount of caffeoylquinic acid derivatives is 3−6.5% (w/w).[24] To clarify the roles of cynarin, the pure compound was evaluated in the antioxidant/antiglycative models. In antioxidant assays, cynarin showed potent free radical scavenging effects (Table 1), and such findings were consistent with previous reports.[24,25] In the

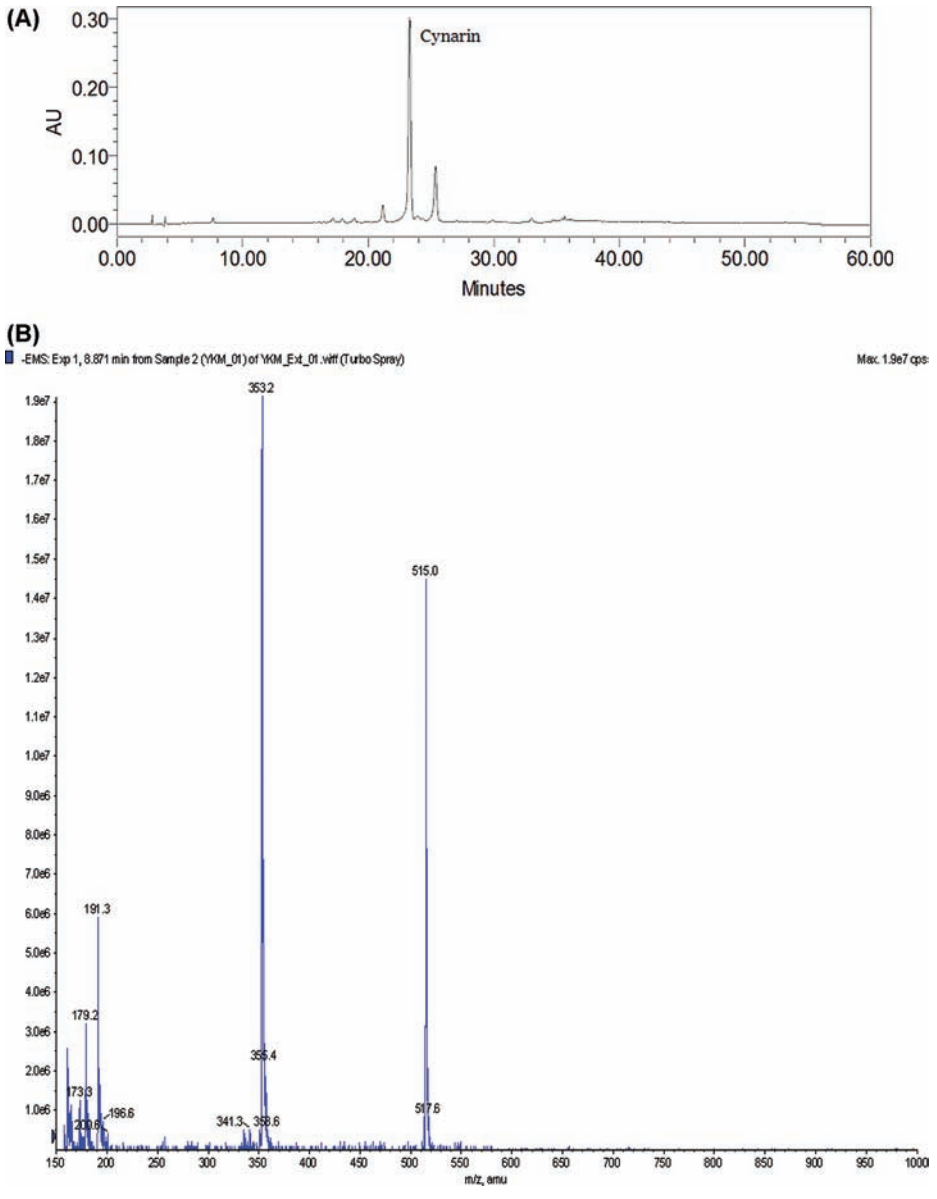

Figure 3. (A) HPLC chromatogram of the *H. annuus* extract at 320 nm. (B) Negative ESI−MS mass spectrum of cynarin.

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BSA−glucose system, cynarin exhibited significant AGE inhibitory abilities in a dose-dependent manner (Figure 4).

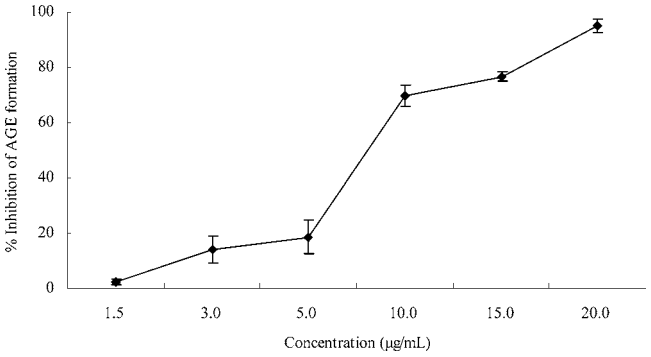

Figure 4. Inhibitory effects of cynarin against the formation of AGEs. Each value represents the mean ± SD (n = 3).

Its EC_{50} concentration was 9.38 μg/mL. These results clearly proved that cynarin is an active ingredient contributing to the antioxidant and antiglycative activities of *H. annuus*. As a phenolic compound, cynarin (Figure 5) has been determined to

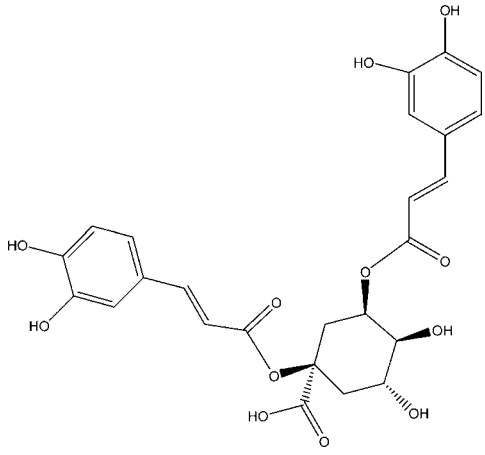

Figure 5. Structure of cynarin.

possess cholesterol/triglyceride-lowering effects and could be potentially beneficial for patients with hyperglycemia or hyperlipidemia.[26−28] However, there are no previous reports on its effects on glycoxidation. Results of the present work may provide some good evidence.

In conclusion, in the present study, we reported the presence of a high level of cynarin in the sunflower sprout *H. annuus* and its novel function for the first time. This phenolic compound provided both antioxidant and antiglycative effects greatly, making *H. annuus* a food choice with potential benefits for diabetic patients. Because AGEs represent a huge source of complex and heterogeneous molecules, of which many have distinct structures or characters, systematic investigation using more specific methods needs to be conducted in the future.

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Notes

The authors declare no competing financial interest.

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