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Antioxidant and Xanthine Oxidase Inhibition Activities of *Cynomorium songaricum* Extracts

Soo-Jung Seo, Mi-Ra Han, and Yang-Suk Lee†

Department of Herbal Biotechnology, Daegu Haany University, Gyeongbuk 712-715, Korea

Abstract

In this study, we evaluated the antioxidant activities and xanthine oxidase inhibition effects of water and ethanol extracts of *Cynomorium songaricum*. The ethanol extract of *C. songaricum* (EE) contained more phenolic and flavonoid compounds than the water extract (WE). The antioxidant activities of the extracts were increased as the concentration of the extract increased. The WE has better effectiveness than the EE for DPPH free radical scavenging activity and nitrite scavenging ability. The nitrite scavenging abilities of WE were 90.02% (EC_{50} 653.15 μg/mL) at conditions of pH 1.2 and 2,000 μg/mL, and 84.34% (EC_{50} 817.17 μg/mL) at pH 3.0. The EE has more effective SOD-like activity and XO inhibition than WE. The SOD-like activity of EE was 81.47% at a concentration of 2,000 μg/mL, EC_{50} was 951.70 μg/mL. The xanthine oxidase inhibition of the EE, with an EC_{50} of 112.47 μg/mL, is greater than that of ascorbic acid, which was 192.50 μg/mL ($p<0.05$). These results suggest that the *C. songaricum* is a potentially useful antioxidant source for the development of nutraceuticals and medicines.

Key words: *C. songaricum*, antioxidant, nitrite scavenging, xanthine oxidase inhibition

INTRODUCTION

Reactive oxygen species (ROS) including free radicals such as superoxide anion radicals ($\cdot O_2^-$), hydroxyl radical species ($\cdot OH$), singlet oxygen ($^1O_2$) and hydrogen peroxide (H_2O_2) are active oxygen compounds that are often generated by biological oxidation reactions of exogenous factors (1,2). Oxidative damage caused by free radicals may be related to aging and disease, such as atherosclerosis, diabetes, cancer and cirrhosis (1). Although almost all organisms possess antioxidant defense and repair systems that have evolved to protect against oxidative damage, these systems are insufficient to completely prevent all damage (3). The protective effects in plants are due to the presence of several components that have distinct mechanisms of action, such as natural antioxidant phytochemicals (4). It has been shown that a wide variety of phenolic compounds, including flavonoids, isoflavones, flavones, anthocyanins, and catechins, act to prevent or reduce oxidative stress by scavenging free radicals (5,6). In addition, vitamins such as ascorbic acid, α-tocopherol, and β-carotene have been shown to have antioxidant properties (7). However, the use of common synthetic antioxidants such as butylated hydroxyanisole (BHA) and butylated hydroxy toluene (BHT) are restricted because they are suspected to have some toxic effects and are also possible carcinogens (8,9). Hence the development of alternative antioxidants from natural origins has attracted attention.

The stem of the plant *Cynomorium songaricum* Rupr. (Cynomoriaceae), known as "Suoyang" in traditional Korean medicine, is an achlorophyllous holoparasite plant species (10). Its chemical constituents include steroids, triterpenes, fructosides, flavonoids, lignans and tannins (11-13). The *C. songaricum* is generally used to regulate endocrinopathy, improve sexual function, as a remedy for kidney and intestinal ailments, as a laxative, and to treat lumbar weakness (14). This herb has other potential activities, including anti-hypoxic and anti-anoxic actions (15). Previous studies reported that the plant had inhibitory activity against HIV protease (16), and it improved physical endurance and antioxidant status (17,18).

In this study, we evaluated the total phenolic and flavonoid content of three extracts of *C. songaricum* by different solvent systems. Plus, we investigated the DPPH free radical scavenging activity, nitrite scavenging activity and superoxide dismutase-like activity, as well as xanthine oxidase inhibitory and tyrosinase inhibitory activities of the *C. songaricum* extracts.

MATERIALS AND METHODS

Preparation of extract

The dried *C. songaricum* was purchased from the Herbal Resource Market of Korea. The *C. songaricum*

†Corresponding author. E-mail: yslee@dhu.ac.kr
Phone: +82-53-819-1436, Fax: +82-53-819-1440

was cut into small pieces and extracted with 10 volumes of water at 80°C and 80% ethanol at 60°C for three hours. The sample extraction was replicated three times. The water and ethanol extracts were filtered through Whatman No. 2 filter paper, concentrated using a rotary vacuum evaporator (Eyela 400 series, Tokyo, Japan) under reduced pressure, and freeze-dried (FD 5510 SPT, Ilshin, Yangju, Korea). The dried powders were used for preparing solutions of various concentrations that were then used to analyze the antioxidant activities. As a control, ascorbic acid was prepared at the same concentrations of *C. songaricum* extract, and its antioxidant activities were also determined by the same experiments described below. The water and ethanol extracts of *C. songaricum* were named WE, and EE, respectively.

Total phenolic compound contents

Freeze-dried samples of water and ethanol extracts from *C. songaricum* were dissolved in deionized water (10 mg/mL), and used to determine the content of total polyphenol compound using the Folin-Denis method (19). Briefly, the extracts (0.2 mL) were mixed with 1.8 mL of distilled water and 0.2 mL of Folin-ciocalteu's phenol reagent (Junsei Chemical Co, Tokyo, Japan) and allowed to react for 3 min, after which 0.4 mL of saturated Na_2Co_3 solution was added. The mixture was kept at room temperature for 1 hr and then its absorbance was measured at 725 nm (Shimadzu U-1201, Kyoto, Japan). Tannic acid (Sigma-Aldrich Co, St. Louis, MO, USA) was used as a standard for preparing of the calibration curve ranging 1～300 μg/mL assay solution.

Total flavonoid compound content

The total flavonoid compound contents were measured by the method of Nieva Moreno et al. (20) with slight modification. The water and ethanol extracts of *C. songaricum* were dissolved in 80% ethanol (10 mg/mL). An aliquot of 0.5 mL was mixed with 10% aluminum nitrate (0.1 mL), 1 M potassium acetate 0.1 mL and added 80% ethanol (4.3 mL). The mixture was kept at 25°C for 40 min, and then its absorbance was measured at 415 nm. Total flavonoid concentration was calculated using quercetin as a standard (Sigma-Aldrich Co.).

DPPH free radical scavenging activity

Free radical scavenging activity was determined by using a stable free radical, 1,1-diphenyl-2-picryl-hydrazil (DPPH), according to a slightly modified method of Blois (21). DPPH solution was prepared at the concentration of 0.2 mM in ethanol. Two mL of the sample was vortex-mixed with 1 mL of DPPH solution, and incubated at a room temperature for 30 min. The absorbance was measured at 517 nm against a blank and the activity was expressed as DPPH scavenging activity (%) = $\{1-(A_2-A_1)/A_0\}\times100$, where A_0 is the DPPH without sample (control), A_1 is the sample without DPPH (Blank), and A_2 is the sample with DPPH. The scavenging activity of DPPH radical was expressed as EC_{50}.

Nitrite scavenging activity

Nitrite scavenging activity was measured by the colorimetry method of Kato et al. (22), using Griess reagent. The WE and EE extracts of *C. songaricum* (1 mL) were added to 2 mL of 1 mM $NaNO_2$ and the pH of the solution (7 mL) was adjusted to 1.2, 3.0 and 6.0 with 0.1 N HCl and then was incubated at 37°C for 1 hr. The incubated solution (1 mL) was added to 5 mL of 2% acetic acid and 0.4 mL of Griess reagent (1% sulfanilic acid and 1% naphthylamine in a methanol solution containing 30% acetic acid) and then the mixture was kept at room temperature for 15 min. The color intensity was measured by a spectrophotometer at 520 nm, and activity was expressed as nitrite scavenging activity (%) = $\{1-(A_2-A_1)/A_0\}\times100$, where A_0 was the absorbance of the control, A_1 was the absorbance of the sample without Griess reagent, and A_2 was the absorbance of the sample with Griess reagent. The nitrite scavenging activity was expressed as EC_{50}.

Superoxide dismutase (SOD)-like activity

SOD-like activity was determined by measuring the inhibitory activity of pyrogallol autoxidation, which is similar to superoxide dismutase, according to the modified method of Marklund and Marklund (23). Then, 0.5 mL of each sample, 3 mL of Tris-HCl buffer (pH 8.5), and 0.2 mL of 7.2 mM pyrogallol were mixed and kept at 25°C for 10 min. The reaction was stopped with 0.1 mL of 1 N HCl, and the absorbance was measured at 420 nm. SOD-like activity was expressed as follows: SOD-like activity (%) = $\{1-(A_2-A_1)/A_0\}\times100$, where A_0 was the absorbance of the control, A_1 was the absorbance of the sample without reagent, and A_2 was the absorbance of the sample with reagent. The SOD-like activity of pyrogallol was expressed as EC_{50}.

Xanthine oxidase inhibition

Xanthine oxidase inhibitory activity was measured by the method of Stirpe and Corte (24) with some modification. The assay mixture consisted of 0.1 mL of each extract and 0.6 mL of 2 mM xanthine solution dissolved into 0.6 mL of 0.1 M potassium phosphate buffer (pH 7.5), and 0.1 mL of enzyme solution (0.2 units/mL xanthine oxidase (XO) in phosphate buffer, pH 7.5). After incubation at 37°C for 5 min, the reaction was stopped by the addition of 1 mL of 1 N HCl, and the absorbance was measured at 292 nm using a spectrophotometer. The

blank sample contained the assay mixture without XO. The xanthine oxidase inhibitory activity of pyrogallol was expressed as EC_{50}.

Tyrosinase inhibition

Tyrosinase inhibitory activity was determined according to the method of Yagi et al. (25) using L-dihydroxy phenylalanine (L-DOPA) as a substrate. The reaction mixture consisted of 175 mM sodium phosphate buffer (pH 6.8, 0.6 mL), 0.5 mM L-dopa solution (0.2 mL), water and ethanol extract solution (0.1 mL), and mushroom tyrosinase (110 units/mL, 0.2 mL). The reaction mixture was incubated at 25°C for 2 min, and the absorbance was measured at 475 nm using a spectrophotometer. The absorbance of the same mixture without tyrosinase was used as the control.

Statistical analysis

Data are expressed as mean±SD of at least three separate experiments. Statistical analyses were performed by one-way analysis of variance (ANOVA) followed by Duncan's multiple range tests using version 17.0 for windows (Statistical Package for the Social Science, SPSS INC, Chicago, IL, USA). A value of $p<0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

Extract yields, total phenolic and flavonoid contents

As phenolic compounds have been shown to possess strong antioxidant activity, and flavonoids are one of the most diverse and widespread group of natural phenolic compounds, flavonoids are likely to be the most important natural phenolics (26). We decided to measure the phenolic and flavonoid contents of these extracts, as they likely contribute to the antioxidant activity of the *C. songaricum*.

The extraction yields (%), total phenolic and flavonoid compound content of the *C. songaricum* are shown in Table 1. The EE had a higher extraction yield, a higher total phenolic and flavonoid compound contents than those of the WE. The extract yield of WE and EE were 3.04% and 7.54%, respectively, and the total phenolic compound contents were 35.58 mg/g and 95.65 mg/g, respectively. Notably, the flavonoid content of EE (57.22 mg/g) was about 5 times greater than that of WE (11.58 mg/g). Bang et al. (27) reported that the ethanol extracts of soluble solid contents of mulberry were consistently higher than the level of the water extracts. High phenolic content is thus an important factor in determining the antioxidant activity (26,28). This suggests that the EE of *C. songaricum*, which contained a higher level of phenolic and flavonoid compounds than the WE, might have high antioxidant properties.

Table 1. The extract yields, total phenolic and flavonoid contents of water and ethanol extracts from *C. songaricum*

	Water	Ethanol
Yield (%)	3.04	7.54
Total polyphenol (mg/g)	36.58±1.97[1)]	95.65±0.50
Total flavonoid (mg/g)	11.56±0.58	57.22±0.21

[1)]Values represent the mean±SD.

DPPH free radical scavenging activity

The free radical scavenging activities of *C. songaricum* extracts were evaluated using the DPPH assay. DPPH possesses a proton free radical having characteristic absorption, which decreases upon exposure to a radical scavenger (29). The electron-donating ability of antioxidants allows them to scavenge the DPPH radicals. The DPPH free radical scavenging activity of WE and EE from *C. songaricum* is shown in Table 2. The activities of WE, EE and ascorbic acid increased with increasing of extract concentrations. The free radical scavenging activities of WE were higher than the activities of EE below the concentrations of 300 μg/mL; however, no significant difference was shown between the WE and EE at concentrations of 500～1,000 μg/mL. The EC_{50} value of WE, EE and ascorbic acid were 49.46 μg/mL, 53.72 μg/mL and 34.97 μg/mL, respectively. The EC_{50} of the WE was significantly higher that of the EE, which indicates that the free radical scavenging activity of the WE is higher than EE. Previous studies report that free radical scavenging activity is an index for the antioxidant effectiveness of phenolic compounds, and that this activity increases with the increment of the extract concentration (25,28). In these results, WE had lower phenolic and flavonoid compound contents than EE, but the DPPH free radical scavenging activity of WE was higher than EE; in other words, there is a negative correlations between total phenolic content and DPPH free radical

Table 2. DPPH radical scavenging ability of water and ethanol extracts from *C. songaricum*

Concentration (μg/mL)	Extract		Control
	Water	Ethanol	Ascorbic acid
30	35.85±0.76[b1)]	32.24±1.65[c]	42.29±1.78[a]
50	57.66±1.15[b]	54.70±0.99[c]	88.80±0.63[a]
100	76.48±0.58[b]	69.11±0.95[c]	91.88±0.54[a]
300	82.05±0.46[b]	77.88±1.64[c]	94.58±0.48[a]
500	86.30±0.55[b]	87.34±0.89[b]	96.56±0.31[a]
1,000	87.40±0.67[b]	89.72±1.36[b]	96.88±0.31[a]
EC_{50}	49.46±0.37[b]	53.72±0.46[c]	34.97±0.94[a]

[1)]Values represent the mean±SD and mean with different superscripts within the row are significantly different at $p<0.05$ by Duncan's multiple range test. EC_{50} values were determined by linear regression analysis.

Table 3. Nitrite scavenging ability of water and ethanol extracts from *C. songaricum*

Concentration (μg/mL)		Extract		Control
		Water	Ethanol	Ascorbic acid
pH 1.2	50	3.26±1.45[b1)]	2.97±1.59[b]	14.23±0.36[a]
	100	6.27±0.91[b]	5.09±0.55[b]	27.33±0.10[a]
	300	24.18±0.33[b]	19.03±1.57[c]	70.21±0.58[a]
	500	40.75±0.95[b]	27.15±0.21[c]	76.40±0.37[a]
	1,000	70.95±0.29[b]	46.61±0.21[c]	84.32±1.26[a]
	2,000	90.02±0.19[a]	73.03±0.52[c]	92.07±0.65[a]
	EC_{50}	653.15±11.03[b]	1,128.31±7.01[c]	152.87±0.06[a]
pH 3.0	50	4.48±0.34[b]	3.58±0.68[b]	9.29±0.40[a]
	100	9.29±0.79[b]	4.96±0.66[c]	12.49±1.29[a]
	300	23.65±0.68[b]	16.57±1.47[c]	55.22±0.67[a]
	500	34.96±0.23[b]	26.11±0.11[c]	64.12±1.19[a]
	1,000	58.67±0.89[b]	39.23±0.29[c]	80.79±0.29[a]
	2,000	84.34±0.30[b]	60.39±0.61[c]	88.68±0.29[a]
	EC_{50}	817.17±1.73[b]	1,508.98±9.73[c]	183.62±0.11[a]
pH 6.0	50	1.97±0.70	ND[2)]	ND
	100	3.50±1.53[a]	0.78±1.11[b]	ND
	300	6.60±1.50[a]	3.72±1.58[b]	4.06±0.47[b]
	500	9.31±1.50[a]	4.94±0.96[b]	22.97±0.47[a]
	1,000	9.48±1.22[b]	6.49±1.85[c]	53.25±1.03[a]
	2,000	11.22±1.27[b]	7.65±0.50[c]	71.47±0.60[a]

[1)]Values represent the mean±SD and mean with different superscripts within the row are significantly different at $p<0.05$ by Duncan's multiple range test. EC_{50} values were determined by linear regression analysis.
[2)]ND: not detected.

scavenging activity. This might be due to the WE having a high content of antioxidative materials capable of repressing the reactivity of free radicals.

Nitrite scavenging activity

Nitrite scavenging activity was measured by the capacity of samples to remove nitrosamine, which is known to increase the risk of cancer by converting to diazoalkane, proteins and intracellular components (30). In general, nitrosamines are produced from nitrites and secondary amines, which often occur in proteins under acidic conditions, such as in the stomach. Nitrite scavenging activities of the prepared WE and EE of *C. songaricum* were assessed at pH 1.2, 3.0 and 6.0 (Table 3). Ascorbic acid had the highest scavenging ability of all samples under the same conditions. The WE had a higher nitrite scavenging activity than that of EE at all concentrations and pHs. The nitrite scavenging activity of WE was 90.02% at 2,000 μg/mL at pH 1.2, this value was not significantly different than ascorbic acid (92.07%). The EC_{50} values of WE and EE at pH 1.2 were 653.15 μg/mL and 1,128.31 μg/mL, and at pH 3.0, 817.17 μg/mL and 1,508.98 μg/mL, respectively. The nitrite scavenging activity of the sample was increased as the pH decreased in a dose-dependent manner.

Superoxide dismutase (SOD)-like activity

Superoxide dismutase (SODs) are known to catalyze the conversion of $O2^-$ to H_2O_2 plus O_2, and to provide a defense system under oxidation conditions, in which O_2^- appears to play an important role (31). The enzyme is preventing to be a useful probe for studying the participation of radicals in reactions involving oxygen, such as autoxidations (23). The SOD-like activities of the WE, EE and ascorbic acid increased with increasing sample concentration (Table 4). In this study, ascorbic acid showed a high SOD-like activity greater than 96% at and above concentrations of 100 μg/mL. The EE showed the highest activity of 81.47% at 2,000 μg/mL and EC_{50} was 951.70 μg/mL. The activity of WE was 55.56% at 2,000 μg/mL and EC_{50} was 1,787.03 μg/mL. The EC_{50} of ascorbic acid was 41.83 μg/mL, which did not change over the concentration ranging from 100 to

Table 4. SOD-like activity of water and ethanol extracts from *C. songaricum*

Concentration (μg/mL)	Extract		Control
	Water	Ethanol	Ascorbic acid
50	3.24±1.41[c1)]	6.00±0.95[b]	77.48±1.24[a]
100	5.39±0.49[c]	8.18±1.29[b]	96.95±0.38[a]
300	12.51±1.14[bc]	14.08±1.59[b]	99.17±0.14[a]
500	14.78±0.49[c]	30.64±1.09[b]	99.01±0.00[a]
1,000	30.53±1.63[c]	52.07±1.00[b]	99.17±0.14[a]
2,000	55.56±1.14[c]	81.47±1.26[b]	99.50±0.14[a]
EC_{50}	1,787.03±14.35[c]	951.70±2.45[b]	41.83±1.00[a]

[1)]Values represent the mean±SD and mean with different superscripts within the row are significantly different at $p<0.05$ by Duncan's multiple range test. EC_{50} values were determined by linear regression analysis.

Table 5. Xanthine oxidase inhibition activity of water and ethanol extracts from *C. songaricum*

Concentration (μg/mL)	Extract		Control
	Water	Ethanol	Ascorbic acid
50	ND[1)]	39.34±0.36[a]	20.67±1.15[b]
100	8.16±1.77[c2)]	46.58±0.00[a]	25.33±1.15[b]
300	31.97±0.59[c]	71.01±0.36[a]	52.00±0.00[b]
500	47.96±2.04[c]	81.99±1.24[a]	66.67±1.15[b]
1,000	64.63±0.59[c]	85.51±0.36[b]	88.67±1.15[a]
2,000	84.69±1.02[b]	91.93±0.00[b]	96.67±1.15[a]
EC_{50}	561.19±26.99[c]	112.47±0.11[a]	192.50±0.32[b]

[1)]ND: not detected.
[2)]Values represent the mean±SD and mean with different superscripts within the row are significantly different at $p<0.05$ by Duncan's multiple range test. EC_{50} values were determined by linear regression analysis.

2,000 μg/mL. The EE had significantly higher SOD-like activity compared to the WE at all concentrations. The remarkable effectiveness of EE in comparison to WE might be due to the higher content of antioxidative materials capable of repressing the reactivity of superoxides in the EE.

Xanthine oxidase inhibition

Xanthine oxidase (XO) is the enzyme that catalyzes hypoxanthine and xanthine into uric acid, and is responsible for the medical condition known as gout, which is caused by the deposition of uric acid in the joints, leading to painful inflammation and kidney stones (32). The effects of EE and WE on the inhibition of XO were assessed at six different concentrations, as shown in Table 5. The XO inhibitory activity of the EE extracted from *C. songaricum* was significantly higher than that of the WE at all concentrations, and a positive correlation was found between XO inhibitory activity and the sample concentration. Notably, the activity of EE was higher than ascorbic acid at a concentration of 500 μg/mL, and the EC_{50} of EE displayed the highest XO inhibition activity (112.47 μg/mL, compared with ascorbic acid with 192.50 μg/mL); whereas the ascorbic acid showed a higher activity at 1,000 μg/mL and 2,000 μg/mL concentration than EE.

The treatments of gout are to either increase the excretion of uric acid, or reduce uric acid production. XO also serves as a major component in oxidative damage in living tissue and is involved in many pathological processes (33); therefore, XO inhibition would be beneficial not only to treat gout, but also to combat other diseases (34). These results suggest that *C. songaricum* may potentially be useful for treating gout, inflammation, and other XO induced diseases.

Tyrosinase inhibition

Tyrosinase is known to be a key enzyme for melanin biosynthesis through the hydroxylation of tyrosine and the oxidation of L-DOPA. The inhibition of tyrosinase by natural products has been widely studied with regard to the prevention of skin melanogenesis and in the development whitening cosmetic agents (35). The inhibitory activities of WE, EE and ascorbic acid are summarized in Table 6. The EE showed the highest tyrosinase inhibition activity (19.10%) at 2,000 μg/mL compared to the WE (16.28%), but the inhibitory activities of WE were higher than those of the EE at the concentration of 1,000 μg/mL. However, compared to ascorbic acid's tyrosinase inhibition activity, the WE and EE showed moderate to poor results. The activity of tyrosinase inhibition might depend on the hydroxyl groups of the phenolic compounds, which may form hydrogen bonds with an enzyme site, leading to lower enzymatic activity. Some tyrosinase inhibitors act through hydroxyl groups that bind to the tyrosinase activity site, resulting in steric hindrance of altered conformation (36).

Table 6. Tyrosinase inhibition activity of water and ethanol extracts from *C. songaricum*

Concentration (μg/mL)	Extract		Control
	Water	Ethanol	Ascorbic acid
50	2.79±0.93[b1)]	−	31.23±1.01[a]
100	3.41±0.71[b]	1.98±1.13[bc]	62.14±0.97[a]
300	6.36±1.17[b]	3.96±0.31[c]	96.60±0.49[a]
500	10.08±0.97[b]	5.77±0.62[c]	98.06±0.49[a]
1,000	12.87±0.71[b]	9.73±0.54[c]	98.71±0.56[a]
2,000	16.28±0.93[c]	19.10±0.31[b]	99.78±0.19[a]

[1)]Values represent the mean±SD and mean with different superscripts within the row are significantly different at $p<0.05$ by Duncan's multiple range test.

CONCLUSION

The results of this study provide a basic understanding of some of the functional properties and antioxidative activities of the *C. songaricum*, which could be considered an ingredient of traditional Korean medicine. Our results verified that water extract of *C. songaricum* has very strong antioxidant activities, especially the EC_{50} of XO inhibition activity, which was higher than that of ascorbic acid, suggesting WE of *C. songaricum* can be used as an effective antioxidant source for nutraceuticals and medicine.

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