

Original Article

In vitro and vivo antioxidant activities of daylily flowers and the involvement of phenolic compounds

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Daylily (*Hermercallis fulva* Linn.) flowers were hot air-dried and freeze-dried after harvest. Antioxidant properties of water and ethanol extracts prepared from these dried flowers were evaluated in terms of total antioxidant activity, reducing capacity, and metal chelating activity. Extracts from daylily flowers exhibited strong antioxidant activity. Ethanol was more efficiency to extract antioxidants than water, and freeze-drying preserved higher activities than air-drying. Rutin, (+)-catechin, and gallic acid were identified in the extracts by HPLC, and were highly related to the antioxidant activities. The antioxidant activity was further evaluated by feeding mice with ethanol extract from freeze-dried daylily flowers for 60 days. The results demonstrated that the extract at dosage of 40-225 mg/100 g significantly increased the activity of SOD (superoxide dismutase) and reduced the lipid peroxidation in both blood and liver of rat.

Key Words: daylily flower, antioxidant activity, phenolic compounds, superoxide dismutase, lipid peroxidation

Introduction

Daylily flowers (*Hermercallis fulva* Linn.) have been used as a vegetable and medicinal herb for thousands of years in eastern Asia. The roots and leaves of daylily were used in the treatment of inflammation and jaundice.¹ Pharmacological studies have shown that daylily flowers can facilitate neurological changes in sleeping mice and impact motor activity in rats as a result of alteration to the normal levels of several central nervous system neurotransmitters.¹ Extracts from daylily flowers have been shown to inhibit cancer cell proliferation and induce cells to undergo differentiation.²

Generally, it is supposed that an oxidation process is involved in the initial development steps of cancer and cardiovascular diseases. Indeed, reactive oxygen species (ROS), naturally formed during normal metabolism, can damage biological structures such as proteins, lipids or DNA. To protect cells against oxidative damage by free radicals, an antioxidant system, including SOD (superoxide dismutase), CAT (catalase), GP (glutathione peroxidase), and GR (glutathione reductase) enzymes has evolved in aerobic organisms.³⁻⁶ These enzymes work in tandem to scavenge ROS. SOD, catalyzing the dismutation of superoxide anion ($O_2^{\cdot-}$) to H_2O_2 , presents in the cytoplasm, on the endothelial cell surface (Cu or ZnSOD), and in the mitochondria (MnSOD).⁷ Under conditions of elevated ROS production or when the antioxidant system is compromised, cells are unable to efficiently scavenge the free radicals, leading to ROS accumulation. It is accepted that the intake of antioxidant substances reinforces the defence against ROS. Therefore, the role of antioxidant nutrients such as vitamins (A, E and C) and other food components has been raised. Polyphenolic compounds, naturally pre-

sent in vegetable sources, can contribute to the dietetic intake due to their antioxidant nature.⁸ The potential antioxidant effect in vivo of individual vegetable polyphenols or concentrated extracts has been widely investigated in cultured cells⁹⁻¹³, live animals¹⁴⁻¹⁷ and humans¹⁸⁻²². However data of the antioxidant properties of daylily flowers in vitro and vivo are very limited.

In this study, the antioxidant properties of water and ethanol extracts from dried daylily flowers were determined both in vitro and vivo. Individual phenolic compounds were identified and quantified by HPLC to analyze their contributions to the antioxidant activities. The difference in extracts from hot air-drying and freeze-drying on antioxidant activities were also compared.

Materials and methods

Design

In this study, four kinds of daylily flower (*Hermercallis fulva* Linn.) extracts including ethanol extract with freeze-drying (EF), ethanol extract with hot air-drying (EH), water extract with freeze-drying (WF) and water extract with hot air-drying (WH) were prepared and their antioxidant properties were evaluated in terms of total antioxidant activity, reducing capacity, metal chelating activity. Afterwards the phenolic compounds in the extracts were identified by HPLC.

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Each rat was given EF daily by gavage for 60 consecutive days. Superoxide dismutase (SOD) and malondialdehyde (MDA) levels in serum and liver of rat were analysed.

Subjects

SPF Kunming strain mice (50 male and 50 female) weighing 20 ± 2 g were kept separately in an animal room and fed with commercial mice diet for 6 days. After acclimatization, mice were randomly divided into five groups with 20 mice each (10 male and 10 females separately). EF (40, 70, 150, 225 mg/100 g) was given daily by gavage to the animals for 60 consecutive days. One group was chosen as control group and was sham treated. On day 61, the blood and livers were collected.

Preparation of plant material

The flowers of daylily (*Hemerocallis fulva* Linn.) grown at the pilot farm in Zhejiang University were harvested at their commercial maturity based on color and shape. The day of harvest was about 27-35 d after tassel varying with cultivars. All flowers were transported to the lab within 30 min after harvest. Flowers were hot air-dried at 55°C for 24 h or freeze-dried in a lyophilizer (Savant VLP-200, USA) for 12 h. The dried flowers were ground with a blender and sieved through a 100-mesh screen to obtain daylily flower powder which was sealed in polyester bottles and stored at -20°C .

Preparation of water and ethanol extracts

A mixture was prepared by macerating 10 g of powder with 100 mL of distilled water or ethanol, kept in a rotary shaker overnight and then filtered through Whatman No.1. Extracts were obtained after the filtrates were freeze-dried. Thus four extracts (EF, EH, WF, WH) were obtained as water and ethanol extracts from hot air-dried and freeze-dried daylily flowers, respectively.

Antioxidant properties of daylily flowers in vitro

Total antioxidant activity:

The antioxidant activity was determined according to the thiocyanate method with some modifications.²³ Different amounts of extracts (20, 50, and 100 μg) were added in 2.5 mL of 0.04 mol/L potassium phosphate buffer (pH 7.0) and 2.5 mL of linoleic acid emulsion. Linoleic acid emulsion (50 mL) was prepared with 350 mg of Tween-20 and 310 μL of linoleic acid and 0.04 mol/L potassium phosphate buffer (pH 7.0). The mixed solution (5 mL) was incubated at 37°C in dark. The peroxide level was determined by reading the absorbance at 500 nm in a spectrophotometer (UV-120-02, Shimadzu, Japan) after reaction with FeCl_2 and thiocyanate every 12 h. The solutions without extracts were used as control samples. The inhibition of lipid peroxidation in percent was calculated as: Inhibition (%) = $(1 - A_1 / A_0) \times 100\%$. A_0 was the absorbance of the control reaction and A_1 was the absorbance in the presence of extracts. Butylated hydroxyanisole (BHA) and α -tocopherol were used as positive standards.

Superoxide anion scavenging activity:

The superoxide anion scavenging activity was measured using the xanthine/xanthine oxidase method.²⁴⁻²⁵ One

milliliter of water containing 200, 400, 800 μg of extracts were separately added to a 1.0 mL mixture of 0.4 mmol/L xanthine and 0.24 mmol/L nitro blue tetrazolium chloride (NBT) in 0.1 mol/L phosphate buffer (pH 8.0). A 1.0 mL solution of xanthine oxidase (0.049 units/mL), diluted in 0.1 mol/L phosphate buffer (pH 8.0), was added and the resulting mixture incubated in a water bath at 37°C for 40 min. The reaction was terminated by adding 2.0 mL of an aqueous solution of 69 mmol/L sodium dodecylsulphate (SDS) and the absorbance of NBT was measured at 560 nm. The scavenging of superoxide anion in percent was calculated by the equation: scavenging activity (%) = $(1 - A_1 / A_0) \times 100\%$. A_0 was the absorbance of the control (without extract) reaction and A_1 was the absorbance in the presence of extracts. Vitamin C was used as the positive standard.

Chelating activity on Fe^{2+} ions:

The chelating activity on Fe^{2+} was measured using the method of Decker and Welch.²⁶ One milliliter solution containing extracts (200, 400, 800 μg) was mixed with 3.7 mL of distilled water and then the mixture was reacted with 0.1 mL of 2 mmol/L FeCl_2 and 0.2 mL of 5 mmol/L 3-(2-pyridyl)-5,6-bis (4-phenyl-sulfonic acid)-1, 2, 4-triazine (ferrozine) for 20 min. The absorbance was read at 562 nm in a spectrophotometer. One milliliter of distilled water, instead of extraction of daylily flower, was used as control. Lower absorbance of the reaction mixture indicated higher chelating activity. The percentage of chelating activity on inhibition of ferrozine- Fe^{2+} complex formation was calculated by the formula: chelating activity (%) = $(1 - A_1 / A_0) \times 100\%$. A_0 was the absorbance of the control reaction and A_1 was the absorbance in the presence of extracts. Ethylenediaminetetraacetic acid (EDTA) and citric acid were used as positive standards.

Analysis of phenolic compounds by HPLC:

The extract (1.0 g) was dissolved in 1 L of ethanol or water into a bottle. HPLC analysis of phenolic compounds was performed on a Waters 2695 system controller connected to a photodiode array detector Waters 2996 which was programmed to take data from 240 to 450 nm with a 2.4 nm resolution. The column was Inertsil ODS-3 (250-4.6 mm) protected by a guard column, Easyguard C18. Sample (50 μL) filtrated through 0.22 μm low-extractable polyvinylidene fluoride membrane was injected. The chromatographic conditions were modified based on Roggero, Coen and Archier.²⁷ A gradient of three solvents of A (acetic acid-water, 1:99), B (acetic acid-water, 6:94) and C (acetic acid-water-acetonitrile, 5:65:30) was programmed as: 0 min (100% A), 20 min (100% B), 55 min (90% B + 10% C), 65 min (80%B + 20%C), 90 min (30%B + 70%C), 100 min (100% C), and 120 min (100 % C) with a flow rate of 0.7 mL/min at 22.5°C . Phenolic compounds were identified by comparing retention time and UV (ultraviolet rays) spectral matching with authentic standards, and quantitative analysis was performed in triplicate using external calibration curves.

Antioxidant properties of daylily flowers in vivo

Animals and treatment:

Fifty male and fifty female SPF Kunming strain mice

weighing 20±2 g were obtained from Experimental Center of Medical Scientific Academy of Zhejiang Province. The male and female mice were kept separately in an animal room controlled at temperature of 22±2°C, humidity of 55±15%, ventilation, more than 10 air changes per hour (all-fresh-air system), 12-h light/12-h dark cycle (lights on: 07:00–19:00 h), and free access to food and water throughout the study. This experiment was undertaken in the Animal Experiment Center of Zhejiang University.

Since ethanol extract from freeze-dried daylily flowers (EF) shows the highest antioxidant activity and has the highest phenolic content than other three extracts, it was selected for feeding the mice to evaluate the antioxidant properties of daylily flowers in vivo. After acclimatization (6 days), mice were randomly divided into five groups with 20 mice each (10 male and 10 females separately). EF (40, 70, 150, 225 mg/100 g) was given daily by gavage using a stainless steel gavage tube to the animals for 60 consecutive days. One group was chosen as control group and was sham treated with an equivalent volume of distilled water by gavages daily.

On day 61, mice blood was drawn from the eyeball before killing and centrifuged at 1000 g for 10 min within 1 h after collection. The serum was frozen at -80 °C before analyzed. Livers were removed quickly into ice-cold saline as soon as the mice were killed by decapitation. The livers were homogenized with saline to be 10% (w/v) homogenates which were centrifuged at 3,000 g for 10 min and the supernatants were centrifuged at 1000 g at 4°C for 15 min. The resultant supernatants were used as the cytosol and stored at -30°C for the further assays.

Biochemical analyses:

Superoxide dismutase (SOD) levels in serum and liver were determined by using spectrophotometric diagnostic kits (Nanjing Jiancheng Biotechnology Institute, China) based on the methods of Beyer and Fridovich and Nebot et al.^{28, 29} Absorbances were determined by using a spectrophotometer at 525 nm. The SOD activity was then determined from the ratio of auto-oxidation rates measured in the presence (sample) and in the absence (blank) of the sample. The enzyme activity was expressed as SOD units/mL (serum) and units/mg protein (liver). The protein concentration was measured by the Lowry method.

The extents of lipid peroxidation in serum and liver were monitored by measuring malondialdehyde (MDA) levels according to Bagchi and Ray and Fariss.^{30, 31} Briefly, the samples were reacted with thiobarbituric acid (TBA) to determine TBARS and the absorbance were extrapolated from a standard curve generated by using pure MDA. Absorbances were read at 532 nm by the same spectrophotometer. The results were expressed as MDA formation per mL in serum and per mg protein in liver.

Statistical analysis:

Experimental results were means ± SD of three parallel measurements. Analysis of variance was performed by ANOVA procedures (SAS 8.0 for Windows). Significant differences between means were determined by Duncan's Multiple Range tests. *p* values were two tailed and *p* <0.05 were regarded as significant and *p* values <0.01 very significant. Bivariate correlation was employed to

Table 1. Correlation matrix between the chromatographic results and the antioxidant activities in the extracts from daylily flowers.

Phenolics	Total antioxidant activity †	Superoxide anion radical scavenging activity ‡	Chelating activity on Fe ²⁺ ions ‡
Gallic acid	0.7849*	0.7823*	0.6491
(+)-Catechin	0.8801**	0.8339**	0.7811*
Rutin	0.8778**	0.9158**	0.9717**
Total Phenolics	0.8689**	0.9335**	0.9755**

† Activity determined after 120 h of incubation; ‡Activity at the concentration of 160 µg/mL; Values are mean ± SD. * indicates that *p* <0.05 and ** *p* <0.01

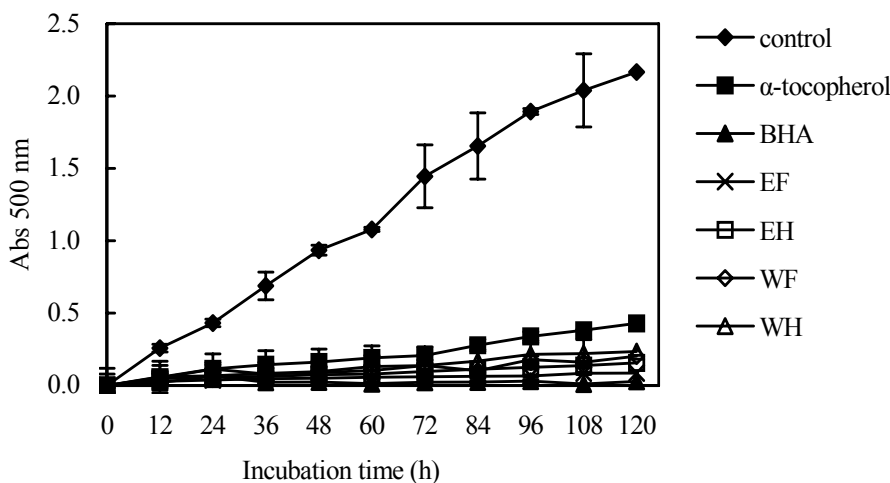


Figure 1. Antioxidant activity in water and ethanol extracts from hot air-dried and freeze-dried daylily flowers (WH, WF, EH, EF), α-tocopherol, and butylated hydroxyanisole (BHA) at the amount of 100 µg in the 2.5 mL of linoleic acid emulsion during 120 h of incubation.

Table 2 Effect of oral administration with EF on MDA and SOD in mice serum and liver

Dose (mg/100g)	Mice number	MDA in serum (nmol/mL)	MDA in liver (nmol/mg protein)	SOD in serum (U/mL)	SOD in liver (U/mg protein)
225	20	10.31 ± 0.68**	8.38 ± 0.63**	44.70 ± 2.7**	286.08 ± 5.1**
150	20	11.98 ± 0.72**	9.64 ± 0.69**	44.63 ± 2.3**	271.81 ± 3.9**
70	20	13.21 ± 0.77**	10.98 ± 0.71**	35.41 ± 2.1**	258.56 ± 4.5**
40	20	15.94 ± 0.86*	12.31 ± 0.82*	31.15 ± 1.8*	223.65 ± 4.2*
0	20	18.67 ± 0.82	14.41 ± 0.73	29.11 ± 1.9	206.76 ± 3.8

Values are mean ±SD. * indicates that $p < 0.05$ and ** $p < 0.01$ compared with control group.

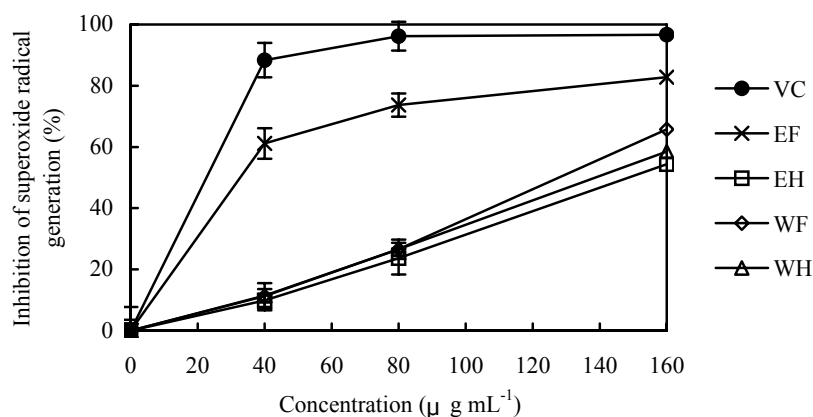


Figure 2. Superoxide anion radical scavenging activity in water and ethanol extracts from hot air-dried and freeze-dried daylily flowers (WH, WF, EH, EF), and vitamin C (VC)

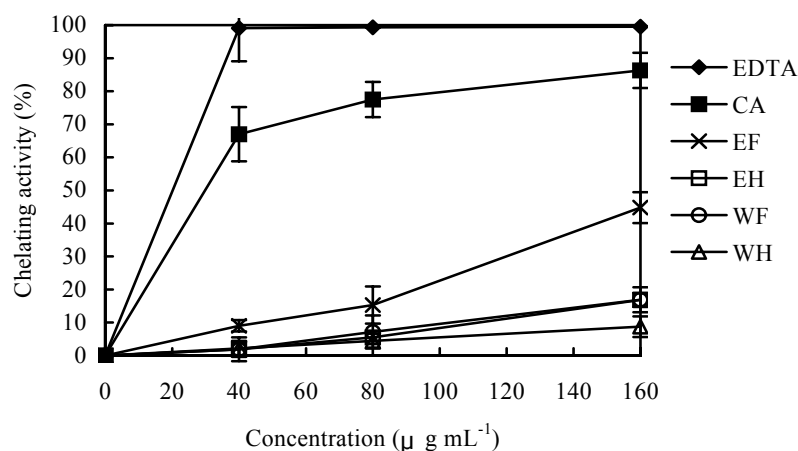


Figure 3. Metal chelating activity of different amounts of water and ethanol extracts from hot air-dried and freeze-dried daylily flowers (WH, WF, EH, EF), ethylenediaminetetraacetic acid (EDTA) and citric acid (CA) on ferrous ions

determine the relationship between the chromatographic results and the antioxidant activities.

Results and discussions

Total antioxidant activity

Both water and ethanol extracts from hot-dried and freeze-dried daylily flowers showed effective total antioxidant activity during the whole incubation time of 120 h (Fig. 1). Ethanol extracts from both hot-dried and freeze-dried flowers exhibited similar strong antioxidant activity. The antioxidant activity in freeze-dried flowers was a little higher than that in hot-dried flowers. All extracts from daylily flowers showed higher antioxidant activities than α -tocopherol, but lower than BHA in the whole test. The order of antioxidant activity in dried daylily flowers was ethanol extract with freeze-drying (EF) > ethanol extract with hot air-drying (EH) > water extract with freeze-drying (WF) > water extract with hot air-drying (WH).

Superoxide anion scavenging activity

Superoxide anion is an oxygen-centred radical with selective reactivity. This species is produced by a number of enzyme systems in auto-oxidation reactions and by nonenzymatic electron transfers that univalently reduce molecular oxygen. It can also reduce certain iron complex such as cytochrome.²³ Superoxide anion radical scavenging activities increased with amounts of extract from daylily flowers (Fig. 2). Ethanol extract from freeze-dried daylily flowers (EF) exhibited higher activity than other extracts. At the extract concentration of 160 $\mu\text{g/mL}$, superoxide anion radical scavenging activities, in the decreasing order, were vitamin C, EF, EH, WF and WH.

Chelating activity on Fe^{2+} ions

Metal chelating capacity is claimed as one of the antioxidant mechanisms, since it reduces the concentration of the catalysing transition metal in lipid peroxidation.³² Ferrozine can quantitatively form complexes with Fe^{2+} . In the

presence of extract of daylily flowers, the complex formation is disrupted with the result that the red color of the complex is decreased. Measurement of color reduction therefore allows estimation of the chelating activity of the

extract of daylily flowers. The chelating activity was linearly increased within 40-160 $\mu\text{g/mL}$ extract (Fig. 3). Again, extraction solvent showed significant influence on metal chelating capacity in extracts from daylily flowers.

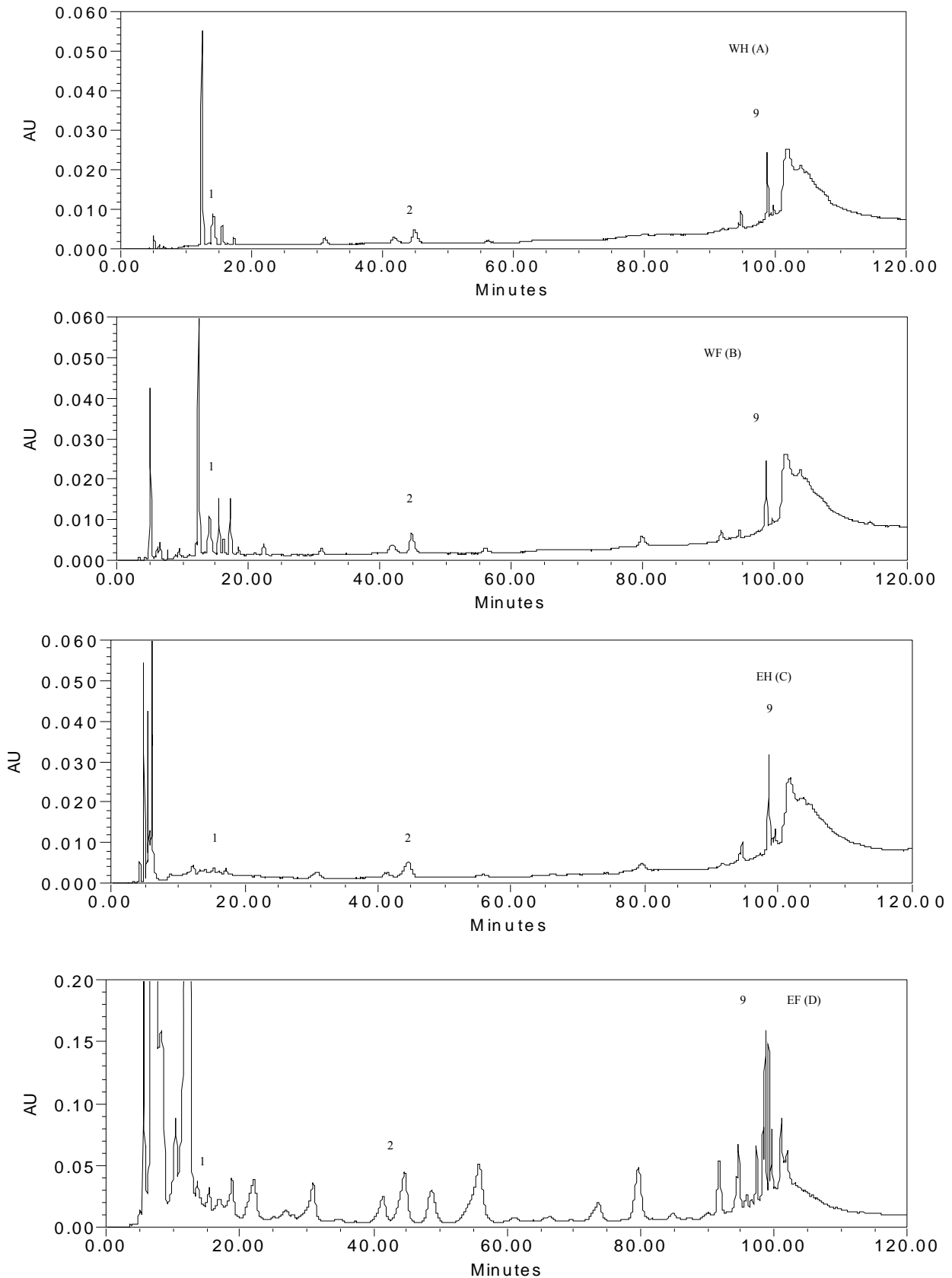


Figure 4. Chromatographic profiles of phenolic compounds in water and ethanol extracts from hot air-dried and freeze-dried daylily flowers (WH, WF, EH, EF), at 280 nm. The peaks correspond to: 1, gallic acid; 2, (+)-catechin; 9, rutin.

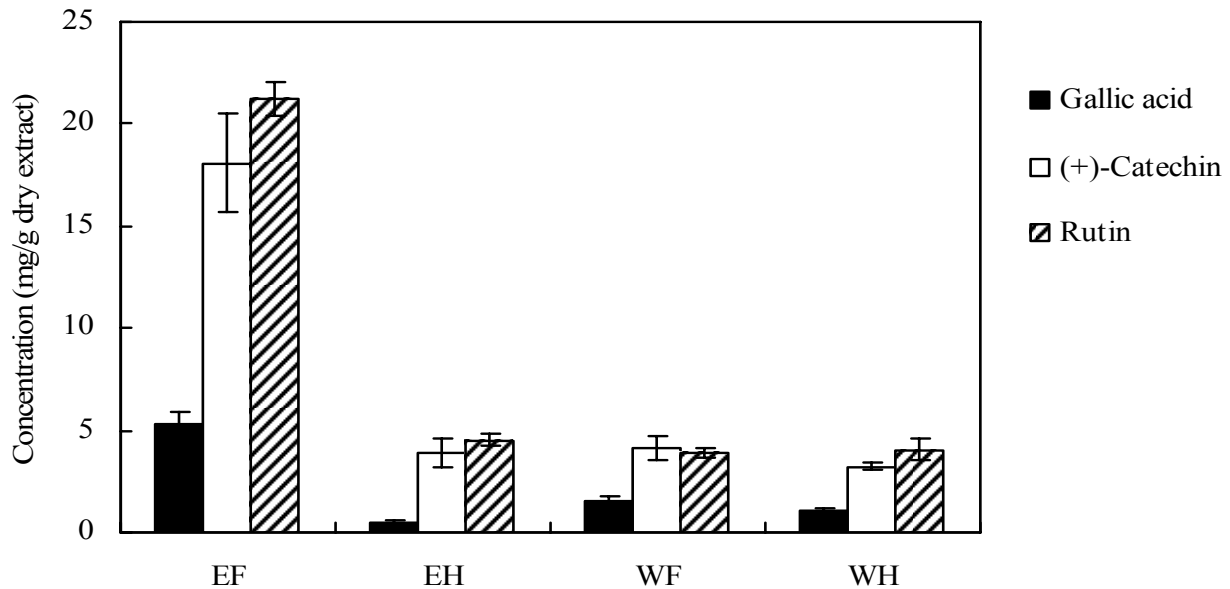


Figure 5. Concentrations (mg/g dry extract) of identified phenolic compounds in water and ethanol extracts from hot air-dried and freeze-dried daylily flowers (WH, WF, EH, EF) by HPLC.

EF showed higher metal chelating capacity than other extracts with a significant difference ($p < 0.01$).

However, extracts of daylily flowers exhibited much lower metal chelating capacity than such standards as ethylenediaminetetraacetic acid (EDTA) and citric acid. The decreasing order of the metal scavenging effect was EDTA > citric acid > EF > WF > EH > WH.

Phenolic compounds in extracts from daylily flowers

HPLC identification of phenolic compounds was done by comparing the retention time and UV spectral matching with authentic standards. Three phenolic compounds were successfully appeared at 15.28, 45.19 and 98.85 min after the injection of the sample, and were proposed to be gallic acid, (+)-catechin and rutin, respectively (Fig. 4 and Fig. 5). EF preserved more phenolic compounds than other extracts.

Correlation between individual phenolic compounds and antioxidant properties was shown in Table 1. There was a high correlation between the contents of gallic acid, and (+)-catechin, rutin and the antioxidant activities in daylily flower extracts. This result confirmed that phenolic compounds play an important role in the antioxidant activities of daylily flowers.

SOD and MDA content in serum and liver of mice

Superoxide dismutase (SOD) is one of the crucial components in the antioxidant defense system through the involvement in the reduction of reactive oxygen species (ROS) and peroxides produced in the living organism as well as in the detoxification of certain compounds of exogenous origin, thus playing a primary role in the maintenance of a balanced redox status.³³ MDA is the major oxidation product of peroxidized poly-unsaturated fatty acids and increased MDA content is an important indicator of lipid peroxidation.³⁴

The liver is the main detoxifying organ in the body, and as such it possesses a high metabolic rate and it is sub-

jected to many insults potentially causative of oxidative stress. Consequently, a correct status of the hepatic antioxidant defense system is of major importance for the maintenance of health. The liver is also the main organ involved in the metabolism of absorbed phenolics, where they arrive via the portal vein after absorption along the gastrointestinal tract. Therefore, the potential beneficial effects of food phenolics would take place primarily in the liver and also in blood.³³

The present study was undertaken to assess the effect of oral administration with EF on the concentration of MDA and the activity of SOD in the serum and liver of mice (Table 2). MDA concentration was significantly reduced (compared to the control group, $p < 0.05$) with the increasing of EF dose. Whereas, SOD activities increased with the concentration of EF (compared to the control group, $p < 0.05$). The data indicated that oral administration with EF could significantly reduce the lipid peroxidation and enhance the activity of antioxidant enzyme in mice. Similar results were observed by administration of green tea leaves,³⁴ thyme oil and thymol,³⁵ lycopene,³⁶ which were abundant of phenolic compounds. An induction of the antioxidant enzymes has been suggested to reflect an enhancement in cellular protection, ensuring that potential oxidants are metabolized and eliminated more rapidly.³⁸ Improvement of the redox status as determined by the antioxidant capacity and SOD activity in the present study would have added evidence supporting the beneficial health effect of daylily flowers.

Conclusion

Both water and ethanol extracts from hot air-dried and freeze-dried daylily flowers exhibited strong antioxidant activity, in terms of total antioxidant activity, reducing capacity, superoxide anion scavenging activity and chelating activity. EF had the strongest antioxidant activity with the highest content of phenolic compounds. Rutin, (+)-catechin, and gallic acid were identified in daylily flowers

and highly correlated with the antioxidant activities. Animal experiment also indicated that EF significantly reduce the lipid peroxidation and enhance the activity of antioxidant enzyme in blood and liver of mice.

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