In Vitro Antioxidant Study of Aqueous and Ethanol Extracts of Cercidiphyllum japonicum Postprint

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Abstract

Abstract: This studyinvestigated the main components and antioxidant effects of aqueous and ethanol extracts from Cercidiphyllum japonicum leaves to provide a theoretical basis for their further development and utilization. Metabolites were extracted from C. japonicum leaves using aqueous and ethanol extraction methods, and their main components were determined. In vitro antioxidant experiments were conducted to evaluate their antioxidant effects through four indicators: scavenging capacity against hydroxyl radicals (·OH), DPPH radicals (DPPH·), superoxide anion (O ·), and ferric ion (Fe^{3}) reducing capacity. The results showed that both aqueous and ethanol extracts contained kaempferol. Additionally, the aqueous extract contained flavonoids such as tricin and isoquercitrin, while the ethanol extract contained flavonoids such as naringenin and quercetin 3-O- -D-glucoside. Both extracts demonstrated the ability to scavenge hydroxyl radicals, DPPH radicals, superoxide anion, and reduce ferric ions. The antioxidant effects increased with increasing extract concentration. Notably, the superoxide anion scavenging capacity (with IC values of 0.092 mg·mL ^{1} and 0.002 mg·mL ^{1}, respectively) was stronger than that of the positive control vitamin C (IC value of 0.241 mg·mL ^{1}), and the IC values for ferric ion reducing power (0.014 mg·mL ^{1} for the aqueous extract and 0.001 mg·mL ^{1} for the ethanol extract) were relatively low, indicating strong total antioxidant activity. Therefore, both aqueous and ethanol extracts from C. japonicum exhibit good antioxidant effects and may serve as potential natural antioxidants.

Full Text

Preamble

Study on the In Vitro Antioxidant Activity of Aqueous and Ethanol Extracts of *Cercidiphyllum japonicum*

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Abstract

This study investigated the main chemical components and antioxidant activities of aqueous and ethanol extracts from *Cercidiphyllum japonicum* to provide a theoretical basis for their further development and utilization. Secondary metabolites were extracted from *C. japonicum* leaves using both aqueous and ethanol extraction methods, and their primary constituents were determined. The antioxidant effects were evaluated through in vitro assays measuring the capacity to scavenge hydroxyl radicals (· OH), DPPH radicals (DPPH ·), superoxide anions (O ·), and reduce ferric ions (Fe^{3}).

The results demonstrated that both extracts contained kaempferol. Additionally, the aqueous extract contained flavonoids such as tricin and isoquercitrin, while the ethanol extract contained naringenin and quercetin 3-O- -D-glucoside. Both extracts exhibited scavenging activities against hydroxyl radicals, DPPH radicals, and superoxide anions, as well as ferric ion reducing capacity. Antioxidant activity increased with extract concentration in a dose-dependent manner. Notably, the superoxide anion scavenging capacity (IC values of 0.092 mg · mL ^{1} and 0.002 mg · mL ^{1} for aqueous and ethanol extracts, respectively) exceeded that of the positive control vitamin C (IC = 0.241 mg · mL ^{1}). Furthermore, the IC values for ferric ion reducing power were relatively low (0.014 mg · mL ^{1} for aqueous extract; 0.001 mg · mL ^{1} for ethanol extract), indicating strong total antioxidant activity. These findings suggest that both aqueous and ethanol extracts of *C. japonicum* possess excellent antioxidant properties and represent potential natural antioxidant sources.

Keywords: *Cercidiphyllum japonicum*, aqueous extract, ethanol extract, antioxidant activity

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Introduction

Cercidiphyllum japonicum, commonly known as "mountain ginkgo," is an ancient and rare deciduous tree species belonging to the family Cercidiphyllaceae. Listed

as a second-class nationally protected plant in China, it is primarily distributed in Japan and in mixed evergreen and deciduous broad-leaved forests at elevations of 400–2500 meters in western and southeastern Sichuan, southern Shanxi, and western Hubei provinces. The species emits a fragrant aroma detectable from hundreds of meters away and features an imposing stature with symmetrically attractive foliage. Its roots, stems, and leaves have medicinal value, particularly the bark, which when decocted is effective for treating colds and dysentery. Both bark and leaves contain tannins suitable for tannin extraction, giving *C. japonicum* significant ornamental and economic value.

Current research on *C. japonicum* has primarily focused on resource restoration and expansion through tissue culture and rapid propagation methods. However, studies on its secondary metabolites and functional development remain incomplete, particularly regarding antioxidant and anticancer properties. This investigation explores the main components of leaf secondary metabolites and their antioxidant activities.

Natural plant antioxidants represent a class of non-toxic, safe compounds with important biological activities that effectively scavenge excess free radicals produced during oxidative stress, maintaining redox homeostasis. Synthetic antioxidants such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) may pose potential toxicity risks, creating an imperative to identify low-toxicity, safe, and efficient natural alternatives. Numerous plant extracts demonstrate antioxidant activity, though effects vary with extraction method and solvent. This study employs aqueous and ethanol extraction of *C. japonicum* leaves to evaluate antioxidant capacity through hydroxyl radical, DPPH radical, and superoxide anion scavenging assays, along with ferric ion reduction, providing a foundation for further development.

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1. Materials and Instruments

1.1 Sample Collection and Preparation

Cercidiphyllum japonicum leaves were collected in mid-July 2017 from the Yanjinghe Forest Farm in Wangcang County, Guangyuan City, Sichuan Province, at an elevation of 1800 meters. The species was authenticated by Professor MA Dan-wei of the College of Life Sciences, Sichuan Normal University. Leaves were processed into powder in the laboratory and stored for subsequent use.

1.2 Chemicals and Reagents

Ferrous sulfate (FeSO), hydrogen peroxide (H O , 0.3%), Tris(hydroxymethyl)aminomethane (Tris), hydrochloric acid (HCl), pyrogallol (1,2,3-trihydroxybenzene), 1,1-diphenyl-2-picrylhydrazyl (DPPH), potassium ferricyanide (K Fe(CN)), sodium dihydrogen phosphate (NaH PO), disodium hydrogen phosphate (Na HPO), sodium chloride (NaCl), trichloroacetic acid (TCA), ferric chloride

(FeCl), ascorbic acid (Vc), dimethyl sulfoxide (DMSO), anhydrous ethanol, and 95% ethanol.

1.3 Equipment

AR224CN electronic balance (Shanghai Ohaus Instrument Co., Ltd.); XH-300A Xianghao computer-controlled ultrasonic synthesis/extraction apparatus (Beijing Xianghao Technology Development Co., Ltd.); RE-52CS rotary evaporator (Shanghai Yarong Biochemical Equipment Co., Ltd.); CHASHB-3A circulating water vacuum pump (Beijing Heng' ode Technology Co., Ltd.); HH-4 constant temperature water bath (Jiangsu Jincheng Guosheng Experimental Instrument Factory); TGL-16B centrifuge (Shanghai Anting Scientific Instrument Factory); Multifunctional cell analyzer (MD/Spectra Max M2); Laobenhang 400Y grinder; UPLC-MS system (Waters Xevo G2-XS TOF).

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2. Methods

2.1 Extract Preparation

2.1.1 Aqueous Extract Preparation Aqueous extraction was performed following the decoction method. Briefly, 1150 g of *C. japonicum* leaf powder was soaked in 3200 mL distilled water (1:3 ratio) for 8 hours, then simmered for 4 hours. The mixture was filtered to obtain 500 mL of aqueous extract, with a yield of 15.62%.

2.1.2 Ethanol Extract Preparation Ethanol extraction was adapted from Jiao (2014) and Tang et al. (2011) with minor modifications. Fifty grams of leaf powder was mixed with 300 mL of 80% ethanol (1:6 solid-liquid ratio) and sonicated at 302 W for 30 minutes at 60°C. This process was repeated five times, after which the combined extracts were filtered and concentrated using a rotary evaporator to obtain 20 g of extract, yielding 8%.

2.2 Chemical Composition Analysis

Extract composition was analyzed using ultra-high performance liquid chromatography-time-of-flight mass spectrometry (UPLC-TOF/MS). Chromatographic and mass spectrometric conditions followed Sun et al. (2017), with a mass scan range of 0–800 m/z and data acquisition time of 42 minutes.

2.3 Antioxidant Activity Assays

2.3.1 Hydroxyl Radical (· OH) Scavenging Capacity Aqueous extract and Vc positive control solutions were prepared in distilled water at final concentrations of 1.6, 0.8, 0.4, 0.2, and 0.1 mg · mL ^{1} (extract) and 0.016, 0.008, 0.004, 0.002, and 0.001 mg · mL ^{1} (Vc). Ethanol extract solutions were prepared

in DMSO at 0.16, 0.08, 0.04, 0.02, and 0.01 mg · mL ^{1}. Hydroxyl radical scavenging activity was measured using the salicylic acid method following Ren et al. (2017).

2.3.2 DPPH Radical Scavenging Capacity Aqueous extract and Vc solutions were prepared in distilled water at 0.8, 0.4, 0.2, 0.1, and 0.05 mg · mL ^{1} (extract) and 0.016, 0.008, 0.004, 0.002, and 0.001 mg · mL ^{1} (Vc). Ethanol extract solutions were prepared in DMSO at 0.08, 0.04, 0.02, 0.01, and 0.005 mg · mL ^{1}. DPPH radical scavenging activity was assessed using the DPPH method according to Li et al. (2012) and Ren et al. (2017).

2.3.3 Superoxide Anion (O ·) Scavenging Capacity Aqueous extract and Vc solutions were prepared in distilled water at 0.4, 0.2, 0.1, 0.05, and 0.025 mg · mL ^{1} (extract) and 3.2, 1.6, 0.8, 0.4, and 0.2 mg · mL ^{1} (Vc). Ethanol extract solutions were prepared in DMSO at 0.008, 0.004, 0.002, 0.001, and 0.0005 mg · mL ^{1}. Superoxide anion scavenging activity was determined using the pyrogallol method described by Ren et al. (2017).

2.3.4 Ferric Ion (Fe^{3}) Reducing Power Aqueous extract and Vc solutions were prepared in distilled water at 0.8, 0.4, 0.2, 0.1, and 0.05 mg · mL ^{1} (extract) and 0.016, 0.008, 0.004, 0.002, and 0.001 mg · mL ^{1} (Vc). Ethanol extract solutions were prepared in DMSO at 0.08, 0.04, 0.02, 0.01, and 0.005 mg · mL ^{1}. Ferric ion reducing power was measured following Qi et al. (2012), where higher absorbance values indicate stronger reducing capacity.

2.4 Statistical Analysis

Data were processed using SPSS 17.0 software. Significant differences were analyzed using LSD test, correlations were assessed using bivariate analysis, and IC values were calculated using Probit regression.

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3. Results and Analysis

3.1 Chemical Composition of Extracts

UPLC-MS analysis generated total ion chromatograms and UV spectra for both extracts [FIGURE:1, FIGURE:2]. In the aqueous extract chromatogram, compounds with strong UV absorption were analyzed individually. For example, the peak at 4.37 minutes showed a molecular weight of 286.1069. Secondary mass spectrometry revealed fragment ions including 153 [M+H-C H O] , consistent with kaempferol fragments reported by Sun et al. (2017), suggesting the compound was 3,5,7,4' -tetrahydroxyflavone (kaempferol, C H O). Similarly, the aqueous extract was found to contain flavonoids including tricin (Wang et al., 2016) and isoquercitrin (Huang et al., 2009).

In the ethanol extract chromatogram, a compound with molecular weight 286.1032 was detected at 3.754 minutes. Secondary mass spectrometry identified five major fragment ions matching those of kaempferol reported by Sun et al. (2017): 287 [M+H] , 241 [M+H-CO-H O] , 213 [M+H-2CO-H O] , 165 [M+H-C H O] , and 153 [M+H-C H O] , confirming its identity as kaempferol. The ethanol extract additionally contained naringenin and quercetin 3-O- -D-glucoside (Sun et al., 2017).

3.2 Hydroxyl Radical (·OH) Scavenging Activity

The hydroxyl radical scavenging effects are presented in [Figure 3: see original paper], [Figure 4: see original paper], and . The color reaction diagrams show progressively darker solutions with decreasing concentration, indicating dose-dependent scavenging activity. At low concentrations, scavenging rates were only 6.46–6.88%, increasing to over 70% at high concentrations, with a maximum of 89.11%. Statistical analysis revealed significant positive correlations: aqueous extract (P = 0.000, r = 0.994), Vc (P = 0.026, r = 0.921), and ethanol extract (P = 0.002, r = 0.985).

3.3 DPPH Radical Scavenging Activity

DPPH radical scavenging results are shown in [Figure 5: see original paper], [Figure 6: see original paper], and . Color reactions transitioned from purple to yellow with increasing concentration, confirming scavenging activity. Scavenging rates were dose-dependent, ranging from 2.51–5.53% at low concentrations to over 90% at high concentrations. Statistical analysis showed significant positive correlations: aqueous extract (P = 0.040, r = 0.895), Vc (P = 0.013, r = 0.950), and ethanol extract (P = 0.016, r = 0.944).

3.4 Superoxide Anion (O ·) Scavenging Activity

Superoxide anion scavenging data are presented in [Figure 7: see original paper], [Figure 8: see original paper], and . While color changes were less pronounced, scavenging rates increased with concentration, reaching 77.21–87.48% at maximum concentrations. Statistical analysis indicated a positive correlation for aqueous extract (P = 0.103, r = 0.802) and significant correlations for ethanol extract (P = 0.040, r = 0.895) and Vc (P = 0.013, r = 0.952).

3.5 Ferric Ion (Fe^3) Reducing Power

Ferric ion reducing power results are shown in [Figure 9: see original paper], [Figure 10: see original paper], and . Color reactions demonstrated conversion of yellow Fe^3 to green with increasing concentration. Reducing power was dose-dependent, increasing from 0.017–0.066 at low concentrations to 0.235–0.397 at high concentrations. All correlations were highly significant: aqueous extract (P = 0.000, r = 0.997), ethanol extract (P = 0.000, r = 0.997), and Vc (P = 0.000, r = 0.999).

3.6 Comparative Antioxidant Activity

IC values for all four antioxidant parameters are summarized in . Both extracts showed weaker activity than Vc in scavenging hydroxyl radicals, DPPH radicals, and reducing ferric ions, but stronger superoxide anion scavenging capacity. Homogeneity of variance tests yielded p-values of 0.827, 0.958, 0.052, and 0.417 (all > 0.05), permitting LSD one-way ANOVA. Comparison of scavenging rates revealed no significant differences between the two extract groups and the positive control ($p = 0.802$, 0.914, 0.783, and 0.565, respectively).

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4. Discussion

UPLC-TOF/MS analysis identified three flavonoids in each extract: kaempferol, tricin, and isoquercitrin in the aqueous extract; and kaempferol, naringenin, and quercetin 3-O- -D-glucoside in the ethanol extract. Kaempferol was common to both, consistent with its previous identification in ethanol extracts of *C. japonicum* bark (Wang et al., 1999), establishing it as a major constituent. Extensive research indicates that flavonoids, phenolics, terpenes, and nitrogen-containing compounds are primary antioxidant agents in plant essential oils, aqueous extracts, and ethanol extracts. The antioxidant activity observed in *C. japonicum* extracts likely results from synergistic effects among multiple flavonoid compounds.

Cercidiphyllum japonicum contains diverse chemical constituents with significant research value regarding secondary metabolite functions. This study extracted leaf secondary metabolites and evaluated their antioxidant capacity through in vitro assays. The extracts effectively scavenged hydroxyl radicals, DPPH radicals, and superoxide anions, and reduced ferric ions. While only superoxide anion scavenging exceeded that of Vc, all four antioxidant parameters showed relatively low IC values compared to other natural antioxidants reported in literature, indicating strong total antioxidant activity. These results demonstrate that *C. japonicum* extracts possess potent antioxidant properties and represent a promising source of natural antioxidants.

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Note: Figure translations are in progress. See original paper for figures.