

THE SYNTHESIS MECHANISM OF CHLOROGENIC ACID IN LEAVES OF *EUCOMMIA ULMOIDES* OLIVER

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Abstract. This study revealed intraday variation of chlorogenic acid (CGA) and synthesis mechanism in leaves of *Eucommia ulmoides* Oliver by investigating relation of CGA content in leaves and the light intensity. CGA was quantified by High performance liquid chromatography (HPLC) with diode array detection (DAD). Illumination intensity was determined by portable photosynthesis system. It was shown that CGA content in leaves enhanced with the increase of light intensity and the highest point of CGA content in leaves got behind the highest point of illumination intensity. The fact that dark spots of blue formazan appeared in leaves which had been illuminated by sun light and were treated with nitroblue tetrazolium (NBT) showed that sunlight induces O₂^{•-} production in leaves. In addition, CGA possessed higher antioxidant activity and free radical scavenging activity in vitro experiment. DPPH radical scavenging activity was 2.43 ± 0.092 times of vitamin C equivalents (molar ratio), and ABTS radical cation scavenging activity was 3.61 ± 0.14 time of trolox equivalents (molar ratio), and reducing powers was 3.40 ± 0.11 times of vitamin C equivalents (molar ratio). In plant cells, free radicals produced by photo oxidation can damage the cells, and however, CGA can protect cells by eliminating free radicals. This may be the mechanism of the synthesis of chlorogenic acid in plants.

Keywords: *chlorogenic acid, Eucommia ulmoides Oliver, oxygen free radical*

Introduction

Eucommia ulmoides Oliver (*E. ulmoides*) is a traditional medicinal plant of China, and is also one of the main sources of chlorogenic acid (CGA). The pharmacological functions of CGA have been studied extensively. Potentially beneficial properties to human such as antimicrobial, antiinflammatory, antioxidant, anticancer, antiviral, hepatoprotective activities, antihypertension and protection against UV radiation have been attributed to CGA in vitro (Bhattacharyya et al., 2014; Chen et al., 2010; Shao et al., 2015). In plants, CGA performs anti-oxidant functions, and increased CGA accumulation is also associated with increased UV-protection (Cle et al., 2008; Doring and Petersen, 2014; Mudge et al., 2016). In this paper, the effect of illumination intensity on produce of CGA in leaves of *E. ulmoides* was investigated.

The quantitative determination methods of CGA have been reported, including gradient HPLC method, new flow injection chemiluminescent method, nano-LC-ESI mass spectrometry and electrochemical sensor (Hu et al., 2009; Santos et al., 2011; Wang et al., 2007; Zhang et al., 2007). However, a rapid, reliable and convenient determination of CGA is required. High performance liquid chromatography (HPLC) with diode array detection (DAD), more common and less costly, currently represents the most prevalent and reliable analytical method for the characterization of compounds, which need to be simultaneously acquired of chromatograms at any wavelength accompanied by the absorption spectrum of each eluted band and verified

of separation quality with peak purity analysis (Huang et al., 2015; Luo et al., 2011; Sriseadka et al., 2012; Zeraik and Yariwake, 2010). Consequently, a normally manageable HPLC-DAD process was proposed in this research.

Materials and methods

Materials

Fresh leaves of *E. ulmoides* were obtained in campus of Henan University in summer and ground into dry powder, followed by sieving through 200 mesh screen to control the particle size.

Reagents

1,1-diphenyl-2-picrylhydrazyl (DPPH) and 2,2'-azinobis-(3-ethylbenzthiazoline-6-sulphonate (ABTS) were purchased from Sigma Chemical Co. Trolox was purchased from MP Biomedicals. Nitroblue tetrazolium (NBT) and CGA were purchased from Bomei Biotechnology Co. Methanol was chromatographically reagent grade. All other chemicals used in this study were of analytical reagent grade.

Determination of sunlight intensity

Sunlight intensity and temperature of leaves were determined by portable photosynthetic apparatus (LI-6400XT, LICOR USA) in a clear day.

Determination of CGA in leaves

CGA in leaves was separated and quantified using a Waters HPLC (Milford, Massachusetts, USA) equipped with a Waters1525 pump, a Waters 2707 autosampler, and Waters diode array detector 2998. Breeze Software was used for the data processing. C18 column (Waters, 250 × 4.6 mm i.d., 5 µm particle size) was used to identify CGA. The chromatographic separation was operated with solvent A (MeOH) and B (water). The separated flavonoid peaks were identified by comparing the retention time of individual standards. The optimum procedure elution used in this study was as follows: a gradient of 70-30% A (0-15 min), 30-70% A (15-20 min). The samples were injected automatically (20.0 µL). The column and guard column were controlled at 30 °C and a 1.0 mL min⁻¹ flow rate was applied, and the CGA were scanned from 210 to 400 nm in HPLC.

O₂⁻ localization in situ

Fresh leaves from control (dark-treated leaves) and light-treated leaves were excised and immersed in a 0.1% solution of nitroblue tetrazolium (NBT) in 50 mM Kphosphate buffer (pH 6.4), containing 10 mM Na-azide, and were vacuum-infiltrated for 5-10 min and illuminated until appearance of dark spots, characteristic of blue formazan precipitates. Leaves were bleached by immersing in boiling ethanol (Romero-Puertas et al., 2004).

Analytical method of antioxidant capacity

The antioxidant capacity of CGA was determined by the Thermo Multiskan GO (Thermo Fisher Scientific Oy, Finland) (Shown as *Table 1*). The free radical scavenging

activity was evaluated using the 2,2-diphenyl-1-picrylhydrazil (DPPH) assay and the absorbance values were measured at 518 nm and converted into the percent antioxidant activity (AA) using the following formula: $AA\% = [(absorbance\ of\ the\ control - absorbance\ of\ the\ sample) / absorbance\ of\ the\ control] \times 100$, The Vitamin C equivalents (VCEs) values of CGA were calculated from a linear regression of the AA of Vitamin C (VC, ascorbic acid); the scavenging activity against 2,2'-azino-bis(3-ethylbenzo thiazoline)-6-sulfonic acid (ABTS) radical cation was measured by the method that ABTS radical cation was produced directly by reacting ABTS solution with potassium persulfate and allowing the mixture to stand for 12-16 h in dark at the room temperature and prior to beginning the assay, and ABTS solution was diluted with methanol to an absorbance of at 734 nm, and then sample solution was added to ABTS solution and read at 734 nm after mixture and 30 min incubation at room temperature, and then The trolox equivalents (TEs) values of CGA were calculated from a linear regression of the AA of trolox equivalents; the determination of reducing powers of CGA was changed according to the method described by Ozsoy: 0.1 ml VC (20-100 µg/ml) and 0.1 ml CGA (20 µg/ml) were mixed with an equal volume of 0.2 M phosphate buffer (pH 6.6) and 1% potassium ferricyanide and then incubated at 50 °C for 20 min, and then 0.25 ml of 1% trichloroacetic acid was added to the mixture to stop the reaction, and then the mixture was centrifuged at 3000 g for 10 min, and the supernatant (0.25 ml) was mixed with 0.25 ml distilled water and 0.1% FeCl₃ (0.5 ml) and then the absorbance was measured at 700 nm, and the VCEs values of CGA were calculated from a linear regression of the AA of Vitamin C (Du et al., 2009; Dudonne et al., 2009; Ma et al., 2017; Ozsoy et al., 2008; Reis et al., 2015; Zengin et al., 2015).

Table 1. Antioxidant capacity of chlorogenic acid (CGA)

	VCEs (mass ratio)	VCEs (molar ratio)	TEs (mass ratio)	TEs (molar ratio)
DPPH radical scavenging activity	1.21±0.046	2.43±0.092	-	-
ABTS radical cation scavenging activity	-	-	2.55±0.099	3.61±0.14
Reducing powers	1.69±0.054	3.40±0.11	-	-

VCEs: vitamin C equivalents; TEs: trolox equivalents

Statistical analysis

Results were described as means ± standard deviation (SD) of three measurements. Statistical analysis was carried out using Student's t-test and significant difference was statistically considered at the level of $p < 0.05$.

Result and discussion

The CGA peaks were identified by their UV/DAD spectra due to their characteristic UV spectral pattern (Band I, λ max around 320 nm and Band II, λ max around 220 nm) in *Figure 1*. The absorption spectrum of each eluted band of leaves of *E. ulmoides* at any wavelength was consistent with that of CGA standard (*Fig. 1a* and *b*). Practically, UV/DAD scanning of herbal medicines without grinding makes CGA determination much more convenient.

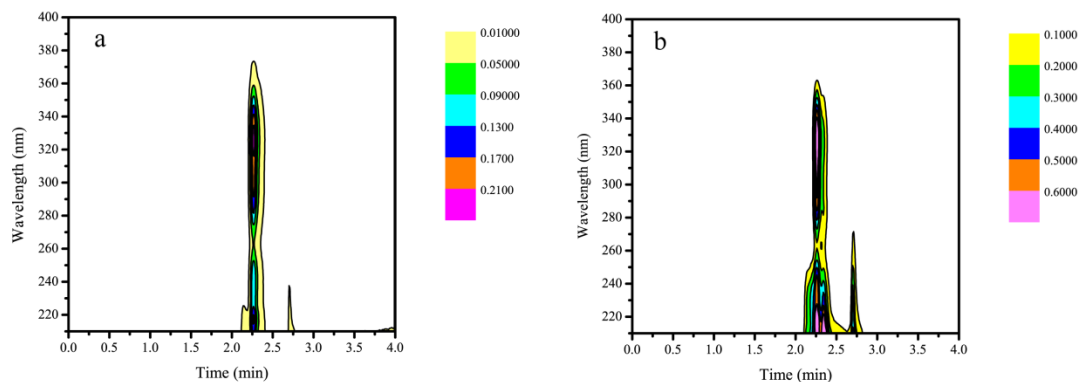


Figure 1. HPLC-UV/DAD of chlorogenic acid (CGA). a: CGA standard, b: leaves of *E. ulmoides*

Under clear day solar radiation condition, the change of CGA content in leaves of *E. ulmoides* with the illumination intensity was shown in *Figure 2*. The highest CGA content in leaves appeared at the decrease point of illumination intensity (14:00) but not the top of illumination intensity (12:00). And then CGA content in leaves dropped fast with illumination intensity after 14:00.

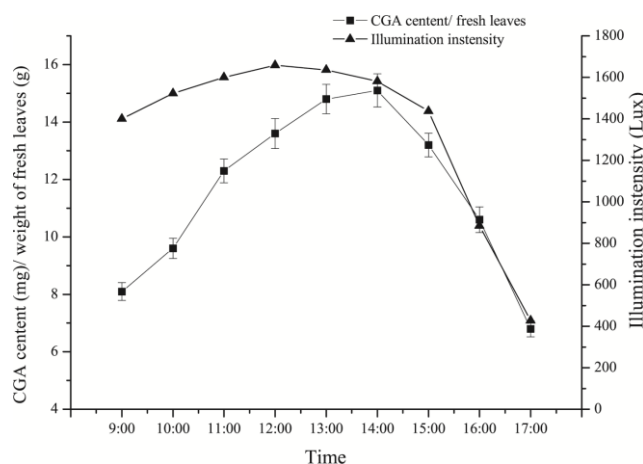


Figure 2. Intraday variation of chlorogenic acid (CGA) content in leaves of *E. ulmoides*

Fresh leaves of *E. ulmoides* had been illuminated by sun light and then were treated with nitroblue tetrazolium (NBT). In *Figure 3*, the dark spots of blue formazan appeared in leaves, while no dark spots appearing in the control (leaves in dark). This showed that sunlight induces $O_2^{\cdot -}$ production in leaves.

CGA performed higher antioxidant capacity: DPPH radical scavenging activity was 2.43 ± 0.092 times of VCEs (mmol/mmol), and ABTS radical cation scavenging activity was 3.61 ± 0.14 time of TE_s (mmol/mmol), and reducing powers was 3.40 ± 0.11 times of VCEs (mmol/mmol).

In plant, reactive oxygen species (ROS) are involved in plant responses to various biotic and abiotic stresses (Ma et al., 2012; Wang et al., 2013; Qi et al., 2018). Plants requires light for photosynthesis, but absorption of too much light can lead to photo-oxidative damage to the photosynthetic apparatus, and then this phenomenon occurs due

to imbalances which result from the overproduction of ROS (Chung et al., 2015; Ramabulana et al., 2016). However, accumulation of ROS can damage various cellular components such as proteins, nucleic acids, carbohydrates, and unsaturated lipids (Yu et al., 2018). Here, increased illumination intensity accelerated photooxidation reaction rate, which lead to enhancement of ROS level in cells. And then ROS heightened the content of CGA in the cell by increasing the expression of the genes related to the biosynthesis of CGA, which was completed over a period of time. Probably, this was why the top CGA content in leaves was late for a few hours than illumination intensity.

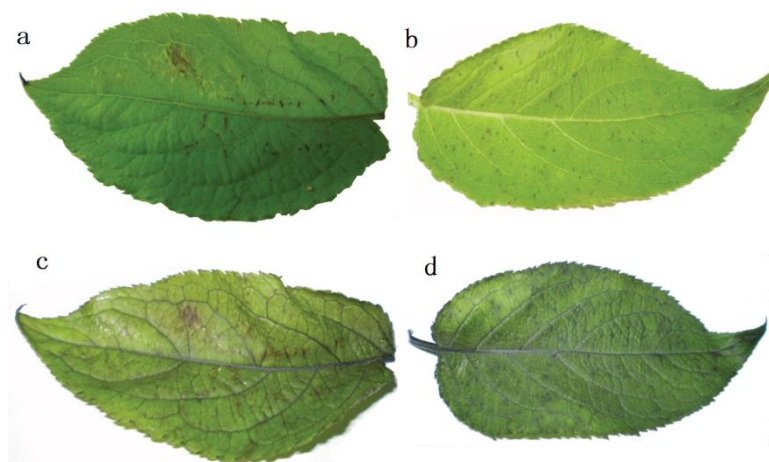


Figure 3. O_2^- localization in situ of fresh leaves of *E. ulmoides*. Control leaf and sample leaf illuminated by sun light were treated with NBT and then bleached by immersing in boiling ethanol; a: control leaf; b: sample leaf c: control leaf treated by boiling ethanol; d: sample leaf treated by boiling ethanol

In plant cells, free radicals produced by photo oxidation can damage the cells, and however, CGA can eliminate free radicals, which can protect cells. This may be the mechanism of the synthesis of chlorogenic acid in plants.

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