Postprint: HPLC-ECD-Based Investigation of the Spectrum-Effect Relationship for Antioxidant Activity of Vitex negundo Leaves

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Abstract

To investigate the spectrum-effect relationship between the fingerprint chromatogram and antioxidant activity of Vitex negundo leaves, this study first established high-performance liquid chromatography-electrochemical detection (HPLC-ECD) fingerprint chromatograms for 18 batches of Vitex negundo leaves, and performed cluster analysis on medicinal materials from different sources; identified the main phenolic compounds and determined their contents; analyzed the total phenolic and total flavonoid contents of Vitex negundo leaves, evaluated their in vitro antioxidant activity using DPPH free radical scavenging assay, ABTS free radical scavenging assay, oxygen radical absorbance capacity assay, and ferric ion reducing antioxidant power assay, and investigated the spectrum-effect relationship of Vitex negundo leaves through Pearson correlation analysis, grey relational analysis, and partial least squares regression analysis. The results showed that: (1) The fingerprint chromatogram of Vitex negundo leaves was calibrated with 21 common peaks, among which 10 peaks were identified, with the content order being chlorogenic acid > isoorientin > luteolin-7-glucoside > isovitexin > isochlorogenic acid A > isochlorogenic acid C > protocatechuic acid > orientin > isochlorogenic acid B > neochlorogenic acid; the similarity between samples from different origins was relatively high, with similarity results ranging from 0.816 to 0.983; (2) Cluster analysis indicated that sample content had a certain influence on classification, and samples from different sources were divided into 3 categories, with certain differences observed between southern and northern samples; (3) The total phenolic and total flavonoid contents in Vitex negundo leaves ranged from 15.82~61.83 mg · g-1 and 27.85~157.65 mg · g-1, respectively, and all samples exhibited varying degrees of antioxidant activity; (4) The spectrum-effect relationship revealed that the antioxidant activity of Vitex negundo leaves was the result of synergistic effects of multiple compounds, with compounds such as peak 9 (isoorientin),

peak 4 and peak 5 (chlorogenic acid) contributing the most to the antioxidant activity of the medicinal material. This study can provide a reference basis for the screening of antioxidant active components and quality control of Vitex negundo leaves.

Full Text

Preamble

Spectrum-Effect Relationship of Antioxidant Activity in *Vitex negundo* var. *cannabifolia* Based on HPLC-ECD

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Abstract

To investigate the spectrum-effect relationship between the HPLC fingerprint and antioxidant activity of *Vitex negundo* var. *cannabifolia* leaves, we established HPLC-ECD fingerprints for 18 batches of samples and performed hierarchical cluster analysis. Ten major phenolic compounds were identified and quantified. Total phenolic and flavonoid contents were measured, and in vitro antioxidant activity was evaluated using DPPH radical scavenging, ABTS radical scavenging, oxygen radical absorbance capacity (ORAC), and ferric ion reducing antioxidant power (FRAP) assays. The spectrum-effect relationships were analyzed through Pearson correlation analysis, gray relational analysis, and partial least squares regression. The results showed: (1) The fingerprints contained 21 common peaks, of which 10 were identified. The content order was: chlorogenic acid > isoorientin > luteoloside > isovitexin > isochlorogenic acid A > isochlorogenic acid C > protocatechuic acid > orientin > isochlorogenic acid B > neochlorogenic acid. Inter-sample similarity was high (0.816–0.983) across different producing regions. (2) Cluster analysis revealed that compound content influenced classification, dividing samples into three categories with distinct north-south differences. (3) Total phenolic and flavonoid contents ranged from 15.82–61.83 $mg \cdot g^{-1}$ and 27.85–157.65 $mg \cdot g^{-1}$, respectively, with all samples showing varying antioxidant capacities. (4) The spectrum-effect relationship demonstrated that antioxidant activity resulted from synergistic effects of multiple compounds, with peak 9 (isoorientin), peak 4, and peak 5 (chlorogenic acid) contributing most significantly. This study provides a reference for screening antioxidant components and quality control of *V. negundo* leaves.

Keywords: *Vitex negundo* var. *cannabifolia*; HPLC; electrochemical detection; antioxidant activity; spectrum-effect relationship; total phenolics; total flavonoids

Introduction

Vitex negundo var. *cannabifolia* (family Verbenaceae) is widely distributed across eastern China, Guangxi, Guangdong, Hebei, Guizhou, and Sichuan. Its leaves have been traditionally used for resolving the exterior, transforming dampness, dispelling phlegm, and relieving asthma (Compilation Group of National Chinese Herbal Medicine, 1996; Chinese Pharmacopoeia Commission, 2020). The plant contains phenolic acids, flavonoids, lignans, and terpenoids, and exhibits antioxidant, anti-inflammatory, analgesic, antibacterial, and antitumor activities (Shu et al., 2020). Antioxidant activity is a primary bioactivity of *V. negundo*; both aqueous and ethanol extracts demonstrate notable antioxidant capacity, with phenolic acids and flavonoids as the main active constituents (Xiang et al., 2021). Hu et al. (2015) confirmed that most phenolic acids possess strong ABTS radical scavenging activity. However, the specific material basis for its antioxidant activity remains unclear, and no studies have integrated fingerprinting with bioactivity evaluation for quality control.

Spectrum-effect relationship studies combine chromatographic fingerprints with pharmacological data to establish mathematical models reflecting the intrinsic quality of medicinal plants, and have been widely applied for quality evaluation (Wang et al., 2017; Yan and Zhang, 2020). HPLC is the preferred method for fingerprint analysis due to its high sensitivity, resolution, reproducibility, and broad applicability. Electrochemical detection (ECD) selectively detects redox-active compounds containing nitro, thiol, or phenolic hydroxyl groups, making HPLC-ECD particularly suitable for screening antioxidant components in medicinal plants (Luo et al., 2020; Zhang et al., 2021). Among various analytical methods for spectrum-effect studies, gray relational analysis (GRA) can analyze trends between common peak areas and efficacy indicators, while partial least squares regression (PLSR) better distinguishes system information from noise and compensates for the limitation of GRA' s exclusively positive correlations (Liu et al., 2020). Therefore, combining GRA and PLSR is commonly employed for comprehensive spectrum-effect analysis.

In this study, we collected 18 batches of *V. negundo* leaves from different sources and employed HPLC-ECD fingerprinting combined with in vitro antioxidant assays. Through hierarchical cluster analysis (HCA) and spectrum-effect relationship analysis, we aimed to: (1) characterize the chemical constituents and their contents in *V. negundo* leaves, and (2) evaluate the contribution of different chemical components to antioxidant activity.

Materials and Methods

1.1.1 Materials and Reagents

Reference standards of protocatechuic acid, chlorogenic acid, luteoloside, gallic acid, and trolox (Aladdin Biochemical Technology, batch numbers K1717091, J1523050, 10122401, L1810248, A2010059; purity $ $97%); orientin, isoorientin, neochlorogenic acid, isochlorogenic acid A, isochlorogenic acid B, and isochlorogenic acid C (Chengdu Preferred Biotechnology, batch numbers 20031202, 20052201, 19042305, 20032601, 20032602, 20080802; purity $ $98%); and rutin (Sinopharm Chemical Reagent, batch number 2016092; purity $ $95%) were used. Methanol and acetonitrile (HPLC grade) were purchased from Beijing InnoChem Science & Technology; all other reagents were analytical grade. Fresh *V. negundo* leaves were collected from Guangxi, Guangdong, and Hebei, and identified by Dr. Meng Lingjie at Zunyi Medical University as leaves of *Vitex negundo* var. *cannabifolia* according to the 2020 Chinese Pharmacopoeia standards. Sample details are provided in Table 1 . Fresh leaves were dried at 40 °C, pulverized, passed through a 50-mesh sieve, and stored at 4 °C.

1.1.2 Instruments

The HPLC system consisted of a Thermo UltiMate 3000 bio-RS with an ECD-3000 RS detector. Additional equipment included a Sorvall ST 8R high-speed centrifuge (Thermo Fisher Scientific), SpectraMax i3x multi-mode microplate reader (Molecular Devices), ME104E electronic balance (Mettler-Toledo), Purelab Chorus 2 ultrapure water system (ELGA), and GZX-9070MBE electric drying oven (Boxun).

1.2.1 Preparation of Standard Solutions

Individual stock solutions (2 $mg \cdot mL^{-1}$) of protocatechuic acid, neochlorogenic acid, chlorogenic acid, orientin, isoorientin, isovitexin, luteoloside, isochlorogenic acid B, isochlorogenic acid A, and isochlorogenic acid C were prepared in methanol and stored at -20 °C.

1.2.2 Preparation of Sample Solutions

Powdered *V. negundo* leaf samples were accurately weighed and extracted with 80% methanol at a solid-to-liquid ratio of 1:30 ($g \cdot mL^{-1}$) by ultrasonication at 25 °C for 30 min. After centrifugation at 9,000 rpm for 5 min, the supernatant was diluted 1:1 with 80% methanol and filtered through a 0.22 m organic membrane to obtain the test solution.

1.2.3 Chromatographic Conditions

Separation was performed on an XBridge BEH Shield RP18 column (3.0 mm × 150 mm, 2.5 m) with gradient elution using mobile phase A (acetonitrile) and mobile phase B (25 $mmol \cdot L^{-1}$ ammonium formate and 25 $mmol \cdot L^{-1}$ citric acid

mixed 1:1, pH adjusted to 2.6 with formic acid) at a flow rate of 0.6 mL · min^{-1}. The gradient program was: 0–9.5 min, 5%$→7.5→12→19→45→$80% A. The ECD detection voltage was 700 mV, column temperature 45 °C, autosampler temperature 12 °C, and injection volume 1 L.

1.2.4 Determination of Total Phenolics, Total Flavonoids, and Antioxidant Activity

1.2.4.1 Total Phenolic Content: Following Małgorzata et al. (2018) with modifications, 250 L of diluted test solution (40-fold dilution in 80% methanol) was mixed with 250 L of 0.25 mol · L^{-1} Folin-Ciocalteu reagent. After 3 min, 500 L of 15% Na_2CO_3 solution was added, mixed, and reacted in darkness for 30 min. Absorbance was measured at 760 nm using a microplate reader. Results were expressed as gallic acid equivalents (mg · g^{-1}) based on the calibration curve y = 0.0142x + 0.0625 (R^2 = 0.9988).

1.2.4.2 Total Flavonoid Content: Following He et al. (2015) with modifications, 500 L of diluted test solution (5-fold dilution), 1000 L of 80% methanol, and 250 L of 5% $NaNO_2$ solution were mixed and incubated for 6 min. Then 250 L of 10% $Al(NO_3)_3$ solution was added, mixed, and after 6 min, 2000 L of 4% NaOH solution was added. After 15 min, absorbance was measured at 510 nm. Results were expressed as rutin equivalents (mg · g^{-1}) using the calibration curve y = 0.00091x + 0.046 (R^2 = 0.9992).

1.2.4.3 Ferric Ion Reducing Antioxidant Power (FRAP): Following Wang et al. (2017) with modifications, 80 L of diluted test solution (60-fold dilution) was brought to 100 L with 80% methanol, mixed with 300 L of TPTZ working solution, and reacted for 5 min before measuring absorbance at 593 nm. Results were expressed as trolox equivalents (mg · g^{-1}) using y = 0.0192x + 0.0222 (R^2 = 0.9969).

1.2.4.4 DPPH Radical Scavenging Assay: Following Zhang et al. (2015) with modifications, 80 L of diluted test solution (20-fold dilution) was brought to 200 L with 80% methanol, then 400 L of DPPH solution (DPPH:80% methanol = 1:10) was added. After 10 min in darkness, absorbance was measured at 517 nm. Scavenging rate was calculated as: (A_{blank} - A_{sample})/A_{blank} × 100%, with trolox as reference.

1.2.4.5 ABTS Radical Scavenging Assay: Following Aati et al. (2018) with modifications, 100 L of diluted test solution (30-fold dilution) was mixed with 200 L of ABTS working solution, reacted in darkness for 20 min, and absorbance measured at 734 nm. Scavenging rate was calculated as: (A_{blank} - A_{sample})/A_{blank} × 100%, with trolox as reference.

1.2.4.6 Oxygen Radical Absorbance Capacity (ORAC): Test solution was diluted 500-fold in phosphate buffer. Following Xu et al. (2014), fluorescence intensity was measured every 5 min for 3 h. Results were expressed as trolox equivalents (mg · g^{-1}) using equations (1) and (2):

$$AUC = 0.5 \times [2 \times (f_0 + f_1 + \cdots + f_{n-1} + f_n) - f_0 - f_n] \times \Delta t$$

where AUC is the area under the fluorescence decay curve, f is relative fluorescence intensity at point n, and Δt is the time interval.

$$ORAC\ value = \frac{AUC_{sample} - AUC_{blank}}{AUC_{trolox} - AUC_{blank}} \times \frac{[trolox]}{[sample]}$$

The trolox calibration curve was y = 1.368x + 8.3019 (R^2 = 0.9946).

1.2.5 Data Processing and Analysis

Data processing and gray relational analysis were performed using Excel 2013. Pearson correlation and hierarchical cluster analysis were conducted with SPSS 26. PLSR analysis used SIMCA 14.1. Chromatograms and PLSR results were plotted using Origin 2021.

Results

2.1 Method Validation

Precision: Six consecutive injections of sample S2 solution yielded RSDs $ $0.45% for retention times and $ $1.31% for peak areas of 21 common peaks. **Repeatability:** Six independently prepared solutions of sample S2 showed RSDs $ $1.72% for retention times and $ $4.81% for peak areas. **Stability:** Sample S2 solution analyzed at 0, 4, 8, 12, 16, 18, and 24 h exhibited RSDs $ $0.58% for retention times and $ $3.94% for peak areas, confirming stability within 24 h. These results demonstrate the method meets analytical requirements.

2.2 Establishment of HPLC-ECD Fingerprints and Peak Identification

Eighteen batches of *V. negundo* leaves were analyzed under the conditions described in sections 1.2.2 and 1.2.3. Chromatograms were imported into the "Similarity Evaluation System for Chromatographic Fingerprints of Traditional Chinese Medicine (2012 edition)" using S8 as the reference to generate the HPLC-ECD fingerprint [Figure 1: see original paper]. The mixed standard chromatogram (A) and reference fingerprint (B) are shown in [Figure 2: see original paper]. Twenty-one common peaks were identified through multi-point correction and Mark peak matching. Peaks 1, 2, 5, 8, 9, 11, 12, 14, 15, and 17 corresponded to protocatechuic acid, neochlorogenic acid, chlorogenic acid, orientin, isoorientin, isovitexin, luteoloside, isochlorogenic acid B, isochlorogenic acid A, and isochlorogenic acid C, respectively. Similarity values between samples and the reference fingerprint ranged from 0.816 to 0.983 (S1–S18: 0.843,

0.983, 0.967, 0.959, 0.953, 0.916, 0.969, 0.966, 0.968, 0.980, 0.978, 0.953, 0.974, 0.947, 0.964, 0.847, 0.816, 0.950).

2.3 Standard Curves and Limits of Detection

Mixed standard solutions were prepared from the stock solutions described in section 1.2.1. Calibration curves were constructed by plotting concentration versus peak area. The limit of detection (LOD, S/N = 3) and limit of quantification (LOQ, S/N = 10) were determined. All compounds showed good linearity (R^2 > 0.999) within their respective concentration ranges, with LODs of 2.2–30.4 $ng \cdot mL^{-1}$.

2.4 Recovery Experiments

Six parallel samples of known composition were spiked with mixed standard solutions and analyzed. Recoveries for the 10 compounds ranged from 90.13% to 99.56% with RSDs $ $6.41%, confirming good accuracy .

2.5 Quantitative Analysis

The 10 identified compounds were quantified in samples from different regions . *V. negundo* leaves were rich in phenolic acids and flavonoids, though contents varied significantly among batches and origins. Based on mean values across all batches, the content order was: chlorogenic acid > isoorientin > luteoloside > isovitexin > isochlorogenic acid A > isochlorogenic acid C > protocatechuic acid > orientin > isochlorogenic acid B > neochlorogenic acid.

2.6 Cluster Analysis

To investigate the relationship between origin and chemical composition, hierarchical cluster analysis (HCA) was performed using relative peak areas of 21 common peaks as variables, with squared Euclidean distance as the metric in SPSS 26 [Figure 3: see original paper]. At a Euclidean distance of approximately 4, samples clustered into three groups with total compound contents of 13.06–31.69 $mg \cdot g^{-1}$, 38.01–41.03 $mg \cdot g^{-1}$, and 78.99 $mg \cdot g^{-1}$. Hebei samples fell into the 13.06–31.69 $mg \cdot g^{-1}$ range, while Guangdong and Guangxi samples showed overlapping distribution.

2.7 Antioxidant Activity

Total peak areas, total phenolic and flavonoid contents, and antioxidant activity results for 18 batches are summarized in . Total phenolic and flavonoid contents varied significantly, ranging from 15.82–61.83 $mg \cdot g^{-1}$ and 27.85–157.65 $mg \cdot g^{-1}$, respectively. All samples exhibited DPPH and ABTS radical scavenging capacity, ORAC, and FRAP, indicating that 80% methanol extracts of *V. negundo* leaves possess antioxidant activity.

2.8 Spectrum-Effect Relationship Analysis

2.8.1 Pearson Correlation Analysis Pearson correlation coefficients between total peak area, total phenolic content, total flavonoid content, and antioxidant activities were calculated using SPSS . Total phenolic and flavonoid contents showed significant positive correlations with DPPH, ABTS, ORAC, and FRAP ($r > 0.77$, $P < 0.01$). The ECD detector selectively detects redox-active compounds, and total peak area also correlated significantly with total phenolics, total flavonoids, and antioxidant activity.

2.8.2 Gray Relational Analysis Original data were normalized using the mean value method. Antioxidant activity results served as the parent sequence, while common peak areas were the sub-sequence (resolution coefficient = 0.5). Gray relational coefficients (r) were calculated following published methods (Tie et al., 2021; Du et al., 2021) . All common peaks showed strong correlation with antioxidant activity ($r > 0.79$), confirming that antioxidant activity results from synergistic effects of multiple compounds. Based on average correlation across four assays, the top 10 peaks were: peak 9 (isoorientin) > peak 4 > peak 2 (neochlorogenic acid) > peak 5 (chlorogenic acid) > peak 6 > peak 11 (isovitexin) > peak 20 > peak 12 (luteoloside) > peak 15 (isochlorogenic acid A) > peak 14 (isochlorogenic acid B).

2.8.3 PLSR Analysis PLSR, based on canonical correlation, principal component, and multiple linear regression analysis, reflects the comprehensive contribution of common peaks to efficacy. Positive regression coefficients indicate positive correlation, while VIP values >1 signify significant contributions to the model (Liu et al., 2020). Using peak areas of 21 common peaks as independent variables (X) and antioxidant activity results as dependent variables (Y), PLSR analysis was performed using SIMCA 14.1 [Figure 4: see original paper]. Peak 1 (protocatechuic acid) and peak 16 showed negative correlation with antioxidant activity, while all other peaks were positively correlated. Peaks with positive correlation and VIP >1 were peaks 3, 4, 5 (chlorogenic acid), 6, 8 (orientin), 9 (isoorientin), 19, and 20.

Discussion and Conclusion

This study established HPLC-ECD fingerprints for 18 batches of *V. negundo* leaves, identifying 21 common peaks with similarities $ $0.816, indicating relatively consistent quality across sources. Ten peaks were identified by comparison with UPLC-MS/MS data and reference standards (Luo et al., 2011; Huang et al., 2015), revealing chlorogenic acid, isoorientin, luteoloside, and isovitexin as major components. HCA suggested that harvest season, climate, and leaf age may influence classification, with overlapping distribution between Guangdong and Guangxi samples, while compound content differences played a relatively important role in sample grouping.

Pearson correlation analysis confirmed strong antioxidant activity in *V. negundo* leaves, with significant correlations among total peak area, total phenolics, total flavonoids, and antioxidant capacity, consistent with previous reports (Hu et al., 2015; Wang et al., 2022). This suggests that phenolics and flavonoids are the primary antioxidant compounds, and HPLC-ECD effectively detects these active substances.

GRA and PLSR analysis of the spectrum-effect relationship revealed that antioxidant activity arises from synergistic effects of multiple components. PLSR identified peak 9 (isoorientin) as the greatest contributor, followed by peaks 4 and 5 (chlorogenic acid). Chlorogenic acid has been reported as a potential chemical marker for distinguishing different medicinal parts of *Vitex* species and a quantitative indicator for quality control (Hu et al., 2015), possessing diverse bioactivities including antioxidant, anti-inflammatory, and antibacterial effects (Wang et al., 2017). Deepha et al. (2014) demonstrated that isoorientin exhibits strong antioxidant activity due to its B-ring 3' -OH group, intramolecular hydrogen bonding, and O-H structure, validating our spectrum-effect findings.

In summary, this study is the first to establish HPLC-ECD fingerprints for *V. negundo* leaves, identifying chlorogenic acids and flavonoids as the main antioxidant compounds. Content variations influenced sample classification, with isoorientin and chlorogenic acid being the most significant contributors to antioxidant activity. This approach provides a reference for identifying key quality components and markers, contributing to the establishment of a comprehensive quality evaluation system for *V. negundo* leaves.

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