Tissue and Growth Specific Chemical Profiles and Antimicrobial Activity of Libanotis buchtormensis (Fisch.) DC Extracts

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Abstract

Libanotis buchtormensis (Fisch.) DC. is a traditional Chinese medicinal herb used for the treatment of rheumatism, articular pain, and symptoms of the common cold. To evaluate the possible medicinal value of the aerial parts of L. buchtormensis, the chemical composition and antibacterial activity were assessed on a tissue and growth specific basis. Results based on high performance liquid chromatography (HPLC) showed that the samples were divided into two main clusters, one associated with roots and the other associated with the aerial parts. Analysis of the bioactive chemical contents revealed that coumarin was the most abundant compound in leaves (0.53 to 0.82%), while osthole and isoimperatorin exhibited relatively high concentrations in roots. Two-year growth samples, which correspond with the harvest period of L. buchtormensis, contained the highest amounts of total coumarin, osthole, and isoimperatorin. Broth microdilution tests showed that Gram-positive bacterial strains (Staphylococcus aureus, S. epidermidis and Streptococcus agalactiae) were more sensitive to L. buchtormensis extracts than the Gram-negative bacterial strain (Escherichia coli). Staphylococcus aureus and S. epidermidis were more sensitive to the extracts, exhibiting significant correlation with osthole and isoimperatorin contents. Comparative analysis of the chemical profiles and antibacterial activity revealed significant differences between the root and the aerial parts, which implied that the clinical application of the aerial parts needed further study. In addition, the aerial parts could serve as a material source for osthole, having comparable content to that of the root.

Keywords

Libanotis buchtormensis, HPLC fingerprint, total coumarin, osthole, antimicrobial activity

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of *L. buchtormensis*. However, little is known about the chemical profiles of the aerial parts of *L. buchtormensis*. In the present study, the chemical profiles and quantitative analysis of *L. buchtormensis* on a tissue and growth specific basis were conducted using high performance liquid chromatography (HPLC). Furthermore, the antimicrobial activity of extracts of the root and aerial parts was evaluated using the broth microdilution method against four bacterial strains. The objective of the present study was to assess the potential use of the aerial parts of *L. buchtormensis* in clinical applications, which would provide scientific support for more efficient utilization of this resource.

**Materials and Methods**

**Plant Material**

*L. buchtormensis* plants were collected from Wanhuashan Medicinal plantation in Taibai county, Shaanxi, China (107° 35′ 46.75″ E, 33° 49′ 10.44″ N) from June 2018 to June 2020. To study comprehensively the chemical profiles of *L. buchtormensis*, plants at different growth-stages were sampled, including one-year (YF1), two-year (YF2) and three-year old plants (YF3, flowering stage). Samples were further divided into root (R), leaf (L), and stem (S) for the study. The samples were authenticated by Dr. Huaizhu Li, and all voucher specimens were deposited at the Herbarium of Xianyang Normal University.

**Preparation of Sample and Standard Solutions**

The dried roots and aerial parts (leaves and stems) of *L. buchtormensis* were ground into powder and passed through a 20-mesh (0.9 mm) sieve. The powder (∼0.1 g) was macerated in 20 mL 80% ethanol for 2 h at room temperature, and then sonicated (250 W, 40 kHz) for 3 h at 55 °C. Extracts used for detecting the total coumarin content were diluted to 25 mL with 80% ethanol. The extracts for HPLC analysis were filtered, evaporated, and dissolved in 25 mL HPLC-grade methanol. The solution (2 mL) was filtered through a 0.22 μm membrane filter, after which 20 μL of solution was injected for HPLC analysis. Extracts for antibacterial analysis were filtered, evaporated, then diluted to 25 mL with dimethyl sulfoxide (DMSO, Merck, Darmstadt, Germany).

Osthole and isoimperatorin standards were purchased from National Institutes for Drug Control, Beijing, China. The concentrations of osthole and isoimperatorin stock solutions were 0.4 mg/mL and 0.5 mg/mL, respectively. An appropriate quantity of osthole and isoimperatorin standard solutions were diluted with HPLC-grade methanol in 10 mL volumetric flasks to produce a series of mixed standard solutions for plotting the calibration curve.

**Chromatography and Quantitative Analysis**

HPLC analysis was performed using an Agilent HPLC 1200 system equipped with a diode-array detector (Agilent Technologies, USA), and a reversed phase column (Kromasil C18, 250 mm × 4.6 mm, 5 μm). A binary gradient elution system, using acetonitrile as solvent A and 1% (v/v) aqueous acetic acid as solvent B, was programmed as follows: 0 to 18 min, 20 to 40% A, 18 to 28 min, 40 to 50% A, 28 to 30 min, 50 to 55% A, 30 to 35 min, 55 to 65% A, 35 to 55 min, 65 to 80% A, and 55 to 65 min, 80 to 60% A. The absorbance detector and flow rate were set at 320 nm and 0.8 mL/min, respectively.

The total coumarin content was measured using a UV-1800 spectrophotometer (Shimadzu Technologies, Japan) at 322 nm. Osthole (0.0016-0.0096 mg/mL) was used to determine the coumarin content with respect to the calibration curve (y = 0.0181x-0.0003, R² = 0.999).

**Antimicrobial Activity**

Gram-positive bacterial strains [Staphylococcus aureus (ATCC 6538), S. epidermidis (ATCC 12228) and Streptococcus agalactiae (ATCC13813)] and a Gram-negative bacterial strain

![HPLC chromatograms of *Libanotis buchtormensis* root (YFR) leaf (YFL), and stem (YFS) extracts, and the reference chromatogram (R).](image-url)
*Escherichia coli* (ATCC 25922) were purchased from the Microbiological Culture Collection Center, Beijing, China. Antibacterial activity was evaluated using the broth microdilution method, and minimal inhibitory concentrations (MIC) were determined. Two-fold dilution was used to set the concentration range, with initial extract and bacterial concentrations (MIC) being adjusted to 20 mg/mL and 10^7 to 10^8 CFU/mL, respectively. DMSO was used as a negative control and Norfloxacin as a positive control. After incubation at 37 °C for 24 h, the turbidity of the serially diluted cultures was determined. To avoid subjective

|          | YF1R | YF2R | YF3R | YF1L | YF2L | YF3L | YF1S | YF2S | YF3S | R   |
|----------|------|------|------|------|------|------|------|------|------|-----|
| YF1R     | 1    |      |      |      |      |      |      |      |      |     |
| YF2R     | 0.998| 1    |      |      |      |      |      |      |      |     |
| YF3R     | 0.986| 0.994| 1    |      |      |      |      |      |      |     |
| YF1L     | 0.455| 0.414| 0.337| 1    |      |      |      |      |      |     |
| YF2L     | 0.48 | 0.439| 0.361| 0.998| 1    |      |      |      |      |     |
| YF3L     | 0.495| 0.454| 0.374| 0.992| 0.997| 1    |      |      |      |     |
| YF1S     | 0.643| 0.6   | 0.514| 0.917| 0.93 | 0.942| 1    |      |      |     |
| YF2S     | 0.772| 0.736| 0.661| 0.86  | 0.878| 0.892| 0.983| 1    |      |     |
| YF3S     | 0.802| 0.768| 0.696| 0.827| 0.847| 0.864| 0.969| 0.998| 1    |     |
| R        | 0.936| 0.917| 0.874| 0.737| 0.757| 0.768| 0.861| 0.935| 0.946| 1   |

Table 1. Similarity Coefficients of *Libanotis buchtormensis* Based on HPLC Data.

![Dendrogram of hierarchical cluster analysis (HCA) based on HPLC data.](image-url)
misjudgment, the culture medium was spread on agar medium to test the authenticity of turbidity. The MICs of tested extracts were determined with triplicate tests.

Data Analysis

The Similarity Evaluation System for Chromatographic Fingerprint of TCM (Version 2012A) was introduced to generate a reference fingerprint (R) with the median of nine chromatograms and to calculate the correlation coefficients between chromatograms and R. Hierarchical clustering analysis (HCA) was performed with SPSS 22 software using Ward’s method and the Square Euclidean distance. Principal component analysis (PCA) was performed using SIMCA-P software (version 14.1) for further classification of the tested samples. Based on the PCA classification, partial least squares-discrimination analysis (PLS-DA) was used to evaluate variants with variable importance for projection (VIP) scores. Components with VIP values >1 are regarded as significant biomarkers. Significant differences between samples were measured using one-way analysis of variance (ANOVA) and the Student’s t-test ($P<0.05$) with SPSS 22 software. The relationship between the chemical contents and antibacterial activity was studied by Pearson’s correlation coefficients ($r$) using MANOVA. Values of $r \leq 0.35$ represent weak correlation, $r=0.36$ to 0.67 represent moderate correlation, and $r=0.68$ to 1.0 represent strong correlation.

Results and Discussion

Chemical Profiling Based on HPLC Data

The chemical fingerprints conducted by HPLC can reflect the whole information of the TCM and can be used for its identification and classification. In the study, HPLC fingerprints were performed for root, leaf and stem samples of *L. buchtormensis*. The RSD values of precision, repeatability, stability, and sample recovery were all below 3% (Table S1), indicating that the developed fingerprints were stable and reliable. A total of 18 well-resolved common peaks formed the HPLC fingerprints of *L. buchtormensis* samples taken from different plant parts and at different growth years (Figure 1). Peaks 14 and 15 were identified as osthole and isoimperatorin, respectively. Compared to the reference chromatogram (R), the similarity coefficients of the root, leaf, and stem chromatograms ranged from 0.874 to 0.936, 0.737 to 0.768 and 0.861 to 0.946, respectively (Table 1). The similarity coefficients between roots and leaves were 0.337 to 0.495, while those between roots and stems were 0.514 to 0.802 (Table 1). The results revealed that the chemical profiles of *L. buchtormensis* roots were distinct from the other parts. In general, chemical profiles of leaves and stem extracts taken from the same individual were more similar, having higher similarity coefficients (Table 1). For convenience, we suggest leaves and stems be considered together as the aerial parts of the species.

Based on the HCA analysis, nine samples taken from different parts of *L. buchtormensis* were divided into two main clusters (Figure 2). The root samples formed cluster I, while the others

![Figure 3. Clustering based on principal component analysis (PCA).](image-url)
were in cluster II. Leaf and stem samples were further clustered into two subgroups. Thus, the nine samples could be divided into three clusters, according to tissue origin. As a result of the regulation of biosynthesis and transportation, the accumulation and distribution of secondary metabolites in medicinal plants differed on a tissue and organ level basis.\textsuperscript{13,14} Tissues with the highest concentration of active ingredients are typically considered to be the medicinally used parts. The resulting PCA graph further demonstrated the differences in chemical profiles between the root and aerial parts of \textit{L. buchtormensis} (Figure 3). From a chemodiversity perspective, the clinical application of extracts of the aerial plant parts in place of root extracts should be approached with caution. The chemical components assessed were characterized by VIP values under two classifications with high model predictivity (Figure S1). According to the VIPs, 8 components were identified as potential biomarkers contributing to the clustering, including peaks (components) 18, 15 (isoimperatorin), 13, 4, 11, 2, 9 and 3 (Figure 4). However, osthole (peak 14) was not the main differentiating marker, which hinted that osthole in tissue specific extracts exhibited no significant difference with regards to tissue origin. The established HPLC fingerprints were essential for the characterization of the chemical profiles of the different parts of \textit{L. buchtormensis}, which could reflect the comprehensive components in the species.

**Content of Active Components**

The total coumarin, osthole, and isoimperatorin contents are detailed in Table 2. Leaf extracts contained the highest total coumarin contents, followed by root and stem extracts. Osthole contents in roots varied from 0.53 to 0.82\% across different growth stages of the plant, while the contents in leaves and stems varied from 0.48 to 0.54\% and 0.47 to 0.50\%, respectively. The values were lower than those reported by Zhang et al (1.29-1.45\%),\textsuperscript{15} partly because the tested samples in this study

![Variable importance in the project value (VIP) of each compound based on PLS-DA analysis.](image)

**Table 2.** Total Coumarin, Osthole and Isoimperatorin Contents (%) of the Root (R), Leaves (L) and Stem (S) Parts of \textit{L. buchtormensis}.

| Samples | Total coumarin | Osthole | Isoimperatorin |
|---------|----------------|---------|----------------|
| YF1R    | 2.25 ± 0.38\textsuperscript{b} | 0.53 ± 0.03\textsuperscript{e} | 0.042 ± 0.0050\textsuperscript{b} |
| YF2R    | 3.11 ± 0.31\textsuperscript{b} | 0.82 ± 0.11\textsuperscript{a} | 0.050 ± 0.0081\textsuperscript{a} |
| YF3R    | 2.92 ± 0.22\textsuperscript{b} | 0.54 ± 0.06\textsuperscript{b} | 0.036 ± 0.0033\textsuperscript{bc} |
| YF1L    | 5.00 ± 0.10\textsuperscript{a} | 0.48 ± 0.30\textsuperscript{d} | 0.014 ± 0.0009\textsuperscript{e} |
| YF2L    | 5.40 ± 0.32\textsuperscript{a} | 0.54 ± 0.03\textsuperscript{e} | 0.017 ± 0.0003\textsuperscript{d} |
| YF3L    | 5.26 ± 0.48\textsuperscript{a} | 0.52 ± 0.05\textsuperscript{e} | 0.010 ± 0.0001\textsuperscript{ef} |
| YF1S    | 2.07 ± 0.14\textsuperscript{c} | 0.47 ± 0.02\textsuperscript{d} | 0.011 ± 0.0006\textsuperscript{ef} |
| YF2S    | 2.28 ± 0.4\textsuperscript{ce} | 0.50 ± 0.09\textsuperscript{e} | 0.011 ± 0.0005\textsuperscript{ef} |
| YF3S    | 2.25 ± 0.03\textsuperscript{a} | 0.49 ± 0.01\textsuperscript{d} | 0.009 ± 0.0008\textsuperscript{f} |

Different superscript letters in each column indicate significant difference (\textit{P} < 0.05) between samples using one-way ANOVA.
were cultured. Isoimperatorin contents of roots were relatively higher than those in leaves and stems, reaching 0.04% at one-year, 0.05% at two-years and 0.036% at three-years. Osthole and isoimperatorin had relatively higher concentrations in roots than that in leaves and stems, which may explain why roots are primarily used as the medicinal part. Coumarins are presumed to be synthesized in leaves, but accumulate in roots. This might account for the high level of total coumarin in leaves. However, osthole contents did not exhibit a significant tissue-specific difference. Considering the high dry weight of the aerial parts, we suggested that these could be used as a suitable material source for osthole. Overall, the two-year-old samples contained the highest amount of total coumarin, osthole, and isoimperatorin, followed by the three-year-old and one-year-old samples, with the exception of isoimperatorin content. This was in accordance with the life-history and usage pattern of *Libanotis buchtormensis*. With the onset of flowering, the roots of *L. buchtormensis* begin to lignify, and their quality decreases. Thus, the optimal harvest period for *L. buchtormensis* is at the two-year stage during the vegetative growth, similar to *Peucedanum praeruptorum*.16

**Antimicrobial Activity**

The antibacterial activity of *L. buchtormensis* extracts was tested using the broth microdilution method to determine MIC values against four bacterial species. The results revealed that *L. buchtormensis* extracts had varying degrees of inhibition against the different strains (Table 3). Gram-positive bacteria were found to be more sensitive than Gram-negative ones. This was in agreement with a majority of antibacterial tests, and might relate to the compositional differences of the outer envelope of the bacteria.11,17-18 *E. coli* was relatively more sensitive to extracts of the aerial parts, which had MIC values of 10 mg/mL, while the MICs of root extracts were 20 mg/mL (Table 3). *Streptococcus agalactiae* displayed high sensitivity to all tested extracts, with MICs between 1.25 and 2.5 mg/mL. As shown in Table 4, the antibacterial activity against *Streptococcus agalactiae* seemed to have no significant correlation with the total coumarin, osthole, and isoimperatorin contents of *L. buchtormensis*. Compared with leaves and stems, root extracts were the more potent antibacterial agent against *S. aureus* and *S. epidermidis*, with MICs of 1.25 to 5 mg/mL and 2.5 to 5 mg/mL, respectively. Antibacterial activity against *S. aureus* and *S. epidermidis* showed significant correlation with the contents of osthole and isoimperatorin, which have been demonstrated to have antimicrobial activity in previous studies.19,20 Osthole especially exhibited promising antifungal and antibacterial activities, and has been used in the control of agricultural pests and diseases.6,21 The MICs of the extracts were relatively higher than that of the positive control Norfloxacin (0.05-0.125 mg/mL), and thus *L. buchtormensis* showed potent antibacterial ability against the four common bacteria tested.

**Conclusions**

In this study, the chemical contents and antimicrobial activity of the root and aerial parts of *Libanotis buchtormensis* were assessed. The HPLC fingerprints of different parts of *L. buchtormensis* indicated that the chemical profiles of root and aerial extracts were distinct, forming two main groups associated with their different tissue origins. In general, the samples from two-year

### Table 3. MIC Values Against Four Bacterial species.

| Samples | *Escherichia coli* (mg/mL) | *Staphylococcus aureus* (mg/mL) | *Staphylococcus epidermidis* (mg/mL) | *Streptococcus agalactiae* (mg/mL) |
|---------|---------------------------|-------------------------------|----------------------------------|-------------------------------|
| YF1R    | 20                        | 2.5                           | 5                                | 2.5                            |
| YF2R    | 20                        | 1.25                          | 2.5                              | 1.25                           |
| YF3R    | 20                        | 1.25                          | 2.5                              | 1.25                           |
| YF1L    | 10                        | 5                             | 5                                | 1.25                           |
| YF2L    | 10                        | 5                             | 5                                | 1.25                           |
| YF3L    | 10                        | 5                             | 5                                | 1.25                           |
| YF1S    | 10                        | 5                             | 5                                | 2.5                            |
| YF2S    | 10                        | 5                             | 5                                | 2.5                            |
| YF3S    | 10                        | 5                             | 5                                | 2.5                            |
| Norfloxacin | 0.05                  | 0.0125                        | 0.0125                           | 0.0125                         |

### Table 4. The Relationship Between the MICs Against Four Bacterial species and the Contents of Total Coumarin, Osthole and Isoimperatorin Based on Pearson’s Coefficient Correlation (r).

| r       | *Escherichia coli* | *Staphylococcus aureus* | *Staphylococcus epidermidis* | *Streptococcus agalactiae* |
|---------|--------------------|-------------------------|-----------------------------|---------------------------|
| Total coumarin | −0.335          | 0.295                   | 0.151                       | −0.292                    |
| Osthole     | 0.610             | −0.683*                 | −0.729*                     | −0.365                    |
| Isoimperatorin | 0.965**       | −0.947**                | −0.742*                     | −0.246                    |

*indicates significant difference (P < 0.05), **indicates highly significant difference (P < 0.01).
old plants contained the highest amount of total coumarin, osthole, and isoimperatorin. The total coumarin content was highest in leaves, while osthole and isoimperatorin showed relatively higher concentrations in roots. However, osthole content did not differ significantly between the roots and aerial parts. Thus, the clinical application of the aerial parts needs further study, but with the possibility of being a material source for osthole. Additionally, the antibacterial activity of the extracts exhibited varying degrees of inhibition against different bacterial strains. Gram-positive bacterial strains (Staphylococcus aureus, S. epidermidis and Streptococcus agalactiae) were more sensitive to L. buchtormensis extracts than the Gram-negative bacterial strain (Escherichia coli). The antibacterial activity against Staphylococcus aureus and S. epidermidis showed significant correlation with the contents of osthole and isoimperatorin.

Ethical Approval
Ethical approval is not applicable for this article.

Statement of Human and Animal Rights
This article does not contain any studies with human or animal subjects.

Statement of Informed Consent
There are no human subjects in this article and informed consent is not applicable.

Declaration of Conflicting Interests
The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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Trial Registration
Not applicable, because this article does not contain any clinical trials.

Supplemental material
Supplemental material for this article is available online.

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