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Original Research Article

Chromatographic Profiling of *Heracleum bivittatum* H.Boissieu Root Collected in Vietnam

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ABSTRACT

Heracleum bivittatum H.Boissieu, a lesser-studied member of the Apiaceae family endemic to northern Vietnam, remains underexplored in terms of its phytochemical composition and chromatographic properties. This study aims to provide a comprehensive phytochemical and chromatographic characterization of *H. bivittatum* root using high-performance thin-layer chromatography (HPTLC) and Liquid Chromatography – Quadrupole Time-of-Flight – Mass Spectrometry (LC-QToF-MS) combined with molecular networking. Ethanolic extracts of *H. bivittatum* roots were subjected to preliminary phytochemical screening, HPTLC analysis, and high-resolution tandem mass spectrometry. LC-MS/MS data were processed using the GNPS platform and annotated via the Network Annotation Propagation (NAP) tool with support from Reaxys, PubChem, and literature comparisons. Preliminary screening revealed the presence of flavonoids, coumarins, and non-precipitating tannins. HPTLC profiling showed distinct bands suggestive of phenolic and flavonoid compounds. LC-QToF-MS analysis led to the annotation of ten compounds, including peucedanol and zinniol, across 14 molecular families comprising 147 networked features. The combined use of HPTLC and molecular networking approaches allowed for the first-time phytochemical characterization of *H. bivittatum* root. These findings contribute to the chemotaxonomic understanding and medicinal evaluation of the species, highlighting its distinct metabolite profile within the *Heracleum* genus.

Keywords: *Heracleum bivittatum*, phytochemical screening, High-performance thin-layer chromatography, Liquid Chromatography – Quadrupole Time-of-Flight – Mass Spectrometry, Molecular networking

Introduction

The genus *Heracleum* L. (Apiaceae) includes over 120 species, many of which are traditionally used in folk medicine and recognized for their production of essential oils and furanocoumarins. These phytochemicals exhibit diverse biological properties such as antimicrobial, antioxidant, and cytotoxic activities.[1–3] However, the morphological similarities among *Heracleum* species have made taxonomic resolution difficult, prompting the need for phytochemical and chromatographic approaches. Chromatographic fingerprinting has proven to be a powerful tool for species discrimination. A two-dimensional thin-layer chromatography (2D-TLC) technique was established utilizing an adsorbent gradient to efficiently isolate eleven structurally analogous furanocoumarins from diverse *Heracleum* species. Their findings demonstrated that each species produces distinct fingerprints, enabling their chemotaxonomic classification.[4]

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Essential oil (EO) profiling is another approach that has been widely applied to study *Heracleum* species. The essential oil composition of nine *Heracleum* taxa from Southeastern Europe was examined, revealing that the species could be distinguished by the relative abundance of predominant monoterpenes (e.g., α-pinene, β-pinene), sesquiterpenes, and phenylpropanoids. These findings emphasize the chemical diversity of *Heracleum* and support the use of metabolite profiling for taxonomic and pharmacognostic purposes.[5]

Despite the growing interest in *Heracleum* spp., certain species such as *Heracleum bivittatum* H.Boissieu remain poorly characterized. This species, endemic to northern Vietnam, has recently been studied for its essential oil composition. GC-MS analysis revealed that the essential oil from the aerial parts of *H. bivittatum* contains *α*-pinene (22.5%), *β*-pinene (43.2%), sabinene (13.5%), and bornyl acetate (5.1%) as major components. In the root oil, *α*-pinene (70.2%), *β*-pinene (20.0%), and trans-*β*-ocimene (5.0%) were dominant. These constituents were either absent or found in only minor quantities in other *Heracleum* species,[6] suggesting that *H. bivittatum* possesses a unique chemical profile.

It is noteworthy that *Heracleum bivittatum* is currently recognized under the accepted name *Tetrataenium bivittatum* (H.Boissieu) Manden. according to the Plants of the World Online database.[7] Nonetheless, the species is still referred to as *Heracleum bivittatum* in many phytochemical and ethnobotanical contexts, including the present paper.

Given its distinctive essential oil composition and the absence of previous chromatographic fingerprinting studies, the development of a TLC or LC-MS-based fingerprint for *H. bivittatum* is essential. Such work will not only support its chemotaxonomic classification within the genus *Heracleum*, but also provide a scientific basis for evaluating its

medicinal potential, particularly in comparison with other well-studied species such as *H. persicum*, *H. sphondylium*, and *H. mantegazzianum*.[1,3]

Materials and Methods

Plant material

The investigated plant, *Heracleum bivittatum* H.Boissieu (currently accepted as *Tetrataenium bivittatum* (H. Boissieu) Manden. according to POWO), was collected in Sa Pa commune, Sa Pa district, Lào Cai province, Vietnam (22º19'31"N 103º50'01"E), on October 12, 2023. Botanical identification was performed at the Department of Plant Resources, Institute of Ecology and Biological Resources (IEBR), Vietnam Academy of Science and Technology. A voucher specimen was deposited under the code CSCL.03/23-24-SP06. The collected material was air-dried in a ventilated oven, ground to fine powder, and stored in sealed PE bags for chemical analysis.

Chemicals and Reagents

For preliminary phytochemical screening: 10% NaOH, 5% $FeCl_3$, concentrated H_2SO_4, lead acetate, concentrated ammonia, concentrated HCl, diazo reagent, Mayer's reagent, Dragendorff's reagent, Bouchardat's reagent, picric acid, Fehling's reagent A and B, Na_2CO_3, etc. For HPTLC: silica gel 60-F254 TLC plates (Merck, Germany); solvents including toluene, ethyl acetate, *n*-hexane, formic acid, acetone, chloroform, glacial acetic acid, and 70% ethanol; NP/PEG derivatization reagent. For LC-QToF-HRMS: formic acid (0.1%) in water (solvent A) and formic acid (0.1%) in acetonitrile (solvent B). All chemicals and solvents were of analytical grade and met the standards of the Vietnamese Pharmacopoeia (V).

Extraction Procedure

Powdered root material (32 g) was subjected to reflux extraction using 400ml of 70% ethanol twice (each for 1 hour). The combined extracts were filtered and concentrated under reduced pressure to yield an ethanol extract (5.28 g), which was used for chromatographic analyses.

Preliminary Phytochemical Screening

Phytochemical constituents of ethanolic extract were screened using standard qualitative chemical tests to identify groups such as alkaloids, flavonoids, tannins, phenolics, and glycosides.

High-Performance Thin-Layer Chromatography (HPTLC)

The ethanol extract was dissolved in methanol, sonicated for 10 minutes, and filtered to obtain the test solution (A). The HPTLC analysis was performed on silica gel 60-F_{254} plates (Merck) using two solvent systems: system I: toluene – ethyl acetate – formic acid (14:10:1, v/v/v), system II: ethyl acetate – formic acid – glacial acetic acid – water (100:11:11:26, v/v/v/v). After development, the plates were visualized under UV light at 254 nm and 366 nm, then derivatized with NP/PEG reagent for detection of phenolic compounds and coumarins.[8]

LC-QToF-MS Analysis

The ethanol extract was analyzed by liquid chromatography–mass spectrometry (LC-MS) using an Exion LC™ system coupled with a high-resolution QTOF mass spectrometer (X500R, Sciex, USA) equipped with an electrospray ionization (ESI) source. Chromatographic separation was performed using a Hypersil GOLD column (150 mm × 2.1 mm, 3 µm; Thermo Scientific, USA) with gradient elution at a flow rate of 0.4 mL/min. The mobile phases were: solvent A: 0.1% formic acid in water and solvent B: 0.1% formic acid in acetonitrile. Gradient program: 0–1 min: 100% A, 1–20 min: linear gradient to 2% A, 20–25 min: hold at 2% A. Injection volume was 2.0 µL. Mass detection was carried out in negative ion mode over an m/z range of 50–2000 amu. Data acquisition and processing were done using Sciex OS software.

Molecular networking and annotation

LC-MS/MS data were analyzed by molecular networking on the GNPS platform (ID: ce42c135a918421aacb28129b6939b72 (negative ion); collected on April 23, 2024 at https://gnps.ucsd.edu/). MS/MS spectra were processed by filtering the data based on the five most intense ion peaks within ± 50 Da across the spectrum. A molecular network was then generated with a minimum requirement of four peak matches, with connections between nodes filtered by a cosine value greater than 0.70. Nodes in the molecular network were constructed and annotated based on MS2 fragments. The molecular network was organized and visualized using Cytoscape 3.8.2 software (NRNB, USA). The data exported from Cytoscape software provides access to detailed information regarding compound name prediction and compound group classification. Combined with reputable databases such as Reaxys, PubChem and international scientific publications, the identification and confirmation of compounds in the sample is carried out. This includes comparing and verifying compounds based on LC-MS/MS data, including retention times, MS1, MS2 data and ion fragment types.

Results and Discussion

Preliminary Phytochemical Screening

The preliminary phytochemical analysis of *Heracleum bivittatum* root extract revealed the presence of several classes of secondary metabolites. The sample tested strongly positive for flavonoids, as indicated by multiple characteristic reactions including alkaline reagent, ammonia vapor, $FeCl_3$, cyanidin, and diazo reactions. Coumarins were also confirmed with strong lactone ring opening/closing reactions and positive responses to diazo and cis–trans isomerization tests. In contrast, saponins, alkaloids, anthraquinones, carotenoids, and organic acids were not detected. Tannins showed ambiguous results—positive with $FeCl_3$ but negative with gelatin and lead acetate, suggesting the presence of non-precipitating tannins. Additionally, reducing sugars, amino acids, and polysaccharides were detected, while cardiac glycosides yielded weak and inconsistent signals.

Thin layer chromatography

The TLC analysis of the 70% ethanol extract of *Heracleum bivittatum* was performed using solvent systems of different polarity. Figure 1A illustrates the chromatographic profile developed with the solvent system (I) toluene – ethyl acetate – formic acid (14:10:1, v/v/v). As shown in the chromatogram, after derivatization with NP/PEG reagent, multiple fluorescent bands were observed along the plate. Some light blue bands could be observed at $R_f \approx 0.05$, 0.20, 0.26, 0.43, 0.48 and 0.62 and a yellow band at $R_f \approx 0.28$ which could be a flavonoid. Figure 1B annotates the chromatographic fingerprint developed with the solvent system (II) Ethyl acetate – formic acid – glacial acetic acid – water (100:11:11:26). Some blue bands could be observed at $R_f \approx 0.07$, 0.29, 0.40, 0.47, 0.90, and 0.95.

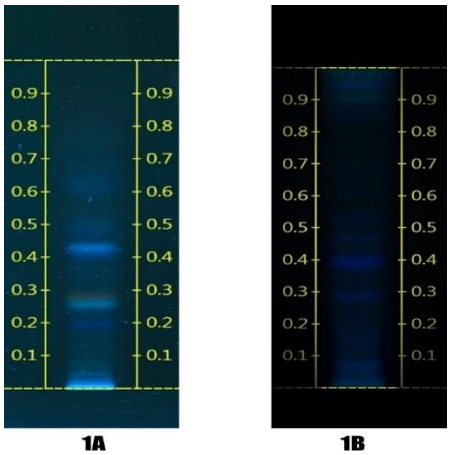

Figure 1: Chromatogram of *H. bivittatum* ethanolic extract observed at 366 nm after derivatization with NP/PEG reagent at two different solvent systems: (I) Toluene – ethyl acetate – formic acid (14:10:1) (1A), (II) Ethyl acetate – formic acid – glacial acetic acid – water (100:11:11:26) (1B)

Comprehensive chemical constituent profiling of Heraceleum

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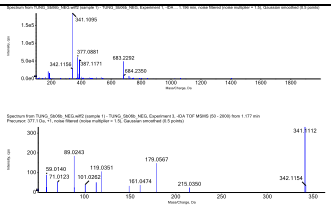
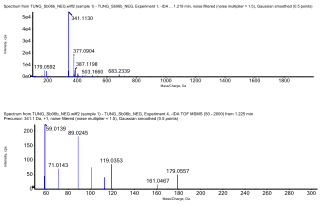
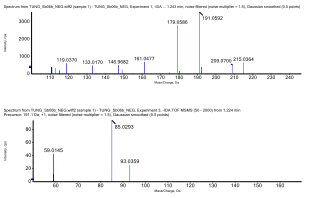
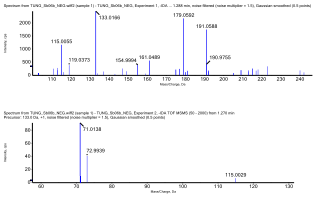
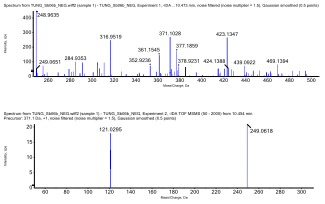
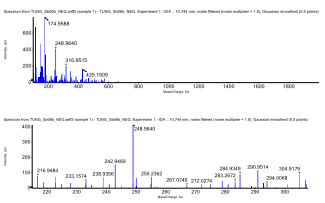
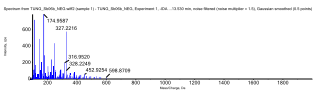
bivittatum root ethanolic extract through advanced tandem mass spectrometry and molecular networking approach

The molecular networks generated via GNPS and visualized through Cytoscape software elucidate numerous clusters comprising annotated chemical entities. These networks are stratified based on component annotations within each cluster, as delineated in Figures 2. Specifically, Figures 2A and 2B showcase the molecular network derived from negative ionization data of the extract, encompassing 147 nodes and 14 molecular families. The classification of these molecular families is visually represented by node coloration in Figure 2B. Using spectral library matching and *in silico* structure prediction tools, the chemical classes of key molecular families were tentatively determined. By integrating the Network Annotation Propagation (NAP) tool with Reaxys data and corroborative literature sources, 10 chemical compounds were successfully annotated and identified in the *H. bivittatum* ethanoli extract, respectively, as summarized in Table 1.

The application of high-resolution LC-QToF-MS combined with the GNPS molecular networking platform provided a robust strategy for dereplication and tentative identification of secondary metabolites in the ethanolic root extract of *Heracleum bivittatum*. A total of 147 nodes grouped into 14 molecular families were visualized, allowing for a detailed view of the metabolomic landscape of this species.

Table 1: The characterized metabolites originating from the ethanolic extract of *Heracleum bivittatum* root

No.	Retention time (min)	Compound (Ref.)	Ion adduct	Precusor/ fragments (m/z)	Formula (error-ppm)	MS and MS/MS spectra
1	1.19	D-saccharose [11]	M+Cl	377.088 (59, 89, 179, 221, 281, 341)	$C_{12}H_{22}O_{11}$ (7.96)	
2	1.22	D-trehalose [12]	M-H	341.113 (59, 89, 119)	$C_{12}H_{22}O_{11}$ (14.66)	
3	1.25	D-(-)-quinic acid [13]	M-H	191.058 (59, 85)	$C_7H_{12}O_6$ (10.47)	
4	1.28	malic acid [14]	M-H	133.016 (71, 115)	$C_4H_6O_5$ (15.04)	
5	10.47	(2S,3S,4S,5R,6R)-6-(3-benzoyloxy-2-hydroxypropoxy)-3,4,5-trihydroxyoxane-2-carboxylic acid [15]	M-H	371.103 (121, 249)	$C_{16}H_{20}O_{10}$ (13.47)	
6	10.75	peucedanol [9]	M-H	263.095 (175, 245)	$C_{14}H_{16}O_5$ (11.40)	
7	13.52	corchorifatty acid F [16]	M-H	327.222 (146, 171, 211, 229)	$C_{18}H_{32}O_5$ (15.28)	

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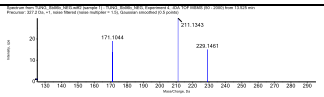
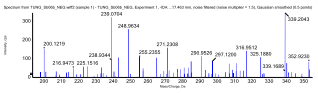
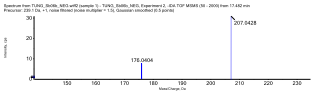
8	17.46	Dinoseb (GNPS libraries)	M-H	239.07 (151, 207, 239)	$C_{10}H_{12}N_2O_5$ (12.55)	
9	17.77	(2-ethylhexyl) hydrogen phthalate (GNPS libraries)	M-H	277.148 (77, 121, 134, 180)	$C_{16}H_{22}O_4$ (14.43)	
10	18.85	zinniol (GNPS libraries)	M-H	265.15 (96)	$C_{15}H_{22}O_4$ (22.63)	

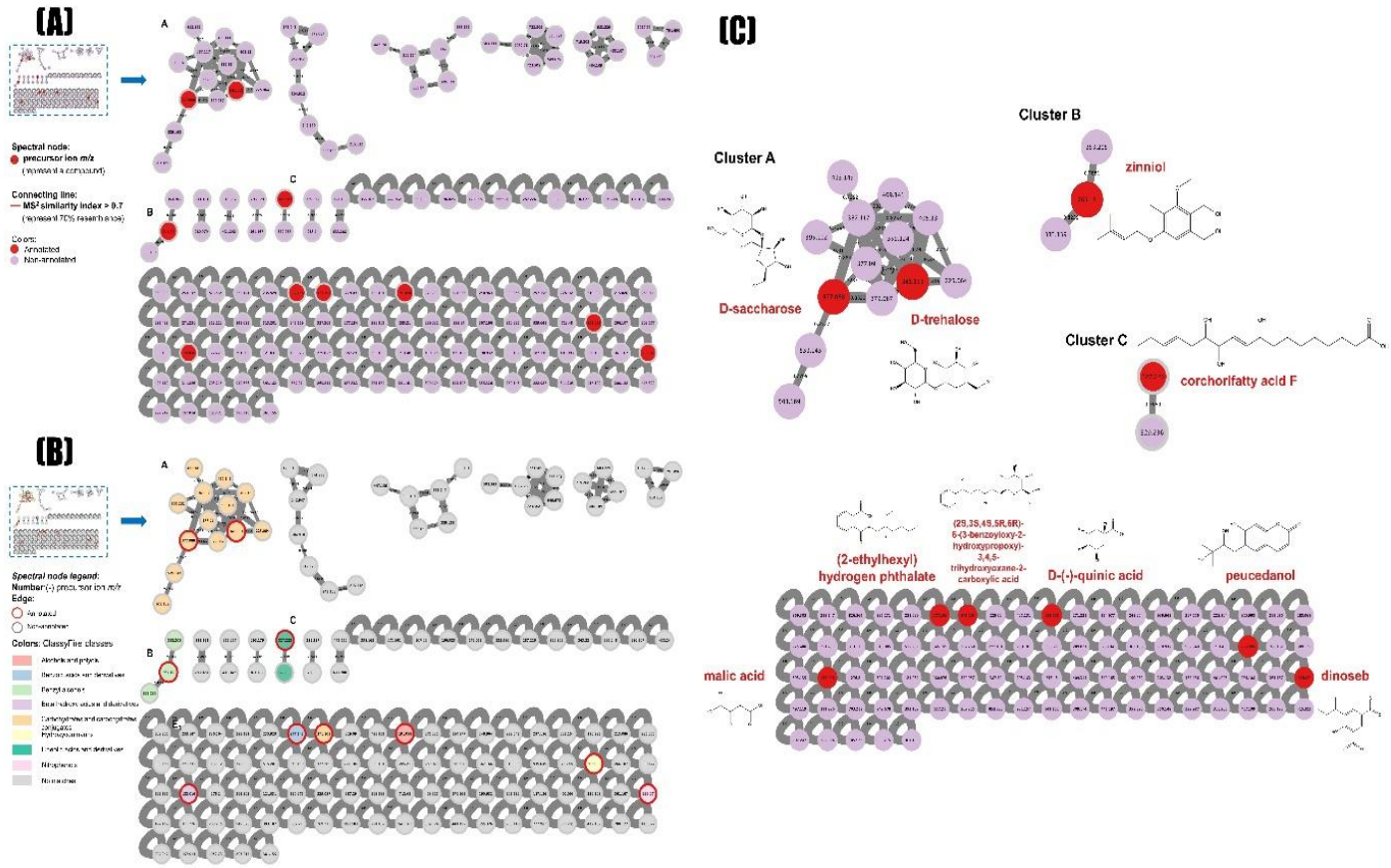


Figure 2: Molecular networks of the *H. bivittatum* ethanol extracts (A); putative chemical classes of major molecular families; and (B) putative annotations of significant representatives (C)

Among the annotated compounds, peucedanol - a coumarin derivative - was identified for the first time in *H. bivittatum*. This compound has previously been isolated from *Angelica pubescens*, another Apiaceae plant, where it exhibited notable neuraminidase inhibitory activity.[9] Furthermore, peucedanol has recently been reported to interact with major human cytochrome P450 enzymes, including CYP1A2, CYP2D6, and CYP3A4, in a dose-dependent manner.[10] Specifically, it acts as a competitive inhibitor of CYP1A2 and CYP2D6, and as a non-

competitive, time-dependent inhibitor of CYP3A4. These enzymes are involved in the metabolism of a wide array of drugs, and the inhibitory properties of peucedanol raise concerns about possible herb–drug interactions, especially in polypharmacy settings.

The presence of peucedanol in *H. bivittatum* adds chemotaxonomic value and indicates potential pharmacological activity, particularly as a bioactive coumarin. In addition, other detected compounds such as quinic acid, zinniol, and sugar alcohols point to a metabolically diverse profile with possible antioxidant, antimicrobial, or cytoprotective functions. These findings reinforce the utility of GNPS-based molecular networking not only for the annotation of known metabolites but also for highlighting novel chemical clusters, many of which remain structurally uncharacterized and deserve further exploration.

Overall, this study demonstrates that the combination of HPTLC and LC-MS/MS, coupled with molecular networking, is a powerful tool for phytochemical profiling and supports the chemotaxonomic distinction of *H. bivittatum* within the *Heracleum* genus. Future research should focus on isolating bioactive compounds such as peucedanol and assessing their pharmacodynamics and potential interactions, especially with enzymes involved in drug metabolism.

Conclusion

The integration of morphological, chromatographic, and molecular networking techniques enabled a comprehensive phytochemical investigation of *Heracleum bivittatum* root. The study successfully established a distinct HPTLC fingerprint and tentatively identified ten metabolites using LC-QToF-HRMS coupled with GNPS-based molecular networking. The presence of peucedanol, quinic acid, and unique sugar derivatives suggests potential pharmacological relevance and supports the chemotaxonomic distinction of *H. bivittatum* from related *Heracleum* species. These results lay the groundwork for further biological and pharmacological studies and advocate for the continued exploration of Vietnamese endemic Apiaceae plants.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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