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Original Article

Polyphenolic Metabolites of Scutellaria Lateriflora Hairy Root Culture

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ABSTRACT

Scutellaria lateriflora, commonly known as American skullcap, has a long history of use in Western traditional herbal medicine for treating insomnia, anxiety, hysteria, and nervous tension. However, the complete metabolite profile of *S. lateriflora* extracts has not been fully characterized due to the complexity of metabolite identification. Liquid chromatography-mass spectrometry (HPLC-MS/MS) provides an effective approach for the non-targeted analysis of polyphenolic compounds in plant extracts. The aim of this study was to characterize the polyphenolic profile of hairy root cultures of *S. lateriflora* using a comprehensive analytical methodology. The metabolome of the hairy root culture was found to be rich in two major classes of bioactive compounds: phenylethanoids (e.g., verbascoside and its derivatives) and flavonoids (e.g., wogonin, 6-OMe-wogonin, and their glucuronides). While the hairy roots produced a similar set of metabolites to those of intact plant roots, quantitative differences were observed, particularly in the enhanced production of phenylethanoids by the hairy root culture. Notably, wogonin and its derivatives were among the most efficiently synthesized flavones. These findings suggest that hairy root cultures of *S. lateriflora* can serve as a promising biotechnological platform for the targeted production of valuable polyphenolic compounds with potential pharmacological applications.

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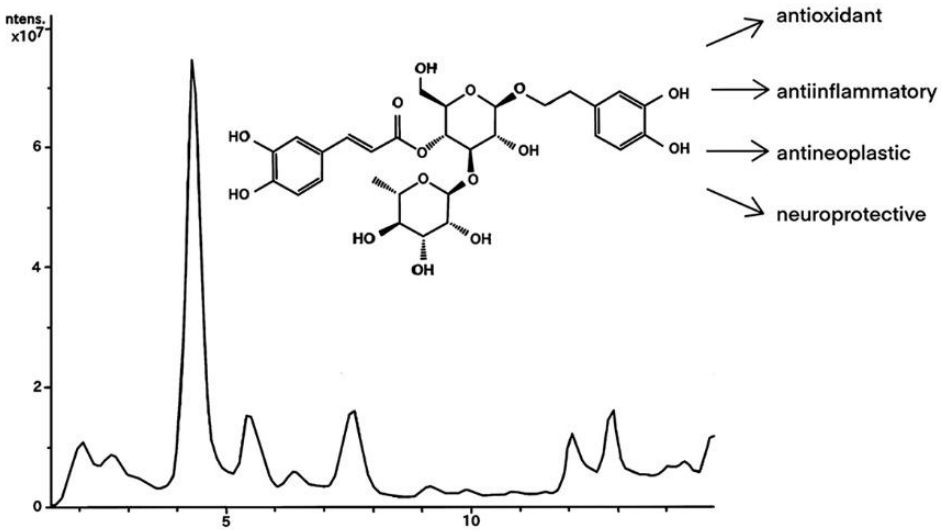
GRAPHICAL ABSTRACT



Introduction

Plants of the genus *Scutellaria* are used in the treatment of various diseases, associating their therapeutic effects with flavonoids. *S. lateriflora* grows on the moist soils of North America [1]. The aerial parts of *Scutellaria lateriflora* (American skullcap) are traditionally utilized in herbal medicine as sedative and antispasmodic agents for managing epilepsy and anxiety disorders. The 4'-deoxyflavones identified in *Scutellaria lateriflora* include baicalein (**3**), wogonin (**4**), and oroxylin A (**5**), alongside products of an alternative flavone biosynthetic pathway. These comprise 4'-hydroxyflavones such as scutellarein (5,6,7,4'-tetrahydroxyflavone) and apigenin (5,7,4'-trihydroxyflavone) [2-4].

Besides the aforementioned flavones, 6-OMe-wogonin, phenylethanoids, and sucrose were found in the roots [5]. Another phenylethanoid verbascoside is found in the stem of the plant [3].

The prospect of creating medicines based on plant raw materials is often obstructed by the complex composition of metabolites, their low or inconsistent levels, as well as the unstable quality of the plant raw materials themselves. The hairy roots culture, obtained by the genetic transformation of plant tissue by the soil agrobacterium *Rhizobium rhizogenes*, aims to address most of the aforementioned difficulties in a biotechnological manner by optimizing the growth conditions of the culture and, consequently, increasing the production of target flavonoids [6]. To date, several authors have noted the promising potential of *S. lateriflora* hairy roots as flavonoid and phenylethanoid producer [7-10]. The previously established polyphenolic metabolome of *S. lateriflora* roots showed a wider range of compounds [5] as compared to previously published results [11]. Thus, the present work continues the use of HPLC-MS/MS method to establish the polyphenolic metabolome of hairy root culture of *S. lateriflora*.

Experimental

The hairy root culture of *Scutellaria lateriflora* L. from the collection of IPP RAS obtained earlier was used as the object of the study [12]. Hairy roots were cultured in hormone-free B_5 nutrient medium as follows [13]: for two weeks in 100 ml flasks containing 40 ml of liquid B5 nutrient medium, then the material was transferred to 300 ml flasks containing 100 ml of medium [14]. Round-bottomed flasks were used instead of conical flasks.

Approximately 100 mg of *S. lateriflora* hairy root culture was extracted twice with 2 mL of aqueous ethanol (96%, v/v) under controlled conditions (50 °C, 2 h). The combined extracts were centrifuged for 3 min at 15,000 rpm and the supernatant was analysed *via* HPLC-MS. Samples were filtered through PTFE syringe filters

(Phenomenex, pore size 0.45 μm, diameter 13 mm).

HPLC-MS analysis of root hair extracts of *S. lateriflora* was conducted at the Instrumental Centre of Biotechnology and Gene Engineering of the Federal Scientific Center of the East Asia Terrestrial Biodiversity FEB RAS. The characterization of secondary metabolites was carried out using an Agilent 1260 Infinity analytical HPLC system (Agilent Technologies, Santa Clara, CA, USA) coupled with a Bruker HCT ultra PTM Discovery System mass spectrometer (Bruker Daltonik GmbH, Bremen, Germany), equipped with an electrospray ionization (ESI) source, following previously established protocols [5]. The data were obtained in negative ionization mode. Chromatographic separation was performed on analytical column (Zorbax C18, 150 mm, i.d 2.1 mm, 3.5 μm particle size, Agilent Technologies, USA), the mobile phase consisted of 0.1% aqueous formic acid (A) and acetonitrile (B). The following elution gradient with a flow rate of 0.2 mL/min was used: 0 min 20% B; 3 min 20% B; 25 min 80% B, 30 min 100% B, followed by eluent B to 40 min, wavelength 275 nm. All solvents were categorized for high-performance liquid chromatography (HPLC).

Results and Discussion

HPLC-MS Data Presentation

The HPLC-MS/MS method for non-targeted analysis of plant metabolites requires some experience and sometimes overlooks compounds that elute together with the major ones but are of a different origin [15]. As a result of a comparative study of the metabolome of the roots and hairy roots culture of *Scutellaria baicalensis*, a protocol for presenting a vast amount of HPLC-MS/MS data (retention times, spectrophotometric data, mass spectra of ions and their fragmentation products) was developed [16]. A key feature of the protocol is visualizing primary instrumental data and making it accessible to readers (Supplementary file).

Having knowledge of the flavone biosynthesis pathways [17] and their subsequent modification (methylation, glycosylation, *etc.*), it is possible to determine their elution sequence in UV and Total Ion Current (TIC) chromatograms of $[M-H]^-$ ions (Figure 1).

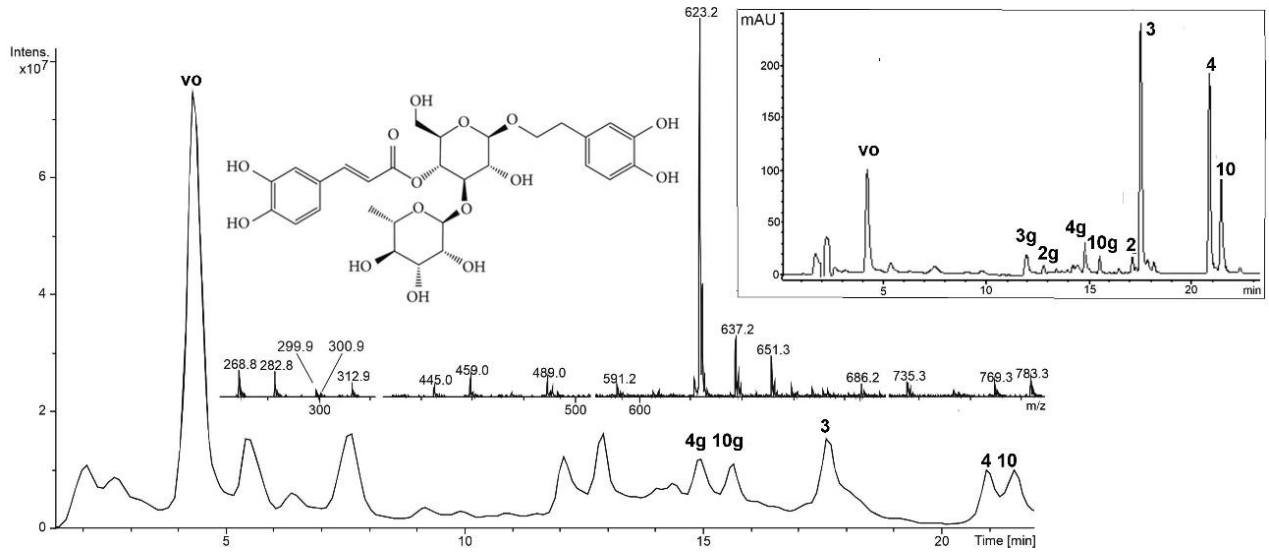


Figure 1: Total mass spectrum and ion profile chromatogram of the hairy root culture extract of *S. lateriflora*. Insert: UVLC profile. Annotation: VO – verbascoside 4 -wogonin, 3 -baicalein, 10 - 6-OMe wogonin, and Prefix g – glucuronide

Thus, the polyphenols are divided by polarity into conditional fractions 1-4. Flavones glycosylated with glucuronic acid are a source of carboxylate anion $[M-H]^-$, whose formation efficiency in the

ion source is higher than that of phenolate ions of polyphenols. Hence, the TIC profile shows an excess of glycosides 4g and 10g, compared to their aglycones 4 and 10, whereas the UV profile reflects their ratio in hairy roots in a more correct manner. The UV profile is characterized by 3 major peaks, demonstrating the priority synthesis of flavones 3, 4 and 10 (Figure 1). The TIC profile is represented by a larger number of peaks, the peak of the ion with m/z 623 being the highest among them (Figure 1). According to the literature, it corresponds to the phenylethanoid (PE) verbascoside, which is common in plants [18]. The latter was previously isolated in significant amounts by Marsh *et al.* (2014) [9] from the culture of hairy roots of *Scutellaria lateriflora*. Along with verbascoside, the other metabolites were identified by the combined retention time, nominal mass of $[M-H]^-$ ions and their characteristic MS/MS spectrum of fragments according to the literature [5].

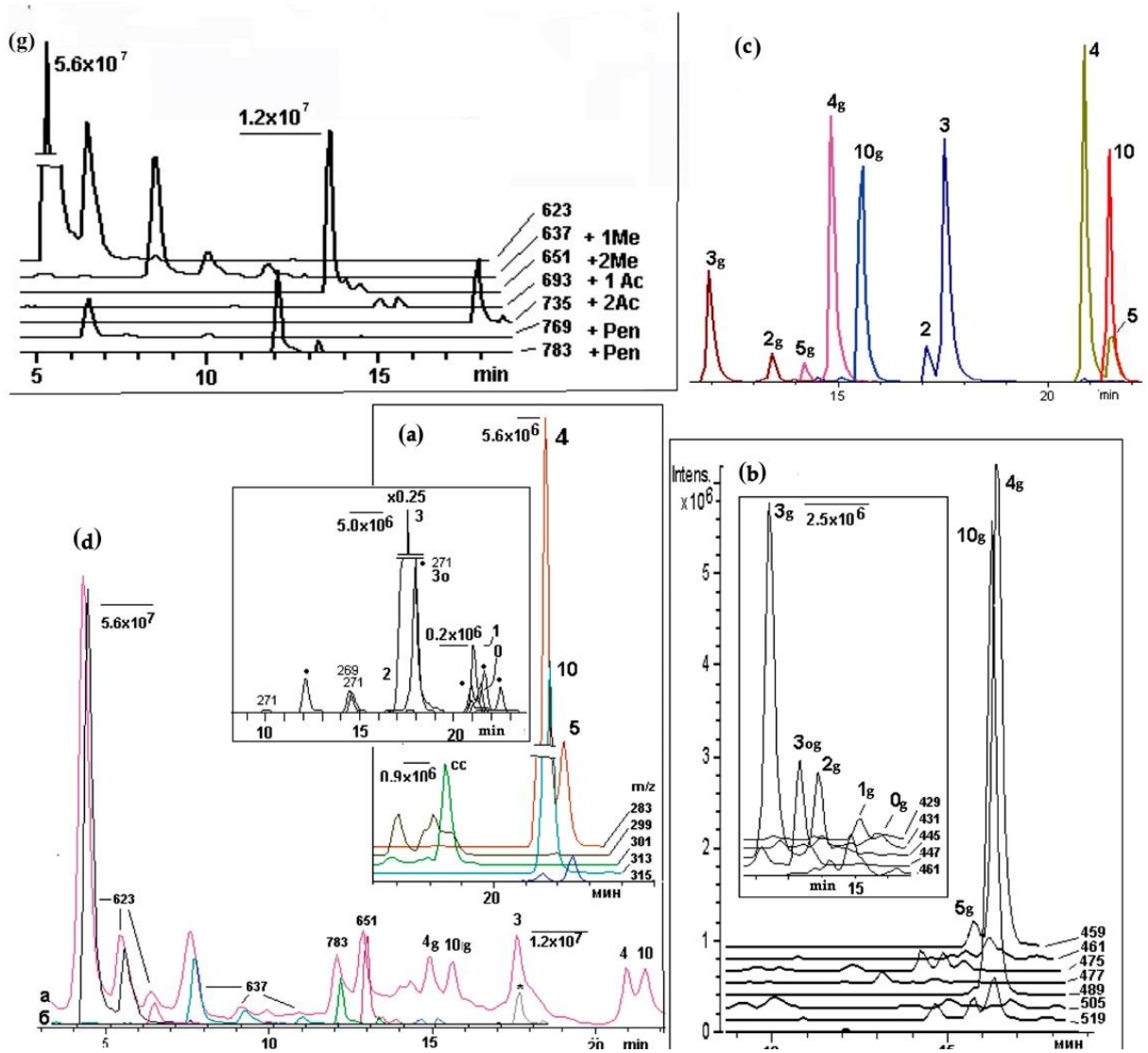


Figure 2: (**a**) Ion chromatograms for main methylated flavones in hairy roots of *Scutellaria lateriflora:***4** – wogonin, **5** – oroxylin A, **cc** – terpene, **10** – 6-OMe wogonin, ions m/z 299 – hydroxylation products of mono-methylated flavones, ions m/z 315 – di-OMe flavanone. Insertion: 0 – pinocembrin, 1 – chrysin, 2 – norwogonin, 3 – baicalein, •- ions m/z 285. (**b**) Annotation: glucuronides: **4g** – wogonin, **10g** – 6-OMe wogonina. Insertion: glucuronides: **0g** – pinocembrin, **1g** –chrysin, **2g** – norwogonin, **3g** – baicalein, **o3g** – flavonone of baicalein. (**c**) Overloaded ion chromatograms of metylated and glycosilated flavones. Annotation: ions of m/z 623 of caffeoyl rutinosides hydroxytyrosol, m/z 637 (mono-OMe), m/z 651 (di-OMe), and m/z 793 (di-OMe + pentose). (b) **10**-6-OMe wogonin, 4-wogonin, 3-baicalein, 10g-glucuronide of 6-OMe wogonin, 4g-wogonin glucuronide

Methylated flavones (MF) are represented by wogonin **4** and 6-OMe wogonin **10** (Figure 2a), along with a smaller content of oroxylin A **5** and presumably sesquiterpene, also found in the roots of the plant. Their ion chromatograms (IC) reveal the notable presence of a series of monomethylated flavone ions m/z 299, products of the second echelon of hydroxylation of molecules in the B ring, as well as a pair of di-OH-di-OMe flavanones, ions m/z 315 [5]. Among the glycosylated MFs, the predominant ions are m/z 459 and 489 of glycosides **4g** and **10g** of flavones **4** and **10**, respectively (Figure 2b).

Free flavones are represented by baicalein **3**, whose content is an order of magnitude higher than chrysin **1** (Figure 2c). Peak 269 at 14.5 min is a possible trace of apigenin. Of the five very low dotted peaks 285 characterizing the molecular weight of the tetra-hydroxy flavones, only at 12 min is there a possible presence of traces of scutellarein, which leaves the column following baicalin **3g**. The conspicuous ion m/z 271 at 18 min was assigned by the MS/MS spectrum to flavonone **3o**, a precursor of baicalein **3** [15].

Peak 271 at 14 min is a possible precursor of one of the flavones 269 (Supplementary file). Flavanones elute slightly later than their dehydration products (Zha et al., 2019). Peaks of 271 at 20-23 min, represent ^{13}C isotopes of abundantly methylated flavones. From the ratios of the content of free flavonoid glycosides, it follows that baicalin **3g** is dominant accompanied by flavanone **3og** (Figure 2c). Flavone biosynthesis proceeds through distinct enzymatic pathways. The primary pathway initiates with pinocembrin (**0**), which is converted to chrysin (**1**) (5,7-dihydroxyflavone) via flavone synthase II (FNSII) and subsequent hydroxylation of chrysin's A- and B-rings by generates diverse flavone derivatives [19,20]. In the absence of FNSII, pinocembrin (**0**) gives rise to the flavanone biosynthesis pathway [17]. The products of 6-hydroxylases, 6-OH and 6-OMe flavones are dominant in plant roots [5]. The ion m/z 270.9 is flavanone.

Ions heavier than m/z 600 in the mass spectrum of the extract (Figure 1) are attributed to a series of verbascoside modification products: mono-, di-O-methylated, acetylated, and over glycosylated by pentose. The relative abundances of phenylethanoids were visualized by overlaying their extracted ion chromatograms (EICs) on the total ion current (TIC) profile (Figure 2d). This comparative representation highlights the predominance of phenylethanoids over flavones, with verbascoside (m/z 623) and its derivatives showing the highest signal intensities. A flat IC assembly of phenylethanoids with baicalein **3** overlaid on the TIC profile shows an excess of this class of polyphenols over flavones **3, 4, 5, 10**, taking the peak of baicalein **3** as an internal standard and its approximately equal content to that of flavones **4** and **10**, as depicted in Figure 2d. The metabolites found in the roots and culture of *S. lateriflora* have a similar composition in the polyphenolic profiles of roots and culture of the closely related *S. baicalensis*, but differ in the ratio. This reflects the fact that the genes encoding enzymes for the biosynthesis of plant metabolites are closely related, but operate depending on environmental conditions.

Conclusion

The established metabolomic profile of polyphenols of *S. lateriflora* hairy root culture, consisting of phenylethanoids and flavonoids, was found to be identical in composition to that of the plant roots. However, the profiles differ in the relative content of metabolites. Derivatives of the phenylethanoid verbascoside, norwogonin, and baicalein are synthesized more efficiently by hairy root culture than their methylated derivatives, according to the ion current. The mentioned polyphenolic compounds of the two classes share similarities in the structural motifs of their molecules. The chemically active catechol and pyrogallol groups are in reaction readiness.

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Authors' Contributions

Y.N.E. developed the concept; Y.N.E., A.Y.M. and A.Y.S developed the methodology; A.Y.M, Y.N.E., and A.Y.S. wrote the article; Y.N.E. and A.Y.M. carried out the HPLC analyses. All authors have read and agreed to the published version of the manuscript.

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