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SCUTELLARIA BAICALENSIS: THE END OF THE FLAVONE BIOSYNTHESIS PATHWAY

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The report demonstrates that plenty of methylated flavones of the plant, as the end product of the flavone biosynthesis pathway, are concentrated in the root bark, which largely remains in the waste that makes up 7% w/w of the commercial root and is discarded. The study of the waste extract showed that the last enriched with monomethylated flavones wogonin and oroxylin A, along with some polymethylated ones represent an end of the biosynthesis pathway of flavones in the plant. In addition to wogonin, 7 known methylated flavones were found for the first time in the root bark. Due to a high content of wogonin and oroxylin A in the extract, these flavones were concentrated using preparative liquid chromatography. As a result, the obtained complex of these two target flavones turned out to be 0.8% w/w of the waste. Such morphologically specific accumulation of methylated flavones at the root boundary raises the question of their role in plant life.

Keywords: Chinese skullcap, LC-MS analysis, OMe flavones, oroxylin A, root bark, wogonin.

INTRODUCTION

Giant volumes of crop waste stimulate development of technologies for their disposal. A special place in plant production is occupied by medicinal plants producing substances used in medicine and food industry. Roots of perennials are the most significant raw material, since they concentrate such substances for years. Drugs from such roots are in demand in countries of the Asia-Pacific region (Chen et al., 2016; Zhao et al., 2016). China's need of the *S. baicalensis* roots reaches 5000 tons, which is barely met only by the cultivated plants. Therefore, Chinese researchers studied the composition of extractable substances in the aboveground parts of the plants (Yan et al., 2017). They found that the content of the target flavones: baicalein **3**, wogonin **4**, oroxylin A **5,** is an order of magnitude lower than in the roots. Natural populations of the plant *S. baicalensis* in Russia are still preserved on the prairies of the Trans-Baikal Territory. The region already has experience in cultivating the plant.

According to the guidance of Chinese Pharmacopoeia, lifted roots must be purified from root debris, shoots and bark (Wagner et al., 2011). An earlier study showed that the bark contains two of three target flavones **3** and **4** (Tani et al., 1985).

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Laser ablation with mass spectrometric visualization of a root cross cut of the cultivated *S. baicalensis* revealed similar results at the edge of the cut, where neither oroxylin A **5** nor inherent polymethylated flavones (Lui et al., 2009; Qiao et al., 2016; Elkin et al., 2018; Xu et al., 2018) were recorded (Feng et al., 2014; Sun et al., 2020). With these results and the fact that the plant treatment residue mainly consist of the bark, the wastes can be an additional source of valuable flavones.

In our research we dealt with the wastes which remained after processing roots of both wild and cultivated *S. baicalensis* of Dauria, an ecoregion to the east of Lake Baikal, which has given its name to this species. DAD LC-MS analysis of the waste composition revealed a wider set of methylated flavones and a significant content of three target flavones.

MATERIALS AND METHODS

PLANT MATERIAL

Wild *S. baicalensis* (over 6-8 years old) were harvested in the vicinity of the town of Orlovsky, in the Trans-Baikal Territory. Cultivated *S. baicalensis* were grown from wildflower seeds on an experimental plot in Ivolginsk village in Buryatia. The wild roots were dried in the open air under awnings and processed in a perforated, horizontally rotating drum. The first rotations removed soil particles from the roots. After removing the soil from the pallet, the process of cleaning was continued with accumulation of the plant waste consisting predominantly of bark and fine rootlets in the pallet. Weight fraction of the waste constituted 6-7% of total processed roots. The peeled roots from inside the drum were used for preparation of ground product and subsequent extraction. The roots of the cultivated plants were manually purified from the soil and peeled.

EXTRACTION

29 g of wild-root waste was twice extracted with 96% ethanol in sonic bath at 50°C. The extraction of 0.5 g of the ground plant product and 0.1 g of the waste of cultivated plants was carried out under similar conditions using flasks corresponding to the sample volumes. Aliquots of the jointed extracts were centrifuged before injecting in a column of the LC-MS instrument.

PREPARATIVE LC

A 23 × 2 cm glass column filled with Teflon powder (0.5-1 mm) was used to isolate the fraction containing flavones **4** and **5**. The extract adsorbed on the powder was applied to the column and eluted with a stepwise water/ethanol gradient. The high content of yellow flavones made it possible to select fractions visually. LC-MS analysis of the fractions showed the highest content of target flavones in 90% ethanol fraction.

LC-MS ANALYSIS

Agilent Technologies 1260 Infinity analytical HPLC system – Bruker HCT ion trap mass spectrometer was used for the analysis. The column Zorbax C18, 150 mm, 2.1-mm i.d., 3.5-μm particle size was applied for separation. Separation was carried out using the following conditions: the column temperature was 40°C, and the mobile phase consisted of 0.1% aqueous acetic acid (A) and acetonitrile (B) and detection on 275 nm. 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, and then eluent B until 40 min. The ESI MS analyses were run in the negative ion mode.

RESULTS AND DISCUSSION

Visual inspection of the waste in the pallet revealed a noticeable amount of bark. The phytochemical composition of roots of the plant *S. baicalensis* had been earlier thoroughly studied using LC-MS methods that allowed for auditing the composition of a large number of methylated flavones (Lui et al., 2009; Qiao et al., 2016; Elkin et al., 2018). Therefore, the content of these lipophilic substances in the waste was previously assessed only by liquid chromatography (Fig. 1). The LC profiles for the studied specimens were subdivided into four fractions according to the substance types: F1 – carbohydrates; F2 – glycosides; F3 – free flavones; F4 – methylated flavones.

Comparison of the LC profiles of the waste and the plant product (cleaned roots) showed a trend in accumulation of methylated flavones **4**-**9** in the root bark (F4, Fig. 1). The waste of the cultivated plants demonstrates a predominance of methylated flavones (F4, Fig. 1c), obviously due to a hand treatment of the roots, which accidentally resulted in an increased, mainly exfoliated bark in the

waste. A noticeable part of glycosides in the waste of the wild roots is linked to the presence of root fragments and shoots (F1 and F2, Fig. 1a). The root heartwood preferably accumulates glycosides of the target flavones, which is in accordance with the earlier study (Tani et al., 1985). The chromatograms showed that wogonin **4,** oroxylin A **5** and skullcapflavone II **8** represent the majority of methylated flavones in the waste (F4, Fig. 1a,c). It is remarkable that both waste specimens exhibit a noticeable content of nor-wogonin **2** and baicalein **3,** the precursors of wogonin **4** and oroxylin A **5**, respectively. The content of both flavones **2** and **3** is negligible in the plant product (F3, Fig. 1b). Presumably, the cambium layer, in which their synthesis from chrysin 1 (5,7-di-OH-flavone) occurs, was lost during processing in the drum.

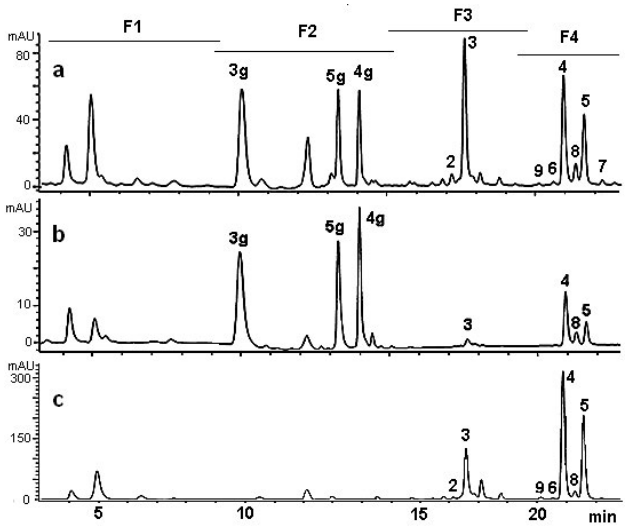

Fig. 1. Chromatogram extracts subdivided into four fractions according to the substance type: waste from wild plant roots (**a**), plant product from treated wild plant roots (**b**), waste of manually peeled roots of cultivated plants (**c**). Annotation: (2) nor-wogonin; (3) 5,6,7-tri-OH-flavone, baicalein; (4) 5,7-di-OH-8-OMe-flavone, wogonin; (5) 5,7-di-OH-6-OMe-flavone, oroxylin A; (8) 6,7,8, 6'-O-tetra-OMe-5,2'-di-OH flavone, skullcapflavone II; (9) 6,7,8, 6',2'-O-penta-OMe-5-OH flavone; 3g, 4g, 5g – glucuronosides of the flavones 3, 4, 5, respectively.

Since the definition of the relative content of methylated flavones, from mono- to penta-methylated ones, by a UV detector is not entirely correct, the flavones were estimated by phenoxide-ions (Xia and Attygalle, 2016; Elkin et al., 2018). The ion reflects the relative content of these flavones with greater certainty, even allowing for some variations in the dissociation constant of OH groups at different positions on the carbon skeleton of the molecules in the ESI ion source. Analysis of mass spectrometry data for both waste specimens revealed eight most abundant methylated flavones. The ion chromatograms of these flavones are collected in a three-dimensional time shift by the descending mass and the number of OMe groups, as shown in Fig. 2. These chromatograms showed the prevailing content of wogonin **4** and oroxylin

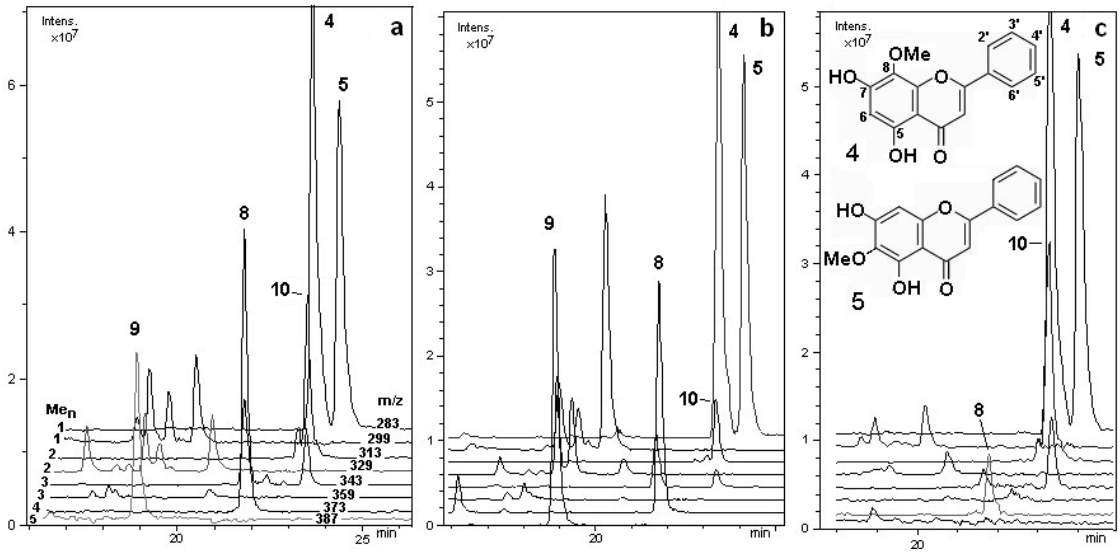

Fig. 2. Ion chromatograms for main methylated flavones of the waste from roots of: wild plant (**a**), cultivated plant (**b**), ion chromatogram of preparative fraction from the extract of the waste of wild plant roots (**c**). Annotation: (4) wogonin; (5) oroxylin A; (8) skullcapflavone II; (9) 6,7,8,2',6'-O-penta-OMe-5-OH flavone; (10) 5,7-di-OH-6, 8-di-OMe flavone.

A **5** amid methylated flavones of both wastes in approximately equal parts (Fig. 2a,b). The commensurate content of tetra- and pentamethylated flavones **8** and **9** was noticeable, which appeared on UV chromatograms in the form of very small peaks (Fig. 1a,c). Owing to their high contents, flavones **4** and **5** were subjected to additional concentration using a preparative liquid chromatography.

The ion chromatogram of 90% ethanol fraction showed a concentrate of methylated flavones with the highest content of wogonin and oroxylin A (Fig. 2c). The dry residue of the fraction was 0.25 g (0.8%) of the waste bulk. Both of these flavones may be involved in prevention of malignant transformation at a low concentration by reducing the formation of carcinogens through inhibition of human cytochrome P450 (CYP) 1A1 (Shao et al., 2012). These specialized flavones are devoid of 4'-hydroxyl groups in the B ring (4'-deoxyflavones) and induce apoptosis in a wide range of human tumor cells *in vitro* and inhibit tumor growth in vivo in various mouse tumor models (Zhao et al., 2016). In this way the inexpensive two-stage processing of the waste roots of *S. baicalensis* accompanied by LC-MS analysis yields a concentrate of wogonin and oroxylin A, which have a high pharmacological value in Chinese traditional medicine. On the other hand, the knowledge gained during the research led to the conclusion that the biosynthesis pathway of flavones in root cambium beginning from chrysin (5,7-di-OH-flavone) stopped at pentamethylated flavon **9** on the root border. Glycosylation followed by biosynthesis of flavones preserves the target flavones as glycuronides in the root core.

CONCLUSION

Thus, an interest in the waste and the use of an appropriate analytical procedure led not only to a source of valuable medicinal flavones wogonin and oroxylin but also to new knowledge about the distribution of the end products of flavone biosynthesis in the roots of *S. baicalensis*. Moreover, among the methylated flavones of the waste, a noticeable content of polymethylated flavones with four and five methyl groups was discovered. The morphological distribution of methylated flavones and their glycosides which has been established by evolution in plant physiology, requires further examination.

AUTHOR'S CONTRIBUTIONS

YNE conceptualization, analytic samples and writing; NIK extraction, preparative chromatography; SMS agricultural technology and specimens preparation; KVM expedition for wild plants, design cleaning machine and root processing, specimens preparation; AYM instrumentation and data curation.

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