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RESEARCH PAPERS

Phenological Fluctuations of Secondary Metabolites in *Dracocephalum charkeviczii*

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Abstract—Plants from the genus *Dracocephalum* are a source of biologically active compounds, including rosmarinic acid and different flavonoids. Their concentration varies during the vegetation period. In order to examine changes in their content in *Dracocephalum charkeviczii* Prob., an endemic species of Sikhote Alin and South Kuriles, wild and cultivated plants were collected in three phenological stages: vegetation, flowering/start of fructification, and preparation for withering. By means of HPLC with UV and mass selective detection, 15 polyphenol compounds were detected in methanol extracts from the leaves. Several new compounds—coumaric acid glycoside, quercetin glycoside and rutinoside, and acacetin coumaroylglycoside—were identified in *D. charkeviczii*. Synthesis of most flavonoids was found to be the highest in the beginning of the vegetation period, and gradually decreased by its end. The concentration of caffeic acid derivatives (chlorogenic acid, rosmarinic acid glycoside, and dehydrorhabdosiin) increased, but the total concentration of compounds decreased by the end of vegetation.

Keywords: *Dracocephalum charkeviczii*, secondary metabolites, polyphenols, phenology

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INTRODUCTION

Plants from the genus *Dracocephalum* L. (family Lamiaceae) were used in phytomedical preparations as early as in the Middle Ages [1]. Curative properties of the species from this genus are caused by the secondary metabolites present therein, such as essential oils, phenolic acids, triterpenoids, flavonoids, and their glucosides [2] whose biological properties have been investigated for many years now [3]. For instance, phenolic acids show anti-inflammatory, antiviral, antibacterial, and antioxidant activities [4]. Among them, chlorogenic, caffeic, and rosmarinic acids were previously identified in species from the genus *Dracocephalum* [5]. Nine derivatives of caffeic acid, with the prevalence of rosmarinic acid and salvianolic acid B, were identified in *D. forrestii* plants in vitro [6]. The two latter compounds protect the cells against injury caused by oxidative stress and promote cancer chemoprophylaxis [4]. These substances are efficient in the therapy of cerebrovascular deceases and rheumatoid arthritis [7–9].

The herbs should be collected during a period when their medicinal properties are at the peak. Investigations have shown that phenological stages affect production of secondary metabolites in plants [10–12]. At present, we know several articles describing qualitative and quantitative composition of secondary metabolites accumulated in different phenological stages in representatives of the genus *Dracocephalum* [13–16]. As to the species *D. charkeviczii,* such reports are lacking. The composition of polyphenols was determined previously in *D. charkeviczii* plants from natural population and in microplants produced *in vitro* [17].

The aim of this work was to examine the effect of phenological stages on the concentration of phenolic compounds in *D. charkeviczii* plants. Information about metabolite synthesis fluctuations in the course of plant development is necessary to arrange the harvest so as to cause a minimal disturbance of the development cycle in natural habitats.

MATERIALS AND METHODS

The profile of polyphenols was investigated in the Federal Scientific Center of the East Asia Terrestrial Biodiversity, Far Eastern Branch, Russian Academy of Sciences (FSCEATB FEB RAS), Vladivostok, in 2021–2023.

We used the leaves of Kharkevich's dragonhead (*Dracocephalum charkeviczii* Prob., family *Lamiaceae*). It is a perennial herbaceous polycarpic plant

with a short rhizome [18], endemic in Sikhote Alin and South Kuriles [19]. This species grows in Primorsky Territory and on the Kunashir island, Sakhalin Region, and also in coastal regions of Japan and China [20].

Sample collection. In order to reveal changes in production of polyphenols during the vegetation period, the leaves of *D. charkeviczii* were collected from plants cultivated on a private plantation in three biotic seasons: May (before flowering/vegetation), July (flowering/onset of fructification), and October (preparing for withering). To test the hypothesis that cultivated plants differ from those grown in natural populations, we studied the leaves collected in an intact natural population of *D. charkeviczii* growing on Zhitkov peninsula (Russkii island) in two biotic seasons: May (vegetation) and July (flowering/start of fructification).

Solvents and standards. Acetonitrile and methanol were purchased from Merck (Germany). Formic acid was supplied by Sigma-Aldrich (Germany). Deionized water was prepared using a Milli-Q Simplicity water purification system (Millipore, France). Standard preparations of kaempferol, rutin, caffeic and chlorogenic acids were purchased from Sigma-Aldrich.

Composition of secondary metabolites. Qualitative and quantitative composition of polyphenols was determined according to the method described previously [17]. Dried and ground samples of leaves were extracted with 80% (v/v) aqueous methanol with ultrasound. The obtained extracts were analyzed by HPLC using an Agilent 1260 Infinity LC system (Agilent, United States) equipped with a diode array detector. Separation was performed on a Zorbax C18 analytical column (150 × 2.1 mm, 3.5 μm, Agilent, United States). As eluents, we used 0.1% (v/v) aqueous solution of formic acid and acetonitrile. UV spectra within a range of wavelengths from 200 to 400 nm were used for identification; for quantification, chromatograms were recorded at wavelengths of 265 and 330 nm. Identification was verified by means of mass spectrometric detection via coupling an HPLC system to a Bruker HCT Ultra PTM Discovery System (Bruker Daltonics, GmbH, Germany). MS was performed in the mode of electrospray ionization and simultaneous registration of negative and positive ions. MS/MS spectra were recorded automatically at a fragmentation voltage of 1.0 V. The research was done using the equipment of the Biotechnology and Genetic Engineering Joint-Use Center of FSCEATB FEB RAS. Quantification of secondary metabolites was performed by means of external calibration using commercial preparations of kaempferol (Sigma, Germany) and caffeic acid (Sigma, United States).

Diagrams were constructed on the basis of means and their standard errors. The results were analyzed using Statistica package, version 13.0. Independent groups were compared using one-way ANOVA (analysis of variances) with Fisher's protected least significant difference test (LSD); statistical hypotheses were tested at a critical significance level of 0.05.

RESULTS AND DISCUSSION

Methanol extracts produced from the leaves of *D. charkeviczii* in different periods of vegetation were investigated by means of HPLC-UV-MS(/MS). A typical HPLC-UV chromatogram of crude extract is shown in Fig. 1. Seventeen biologically active components of a polyphenol nature were identified, and the results are shown in Table 1 (see Supplementary Information).

Among major detected biologically active components of the extracts, we observed those we had reported previously [17]. They include nine phenylpropanoids: caffeic acid (5), chlorogenic acid (3), and its two isomers (1 and 4), rosmarinic acid (11) and its glycoside (8), rhabdosiin (9), dehydrorhabdosiin (16), and salvianolic acid B (15), as well as three flavonoids: glycosylated acacetin (10) and its acetylated derivatives (12 and 13) [17]. Five minor components were additionally detected and identified via comparison of their chromatographic and mass spectrometric behavior with literature data. Compounds corresponding to peaks 6 and 7 showed identical UV profiles and were preliminarily assigned as placed among flavonoids (Fig. 1). Both compounds exhibited intensive signals of both protonated and deprotonated ions (Table 1, Supplementary Information). Retention time, UV profile, and MS/MS spectra of molecular ions of compound 6 were exactly the same as those of authentic standard quercetin rutinoside (rutin). For instance, MS^2 fragmentation of protonated ions of compound 6 showed the formation of daughter ions with m/z 465 and m/z 303, corresponded to elimination of residues of deoxyhexose (−146 D) and hexose (−162 D), as well as rutin. Compound 7 differed from compound 6 by one fragment of deoxyhexose (146 D) and was presumably identified as quercetin hexoside. Compound 14, corresponding to the peak with a retention time of 30.3 min, demonstrated the UV spectrum identical to spectra of acacetin derivatives (10, 12, and 13). Comparison of mass spectrometric pattern of compound 14 with the results published previously [21, 22] made it possible to identify it as acacetin-hexoside acylated with coumaric acid. UV and MS characteristics of compound 17 (33.7 min) turned out to be identical to those of dehydrorhabdosiin (16) and this compound was qualified as its isomer. Peak 2 with a retention time of 18.7 min showed an absorption maximum at 295 nm, characteristic to *p*-coumaric acid [23]. MS/MS^2 data of compound 2 entirely agreed with the previously published results [24, 25] for hexoside of coumaric acid.

In two groups of plants collected in different habitats at any time, the greatest concentrations were observed for glycosylated and acetylated acacetin (13)

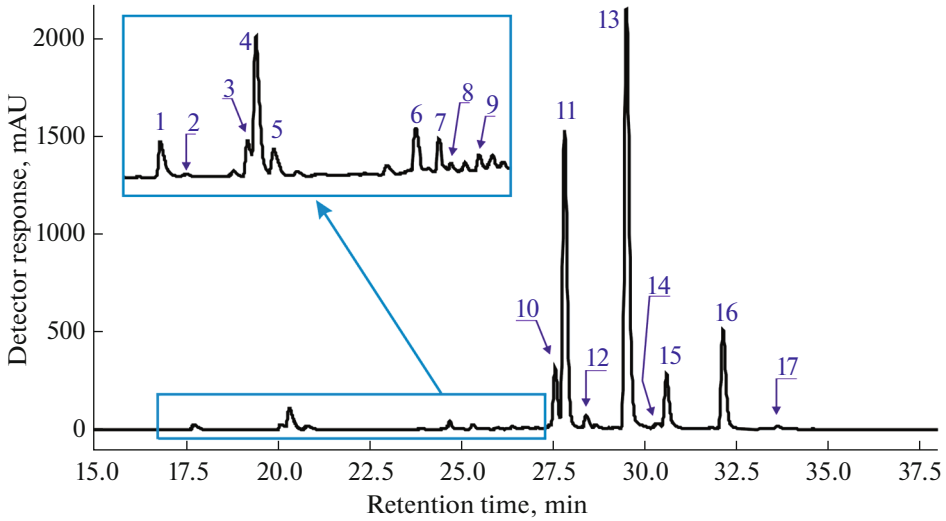

Fig. 1. Typical HPLC-UV profile of methanol extracts from the leaves of *Dracocephalum charkeviczii* recorded at λ = 330 nm. Numeration of peaks corresponds to that given in Table 1 (Supplementary Information): (1) 3-caffeoylquinic acid; (2) *p*-coumaric acid hexoside; (3) chlorogenic acid; (4) 4-caffeoylquinic acid; (5) caffeic acid; (6) quercetin rutinoside; (7) quercetin hexoside; (8) rosmarinic acid hexoside; (9) rhabdosiin; (10) acacetin rhamnosyl-tri-hexoside; (11) rosmarinic acid; (12) acacetin rhamnosyl-tri-hexoside acetylated I; (13) acacetin rhamnosyl-3-hexoside acetylated II; (14) acacetin coumaroyl-hexoside; (15) salvianolic acid B; (16) dehydrorhabdosiin; (17) dehydrorhabdosiin isomer.

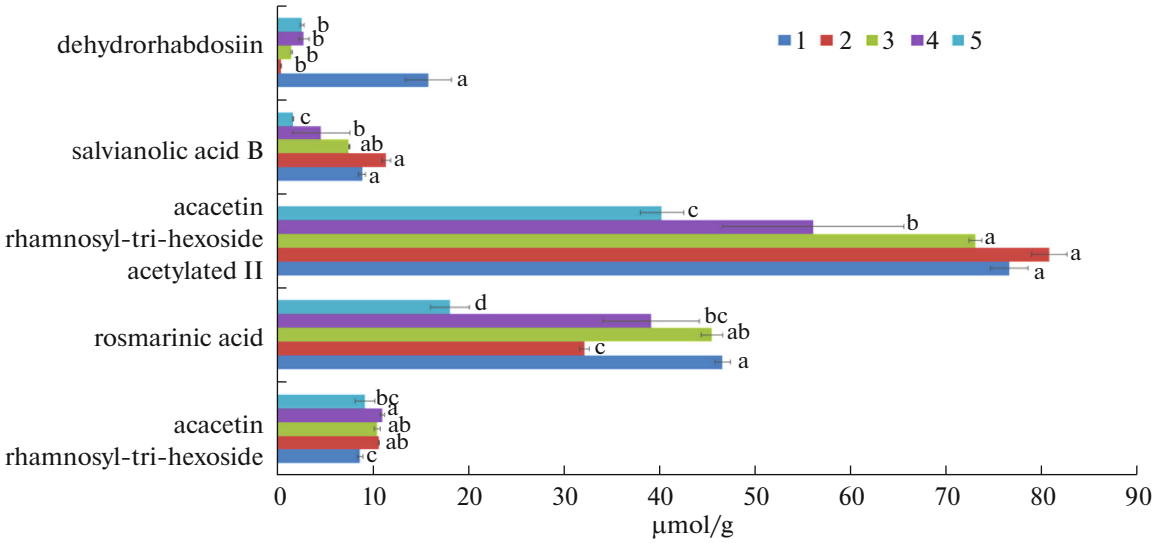

Fig. 2. Distribution of predominant polyphenol compounds in the leaves of *Dracocephalum charkeviczii* in different phenological stages: (1, 2) plants from the natural population and cultivated plants prior to flowering (stage of vegetation), respectively; (3, 4) plants from natural population and cultivated plants in the stage of flowering/start of fructification; (5) cultivated plants in the stage of mature fructification and preparation for withering.

and rosmarinic acid (11). The content of these compounds was ten and more times greater than that of other substances (Figs. 2, 3).

In cultivated plants of *D. charkeviczii*, we observed changes in secondary metabolite concentration associated with phenological stages. For instance, the production of three phenolic acids—two caffeoylquinic (1 and 4) and caffeic (5)—at the end of vegetation decreased by 40, 45, and 64% (Fig. 3), respectively, as compared with their synthesis at the beginning of vegetation. Production of quercetin rutinoside (6) and quercetin hexoside (7), the same as rhabdosiin (9), showed an identical pattern, with the latter lacking in the samples by the end of vegetation. The concentration of acetylglycosylated acacetin (13) and salvianolic acid (15) was the greatest at the beginning of vegetation and decreased by its end 2 and 6.6 times, respectively. The concentration of glycosylated acacetin (10) changed insignificantly. Synthesis of rosmarinic acid (11) also increased during the stage of flowering and decreased

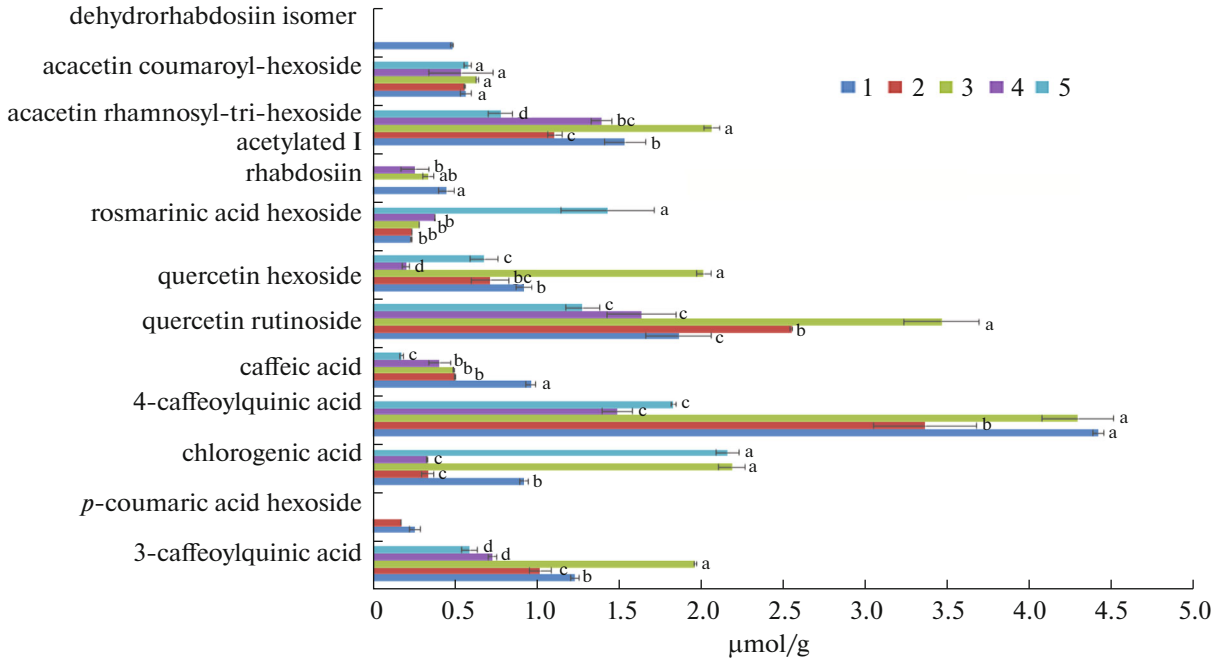

Fig. 3. Distribution of minor polyphenol compounds in the leaves of *Dracocephalum charkeviczii* in different phenological stages. (1, 2) plants from natural population and cultivated plants prior to flowering (stage of vegetation), respectively; (3, 4) plants from natural population and cultivated plants in the stage of flowering/start of fructification; (5) cultivated plants in the stage of mature fructification and preparation for withering.

2.6 times by the end of vegetation. A similar trend was previously observed in *Rosmarinus officinalis* [26]. It was shown that the accumulation of phenol compounds (carnosol, rosmarinic acid, and carnosic acid) was the greatest at the stage of budding and full blossom.

The synthesis of chlorogenic acid (3) in the leaves of *D. charkeviczii* showed an opposite dynamics: it increased 6.4 times by the end of the season. Another species of the genus *Dracocephalum (D. kotschyi)* showed a linear increase in the content of a different class of compounds: methoxylated flavonoids [15]. The authors related this elevation to a gradual temperature rise during the seasonal changes that are considered favourable to production and accumulation of flavonoid aglycons.

In our investigation, synthesis of acetylglycosylated acacetin (12) increased in the stage of flowering with a subsequent decline by the end of vegetation. A similar pattern was previously observed in respect of flavones, flavonols, and essential oil in *D. moldavica* [16, 27]. The authors related a high concentration of polyphenols (phenolic diterpenes) in *Rosmarinus officinalis* in early stages of leaf growth to intense cell divisions observed at that time [28].

As to quercetin hexoside (7) and 4-caffeoylquinic acid (4) concentrations of which in the leaves of *D. charkeviczii* declined during flowering (Fig. 3), it is possible that these compounds may move to other organs. A similar process was observed in *Rosmarinus officinalis* for rosmarinic acid [28]. Its content was the highest in the early stages of leaf growth but sharply decreased when the leaves became 10–15 mm long, probably due to its migration to younger leaves. At the same time, we observed an opposite pattern in our experiment with *D. charkeviczii* with the concentration of rosmarinic acid (11) being the highest in the stage of flowering. This fact may be explained by an active development of leaves upon the formation of inflorescences.

In this work, the concentrations of the majority of secondary metabolites in plants of *D. charkeviczii* collected in the natural population were higher than that in plants taken from the plantation. The content of dehydrorhabdosiin (16) was high in at the beginning of vegetation stage in plants from the natural population and exceeded its concentration in cultivated plants by 37 times. By the time of flowering, we observed a sharp (tenfold) decrease in its concentration. Rhabdosiin (9) and glycosylated acacetin (10) was absent in the leaves of cultivated plants in the beginning of vegetation stage, then was detected in the middle of vegetation and disappeared by the end of it. In plants from natural population rhabdosiin was found at all stages of development. Similar results were obtained on essential oil production in *D. moldavica* plants grown in the field (0.37–0.63%); its content was higher than the content in the green-house plants (0.17–0.24%) [16].

Higher concentrations of compounds in plants taken from natural populations may be explained by a difference in growth conditions. Plants cultivated on a plantation were better supplied with substances necessary for development than wild plants. It is known that the synthesis of secondary metabolites in plants is

more intense under stress conditions [29]. Since the natural population of *D. charkeviczii* inhabits a coastal territory with a saline soil and is exposed to specific winds and other negative factors, it is obvious that the accumulation of secondary metabolites therein may be activated for adaptation to environmental conditions [17]. Previous investigation of the essential oil content in *D. moldavica* in different stages of development showed that the productivity of plants, the content of essential oil, and its composition may depend on stages of growth as well as on environmental and climatic conditions [27]. The disparity in production of compounds in plants from different habitats may be related to differing light conditions. For instance, *D. kotschyi* plants from xeric areas with high illumination intensity accumulated the highest content of methoxylated flavonoids [30].

Our data suggest that the highest antioxidant activity may concur with the highest concentrations of metabolites in plants observed in the stage of flowering and fruit-setting. Similar conclusions were previously made in respect to *D. moldavica* [16]. It was shown that an optimal harvest time was the stage of flowering when the level of essential oil was the highest and, therefore, the content of main terpenes is the greatest. At the same time, it was found that the peak of the secondary metabolite content in *D. kotschyi* occurred in the stage of fructification [15]. In this period, the collection of herbs for medicinal purposes would not cause damage to seed scattering and self-propagation of rare *D. kotschyi* plants in the natural habitat. For *D. charkeviczii,* the best harvest time is the end of June and July when the content of compounds is the greatest as it had been recommended for *D. moldavica* [16]. However, the seeds are still immature in this period and mass collection of the herb may cause an exhaustion of natural populations. Since the cultivated plants did not greatly differ from wild plants in concentration of secondary metabolites, we recommend setting up plantations for growing herbs.

CONCLUSIONS

Current biotechnological investigations of the genus *Dracocephalum* in respect to the production of secondary metabolites (mostly polyphenols with a strong antioxidant activity) have shown that *D. charkeviczii* plants are promising. A wide variety of biologically active polyphenol compounds present therein offers opportunities for the creation of new preparations from the leaves of *D. charkeviczii* collected from the beginning of the vegetation period to the middle of July when the plants bloom and start to fructify. A comparable content of secondary metabolites in *D. charkeviczii* plants taken from the natural population and grown on a plantation makes it possible to recommend their cultivation for medicinal purposes, which will preserve natural populations from extermination.

SUPPLEMENTARY INFORMATION

The online version contains supplementary material available at https://doi.org/10.1134/S1021443723603129.

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ETHICS APPROVAL AND CONSENT TO PARTICIPATE

This work does not contain any studies involving human and animal subjects.

CONFLICT OF INTEREST

The authors of this work declare that they have no conflicts of interest.

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