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Seasonal Dynamics of Riccardin C Accumulation in *Primula macrocalyx* Bge.

YU. S. KOSENKOVA[1], M. P. POLOVINKA[1], N. I. KOMAROVA[1], D. V. KORCHAGINA[1], N. YU. KUROCHKINA[2], V. A. CHEREMUSHKINA[2] and N. F. SALAKHUTDINOV[1]

[1]*Vorozhtsov Novosibirsk Institute of Organic Chemistry, Siberian Branch of the Russian Academy of Sciences, Pr. Akademika Lavrentyeva 9, Novosibirsk 630090 (Russia)*

E-mail: kosen@nioch.nsc.ru

[2]*Central Siberian Botanical Garden, Siberian Branch of the Russian Academy of Sciences, Ul. Zolotodolinskaya 101, Novosibirsk 630090 (Russia)*

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Abstract

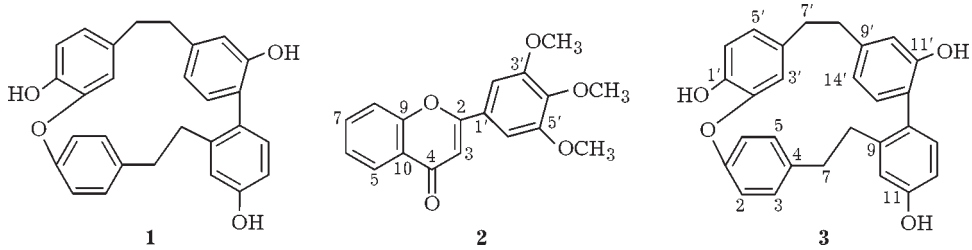
Seasonal dynamics has been studied for a metabolite accumulation in *Primula macrocalyx* Bge. with respect to its major component, bisbibenzyl riccardin C **1**, an inhibitor of inducible NO synthase. Seven introduced populations of the plant have been investigated. A wild-growing *P. macrocalyx* population was used as a reference. It has been demonstrated the harvesting of plant raw material for the purpose of bisbenzyl 1 isolation is appropriate for carrying out after plant flowering, during the fruiting stage. Besides, in the course of the investigation there were 3′, 4′, 5′-trimethoxyflavone **2** and perrottetin E **3** isolated from the acetone extract of *P. macrocalyx*.

Key words: *Primula macrocalyx* Bge., riccardin C, isolation, bioaccumulation, population, 3′, 4′, 5′-trimethoxyflavone, perrottetin E

INTRODUCTION

Primula macrocalyx Bge. (Primrose sp.) represents a herb growing in southern areas of the Western and Central Siberia, as well as in Southern Europe. In order to isolate the secondary metabolites of *P. macrocalyx* we had earlier performed a successive infusing of the aerial part of the plant in hexane and acetone and demonstrated that the major component of the acetone extract represents riccardin C **1** (Scheme 1) [1]. It is known [2], that riccardin C **1** exhibits the properties of inducible NO synthase (iNOS) inhibitor reducing the production of nitrogen oxide in macrophages. The importance of such bioactivity is indicated by the fact that the accumulation of excess NO amount in an organism results in the occurrence of quite a number of pathological states, for example septic shock, neurodegenerative diseases, acute and chronic inflammatory processes [3].

Though metabolite **1** exhibits insignificant inhibiting activity, its methyl ethers are highly active [4]. In connection with the aforesaid we had worked out a technique for isolation riccardin C **1** from aerial and underground parts



Scheme 1.

of wild *P. macrocalyx* [5] for carrying out of its chemical modification and obtaining the derivatives exhibiting the properties of NO synthase inhibitors.

However to solve the synthetic problems posed with the use of riccardin C **1**, isolated only from wild-growing *P. macrocalyx* was impossible due to a low content of compound **1** in the plant. Besides, gathering the underground part means the extermination of the population, which is extremely undesirable. *P.macrocalyx* can be good introduced; due to this the gathering of raw material is appropriate for performing on cultural areas, with prior carrying out a tentative estimation of the quantitative content of riccardine C in plants according to the phases of vegetation.

RESULTS AND DISCUSSION

In order to solve the problem posed, seeds had been gathered in naturally occurring cenotic populations of this species, which seeds were sowed on an experimental plot of the Central Siberian Botanical Garden (CSBG), SB RAS (cenotic populations have been named according to the places where seeds were gathered). Further, the accumulation of compound **1** in the aerial part of plants throughout the seasons was studied. Within two years we performed the analysis of the content of riccardin C in the aerial part of seven introduced and one wild-growing populations of *P. macrocalyx* (Table 1). The gathering of raw material was carried out within the spring-and-summer period with taking into account a stage of a plant development (from the beginning of vegetation up to fructification). Air-dry raw material was extracted *via* shaking of weighed sample portions in acetone, further the extract obtained was centrifuged and then it was analyzed employing an HPLC technique with preliminary quantitative calibration with respect to riccardine C.

As biennial studies have demonstrated, riccardin C **1** is gradually accumulated in the course of vegetation. Within the period of budding there is no riccardin C revealed in the aerial part of *P. macrocalyx*; this substance appears within the plant flowering period a plant being accumulated during the fructification period. So, in the course of the flowering the content of riccardin C **1** in acetoneextracts ranges from 0.05 to 0.17 mass % with respect to air-dry raw material. In the course of fructification, there is an accumulation of compound **1** observed, from 0.18 to 0.40 mass % (see Table 1). According to HPLC data, the content of riccardin C **1** within the fructification period either remains almost at the same level as within the flowering period (for example, population "Yabogan"), or exhibits a 3–4-fold increase (Anos-2). One could explain the results obtained by a small sampling of plants for the analysis as well as by an individual variability in the population.

TABLE 1

Seasonal content of riccardin C in leaves of *P. macrocalyx*, %

Populations	Phenological phases					
	Budding		Flowering		Fructification	
	2007	2008	2007	2008	2007	2008
Kamenushka (wild-growing plants)*	0	0	0.02	0.05	0.19	0.22
Kamenushka*	0	0	0.08	0.10	0.20	0.23
Kamenushka**	0	0	0.10	0.10	0.19	0.24
Yabogan*	0	0	0.12	0.17	0.18	0.20
Yabogan**	0	0	0.12	0.13	0.20	0.18
Gorno-Altaisk**	0	0	0.07	0.05	0.20	0.24
Anos-1**	0	0	0.05	0.06	0.32	0.30
Anos-2**	0	0	0.07	0.11	0.4	0.3

* Plants were transferred directly from the nature.

** Plants were grown from the seeds gathered in the nature.

It should be noted that the gathering of raw material is impeded by the fact that, first, the fructification for different individuals could differ in time a little; second, the aerial part of a plant in this stage uses to die off quickly. In this connection the gathering of an aerial part it is the most appropriate for carrying out right after the flowering period. So, the content of riccardin C **1** in the wild-growing raw material harvested, as a rule, in Altai, usually amounts to 0.1–0.15 % with respect to the mass of dry raw material. Usually, one can isolate about 90 % with respect to this amount [5].

In the course of studying the seasonal accumulation of riccardin C in aerial part of *P. macrocalyx* we carried out the harvesting of plant raw material from introduced populations in the budding stage, which raw did not contain compound **1** and further was used in our studies for the isolation of other secondary metabolites. Owing to a high content of lipophilic substances in the plant, the extract was subjected to additional processing similar to that described in [6]. After doing this, the extract was chromatographed on silica gel. As a result, we have isolated 3′,4′,5′-trimethoxyflavone **2** (see Scheme 1). This compound had already been isolated from *Primula veris* [7] earlier, and the ^{1}H NMR spectral data we have obtained exhibit a good coincidence with literature data.

Moreover, from the acetone extract obtained from the aerial part of wild-growing *P. macrocalyx,* we have isolated compound **3** which basing on ^{1}H and ^{13}C NMR spectral data was ascribed with the structure of perrottetin E. This compound was described earlier in [8]. The ^{1}H NMR spectral data obtained in our studies are close to those presented in the literature. As against a simple listing of chemical shifts in [8], we have performed a complete reference of signals for ^{13}C NMR spectrum. At the same time, the values of chemical shifts we have obtained appeared to be close to the data from [8]. It is mentioned in [9], that perrottetin E exhibits fungicidal properties. Just as riccardin C **1**, perrottetin E **3** belongs to bisbenzyls; it was not isolated earlier from higher plants.

EXPERIMENTAL

High performance liquid chromatography (HPLC) analysis was performed employing a Milichrom A-02 microcolumn chromatograph (EcoNova Co., Novosibirsk) with the use of standard chromatographic columns (2 × 75 mm) packed with a reverse - phase sorbent (Prontosil 120-5-C18, particle size 5 μm, Bischoff, Germany). We used a gradient elution mode with simultaneous multi-wavelength detection at six wavelength values (λ = 220, 240, 260, 280, 320, 360 nm) [12]. The eluent was methyl alcohol with 0.1 % of trifluoroacetic acid (TFA). The gradient amounted to 0–30 % of methanol, 0.1 % of TFA for 5 min, then 30–50 % of methanol, 0.1 % of TFA for 5 min, then 50–70, 70 %, 0.1 % of TFA for 10 min, 70–90, 90 %, 0.1 % of TFA for 10 and 5 min up to pure methanol. The temperature being of 35 °C, pressure 30–36 atm, the flow rate being 150 μL/min.

^{1}H and ^{13}C NMR spectra were recorded on a Bruker DRX-500 spectrometer (500.13 and 125.76 MHz, respectively) for substances dissolved in $CDCl_3$. As the internal standard we used the signals of chloroform (δ_H 7.24 ppm, δ_C 76.90 ppm).

The structure of the compounds isolated was established with the help of NMR method basing on the analysis ^{1}H NMR spectra with the use of ^{1}H–^{1}H double resonance spectra and two-dimensional proton-proton homonuclear correlation spectra, as well as the analysis ^{13}C NMR spectra recorded in *J*-modulation mode (JMOD), with off-resonance proton signal suppression with the use of two-dimensional ^{13}C–^{1}H heteronuclear correlation spectra for direct (C–H COSY, $^1J_{C,H}$ 160 Hz) and long-range spin-spin coupling constants (COLOC, $^{2,3}J_{C,H}$ 10 Hz).

Column chromatography was performed on Merck silica gel 60–200 μm. The analysis of fractions and monitoring the course of the reaction was carried out with the help of thin-layer chromatography technique on Sorbfil plates (PTSH-AF-V-UF, Krasnodar, Russia) in the system of chloroform–ethanol (2–5 % of EtOH).

As a plant raw material we used air-dry aerial and underground part of *P. macrocalyx* from naturally occurring and introduced populations gathered within various vegetation periods.

Isolation of compound 2

The aerial part of *P. macrocalyx* was gathered with no taking into account any popula-

tions at the experimental plot of the CSBG, SB RAS, April 19, 2007. Into a glass vessel of 1 L capacity was loaded 120 g of grinded raw material, then it was flooded with 1 L of hexane. We infused the matter fourfold for 12 h each time until disappearing a bright-green colouring of the solution. The mass of joined hexane extract after the removal of solvent amounted to 0.90. Further we continued infusing in acetone. We filled in 1 L acetone and infused the mixture fourfold for 12 h each time up to a significant reduction of the intensity of solution colouring. The mass of the acetone extract after the removal of solvent amounted to 1.35 g. The extract obtained was treated with 30 mL of hexane. Green hexane solution was successively extracted by 5 % HCl solution (50 mL × 2), then it was washed by 30 mL of water, by 5 % $NaHCO_3$ solution (50 mL × 2) and once again by water. The organic layer was dried over calcined $MgSO_4$. The mass of the extract after the treatment and removal of solvent amounted to 0.98 g.

Further the extract was separated using a column with silica gel (with the ratio extract : SiO_2 = 1 : 30, hexane as an eluent with a gradient of $CHCl_3$ from 70 up to 100 %). As the result of chromatography we have isolated 5 mg of substance **2**. The acetone extract of springtime raw material did not contain riccardin C. The content of the substance **2** amounted to 0.004 % with respect to the mass of dry raw material.

^{1}H NMR spectrum of trimethoxyflavone **2**, δ, ppm (*J*, Hz): 3.92 (3H, s, OCH_3–C4′), 3.95 (6H, s, OCH_3–C3′, OCH_3-C5′), 6.76 (1H, s, H-3), 7.13 (2H, s, H-2′, H-6′), 7.41 (1H, d. d, $J_{6,5}$ 8.1, $J_{6,7}$ 7.2, H-6), 7.56 (1H, d, $J_{8,7}$ 8.4, H-8), 7.69 (1H, d. d. d, $J_{7,8}$ 8.4, $J_{7,6}$ 7.2, $J_{7,5}$ 1.7, H-7), 8.22 (1H, d. d, $J_{5,6}$ 8.1, $J_{5,7}$ 1.7, H-5). The data of ^{1}H NMR spectrum trimethoxyflavonea **2** are close to the corresponding data resulted for this connection in work [7].

Isolation of compound 3

The air-dry aerial part of *P. macrocalyx* (250 g), harvested in August 2008 in Altai, was subjected to successive boiling with hexane and acetone. As a result we have obtained hexane (1.75 g) and acetone (9.98 g) extracts. The acetone extract was chromatographed employing a column with silica gel, with adding neutral aluminium oxide on the top the layer with the thickness of 3 cm (with the ratio extract : silica gel = 1 : 10). As a result, we have isolated the fraction (1.11 g) enriched with riccardin C and the substance similar to the riccardin C in spectral characteristics. The fraction was twice rechromatographed using a column with silica gel, the eluent being hexane with an increasing gradient of chloroform (up to 100 %). Thus, we have isolated 0.38 g of riccardin C and 0.05 g of compounds **3**. The latter, basing on ^{1}H, ^{13}C NMR spectral data and the comparison with literature data was ascribed with the structure of perrottetina E [8]. The content of substance **3** with respect to the mass of dry raw material amounted to 0.02 %.

^{1}H NMR spectrum of perrottetin E **3**, δ, ppm (*J*, Hz): 2.74 (4H, w.s, H-7, H-8), 2.81–2.89 (4H, m, H-7′, H-8′), 6.83 (2H, d, *J* 8.6, H-2, H-6), 7.08 (2H, d, *J* 8.6, H-3, H-5), 6.63 (1H, m, H-10), 6.66 (1H, d. d. d, $J_{12,13}$ 7.8, $J_{12,10}$ 2.5, $J_{12,14}$ 0.8, H-12), 7.12 (1H, t, $J_{13,12} = J_{13,14}$ = 7.8, H-13), 6.73 (1H, d. d. d, $J_{14,13}$ 7.8, $J_{14,10}$ 1.5, $J_{14,12}$ 0.8, H-14), 6.60 (1H, d, $J_{3',5'}$ 2.0, H-3′), 6.81 (1H, d. d, $J_{5',6'}$ 8.2, $J_{5',3'}$ 2.0, H-5′), 6.92 (1H, d, $J_{6',5'}$ 8.2, H-6′), 6.57 (1H, d. d, $J_{10',12'}$ 2.4, $J_{10',14'}$ 1.7, H-10′), 6.64 (2H, m, H-12′, H-14′), 7.07 (1H, t, $J_{13',12'} = J_{13',14'}$ = 7.8, H-13′), 5.67 (1H, br. s) and 5.78 (2H, br. s) – three OH groups).

^{13}C NMR spectrum, δ, ppm: 154.72 (s, C-1), 117.66 (d, C-2, C-6), 129.64 (d, C-3, C-5), 136.64 (s, C-4), 36.59 (t, C-7), 37.55 (t, C-8), 143.28 (s, C-9), 115.39 (d, C-10), 155.53 (s, C-11), 112.81 (d, C-12), 129.34 (d, C-13), 120.68 (d, C-14), 145.21 (s, C-1′), 143.13 (s, C-2′), 118.64 (d, C-3′), 134.04 (s, C-4′), 124.22 (d, C-5′), 115.76 (d, C-6′), 36.69 (t, C-7′), 37.70 (t, C-8′), 143.21 (s, C-9′), 115.31 (d, C-10′), 155.51 (s, C-11′), 112.74 (d, C-12′), 129.28 (d, C-13′), 120.68 (d, C-14′) [8].

*Seasonal riccardin C **1** accumulation in the aerial part*

Seeds and planting stock of *P. macrocalyx* for the introduction have been gathered in the vicinities of the Gorno-Altaisk and in the vicinities of the Anos village (the Shebalino Dis-

trict) in July, 2004; near to the Yabogan Pass in August, 2005 (Republic Altai), as well as in the vicinities of the Kamenushka settlement (Novosibirsk Region) in August, 2005. Plant material for the extraction from introduced populations of *P. macrocalyx* were gathered in the course of the vegetative season in 2007–2008.

Sample preparing and analysis

For the extraction we took 10 mg of the grinded air-dry raw material of each sample. Each sample was infused in acetone (1 mL, 2 h, shaking, centrifugation), five-fold. After joining the five extracts of each sample together into one test tube, acetone was blown off by the flow of argon. Then, after into each test tube were added 500 μL MeOH, we sampled portions of 10 μL in volume for chromatography (HPLC). Basing on data concerning the mass extinction coefficient for riccardin C **1** (total light absorption by the solution of the substance with the concentration of 1 g/L at the length of optical path equal to 1 cm at a preset wavelength) together with HPLC data one could calculate the mass of riccardin C in precisely weighed portion of a plant raw material. Employing this technique we have determined the mass extinction coefficient at the wavelength of 210 nm for the sample of riccardin C with the purity level not less than 99.85 %, whose value amounted to 18.88 arb. units/mg. The calculation of the quantitative content of riccardin C in the extracts was carried out basing on the peak area in HPLC chromatographic profiles for corresponding extracts.

CONCLUSION

The present work represents a continuation of the studies on chemical composition of *Primula macrocalyx* Bge. From the aerial part the plants, we have isolated compounds **2**, **3**, the perrottetin E **3** being for the first time isolated from higher plants. The seasonal dynamics of accumulation riccardin C **1**, a phytogenous iNO synthase inhibitor, has been studied depending on a stage of plant development. It has been demonstrated that the content of riccardin C **1** in the aerial part reaches a maximum during the fructification period.

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