

## Proanthocyanidins in *Rhodiola kirilowii* and *Rhodiola rosea* callus tissues and transformed roots – determination with UPLC-MS/MS method

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## S u m m a r y

Several species of *Rhodiola* genus (*Crassulaceae* family) like *Rhodiola kirilowii* and *Rhodiola rosea* are used in official or traditional medicine. The aim of this study was to determine qualitative and quantitative content of proanthocyanidins using ultra performance liquid chromatograph connected to a tandem mass spectrometer (UPLC MS/MS method) in the callus tissues and in the transformed roots (infected by *Agrobacterium rhizogenes* LBA 9402 strain) of *R. kirilowii* and *R. rosea*. This validated assay allows to determine the content of five flavan-3-ols: (+)-catechin, (-)-epicatechin, (-)-epigallocatechin, (-)-epicatechin gallate (ECG), (-)-epigallocatechin gallate (EGCG). Our results concerning the material from *in vitro* cultivation of *R. kirilowii* and *R. rosea* indicate that *R. rosea* callus can be a better source of catechin when compared with other tested materials, especially when the content of (-)-gallate epigallocatechin is taken under consideration (3.429 mg/100 g of dry powdered material). The application of UPLC MS/MS method allowed to determine the content of proanthocyanidins in tested samples with satisfactory precision and can be used in the phytochemical investigations of *Rhodiola* sp. *in vitro* cultivated tissues.

**Key words:** *Rhodiola kirilowii*, *Rhodiola rosea*, callus tissues, transformed roots, proanthocyanidins, flavan-3-ol content, UPLC-MS/MS method, (-)-epigallocatechin gallate (EGCG)

## INTRODUCTION

*Rhodiola* genus (*Crassulaceae* family) consists of more than 50 species. Some of them are used in official or ethnomedicine, like *Rhodiola kirilowii* and *Rhodiola rosea*. *R. kirilowii* grows in Asian mountains and is used in traditional medicine, for example to prevent damages due to hypoxic environment of high altitude [1]. Among others, the roots contain phenyletanoids: p-tyrosol and salidroside [2, 3], proanthocyanidins

[3-6], phenolic acids [3, 4, 6, 7]. *R. rosea*, growing in Asia, North America and European mountains, is used in supplements to enhance physical and mental work efficiency mainly [8, 9]. The roots contain phenyletanoids: *p*-tyrosol and salidroside [10, 11] and phenylpropanoids (rosavins) [11] as main active substances. There are also reports about high molecular weight polymeric proanthocyanidins in *R. rosea* [12].

The mentioned proanthocyanidins (flavan-3-ols), like catechin, epicatechin, epigallocatechin, epicatechin gallate, epigallocatechin gallate, exhibit antioxidative properties and can protect the organism against harmful effect of free radicals and influenced reactive oxygen forms [13]. According to the fact, that supplements containing *R. rosea* extract are recommended to protect the organism against many pathogenic agents, as well as that use of *R. kirilowii* extract in the Far East ethnomedicine is correlated with protection of human organisms against high altitude reactions, the determination of proanthocyanidins in *Rhodiola* genus could be helpful to explain the activities of the extracts. By the reason of valuable properties of this group of compounds, the main aim of presented study was to determine proanthocyanidins in the material from *in vitro* cultivation (in comparison with roots from field cultivation).

The *in vivo* and *in vitro* cultivation of *Rhodiola* species are carried out in the Institute of Natural Fibres and Medicinal Plants in Poznan and in the Department of Biology and Pharmaceutical Botany, Warsaw Medical University for several years. In some previously published articles we presented the presence of proanthocyanidins in *R. rosea* and *R. kirilowii* roots and callus tissues [4, 5, 7, 14-19]. In this study the qualitative and quantitative determination of proanthocyanidins using ultra performance liquid chromatograph coupled with tandem mass spectrometer (UPLC MS/MS method) in callus tissues and in transformed roots of *R. kirilowii* and *R. rosea* is presented.

## MATERIAL AND METHODS

### Plant material

Investigations were carried out on five kind of plant material: *Rhodiola kirilowii* and *Rhodiola rosea* roots from field cultivation, *Rhodiola kirilowii* callus tissues, *Rhodiola rosea* callus tissues and *Rhodiola kirilowii* transformed roots.

### Roots from field cultivation

The roots of *R. kirilowii* (Regel.) Maxim and *R. rosea* L. were harvested from field cultivation (in the Garden of Medicinal Plants of the Institute of Natural Fibres and Medicinal Plants in Plewiska near Poznań) in October 2009. The roots were dried in the room temperature (about 22–24°C).

## Callus tissues

The callus tissues cultured on solid medium were used in the experiments. Plant material originated from the Garden of Medicinal Plants, Institute of Natural Fibres and Medicinal Plants, Poznań. The callus of *R. kirilowii* was obtained from the cotyledone of sterile seedling; the callus of *R. rosea* was obtained from hypocotyl of the sterile seedling. Tissues were cultivated on the modified Murashige-Skoog (MS) medium [20] supplemented with  $\alpha$ -naphthaleneacetic acid (NAA), benzyladenine (BA) and adenine chloride. The cultures were maintained at  $23 \pm 2^\circ\text{C}$  using fotoperiod – night: 8 h, day: 16 h. The cultures were subcultured every 3–4 weeks. The calluses were collected and air dried ( $25^\circ\text{C}$ ) after 5 weeks of cultivation.

## Hairy root culture

Hairy root culture of *Rhodiola kirilowii*, established in the Department of Biology and Pharmaceutical Botany, Warsaw Medical University, was derived from a single root developed at the wounded site of the internodal shoot segment, as it was described previously [21]. *Agrobacterium rhizogenes* LBA 9402 strain was used to infect the plant explants. The hairy roots were cultivated in 250 ml Erlenmeyer flasks containing 30 mL of hormone-free DCR-M medium. This was the medium used by Gupta and Durzan [22] with the content of  $\text{MgSO}_4$  increased to  $400 \text{ mg/l}^{-1}$  [23], and with the addition of  $500 \text{ mg/l}^{-1}$  of L-glutamine. The culture was maintained at  $25^\circ\text{C}$  in the dark on a GioGyrotorys Shaker (New Brunswick Scientific Co.) at 122 rpm. The used hairy root line has been investigated for integration of the bacteria DNA into the *R. kirilowii* genome, as described by Zych et al. using PCR reaction [21]. The content of secondary metabolites was determined in powdered lyophilized tissue of transformed roots.

## Standard substances

The following comparison substances were used in the experiment: (+)-catechin, (-)-epicatechin, (-)-epigallocatechin, (-)-epicatechin gallate, (-)-epigallocatechin gallate (ChromaDex) and D-(-)-salicine (SIGMA).

## Preparation of test samples: extraction of flavan-3-ols (proanthocyanidins) from dry plant materials

The method of flavan-3-ol extraction by P. Mammela [24] from roots and extracts was used. An exact amount of ca. 0.5 g of dried powdered (0.315) *R. kirilowii*

roots, an exact amount of ca. 0.75 g of dried powdered (0.315) *R. rosea* roots, an exact amount of ca. 2.5 g of dried powdered (0.315) *R. kirilowii* or *R. rosea* callus tissue and an exact amount of ca. 0.25 g of dried powdered (0.315) *R. kirilowii* transformed roots were weighed out and placed in a 20 ml volumetric flasks. Methanol in the amount of 15.0 ml of 80% (v/v) was added and the solutions were subjected to ultrasounds for 60 min at a room temperature (20–25°C). Then the solutions were made up to the mark with the same solvent and filtered on a quantitative filter paper. The filtrates were concentrated to evaporate the methanol up to a volume of about 1/5 in a rotary evaporator in vacuum. The residues were extracted with 4 × 16.0 ml of diethyl ether. The combined ether extracts were dried with anhydrous sodium sulphate and evaporated to dryness in a rotary evaporator in vacuum. The dry residues were dissolved in 4.0 ml of 10% (v/v) methanol and then quantitatively transferred to proper volumetric flasks (in the case of callus tissues 2 ml volumetric flasks were used; in the case of roots or transformed roots 5 ml volumetric flask was used). D-(-)-salicine (IS) in amount of 0.023 ml of 0.5 mg/ml was added to every flasks and the solutions were made up to the mark with 10% (v/v) methanol. The samples were filtered through a membrane filter with a diameter of 0.20 µm.

### LC-MS/MS assay

The validated assay using an ultra performance liquid chromatograph coupled with a tandem mass spectrometer (UPLC-ESI MS/MS; Waters) was worked out in Institute of Natural Fibres and Medicinal Plants [34]. The preparation of calibration curves for flavan-3-ols, the evaluation for precision, linearity and accuracy of this analytical method was described by Gryszczyńska et al. [16].

### Statistical analysis

The study results were statistically verified, determining the relative standard deviation (RSD), n=6.

## RESULTS AND DISCUSSION

The content of flavan-3-ols was investigated in roots, callus tissues and transformed roots (hairy roots) of *R. kirilowii* and in roots and callus tissues of *R. rosea*. The method worked out in the Institute of Natural Fibres and Medicinal Plants UPLC MS/MS [16] allows to determine the content of five flavan-3-ols: (+)-catechin, (-)-epicatechin, (-)-epigallocatechin, (-)-epicatechin gallate (ECG), (-)-epigallocatechin gallate (EGCG). The MRM chromatograms (Multiple Reaction Monitoring) showing fragmentation of flavan-3-ols from *Rhodiola kirilowii* callus tissue and transformed

roots are demonstrated on figures 1 and 2, whereas the MRM chromatograms concerning fragmentation of flavan-3-ols from *Rhodiola rosea* callus tissue are shown on figure 3. The obtained quantitative results are presented in the tables 1 and 2.

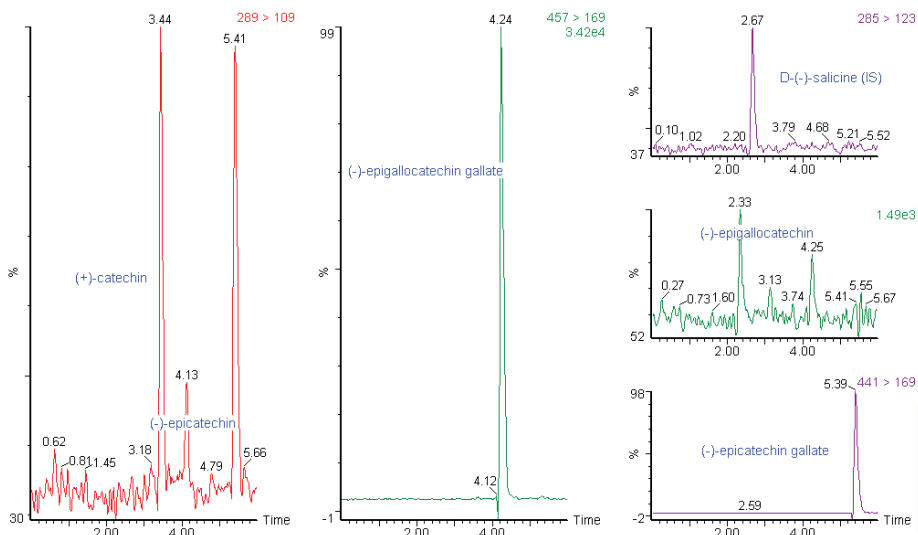


Figure 1.

The MRM chromatogram showing the fragmentation of flavan-3-ols from *Rhodiola kirilowii* callus tissue

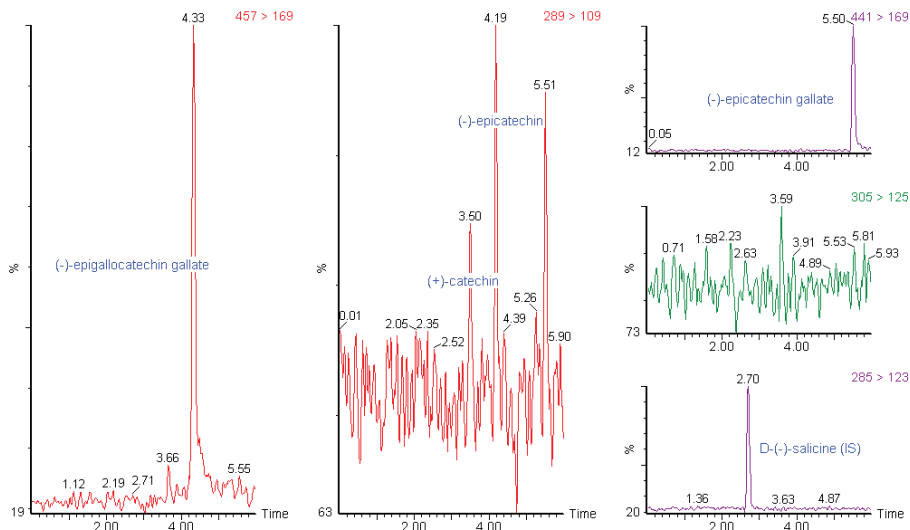


Figure 2.

The MRM chromatogram showing the fragmentation of flavan-3-ols from *Rhodiola kirilowii* transformed roots

Table 1.

Content of catechins in *Rhodiola kirilowii* roots, callus tissues and transformed roots

Sample	(+)-Catechin		(-)-Epicatechin		(-)-Epigallo-catechin		(-)-Gallate epicatechin		(-)-Gallate epigallocatechin	
	content [mg/100 g] <sup>1</sup>	RSD [%] <sup>*</sup>	content [mg/100 g] <sup>1</sup>	RSD [%] <sup>*</sup>	content [mg/100 g] <sup>1</sup>	RSD [%] <sup>*</sup>	content [mg/100 g] <sup>1</sup>	RSD [%] <sup>*</sup>	content [mg/100 g] <sup>1</sup>	RSD [%] <sup>*</sup>
<i>R. kirilowii</i> roots	0.097	2.06	0.288	2.08	19.584	7.53	5.294	4.55	135.435	2.00
<i>R. kirilowii</i> callus tissues	0.039	0.00	0.013	7.69	0.007	14.29	0.309	4.85	0.465	3.87
<i>R. kirilowii</i> transformed roots	0.014	3.18	0.009	5.27	N/o	–	0.251	4.01	0.154	5.71

\* – RSD – relative standard deviation (n=6)

<sup>1</sup> – the content in 100 g of powdered dry material

Table 2.

Content of catechins in *Rhodiola rosea* roots and callus tissues

Sample	(+)-Catechin		(-)-Epicatechin		(-)-Epigallo-catechin		(-)-Gallate epicatechin		(-)-Gallate epigallocatechin	
	content [mg/100 g] <sup>1</sup>	RSD [%] <sup>*</sup>	content [mg/100 g] <sup>1</sup>	RSD [%] <sup>*</sup>	content [mg/100 g] <sup>1</sup>	RSD [%] <sup>*</sup>	content [mg/100 g] <sup>1</sup>	RSD [%] <sup>*</sup>	content [mg/100 g] <sup>1</sup>	RSD [%] <sup>*</sup>
<i>R. rosea</i> roots	0.051	3.92	0.135	1.48	8.526	1.77	0.437	2.29	36.873	2.17
<i>R. rosea</i> callus tissues	0.007	14.29	0.015	0.00	0.078	1.28	0.130	2.31	3.429	2.76

\* – RSD – relative standard deviation (n=6)

<sup>1</sup> – the content in 100 g of powdered dry material

The presented results show that callus tissues of both species contain (+)-catechin, (-)-epicatechin, (-)-epigallocatechin, (-)-epicatechin gallate (ECG), (-)-epigallocatechin gallate (EGCG), as it was detected in the plants from field cultivation, although, the contents are smaller than in the intact plants (tab. 1, 2). Two catechins, namely (-)-gallate epicatechin and (-)-gallate epigallocatechin were present in higher amounts in both tested calluses, especially the second compound, when compared with the rest of catechins, the content of which reached 3.429 mg/100 g in callus of *R. rosea*. The *R. kirilowii* callus line tested here – the line from the cotyledon – although produced EGCG in the higher content than the other catechins (0.465 mg/100 g) shows smaller EGCG contents (as was determined by HPLC method) – in comparison with our previous investigations concerning the lines from hypocotyle or roots of the sterile seedlings, cultivated in dark for 7 days [17]. The

results indicate that *R. rosea* callus is the richer source of EGCG than the callus of *R. kirilowii*, on the contrary to the content determined in roots of intact plants – where the content of EGCG is higher in *R. kirilowii* roots [19].

The transformed roots of *R. kirilowii* contain (+)-catechin, (-)-epicatechin, (-)-epicatechin gallate (ECG) and (-)-epigallocatechin gallate (EGCG) and their amount is lower than in the callus tissue of *R. kirilowii*; in this material (-)-gallate epicatechin is the main proanthocyanidin: 0.251 mg/100 g (tab. 1). Therefore it can be summarized that the determined content of searched catechins was smaller in the hairy roots than in the callus tissues of *R. kirilowii*.

## CONCLUSIONS

The results presented above concerning the material from *in vitro* cultivation of *R. kirilowii* and *R. rosea* indicate that *R. rosea* callus can be a better source of catechin as compared with other tested materials, especially if the content of (-)-gallate epigallocatechin is taken under consideration. The application of worked out ultra performance liquid chromatograph connected to a tandem mass spectrometer (UPLC MS/MS method) allowed to determine the proanthocyanidins content in tested samples with satisfactory precision and can be used in the phytochemical investigations of *Rhodiola* sp. *in vitro* cultivated tissues.

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PROANTOCYJANIDYNY W TKANKACH KALUSOWYCH I W TRANSFORMOWANYCH KORZENIACH  
*RHODIOLA KIRILOVII* I *RHODIOLA ROSEA* – OZNACZENIE ZA POMOCĄ METODY UPLC-MS/MS

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## Streszczenie

Niektóre gatunki z rodzaju *Rhodiola* (rodzina *Crassulaceae*), jak *Rhodiola kirilowii* i *Rhodiola rosea*, są stosowane w medycynie oficjalnej lub ludowej. Celem przedstawionych badań było oznaczenie jakościowe i ilościowe proantocyjanidyn w tkankach kalusowych i w transformowanych (za pomocą szczepu *Agrobacterium rhizogenes* LBA 9402) korzeniach *Rhodiola kirilowii* i *Rhodiola rosea* przy zastosowaniu metodyki wykorzystującej ultrasprawy chromatograf cieczowy sprzężony z tandemowym spektrometrem mas (metoda UPLC MS/MS). Ta zawalidowana metodyka pozwoliła na określenie stężeń pięciu flawan-3-oli: (+)-katechiny, (-)-epikatechiny, (-)-epigalokatechiny, galusanu (-)-epikatechiny (ECG) oraz galusanu (-)-epigalokatechiny (EGCG). Przedstawione w pracy wyniki dotyczące materiału pochodzącego z kultur *in vitro* *R. kirilowii* i *R. rosea* wskazują, że kalus *R. rosea* jest lepszym źródłem katechin w porównaniu do pozostałych badanych surowców, szczególnie, gdy bierze się pod uwagę zawartość galusanu epigalokatechiny (3.429 mg/100 g suchego sproszkowanego surowca). Zastosowanie opracowanej metodyki z wykorzystaniem ultrasprawnego chromatografu cieczowego sprzężonego z tandemowym spektrometrem mas pozwoliło z zadawalającą precyzją oznaczyć zawartości proantocyjanidyn w analizowanych próbkach. Metoda ta może być stosowana w fitochemicznych badaniach hodowanych *in vitro* tkanek rodzaju *Rhodiola*.

**Słowa kluczowe:** *Rhodiola kirilowii*, *Rhodiola rosea*, tkanki kalusowe, korzenie transformowane, proantocyjanidyny, zawartość flawan-3-oli, metoda UPLC-MS/MS, galusan epigalokatechiny (EGCG)