Novel method of detection of phenylpropanoids of *Rhodiola* roots species

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Summary

The aim of the study was the identification and quantitative analysis of phenylpropanoid compounds in the roots of *Rhodiola* species. Rosavin, rosarin and rosin were determined in the roots of *R. kirilowii* and *R. rosea* from the field cultivation, Institute of Natural Fibres and Medicinal Plants. For the quantitative analysis, the ultra performance liquid chromatography - tandem mass spectrometry (UPLC-ESI MS/MS, Waters) was used. The results showed differences in the quantitative and qualitative assessments of these two species. In the root of *R. kirilowii* the presence of phenylpropanoids was not confirmed. In *R. rosea* the most common phenylpropanoid was rosavin (0.022%). The UPLC-MS/MS studies allowed to use this analytical method for determination of phenylpropanoids in the accordance with the requirements of ICH.

Key words: *Rhodiola kirilowii, Rhodiola rosea, phenylpropanoids, UPLC-MS/MS*

INTRODUCTION

Rhodiola genus (*Crassulaceae* family) including 90 species, 70 of which grow in China at an altitude of 1000–5600 m a.s.l. [1,2]. Furthermore, the medicinal properties of *Rhodiola* genus are comparable to those of of *Ginseng* and *Acanthopanax*. The advantage of golden root (*Rhodiola rosea*) is the fact that no side effects are observed [2,3]. Among the all species of *Rhodiola* 20 has been used in the traditional medicine of Asia: *R. rosea*, *R. alterna*, *R. brevipetiolata*, *R. crenulata*, *R. kirilowii*, *R. quadrifida*, *R. sachalinensis* and *R. sacra* [4]. It was observed that the chemical composition and physiological properties of the material are related to the species of the plant, although some of them are similar.

Rhodiola plants grow in North Asia and China, but they are also found in the European mountains. Plants of this family like cold climate and places difficult to grow [5]. *R. kirilowii* is a traditional medicinal plant used in Chinese for centuries [6-8]. It is known for both the adaptogenic properties and applications in alleviating the symptoms of hypoxia [6]. For this reason, the ability to use extracts in chronic heart failure and coronary artery disease are still investigated. Among active components isolated from the material, salidroside, tyrosol, daucosterol, β-sitosterol, EGCG, fructopyrano-1,4-glucopyranose and cyanogenic glucoside named lotaustralin have been described. Number of reports on the biological activity of the extracts are still limited.

Health benefits of *Rhodiola rosea* are much better known than the properties of *Rhodiola kirilowii. R. rosea* is used in traditional Chinese medicine in the treatment of nervous system, improvement of sleep and for cardioprotective characteristics [7,9-13]. The roots enhance physical and mental performance, treats fatigue and depression [7,10-12,14]. It is suggested that the effect on the central nervous system is correlated with changes in the level of neurotransmitters such as serotonin and dopamine [15]. However, the mechanism of this effect is not well documented. *Rhodiola rosea* is known to be adaptogen because it increases the resistance to chemical, biological and physical stress. There are also reports indicating both cardioprotective and anticancer effects in the animal model [4]. Therefore, this raw material is used in dietary supplements such as energy drinks.

The literature review indicates nearly 140 active compounds isolated from *R. rosea*. The roots of the plant mainly contains flavonoids (catechins and proanthocyanidins), organic acids (gallic acid, caffeic acid, and chlorogenic acid), tannins and phenolic glycosides. Apart from the antioxidant properties, the *p*-tyrosol also shows little effect of lipoxygenase inhibition *in vivo*. The chemical composition of both *Rhodiola* species roots used in this study was shown in table 1. Researches on rosavin, rosin, rosarin and rosaridin indicate that they influence the central nervous system as well as adaptogenic and immunostimulating activity (fig. 1) [11].

The aim of presented study was to investigate a selective and specific analytical method to designate the contents of individual compounds of phenylpropanoids.

Table 1.

Typical chemical compounds occurring in *Rhodiola kirilowii* and *Rhodiola rosea*

Name of chemical group	Compound	*Rhodiola kirilowii* [8-12]	*Rhodiola rosea* [16,19-21]
Phenylethanoids			+
	Salidroside	+	
	p-Tyrosol	+	
Phenylpropanoids			+
	Rosavin		+
	Rosarin		+
	Rosin		+
Sitosterol			
	Daucosterol	+	
Cyanogenic glucoside			
	Lotaustralin	+	
Nitrile glucosides			
	Rhodiocyanoside A	+	
Polyphenols glycosides			
	Arbutin	+	

Name of chemical group	Compound	*Rhodiola kirilowii* [8-12]	*Rhodiola rosea* [16,19-21]
Flavano-3-ols			
	Epigallocatechine gallate	+	
Flavonoids			+
	Rodiolin		+
	Rodionin		+
	Tricin		+
Monoterpenes			+
Triterpenes			+
Phenolic acids			+

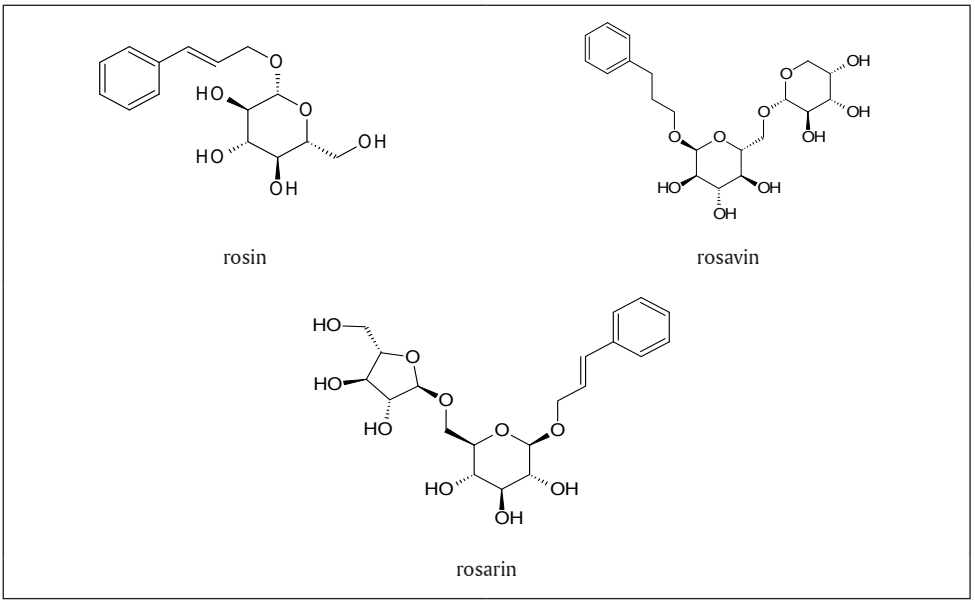

Figure 1.
Structures of phenylpropanoids

MATERIAL AND METHOD

Plant material

In our study *Rhodiola kirilowii* and *Rhodiola rosea* roots were used. Those plants were collected in October 2009 from fields cultivations in the Institute of Natural Fields and Medicinal Plants in Poznań (Plewiska/Poznań). Roots were dried at a room temperature (22–24ºC).

Preparation of plant extracts

Subsequently, dry roots were powdered (0.315) and two kinds of extract were prepared: aqueous extract and 50% (*v/v)* ethanol extract.

Preparation of aqueous extract

The powdered dry roots were extracted with purified water for 3 hours at 90ºC (material to solvent ratio 1:10). After filtering, the extracts were frozen at –55ºC and than lyophilised [2]. The dry plant extracts were stored at a temperature of 20–25ºC.

Preparation of 50% *(v/v)* ethanol extract

The powdered dry roots were extracted with 50% (*v/v)* ethanol using the percolation method at plant material to solvent ratio 1:10. After the evaporation of the alcohol in reduced pressure at a temperature of 40–45ºC the extracts were frozen at –55ºC and than lyophilised [2]. Dry plant extracts were stored at a temperature of 20–25ºC.

Standards substances

The following comparison substances were used in the experiment: rosavin, rosin, rosarin (ChromaDex) and D-(-)-salicine (SIGMA).

Stock solutions

Calibration curves of phenylpropanoids implemented standards from ChromaDex and D-(-)-salicine as an internal standard (Sigma Aldrich) was prepared. All substances were dissolved in methanol and different concentration of stock solutions was prepared. Calibration curves were prepared as 5 different levels of concentration ranging 100–1000 ng/ml.

Sample preparation

Roots of *Rhodiola*

1.0 g of plant material (ca. 1.0 of dried powdered (0.315)) *Rhodiola kirilowii* or *Rhodiola rosea* root was weighed out and placed in a 100 ml round-bottom flask. To 19.0 ml of 10% (*v/v*) methanol a methanolic solution of D-(-)-salicine (IS) was added.

This sample was heated under a reflux condenser in the boiling point of the solvent for 45 min. Then, the sample was filtrated and the extraction of sample was repeated one more time. The filtrate was concentrated to evaporate the methanol up to a volume of about ¼ in a rotary evaporator in vacuum. Sample was transferred quantitatively to 20 ml volumetric flask. Subsequently, the solution was made up to the mark with the 10% (*v/v)* methanol. The sample was filtered through a membrane filter with a diameter of 0.20 μm.

Extracts of *Rhodiola*

0.5 g of *R. kirilowii* or 0.1 g *Rhodiola rosea* extract was weighed out and placed in a 100 ml round-bottom flask. To 19.0 ml of 10% (*v/v*) methanol a methanolic solution of D-(-)-salicine (IS) was added. This sample was heated under a reflux condenser in the boiling point of the solvent for 45 min. Then, the sample was filtrated and the extraction of sample was repeated. The filtrate was concentrated to evaporate the methanol up to a volume of about ¼ in a rotary evaporator in vacuum. Sample was transferred quantitatively to 20 ml volumetric flask. Subsequently, the solution was made up to the mark with the 10% (*v/v)* methanol. The sample was filtered through a membrane filter with a diameter of 0.20 μm.

LC-MS/MS assay

Analyses were conducted by ultra performance liquid chromatography - tandem mass spectrometry (UPLC-ESI MS/MS; Waters). The separation of analytes was performed on an Acquity UPLC BEH C18 column, 1.7 μm, 2.1 · 50 mm (Waters). Mobile phase: phase A: methanol, phase B: acetonitrile. Mobile phase flow rate was: 0.45 ml/min. The assay was performed in gradient elution: 0.0 min. – 97% of phase A, 4.8 min. – 82% of phase A, 4.9 min. – 97% of phase A. Column temperature was 30ºC; ion source temperature: 100ºC; desolvation temperature: 300ºC. Gas flow rate: desolvation gas: 700 l/h; cone gas: 10 l/h. All the substances were analyzed in the negative-ions source.

The phenylpropanoids (rosavin, rosin and rosarin) were identified by fragmentation of parent ion. Following fragmentations were used for detection of the phenylpropanoids rosavin: m/z 427→149 Da, rosin: m/z 295→161 Da and rosarin: m/z 427→149 Da. As an internal standard D-(-)-salicine was used. Figure 1 presents the structure of individual phenylpropanoids.

Validation

The method of extraction of phenylpropanoids was validated in accordance with a requirement of ICH. A linearity of all calibration curves of each compound

was checked in 5 different concentrations of stock solution (the range of concentration was about 100–1000 ng/ml for every compound). In a regression analysis of the calibration curves satisfactory results were obtained making validation of the subsequent steps possible. A precision of extraction was done on 6 samples. An accurancy of this method was conducted for 3 different levels. Percentage of recovery ranged in 89.62-109.67%. All validated parameters allowed to indicate that the extraction and detection methods are validated.

Statistical method

For the phenylethanoids and internal standard, the regression analysis was performed at 5 concentration levels. Concentration of phenylethanoids was carried out for all samples in 6 repeats. The average and relative standard deviations (RSD) for those results were determined.

RESULTS

The fragmentation of particular analytes is presented in table 2. The analytical method employed in our study was evaluated for precision, linearity and accuracy. Precision and linearity were evaluated by using regression analysis for compared substances (tab. 2). The accuracy was analysed using the enrichment method by adding dry hydroalcoholic extract with the determined level of phenylpropanoids to the sample (tab. 3).

Table 2.

Parameters of fragmentation of phenylpropanoids

Compound	Retention time [min]	RSD_t (n=15) [%]	RSD_p (n=5) [%]	Fragmentation m/z [Da]
Rosavin	4.38	0.12	1.33	427→149
Rosin	4.44	0.12	1.66	295→161
Rosarin	4.23	0.14	2.91	427→149
D-(-)-salicine (IS)	2.72	0.39	1.72	285→123

Table 3.

Method of recovery (n=3)

Sample	Rosavin	Rosin	Rosarin
20%	103.15±2.98	93.51±5.32	89.62±6.48
40%	96.52±5.15	103.71±4.74	92.34±4.29
80%	109.67±3.57	98.42±6.11	97.25±4.93

Detection of each analysed compound was conducted in negative ions source. The signals visible in the chromatogram came from the parent ion fragmentation $[M-H]^-$. In order to confirm the recovery method, the method with the use of *R. kirilowii* root sample enriched by the addition of dry hydroalcoholic extract from *R. rosea* with the determined level of individual phenylpropanoids content was used.

DISCUSSION

The MRM chromatogram (fig. 2, 3) presents the fragmentation of phenylpropanoids in two species of *Rhodiola*. The chemical composition of *Rhodiola kirilowii* and *Rhodiola rosea* are different. In the *R. kirilowii* root the presence of phenylpropanoids was not confirmed. The same situation is observed in the extracts from *R. kirilowii*. However, in the *R. rosea* root the presence of all 3 phenylpropanoids was confirmed. The most common compound was rosavin. In aqueous and hydroalcoholic extracts, the contents of all phenylpropanoids was significantly higher than in the raw material. Similar to the root, the main compound in the extracts was rosavin. Total content of phenylpropanoids was 4 087.697 mg/100 g of dry powdered material in the hydroalcoholic extract and 958.517 mg/100 g of dry powdered material in the aqueous extract. The analysis of phenylpropanoid glycosides from *Rhodiola rosea* by UV, MS and NMR methods was also performed by Tolonen et al. [16,17].

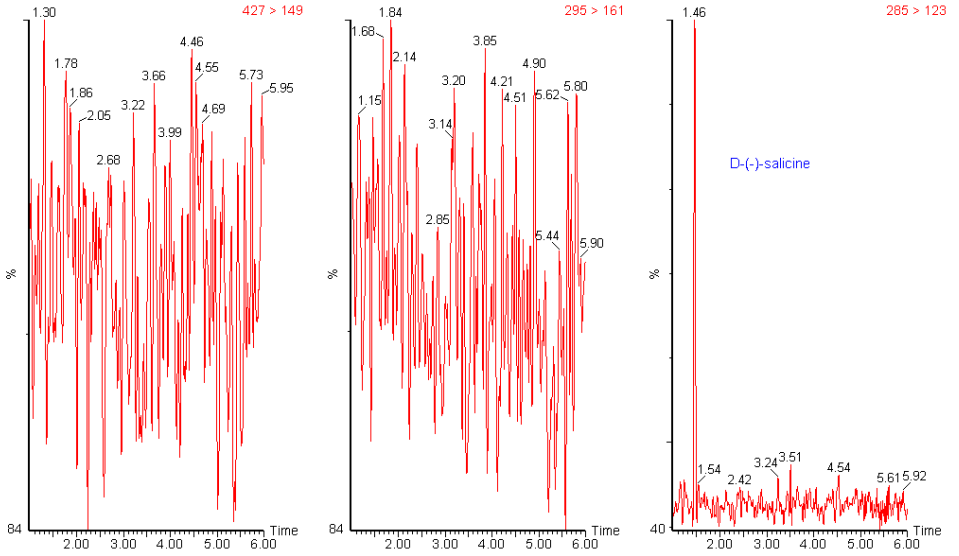

Figure 2.

The MRM chromatogram of fragmentation of phenylpropanoids in *Rhodiola kirilowii*

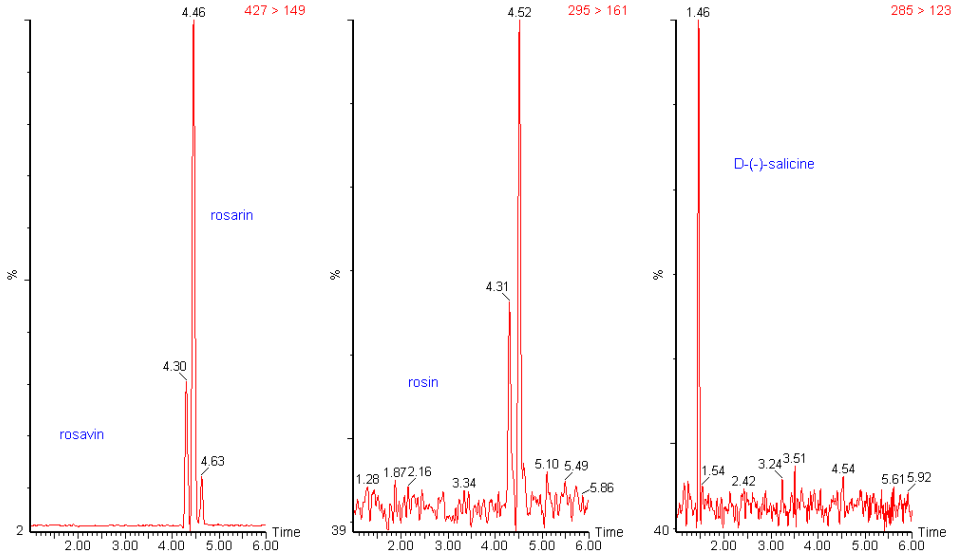

Figure 3.

The MRM chromatogram of fragmentation of phenylpropanoids in *Rhodiola rosea*

Table 4.

Content of phenylpropanoids in *Rhodiola kirilowii* and *Rhodiola rosea* roots

Sample	Rosarin		Rosavin		Rosin	
	Content [mg/100 g of dry powdered material]	RSD [%]*	Content [mg/100 g of dry powdered material]	RSD [%]*	Content [mg/100 g of dry powdered material]	RSD [%]*
Rhodiola kirilowii root	ND	–	ND	-	ND	–
50% ethanol extract	ND	–	ND	-	ND	–
aqueous extract	ND	–	ND	-	ND	–
Rhodiola rosea root	5.423	4.24	21.888	2.92	1.770	2.82
50% ethanol extract	657.355	2.18	3253.340	2.36	177.002	1.29
aqueous extract	198.897	2.90	684.983	1.55	74.637	1.45

* – RSD – relative standard deviation (n=6)
ND – not confirm the presence of the compound

Due to the widespread use of *Rhodiola* material in several countries, the determination of active ingredients such as rosavins, rosin, rosarin and rosaridin is very important for the evaluation of the results of *in vivo* studies. Furthermore, it is necessary to use a sensitive method for the differentiation of the chemical composition of raw materials depending on the plant species and origin.

CONCLUSION

In summary, all the validation tests undertaken an analytical methods for phenylethanoids was confirmed that method the ultra performance liquid chromatography - tandem mass spectrometry (UPLC-ESI MS/MS) can be successfully used for the determination of the compounds of this group.

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NOWA METODA OZNACZANIA ZAWARTOŚCI FENYLOPROPANOIDÓW W KORZENIACH Z GATUNKU *RHODIOLA*

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S t r e s z c z e n i e

Celem przeprowadzonych badań w ramach projektu badawczego było opracowanie metody analitycznej pozwalającej na oznaczenie trzech związków fenylopropanoidów w dwóch gatunkach różeńca. Do detekcji rozawiny, rozyny i rozaryny wykorzystano wysokosprawny chromatograf cieczowy sprzężony z tandemowym spektrometrem mas (UPLC-MS/MS). Obydwa gatunki różeńca *Rhodiola kirilowii* oraz *Rhodiola rosea* zostały zebrane z upraw prowadzonych w Instytucie Włókien Naturalnych i Roślin Zielarskich w Poznaniu w 2009 r. Dodatkowo z tych surowców przygotowano po dwa wyciągi: wyciąg wodny oraz wodno-alkoholowy (50% etanol), które następnie przebadano pod względem zawartości fenylopropanoidów. Wszystkie przeprowadzone analizy potwierdziły możliwość wykorzystania tej metody do oznaczenia zawartości fenylopropanoidów w rodzaju *Rhodiola*.

***Słowa kluczowe*:** *Rhodiola kirilowii, Rhodiola rosea, UPLC-MS/MS, fenylopropanoidy*