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Phytochemical Characterization of an Adaptogenic Preparation from *Rhodiola heterodonta*

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Abstract

The phytochemical constituents of a biologically active, standardized, 80% ethanol extract of *Rhodiola heterodonta* were characterized. The extract was fractionated over a Sephadex LH-20 column to afford two main fractions representing two classes of secondary metabolites: phenylethanoids and proanthocyanidins. This fractionation facilitated the identification and quantification of individual compounds in the fractions and sub-fractions using HPLC, and LC-MS. The major compounds in the phenylethanoid fraction were heterodontoside, tyrosol methyl ether, salidroside, viridoside, mongrhoside, tyrosol, and the cyanogenic glucoside rhodiocyanoside A. These seven compounds comprised 17.4% of the EtOH extract. Proanthocyanidins ranged from oligomers to polymers based on epigallocatechin and gallate units. The main identified oligomeric compounds in the proanthocyanidin fraction were epigallocatechin gallate, epigallocatechin-epigallocatechin-3-*O*-gallate and 3-*O*-galloylepigallocatechin-epigallocatechin-3-*O*-gallate, which constituted 1.75% of the ethanol extract. Tyrosol methyl ether, mongrhoside, and the two proanthocyanidin dimers were reported for the first time from this species in this study. Intraperitoneal injection of the 80% ethanol extract increased survival time of mice under hypoxia by 192%, as an indication of adaptogenic activity.

Keywords

Rhodiola heterodonta; HPLC-ESI/MS; phenylethanoid; proanthocyanidin; adaptogenic activity; anti-hypoxia

Rhodiola spp. (Crassulaceae) have been known for centuries as medicinal herbs in Eastern Europe and Asia. *R. rosea* has been popular as an adaptogenic plant [1-5]. The term adaptogen refers to substances which help the body and brain cells resist physical, chemical, and environmental stresses. Repeated administration of adaptogens leads to a stress-protective effect in a way similar to that produced by repeated physical exercise. Adaptogens have a broad range of therapeutic effects without causing any disturbance to the normal functioning of the organism [6-9]. An important direction in modern pharmacy today is to search for drugs that increase working capacity, stability under stressful conditions, and ability to adapt to rapidly-changing industrial and environmental conditions. The increase in body resistance to external stress conditions, like hypoxia, is considered an indication of adaptogenic activity [10,11]. Previously, a series of *in vivo* experiments to evaluate the

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increase in endurance of animals under intense physical loads was established as a means to gauge the ability of ingested substances to act as natural adaptogens [12].

Wild species of *Rhodiola* have been used in traditional medicine systems [13,14], including *R. heterodonta*, a species endemic to the mountains of Central Asia. *Rhodiola* species were recently reported to contain phenylethanoid/ propanoid compounds along with other biologically active constituents [15]. Analytical methods to determine salidroside and tyrosol have been reported [16-20], but methods to determine the presence of other co-occurring constituents are very limited. To standardize a pharmaceutical preparation, it is essential to fully characterize and quantify the constituents of the preparation. In this study we used simple and efficient analytical methods for characterization and quantification of multiple components in a *R. heterodonta* standardized ethanolic preparation that demonstrated an *in-vivo* antihypoxic efficacy.

Our results showed that administration of the 80% EtOH extract of *R. heterodonta* (40 mg/kg) increased the life span of the treated mice under hypoxic conditions by 1.9 times compared with the untreated control (Table 1). This increase in the mice tolerance to hypoxia is evidence of the drug's adaptogenic activity [10]. Our findings are consistent with the published data on epigalokhin, a polymeric proanthocyanidin isolated from a related species, *R. semenovii*, which exhibited pronounced antihypoxic effect in both *in-vivo* and *in-vitro* experiments [21]. Additionally, the most abundant phenylethanoid glycoside, salidroside, was reported to exert a protective effect on PC12 (neuronal pheochromocytoma cells), where it prevented the morphological changes of these cells under conditions of chemical hypoxia induced by $Na_2S_2O_4$ [22].

R. heterodonta rhizome contains a wide range of secondary metabolites including polymeric proanthocyanidins [15], which interfere with direct HPLC analysis. The presence of proanthocyanidin compounds along with phenylethanoids in the EtOH extract made both qualitative and quantitative analysis of both components difficult, for two reasons: the presence of a polymeric proanthocyanidin spectral hump, and overlapping of the peaks from the two groups because of similarity in UV wavelength absorption. An HPLC-PDA analysis of the 80% EtOH extract acquired at 225 nm and 280 nm displayed a complex hump (Figure 1). This problem was reported before, for the analysis of 5 marker compounds in *R. rosea* [23]. In this study, we separated these compounds on a Sephadex LH-20 column, to two major groups, phenylethanoids (fraction **A**) and proanthocyanidins (fraction **B**). This separation facilitated characterization of individual components. Also, partition of fraction **B** with EtOAc allowed for the separation of three major oligomeric proanthocyanidins (sub-fraction **C**) from the more complex polymers (sub-fraction **D**). This separation allowed accurate quantification of oligomeric proanthocyanidins and overcame the chromatographic hump characteristic for polymeric proanthocyanidin mixtures, which would inevitably affect the quantification of other constituents.

Phenylethanoids isolated from fraction **A** included tyrosol (**1**), viridoside (**2**), salidroside (**3**), tyrosol methyl ether (**4**), heterodontoide (**5**), and mongrhoside (**6**), and the cyanogenic glucoside rhodiocyanoside A (**7**) (Figure 2) [15]. Tyrosol methyl ether and mongrhoside were characterized for the first time in this study. Mongrhoside, the arabinose derivative of viridoside, was recently identified in *R. quadrifida* and *R. rosea* [24, 25]. ^{1}H NMR and ^{13}C NMR spectra were identical to the data recently reported for monrhoside [25].

HPLC-PDA quantification of fraction **A** (Figure 3), using the above isolated compounds as external standards, revealed that heterodontoside was the most abundant phenylethanoid in the 80% EtOH dry extract of *R. heterodonta* (53.2 mg/g), followed by tyrosol methyl ether (41.6 mg/g), salidroside (25.8 mg/g), viridoside (22.6 mg/g), and mongrhoside (18.3 mg/g),

with tyrosol and rhodiocyanoside A as minor components (5.2 and 7.1 mg/g respectively). Collectively, the concentration of the seven compounds was 173.8 mg/g (17.4%) in the 80% EtOH extract of *R. heterodonta*. When completely dried, *R. heterodonta* fresh rhizomes contained 38.2% ± 0.7% dry matter (n = 3), of which 37.5% ± 2.9% constituted the 80% EtOH extract. The 80% EtOH extract constituted 14.3% ± 1.0% (n = 3) of the rhizome fresh weight. The total phenylethanoid/cyanogenic compounds comprised 6.4% and 2.4% of the dry and fresh rhizomes, respectively (Table 2).

Fraction **B** contained a large number of oligomeric and polymeric proanthocyanidins, as indicated by its LC-ESI-MS profile. HPLC-ESI-MS of sub-fraction **C** (the EtOAc partition from fraction **B**) revealed that there were three main components with *m/z* of 763, 915 and 459 $[M+H]^+$ in the positive ion mode. The three components were isolated utilizing semi-preparative HPLC and identified as: epigallocatechinepigallocatechin-,3-*O*-gallate (EGC-EGCG, **9**), 3-*O*-galloyl-epigallocatechin-epigallocatechin-3-*O*-gallate (EGCG-EGCG, **10**), and epigallocatechin gallate (EGCG, **8**). EGCG was identified by comparing its physical and spectral data with a standard compound. EGC-EGCG (**9)** and EGCG-EGCG (**10)** were identified by comparing their physical and NMR characteristics with published data [26]. Compounds **8**, **9** & **10** were used as external standards for the HPLC quantification of oligomeric proanthocyanidins in sub-fraction **C** (Figure 4). The concentrations of EGC-EGCG, EGCG-EGCG, and EGCG in the 80% EtOH extract were 4.35, 4.68, and 8.51 mg/g, respectively. Collectively, the three measured oligomers composed 17.5 ± 0.82 mg/g (n = 3) or 1.75% of the 80% EtOH extract. The three oligomers represented 0.65% and 0.25% of the dry and fresh rhizomes, respectively (Table 3). Sub-fraction **D** contained a mixture of several complex polymeric compounds and, therefore, it was not analyzed further. This is the first report to quantify oligomeric proanthocyanidins in *Rhodiola* plants.

Data on the concentration of phenylethanoids in *Rhodiola* species has rarely been reported outside of publications in Russia, China, and other Central Asian countries. In one study it was reported that the concentrations of salidroside and tyrosol ranged from 1.3-11.1 mg/g and 0.3- 2.2 mg/g, respectively in dry *Rhodiola rosea* rhizomes collected from various parts of China [22]. In another study, the concentration of salidroside in a selection of dried *R. rosea* samples ranged from 0.4 to 2.7 mg/g [23]. In our study, the concentrations of salidroside and tyrosol were 1.0 and 0.2 mg/g dry weight, respectively, while the methyl ether of tyrosol showed higher concentration (1.5 mg/g dry weight).

Experimental

General

Commercial standards of tyrosol, salidroside, and epigallocatechin-3-*O*-gallate were purchased from Chromadex (Laguna Hills, CA). Tyrosol methyl ether, viridoside, mongrhoside, heterodontoside, rhodiocyanoside A, epigallocatechin-epigallocatechin-3-*O*-gallate, and 3-*O*-galloyl-epigallocatechin-epigallocatechin-3-*O*-gallate were isolated from *R. heterodonta* in our laboratory. Lipophilic Sephadex LH-20 (Sigma, St. Louis, MO) and silica gel 60, 230–400 mesh ASTM (Merck, Whitehouse Station, NJ) were used for column chromatography.

Instruments and chromatographic conditions

The NMR spectra were obtained on a Varian Inova spectrometer (Palo Alto, CA), using TMS as internal standard.

HPLC-ESI-MS analyses

These were made with an LCQ Deca XP mass spectrometer (Thermo Finnegan Corp., San Jose, CA), MS version 1.3 SRI, ESI in the positive ion mode (*m/z* 200-2000), with a photodiode array (PDA) detector (200-600 nm), version 1.2, auto-sampler version 1.2, and Xcalibur software for data processing. The HPLC separations were carried out on a C_{18} reversed-phase column (150 mm, 2.1 mm i.d., particle size 5 μm, 90 Å) (VYDAC, Western Analytical, Murrieta, CA). The mobile phase consisted of 95% H_2O, 5% acetonitrile and 0.1% formic acid (solvent A), and 95% acetonitrile, 5% H_2O, and 0.1% formic acid (solvent B) with a step gradient of 0%, 5%, 30%, 60%, 90%, 90%, 0% and 0% of solvent B at 0, 3, 40, 45, 50, 55, 60 and 70 min, respectively. A constant flow rate of 200 μL/min and an injection volume of 10 μL were employed. Standards or samples (2, 5 mg/mL) were prepared in MeOH, filtered and injected.

HPLC-PDA analyses

These were performed using an Agilent 1100 HPLC system (Agilent Technologies Inc., New Castle, DE).

For phenylethanoid quantification, a 150 mm × 4.6 mm × 5 μm Eclipse XDB C_{18} reversed-phase column (Agilent Technologies Inc.) was used. The elution gradient consisted of three solvents: (A) 100% H_2O with 25 mM phosphate buffer and pH 7.0, (B) 100% acetonitrile, and (C) MeOH 100% with 0.1% phosphoric acid [27]. Gradient elution started with 95:5 (A:B), proceeded to 80:20 (A:B) in 30 min, and sustained at this ratio until 37 min. Solvent C was used to flush the column for 5 min, then the elution continued with 95:5 (A:B) for another 5 min to equilibrate the column before injecting the next sample. The column temperature was set at 50°C with a constant flow rate of 0.8 mL/min. The photodiode array detector was set to record absorption at 225 nm.

For proanthocyanidins quantification, a reversed-phase Supelcosel LC_{18} column 250 mm × 4 mm × 5 μm (Supelco, Bellefonte, PA) was used. Solvent systems and elution gradient were as described above (in HPLC-ESI-MS analyses). The column temperature was 250°C with a constant flow rate (1 mL/min) and the UV detection was at 280 nm.

Commercial standards or standards purified in our laboratory were used for this analysis. Three concentrations of each standard were prepared at 0.25, 0.50, and 1.00 mg/mL in MeOH. Fraction A (10 mg/mL) and fraction C (2 mg/mL) were also prepared in MeOH. Samples or standards were filtered (0.22 μm PTFE) and an aliquot (5 μL) was injected.

Plant material

Rhizomes of *Rhodiola heterodonta* (Hook. f & Thomson) Boriss. (ICBG Central Asia voucher UPL_00085, ILLS, MO) were harvested from mountainous regions of Uzbekistan in 2004. Since the traditional use of this plant in herbal therapy has been in the form of a hydro-alcoholic solution, an 80% aq. ethanol extract was prepared and used for this study.

Extraction, isolation and sample preparation

Fine powdered lyophilized rhizomes (96 g) were extracted with 80% aq. ethanol (3 × 1L) at room temperature. The solvent was evaporated and the residual aqueous extract was lyophilized (36 g). For isolation of standard compounds, 16.0 g of the dry 80% EtOH extract was fractionated over Sephadex LH-20 (5 × 25 cm), first eluting with 1L 100% EtOH to give fraction (**A**) and then followed by subsequent elution with a mixture of MeOH-Me_2CO-H_2O (1:1:1, 1L) to obtain fraction (**B**). Fraction **A** was further fractionated to yield the following pure compounds: tyrosol methyl ether, tyrosol, viridoside, salidroside, rhodiocyanoside A, mongrhoside, and heterodontoside. The isolated compounds were

identified through TLC, HPLC, NMR, ESI-MS and comparison of their spectral data with commercial reference compounds (tyrosol and salidroside), and with previously isolated and identified compounds [15]. Mongrhoside was identified by comparing its chemical and spectral data with the published data [25]. Fraction **B** was dissolved in H_2O and partitioned with EtOAc (fraction **C**). A total of 75 mg of sub-fraction (**C**) was separated using a semi-preparative Genesis C_{18} column, 50 mm × 22.5 mm × 15 μm (VYDAC, Chromtech, Apple Valley, MN) using PDA detector at 280 nm. The injection volume was 100 μL with a flow rate of 2 mL/min. Solvent A was 100% H_2O and solvent B was 100% MeOH. The gradient system was 5%, 30%, 60%, 90%, 90%, 5%, and 5% of solvent B at 0, 40, 45, 50, 55, 60, and 70 min, respectively. For each proanthocyanidin peak, a total of 1000 mL was collected from multiple injections and dried under reduced pressure (temperature < 40°C) where about 5 mg, 7 mg, and 10 mg were obtained for the three peaks, respectively. Isolated compounds were identified as epigallocatechin-epigallocatechin-3-*O*-gallate, 3-*O*-galloyl-epigallocatechin-epigallocatechin-3-*O*-gallate, and epigallocatechin gallate. The purities of all isolated compounds were > 95%, as determined by analytical HPLC and NMR analyses.

Quantitative analysis

Three 1.0 g samples of the 80% EtOH dry extract were individually fractionated over Sephadex LH-20 columns (28 × 2 cm), in the same was as above. Fraction **A** was eluted with ethanol (150 mL, 554.0 ±7.2 mg) while Fraction (**B**) was obtained by subsequent elution with $MeOHMe_2CO$-H_2O (1:1:1, 300 mL) (416.0 ± 17.1 mg). Fraction **B** was dissolved in H_2O (20 mL) and partitioned with EtOAc (3 × 40 mL). Evaporation of the EtOAc layer afforded sub-fraction **C** (27.7 ± 2.5 mg), while evaporation of the aqueous layer afforded sub-fraction **D** (365.0 ± 13.2 mg). Fractions **A** and sub-fraction **C** were lyophilized and stored at −20°C for HPLC-PDA analyses, to estimate the concentration of phenylethanoids and oligomeric proanthocyanidins in *R. heterodonta*. HPLC quantification was calculated using a linear regression of the peak area for a known external standard concentration (three levels) with peak area of the corresponding compound in fraction **A**. The correlation coefficient (R^2) between peak area and concentration for the standards was at least 0.98 in the range of 0.25 to 1.00 mg/mL. The concentrations of phenylethanoid and oligomeric proanthocyanidin compounds were expressed as mg/g in the 80% EtOH extract and as % in the fresh and dry rhizome tissue, are presented in Table 2.

Antihypoxic activity

This was determined according to procedures described before [28]. A total of 6 male mice (16-22 g), were intra-peritoneally injected with 40 mg/kg of the 80% ethanol extract dissolved in 0.9% aq. NaCl. The control group (6 male mice of approx. same size) received an equivalent volume of the pure solvent (0.9% NaCl). This treatment was performed 30 min before placing the mice into hermetically closed glass chambers of 550 cm^3 volumes. The treatment did not cause any changes in the general behavior of the mice before they were placed inside the chambers. Time until the last breath was registered for each subject. The investigation was conducted in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication no. 85-23, revised 1996)

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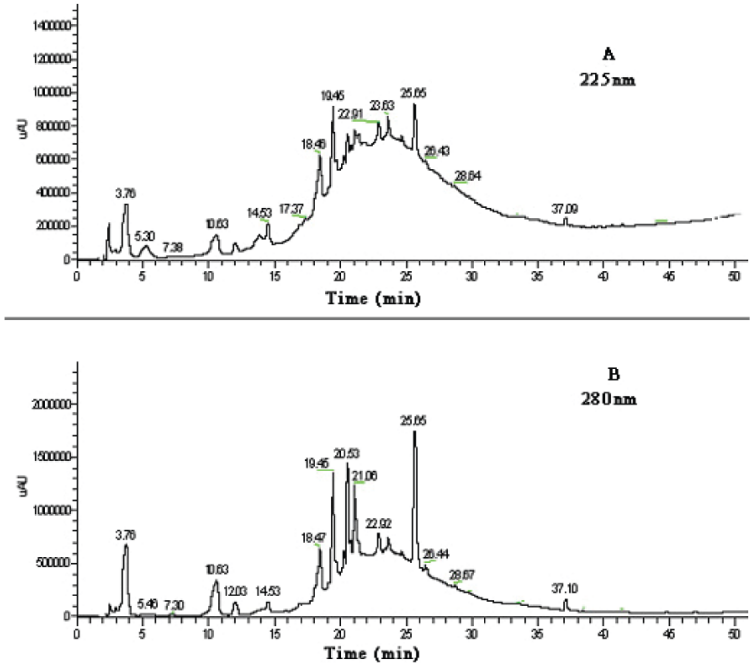

Figure 1.

HPLC chromatogram (from LC-MS) for *R. heterodonta* 80% EtOH extract measured at 225 nm (A), maximum absorption of phenylethanoids, and at 280 nm (B), maximum absorption of proanthocyanidins.

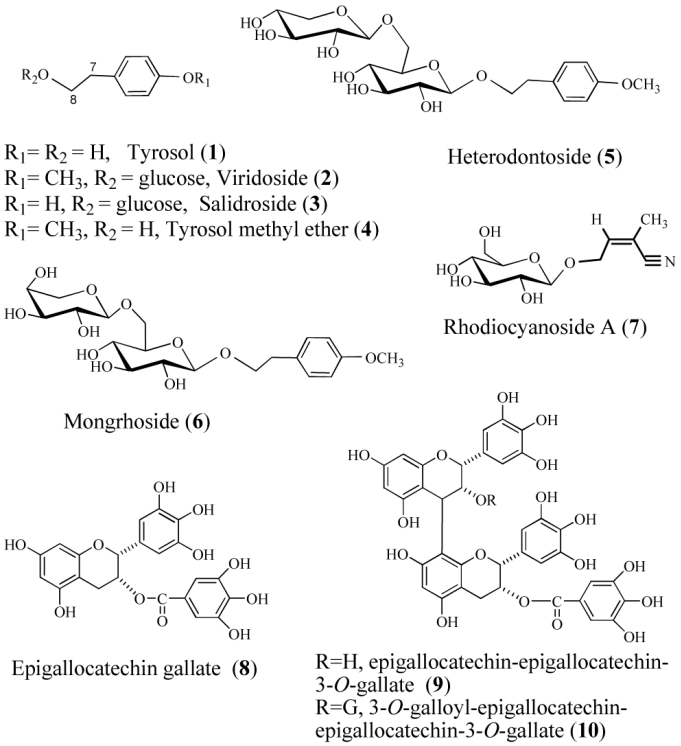

Figure 2.
Compounds isolated and quantified in *R. heterodonta*

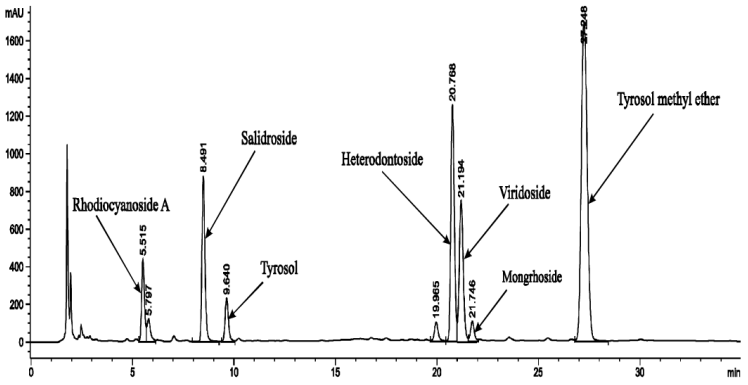

Figure 3.
RP-HPLC chromatogram for phenylethanoids and cyanogenic glycosides in *R. heterodonta* (fraction **A**, UV 225 nm).

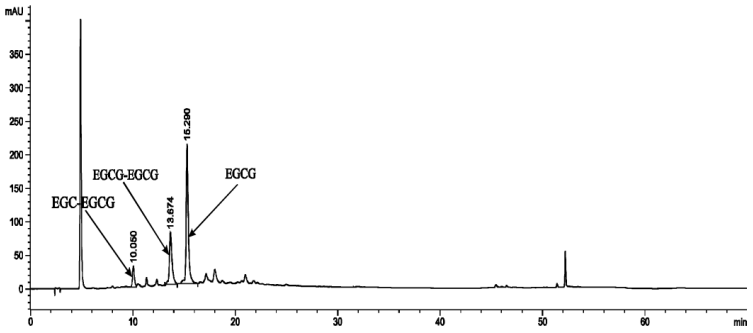

Figure 4.

RP-HPLC chromatogram for oligomeric proanthocyanidins in *R. heterodonta* (sub-fraction **C,** UV 280 nm). EGC-EGCG = epigallocatechin-epigallocatechin-3-*O*-gallate, EGCG-EGCG = 3-*O*-galloyl-epigallocatechin-epigallocatechin-3-*O*-gallate, EGCG = epigallocatechin gallate.

Table 1

Antihypoxic activity of the *R. heterodonta* 80% EtOH extract, intra-peritoneally administered to mice.

	life time (min)	% life prolongation
Control group[1]	28.00 ± 2.14	100%
Treated group[1]	53.80 ± 14:10	192%

[1] Six male mice (16-22 g) were included in control and test groups.

The injected dose of *R. heterodonta* extract was 40 mg/kg. Each mouse was placed into a550 cm^3 hermetically closed glass chamber.

Table 2

Concentrations of the phenylethanoid and cyanogenic glycosides in *R. heterodonta* 80% EtOH extract and the corresponding percentages in the rhizome dry and fresh tissues.

	PG [1] (mg/g) in EXT[2]	% PG			MW[5]	R_t[6]
		EXT[2]	DT[3]	FT[4]		(min)
Rhodiocyano-side A	07.1 ± 0.5[7]	0.7	0.3	0.1	259	5.5
Salidroside	25.8 ± 1.7	2.6	1.0	0.4	300	8.5
Tyrosol	05.2 ± 0.3	0.5	0.2	0.1	138	9.6
Heterodontoside	53.2 ± 2.7	5.3	2.0	0.7	314	20.7
Viridoside	22.6 ± 3.4	2.3	0.8	0.3	446	21.2
Mongrhoside	18.3 ± 3.2	1.8	0.7	0.3	446	21.7
Tyrosol methyl ether	41.6 ± 2.9	4.2	1.5	0.6	152	27.2
Total	173.8 ± 12.0	17.4	6.4	2.4	--	--

[1] PG; phenylethanoid/cyanogenic glycosides

[2] EXT; 80% EtOH extract

[3] DT; dried plant tissue

[4] FT; fresh plant tissue

[5] MW; molecular weight

[6] R_t; retention time (Figure 3)

[7] mean values ± standard deviations (n = 3). Fresh rhizomes contained 38.2% ± 0.7% dry matter (n = 3) or 14.3 % ± 1.0% dry 80% EtOH extract (n = 3). Dry rhizome tissues contained 37.5% ± 2.9% dry 80% EtOH extract.

Table 3

Concentrations of oligomeric proanthocyanidins in *R. heterodonta* 80% EtOH extract and the corresponding percentages in the rhizome dry and fresh tissues.

	PA[1] (mg/g) in EXT[2]	% PA			MW[5]	R_t[6]
		EXT[2]	DT[3]	FT[4]		(min)
EGC-EGCG	4.35 ± 0.50[7]	0.44	0.16	0.06	762	10.0
EGCG-EGCG	4.68 ± 0.50	0.47	0.17	0.07	914	13.7
EGCG	8.51 ± 0.59	0.85	0.32	0.12	458	15.3
Total	17.50 ± 0.82	1.75	0.65	0.25	--	--

[1] PA; oligomeric proanthocyanidin

[2] EXT; 80% EtOH extract

[3] DT dry plant tissue

[4] FT fresh plant tissue

[5] MW; molecular weight

[6] R_t retention time (Figure 4)

[7] mean values ± standard deviations (n = 3). EGC-EGCG = epigallocatechin-epigallocatechin-3-*O*-gallate, EGCGEGCG = 3-*O*-galloyl-epigallocatechin-epigallocatechin-3-*O*-gallate, EGCG = epigallocatechin gallate. Fresh rhizomes contained 38.2% ± 0.7% dry tissues (n = 3) or 14.3% ± 1.0% dry 80% EtOH extract (n = 3). Dry rhizome tissues contained 37.5% ± 2.9% dry EtOH 80% extract