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Comparative phytochemical characterization of three *Rhodiola* species

Gad G. Yousef [a], Mary H. Grace [a], Diana M. Cheng [a], Igor V. Belolipov [b], Ilya Raskin [c], Mary Ann Lila [a,*]

[a] *Department of Natural Resources and Environmental Sciences, University of Illinois, Urbana, IL 61801, USA*
[b] *Tashkent State Agrarian University, Tashkent, Uzbekistan*
[c] *Biotech Center, Cook College, Rutgers University, New Brunswick, NJ 08901, USA*

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Abstract

In comparison to the well-recognized adaptogenic herb *Rhodiola rosea*, phytochemical constituents of two other *Rhodiola* species (*R. heterodonta* and *R. semenovii*) were elucidated and characterized. Two major phytochemical groups; phenolic and/or cyanogenic glycosides and proanthocyanidins, were isolated and identified in the three species. Chemical similarities among the three species were observed; however, each species displayed differences in phytochemical constituents. *R. heterodonta* contained a newly detected phenylethanoid glycoside, heterodontoside, in addition to the known compounds tyrosol, viridoside, salidroside, and rhodiocyanoside A. Both *R. heterodonta* and *R. rosea* contained phenylethanoid/propanoid compounds that were not detected in *R. semenovii*. For *R. semenovii*, the cyanogenic glucosides rhodiocyanoside A and lotaustralin were detected. Although the three species have proanthocyanidins composed of (−)-epigallocatechin and its 3-*O*-gallate esters in common, the degree of polymerization greatly differed between them. In contrast to *R. heterodonta* and *R. semenovii*, *R. rosea* has higher molecular weight polymeric proanthocyanidins. This study resulted in the identification and isolation of phytochemical constituents for direct cross-comparison between three *Rhodiola* species of medicinal and pharmacological value.

Keywords: *Rhodiola heterodonta*; *Rhodiola semenovii*; *Rhodiola rosea*; Crassulaceae; Heterodontoside; Phenylethanoid glycosides; Cyanogenic glucosides; Proanthocyanidins; Prodelphinidins; EGCG; EGC; HPLC-ESI-MS

1. Introduction

Rhodiola rosea L. (golden root), historically used as an adaptogen in Russia, northern Europe, and in China as a traditional herbal medicine, is valued for its ability to enhance human resistance to stress or fatigue, and promote longevity (Spasov et al., 2000; Kurkin and Zapesochnaya, 1986; Tolonen et al., 2003a). Golden root phytochemical extracts are the source of important commercial preparations widely used throughout Europe, Asia, and more recently in the USA, with biological activities including antiallergenic and anti-inflammatory effects, enhanced mental alertness, and a variety of therapeutic applications (Tolonen et al., 2003a). While phenylethanoid derivatives including salidroside (**3**) (rhodioloside) (Fig. 1) were previously used exclusively to standardize medicinal preparations of *R. rosea* extracts, it is now believed that a variety of co-occurring phytochemical constituents in the plant (including the phenylpropanoids [e.g. rosavins] and possibly terpenoids and flavonoids) may be responsible for its unique pharmacological activity (Brown et al., 2002). The phytochemical constituents in *Rhodiola* are species-dependent, and the predominant species screened in efficacy studies has been *R. rosea*, although salidroside (**3**) production in other species including *R. sachalinensis*, *R. kirilowii*, and *R. crenulata* has also been reported (Kurkin and Zapesochnaya, 1986; Wu et al., 2003).

Two central Asian *Rhodiola* genotypes (*R. heterodonta* and *R. semenovii*), Crassulaceae family, have similar rich

* Corresponding author. Tel.: +1 217 333 5154; fax: +1 217 244 3469.
E-mail address: imagemal@uiuc.edu (M.A. Lila).

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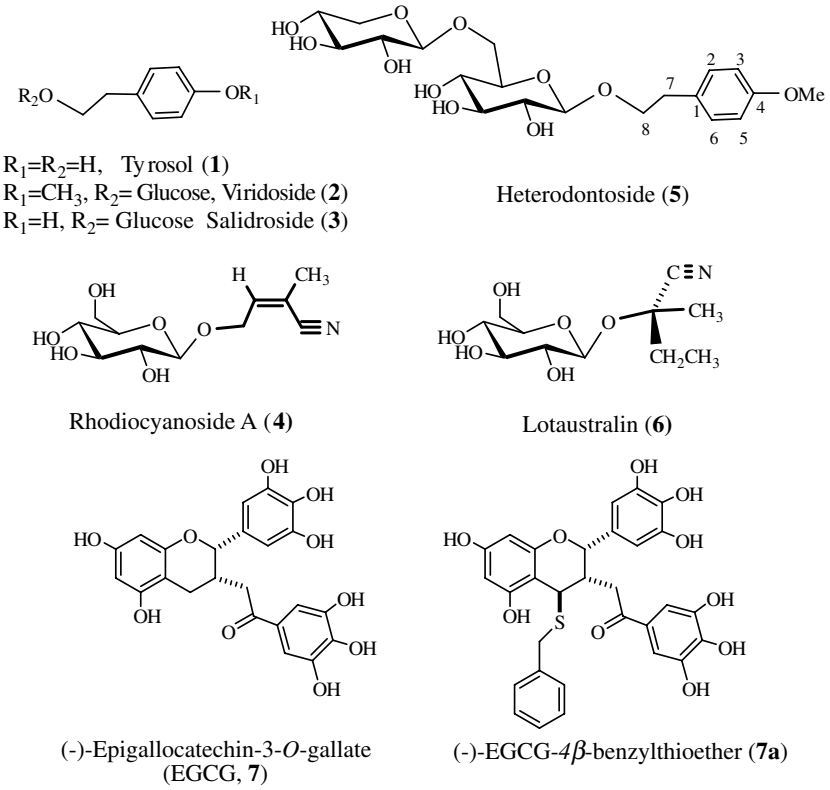


Fig. 1. Isolated and characterized phenolic and cyanogenic glycosides **1**–**6** and proanthocyanidin basic unit **7** and its benzylthioether adduct (**7a**) from the three *Rhodiola* species; *R. heterodonta*, *R. semenovii*, and *R. rosea*.

ethnobotanical histories and are locally valued as adaptogens, but have not been well investigated outside of the former Soviet Union. *R. heterodonta*, indigenous to Uzbekistan, has been found to contain salidroside (**3**) (rhodioloside) in common with golden root, and its extract is included, along with extracts of other *Rhodiola* species, in a medicinal preparation (carpediol) used to treat depression (Krasnov et al., 1976; Wikman and Panossian, 2003). *R. semenovii*, indigenous to Kyrgyzstan and locally valued for endurance-enhancing and anti-hypoxic properties, contains dimeric and oligomeric proanthocyanidins (Kuliev et al., 2004; Matamarova et al., 1999) which have been linked to the hypolipidemic, hypocholesteremic, and anti-inflammatory properties of extracts from this plant. *R. semenovii* was reclassified by some Russian authors as a separate genus (*Clementsia semenovii*), because it did not contain detectable levels of tyrosol (**1**), salidroside (**2**), and herbacetin characteristic of other members of *Rhodiola* (Kurkin et al., 1986).

In previous studies, complete phytochemical characterization of, and cross-comparison between, various species has been complicated by the intermittent use of dried, powdered and/or pulverized plant preparations for analysis. Although the natural isomeric forms, functional groups, and bioactivity of many phytochemical components may be altered during extraction and processing, in many cases the methods used to prepare herbal extracts/dried preparations are unknown or vary widely between processors. The objectives of this study were to compare the phytochemical constituents of the two central Asian *Rhodiola* species (*R. heterodonta* and *R. semenovii*) to the well-characterized golden root (*R. rosea*), using freshly harvested live rhizomes and standardized reproducible extraction and separation methods, in order to examine the inherent constituents potentially responsible for the above mentioned metabolic-enhancing properties. All three species were characterized in terms of phenylethanoid and/or cyanogenic glycosides as well as proanthocyanidin content.

2. Results and discussion

2.1. Identification of phytochemicals in Rhodiola species

Two distinct groups of compounds, which have recognized pharmacological activities were isolated and identified from the three *Rhodiola* species. The first group included phenolic and/or cyanogenic glycosides with anti-allergy, anti-inflammatory, anti-anoxia, anti-fatigue, hepatoprotective, and cognitive-enhancing properties (Brown et al., 2002; Darbinyan et al., 2000; Diaz-Lanza et al., 2001). The second group was proanthocyanidins, which were noted for significant bioactivities including anti-oxidant, anti-cancer, anti-inflammatory, anti-allergic, anti-mutation, anti-aging and improving liver function

(Demeule et al., 2002; Gross, 2004; Kris-Etherton and Keen, 2002; Marchand, 2002; Takahata et al., 2001).

When chromatographically fractionating the 70% aq. acetone crude extract using either silica gel or Sephadex LH-20 columns, phenolic and cyanogenic glycosides along with the lipophylic materials were eluted in the early fractions. The proanthocyanidins with higher polarity and molecular weights were detected in the later fractions, which eluted with a mixture of MeOH–acetone–H_2O. Applying vacuum liquid chromatography to the 70% aq. acetone crude extract using a silica gel column resulted in a wide range of separated fractions (22 fractions) that facilitated characterization of the compounds using TLC and HPLC-ESI-MS, whereas using a Sephadex LH-20 column separated this crude extract into two distinct chemical groups; the phenolic and/or cyanogenic glycosides, and proanthocyanidins.

2.2. Phenolic and cyanogenic glycosides

Subsequent separation of the phenolic glycoside mixture collected from a Sephadex LH-20 column for *R. heterodonta* (Fr. 3–11) by column chromatography on silica gel afforded 2-(4-hydroxyphenyl)ethanol (tyrosol, **1**), 2-(4-methoxyphenyl)ethyl-*β*-D-glucopyranoside (viridoside, **2**), 2-(4-hydroxyphenyl)ethyl-*β*-D-glucopyranoside (salidroside or rhodioloside **3**), and rhodiocyanoside A (**4**) (Fig. 1). The identification of these compounds was consistent with previous reports (Golovina and Nikonov, 1988; Nishimura et al., 1990; LaLonde et al., 1976; Yoshikawa et al., 1996). In addition to the above compounds, a new phenylethanoid glycoside designated "heterodontoside" (**5**) (Fig. 1) was identified. The isolated, previously reported compounds were identified according to their chemical and spectroscopic data (NMR and HPLC-ESI-MS), literature, and comparison to spectra for commercial standards of compounds **1** and **3**.

Heterodontoside (**5**), purified as a white powder, had a molecular formula of $C_{20}H_{31}O_{11}$ according to its HRESI/TOFMS molecular ion peak observed at *m/z* 447.1875, and its ^1H and ^{13}C NMR spectroscopic data. The ^1H NMR spectra (Table 1) exhibited two signals in the aromatic region at δ 7.16 (d, $J = 8.5$ Hz, H-2, 6) and 6.82 (d, $J = 8.5$ Hz, H-3, 5) indicating a *para*-substituted phenyl ring. A singlet observed at δ 3.74 integrated for three protons is characteristic for an *O*-methyl group located at position 4 of the phenyl ring. Two anomeric protons were overlapped at δ 4.29 (d, $J = 7.8$ Hz, H-1′ and d, $J = 6.8$ Hz, H-1″). Chemical shifts and coupling constants of sugar residues showed that they were *β*-glucopyranose and *β*-xylopyranose. The complexity of the upfield signals was resolved by ^1H–^1H COSY spectrum of **5** (Table 1).

The ^{13}C-chemical shifts of the sugar moieties demonstrated that xylose is attached at the 6′ position of glucose. This was shown from the downfield shift of this carbon (δ_c 68.2) compared to viridoside (**2**) (δ_c 61.5). The proposed structure for **5** was further confirmed by comparing its ^{13}C NMR spectrum with that of the monosaccharide (**2**) (Table 1) and other related and reported compounds (Bisset et al., 1989). Acid hydrolysis (HCl) afforded *p*-methoxyphenylethanol, D-glucose and D-xylose. The sugars were identified by co-chromatography with standard samples. The optical rotation of the hydrolyzed sugars indicated that both are in the D-configuration. Acetylation of **5** (Ac_2O, pyr, 25 °C) gave the hexaacetate which was identified by its ^1H and ^{13}C NMR spectra and by ESIMS, in which exhibited molecular ion peaks at *m/z* 721 $[M + Na]^+$ and 716 $[M + NH_4]^+$ corresponding to a molecular

Table 1
^1H and ^{13}C NMR spectroscopic data of two phenolic glycosides; viridoside (**2**) and heterodontoside (**5**) isolated from *R. heterodonta*[a]

Position	Viridoside (**2**)		Heterodontoside (**5**)		
	^1H NMR	^{13}C NMR	^1H NMR	H-H-COSY	^{13}C NMR
1	–	130.7	–	–	130.8
2,6	7.17 (d, $J = 8.7$)	129.7	7.16 (d, $J = 8.7$)	3,5	129.8
3,5	6.82 (d, $J = 8.5$)	113.5	6.82 (d, $J = 8.5$)	2,6	113.6
4	–	158.4	–	–	158.4
7	2.86 (t, $J = 7.4$)	35.1	2.85 (t, $J = 7.4$)	8	35.1
8	4.04 (dd, $J = 15.0, 7.5$)	70.8	4.02 (dd, $J = 14.8, 7.4$), 3.72 (m)	7	70.9
OMe	3.75 (s)	54.4	3.74 (s)		54.4
1′	4.29 (d, $J = 7.8$)	103.1	4.29 (d, $J = 7.8$)	2′	103.2
2′	3.18 (d, $J = 7.8$)	73.9	3.18 (d, $J = 7.8$)	1′,3′	73.8
3′	3.25–3.35 (m)	76.7	3.34 (t, $J = 7.8$)	2′	75.7
4′	3.25–3.35 (m)	70.4	3.30 (m)		70.3
5′	3.25–3.35 (m)	76.7	3.43 (m)		76.7
6′	3.86 (dd, $J = 11.8, 1.8$)	61.5	4.08 (dd, $J = 11.3, 1.8$), 3.72 (m)	6′	68.2
1″	–	–	4.29 (d, $J = 6.7$)	2″	103.9
2″	–	–	3.57 (d, $J = 6.9$)	1″,3″	71.1
3″	–	–	3.48 (m)	2″, 4″	72.9
4″	–	–	3.78 (m)	3″	68.3
5″	–	–	3.84 (dd, $J = 12.4, 3.1$), 3.42 (m)	5″	65.5

[a] ^1H and ^{13}C NMR spectra were acquired in CD_3OD at 500 and 125 MHz, respectively; TMS was used as internal standard; chemical shifts are shown in the δ scale with *J* values (Hz) in parentheses.

formula of $C_{32}H_{42}O_{17}$. From these structural elements we conclude that heterodontoside (**5**) is a phenylethanoid disaccharide and defined as 2-(4-methoxyphenyl)-ethyl-β-*O*-D-glucopyranosyl-6-β-*O*-D-xylopyranoside (Fig. 1).

Re-combined early fractions of *R. semenovii* (Fr. 3–11), fractionated nearly the same way as for *R. heterodonta*, afforded the two cyanogenic glucosides; rhodiocyanoside A (**4**) and lotaustralin (**6**) (Fig. 1). They were identified from their physical, chemical and spectroscopic data (NMR and HPLC-ESI-MS). Lotaustralin (**6**) was previously reported in *R. rosea* (Akgul et al., 2004).

The phenolic glycoside mixture of *R. rosea* obtained from the early fractions of the Sephadex LH-20 column (Fr. 1–11) was characterized by HPLC-ESI-MS. Salidroside (**3**), rosin (**8**), rosarin (**9**), and rosavin (**10**) were identified according to their mass units and by comparing their retention times with commercial standards and reported literature (Tolonen et al., 2003a,b).

2.3. Proanthocyanidins

^{1}H and ^{13}C NMR spectra (acetone-d_6) of the early proanthocyanidin fraction (Fr. 12) of *R. heterodonta* showed a simple spectrum for epigallocatechin-3-*O*-gallate (EGCG). ^{1}H NMR showed two sharp singlets at δ 7.03 and 6.63, each integrated for two protons, characteristic for gallate moiety and pyrogallol B-ring substitution, respectively. Also signals at δ 3.04 (1H, dd, $J = 17.1$, 4.5 Hz, H-4β) and 2.91 (1H, dd, $J = 17.1$, 2.0 Hz, H-4α), in addition to the small coupling constant ($J_{2,3} < 2.0$ Hz) confirmed a relative 2,3-*cis* configuration. The downfield shift of H-3 at δ 5.56 indicated the attachment of the galloyl moiety at this position. The ^{13}C NMR spectrum was consistent with the ^{1}H NMR spectrum, where it showed peaks at δ_c 106.8 (C-2′, 6′) of ring B and 110.0 (C-2″, 6″) of the gallate ester moiety. Also, the upfield chemical shift of C-2 and C-3 (δ_c 78.0 and 69.3), and the downfield shift of C-4 (δ_c 26.6) confirmed the 2,3-*cis* configuration. The 2*R*, 3*R* configuration was based on the negative $[\alpha]_D^{25}$ value (−122, acetone, c 5.0). Both ^{1}H and ^{13}C NMR spectra were identical to the corresponding spectra obtained from the commercial standard and consistent with the reported data for (−)-EGCG (Davis et al., 1996). The ^{1}H NMR spectra of later fractions (Fr. 13–15) were difficult to interpret where they showed broadening and complexity of signals; however, the prominent peaks of the gallate moiety and trihydroxy substitution of the B-ring were clearly recognized in the spectra.

The composition of the proanthocyanidin fractions (Fr. 12–15) was also investigated by HPLC-ESI-MS in the positive ion mode. This soft ionization technique usually gives ion peaks making it useful for molecular mass determination (Guyot et al., 1997). Fraction 12 of both *R. heterodonta* and *R. semenovii* showed the presence of only one peak with *m*/*z* 459 $[M + 1]^+$ corresponding to EGCG, in agreement with its NMR spectroscopic data. Later fractions from both plant extracts showed mixtures of oligomeric and polymeric proanthocyanidins. Representative ESI positive-ion mode spectra for the three species (Fr. 14, Sephadex LH-20) are shown in Fig. 2. A series of singly charged ions $[M + 1]^+$, *m*/*z* 459, 611, 763, 915, 1067, 1219, 1371, 1524 $[M + 2]^+$, 1675, 1827, and 1979 were detected; all of these were consistent with molecular masses of single carbon-carbon linkages. The most abundant ion at *m*/*z* 763 was consistent with a molecular mass of heterogenic dimeric prodelphinidin, suggesting one unit of epigallocatechin linked to one unit of epigallocatechin gallate by a single carbon–carbon bond (B-type). These results were consistent with previous reports on *R. semenovii* (Matamarova et al., 1999; Kuliev et al., 2004). Data also showed similar proanthocyanidin contents for all three species; dimers, trimers, tetramers, and pentamers of heterogenic constitution as well as homogenic oligomers, but with different proportional compositions. The *R. rosea* proanthocyanidin fraction showed different spectra compared to the other two species, where the homogenic oligomeric prodelphinidin gallates were the predominant forms of proanthocyanidins. This was clear from the relative abundance of the peaks at *m*/*z* 915 $[M + 1]^+$ for EGCG dimer, *m*/*z* 1371 $[M + 1]^+$ for EGCG trimer and *m*/*z* 1827 $[M + 1]^+$ for EGCG tetramer (Fig. 2). HPLC-ESI-MS data provided no evidence for the absolute and relative orientation of the many chiral centers or the location of the interflavanyl bond.

The ^{1}H NMR spectra of the proanthocyanidin fractions 12, 13, 14, and 15 from *R. rosea* displayed a complex pattern with broad signals, indicating highly polymerized proanthocyanidins. A subsequent fractionation of the combined fractions (Fr. 12–15) on a Sephadex LH-20 column afforded a purified polymeric proanthocyanidin fraction, obtained after freeze-drying as a light-brown powder, $[\alpha]_D^{25}$ +86.4 (*c* 2.14 MeOH–H_2O 1:1). The ^{13}C NMR spectrum (acetone-d_6-D_2O 1:1 v/v) (Fig. 3) showed a sharp peak at δ_c 110.0 indicating a gallate functionality, and at δ_c 106.3 characteristic for 2′, 6′ nonoxygenated carbons of the pyrogallol B-rings. These two prominent peaks indicated the presence of prodelphinidin gallate esters (Porter et al., 1982). The presence of weak signals around 115.5 ppm indicated also the presence of some units in the polymer with a dihydroxy B-ring substitution (epicatechin-gallate esters, ECG). The upfield resonances at δ_c 33.0–36.0 and 26.0 ppm were attributable to the C-4 of the extender and terminal flavanoid units, respectively.

The flavonoid units were predominantly in the 2,3-*cis* configuration as indicated by the upfield resonance of the heterocyclic ring carbons around δ_c 76 (C-2) and the absence of the corresponding downfield signal for any *trans* isomer (δ_c 84) (Porter et al., 1982; Haslam, 1989). The results also suggest that the terminal unit was predominantly 3-*O*-galloylepigallocatechin as indicated by the absence of resonances in the 27–29 ppm region and the presence of the characteristic upfield C-4 chemical shift at about 26.0 ppm. Also the C-3 resonance of the terminal unit was clearly at 69.5 ppm indicating a flavan-3-*O*-galloyl terminal unit (Foo et al., 1996). This was further confirmed

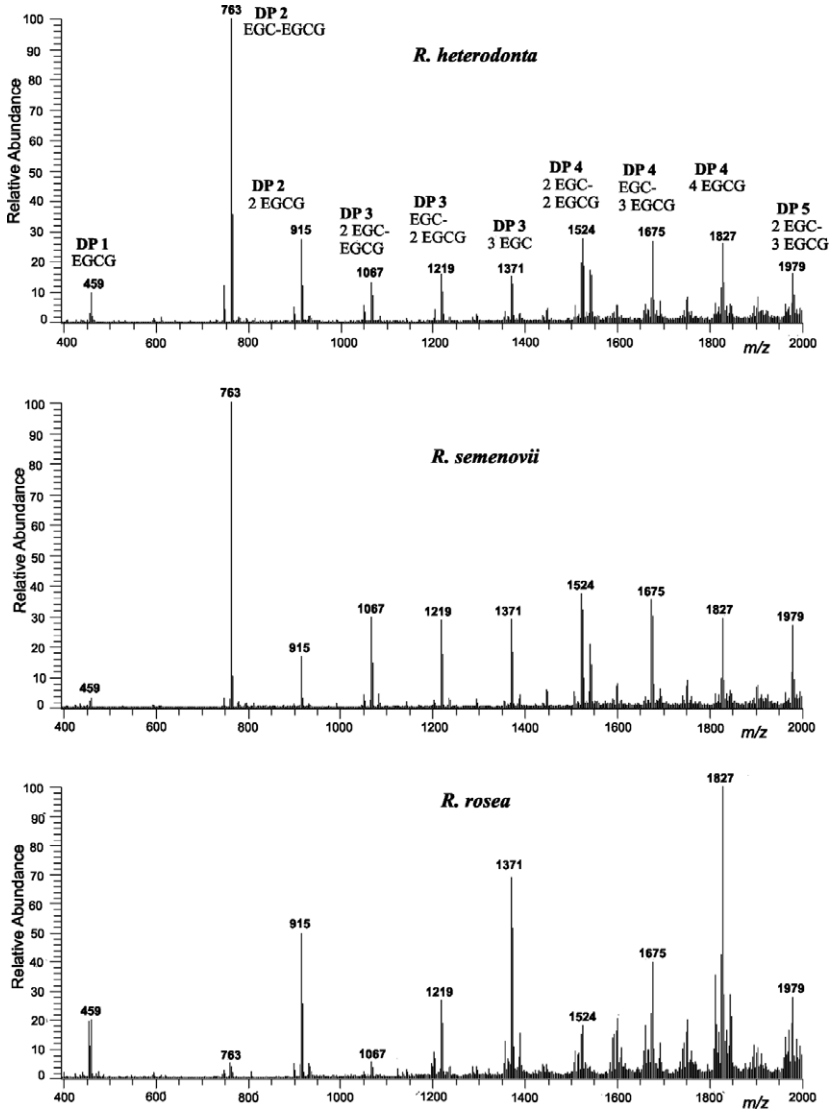


Fig. 2. ESI positive-ion mode mass spectra of proanthocyanidin fraction 14, Sephadex LH-20, from the three *Rhodiola* species. epigallocatechin (EGC), epigallocatechin gallate (EGCG), and degree of polymerization (DP).

by thiolysis degradation (see below) of proanthocyanidin polymers using toluene-α-thiol followed by identification using HPLC-ESI-MS and NMR analysis of the purified thioadducts. Although we had no evidence for the location of the interflavanyl bond, the C-4 to C-8 linkages were favored in these compounds since the alternative C-4 to C-6 linkages were less likely due to their more restricted occurrence (Fletcher et al., 1977; Hemingway et al., 1982).

2.4. Thiolytic degradation of proanthocyanidins

Thiolytic degradation coupled with the reversed-phase HPLC was applied to elucidate the average degree of polymerization (DPn) of the polymeric proanthocyanidins. In this reaction, the flavonoid extension units of the proanthocyanidins are captured by toluene-α-thiol to form the benzylthioether derivatives and only the terminal units are released as the free flavan-ol or flavan-3-*O*-gallates. The reaction was catalyzed by acid under mild conditions; therefore the flavan-3-*O*-gallate units were preserved (Gu et al., 2002). To settle the absolute configuration of the extension units, the benzyl thioether derivative was isolated and purified on Sephadex column using ethanol as eluting solvent to give compound **7a** (Fig. 1). Its ^{1}H NMR spectra (see Section 3) showed the appearance of downfield signals due to the benzene ring. The small coupling constants of the heterocyclic proton resonances are in accord with those of the 2,3-*cis* and 3,4-*trans* configuration in the C-ring (Haslam, 1989). The negative optical rotation (−108) and comparison with reported data (Tanaka et al., 1994; Hashimoto et al., 1989) indicated a 2*R*, 2*R*, 3*S* configuration, and it is identified as (−)-epigallocatechin-3-*O*-gallate-(4β-*S*)-benzylthioether (**7a**). The identities of the individual peaks for the three plants were determined by HPLC-ESI-MS (LCQ) using the same method run conditions as done with the HPLC (Agilent 1100 instrument). The HPLC chromatogram for

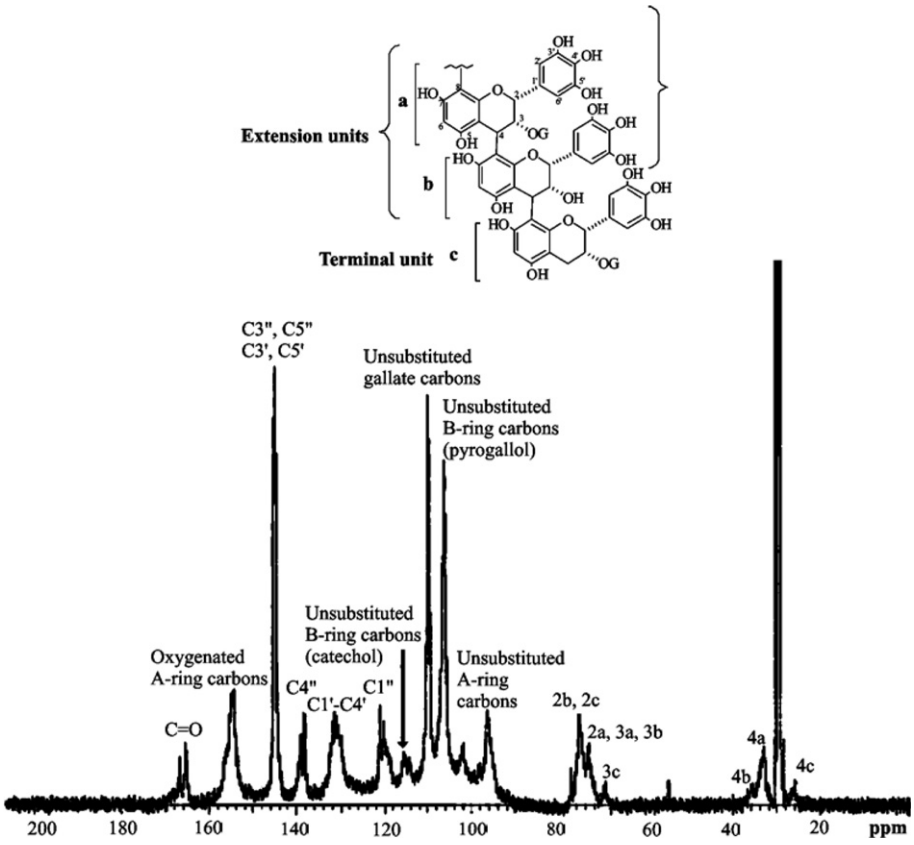


Fig. 3. ^{13}C NMR spectrum of the proanthocyanidin polymer fraction from *R. rosea* (750 MHz, acetone-d_6-D_2O 1:1 v/v).

the three *Rhodiola* species after thiolysis (Fig. 4) showed two main peaks corresponding to m/z 459 $[M+1]^+$ EGCG, terminal unit, R_t 9.1 min, and m/z 581 $[M+1]^+$ EGCG-benzylthioether, extension units, R_t 27.4 min, in addition to other minor peaks and the peak corresponding to the residual reagent at R_t 31.9 min. The estimated degree of polymerization showed that *R. heterodonta*, *R. semenovii*, and *R. rosea* had divergent DPn values of 5 ± 1, 6 ± 1, and 13 ± 3 ($n = 4$), respectively. These results were also in agreement with the HPLC-ESI-MS and NMR spectroscopic data for the polymeric proanthocyanidin fractions from the three species.

2.5. Comparison of phytochemical constituents in Rhodiola species

Characterization of phytochemical compounds present in the three *Rhodiola* species was accomplished using fractions obtained from a 70% acetone plant crude extract and HPLC-ESI-MS and NMR spectroscopic analysis. In general, the two central Asian *Rhodiola* genotypes (*R. heterodonta* and *R. semenovii*) had several phenolic glycosides and proanthocyanidin compounds in common with *R. rosea*. However, an in-depth characterization of *Rhodiola* extracts revealed some unique differences in both the phenyl glycoside and proanthocyanidin constituents in the three *Rhodiola* genotypes as detailed below.

2.5.1. Phenyl and cyanogenic glycosides

The *R. heterodonta* genotype contained a newly discovered phenolic glycoside designated "heterodontoside" (**5**) that has not been previously reported and was not detected in either of the other two species. *R. heterodonta* and *R. rosea* contained phenylethanoid and phenylpropanoid compounds that were not detected in *R. semenovii*. *R. semenovii* contained mainly the two major cyanogenic glucosides; rhodiocyanoside A (**4**) and lotaustralin (**6**). Major phenolic glycosides detected in *R. heterodonta* included, in addition to heterodontoside (**5**), tyrosol (**1**), viridoside (**2**), salidroside (**3**), and rhodiocyanoside A (**4**). The most common phenyl glycosides isolated in this study from *R. rosea* were salidroside (**3**), rosarin, rosavin, and rosiridin. None of these ethanoid or propanoid glycosides were detected in *R. semenovii*. These findings indicated that phytochemical constituents among the three *Rhodiola* species are species-dependent and may account for different phytochemical activities and benefits from each.

2.5.2. Proanthocyanidins

Aqueous acetone (70%) was reported as the most efficient solvent to extract proanthocyanidins from plant tissues (Gu et al., 2003a, 2003b; Yousef et al., 2004). Of the dry 70% acetone extract, pure proanthocyanidin oligomeric and polymeric fractions from Sephadex LH-20 separation collectively constituted *ca.* 28%, 33%, and 35% dry weight

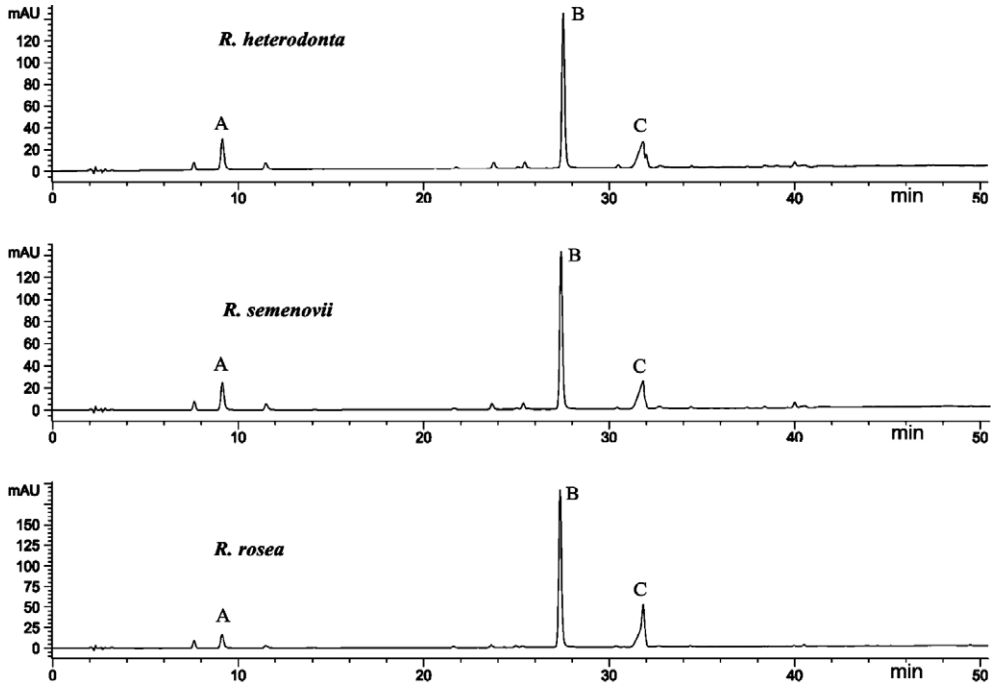


Fig. 4. RP-HPLC chromatogram for *R. heterodonta*, *R. semenovii*, and *R. rosea*, proanthocyanidin polymers detected at 280 nm after thiolysis. Terminal units (−)-EGCG, R_t: 9.1 min (A), (−)-EGCG-benzylthioether, R_t: 27.4 min (B), and reaction reagent, toluene-α-thiol, R_t: 31.9 min (C).

for *R. heterodonta*, *R. semenovii*, and *R. rosea* respectively. This high proportion of proanthocyanidin content is expected to have a significant role in the overall phytochemical activities of the three *Rhodiola* species studied (Brown et al., 2002; Prior and Gu, 2005; Xie and Dixon, 2005). The three *Rhodiola* species contained similar proanthocyanidin constituents including monomers, dimers, trimers, tetramers, and pentamers of epigallocatechin and its gallate esters. However, the relative abundance of these polymers differed among the three species. While the dimer EGC-EGCG (*m*/*z* 763) was the predominant proanthocyanidin in *R. heterodonta* and *R. semenovii*, more abundant homogenic prodelphinidin gallate oligomers were found in *R. rosea*, in particular the EGCG dimer (*m*/*z* 915), EGCG trimer (*m*/*z* 1371), and EGC tetramer (*m*/*z* 1827). The estimated degree of polymerization (DP) of proanthocyanidin was ~5 and 6 for *R. heterodonta* and *R. semenovii*, respectively, but ~13 for *R. rosea* as stated above. In the three species, dimeric and oligomeric proanthocyanidins were only reported before in *R. semenovii* (Matamarova et al., 1999; Kuliev et al., 2004). While proanthocyanidin composition was nearly similar in the three species, *R. rosea* contained a more highly polymerized ratio and this was associated with more difficulties in the isolation and purification during both silica gel and Sephadex LH-20 column chromatography separations.

2.5.3. Conclusion remarks

In summary, this study presented a comprehensive phytochemical characterization of two limitedly-known *Rhodiola* species (*R. heterodonta*, and *R. semenovii*) as compared to the well-known *R. rosea*. Using fresh rhizomes with the same techniques of extraction, isolation, and identification of phytochemical components offered data which elucidated direct cross-comparison between the three *Rhodiola* species. Phytochemical constituents and compounds unique to these species are most likely to be responsible for their pharmaceutical interest in the central Asian region, Europe, and more recently in the USA. A new phenylethanoid glycoside; heterodontoside (**5**), was identified in the *R. heterodonta* species. Proanthocyanidins known to have pharmacological activities, constituting a fairly large portion of the *Rhodiola* extracts (*ca.* 30% of the 70% acetone dry crude extract), were also characterized in all three species. This is the first report to study in detail the chemistry of *R. heterodonta* and to isolate cyanogenic compounds in *R. semenovii*. To the best of our knowledge, this is also the first report on the isolation and characterization of oligomeric and polymeric proanthocyanidins from *R. rosea*.

3. Experimental

3.1. General

HPLC-ESI-MS data were obtained, unless otherwise stated, using a LCQ Deca XP mass spectrometer (150–2000 *m*/*z*) attached to photodiode array (PDA) detector (200–600 nm) (Thermo Finnigan Corp., San Jose, CA). Analyses were performed according to procedures developed by Yousef et al. (2004) with minor modifications.

Briefly, the HPLC separations were carried out on a C_{18}-reversed-phase column (2.1 × 150 mm, VYDAC, Cat. #: 201 SPS215). The mobile phase solvent consisted of 95% DD H_2O and 5% CH_3CN with 0.1% formic acid (A) and 95% CH_3CN and 5% DD H_2O with 0.1% formic acid (B). A step gradient of 0%, 30%, 60%, 90%, and 0% of solvent B was used at 3, 30, 45, 50, and 60 min, respectively, with 200 μl/min flow rate and 10-μl injection volumes. The column was equilibrated with solvent A for 10 min between samples at the same flow rate. Low resolution and high resolution electronic spray ionization mass spectra (LR-ESI-MS and HR-ESI-MS) were recorded on a Micromass ZAB-SE Spectrometer (Waters Corporation, Beverly, MA, USA) in the Mass Spectrometry Laboratory of the School of Chemical Sciences, University of Illinois, Urbana-Champaign.

The ^{1}H NMR, 1D and 2D (500 MHz) and ^{13}C-NMR (125 MHz) spectra (CD_3OD for phenolic glycosides, acetone-d_6 or acetone-d_6-D_2O 1:1 for proanthocyanidins) were recorded on a Varian Inova 500 spectrometer (Varian Instruments, Palo Alto, CA), at a proton frequency of 500.078 MHz, using TMS as internal standard. The ^{13}C NMR spectra of the highly polymerized PA fraction was obtained on the Varian Unity Nova 750 spectrometer with a 10 mm broadband probe at 20°C, at an operating frequency of 188.55 MHz over a sweep width of 45 kHz, using a 90° pulse width of 18.00 μs and an acquisition time of 2.89 s. Optical rotations were measured in CH_3OH solution using a JASCO DIP-370 digital polarimeter (Jasco Limited, UK) at 25 °C. UV spectra were obtained on a Shimadzu UV-2401 PC UV–Vis Recording Spectrophotometer (Shimadzo, North America). Chromatographic separations were achieved by cc using lipophilic Sephadex LH-20 (Sigma, St. Louis, MO) and silica gel 60 230–400 mesh ASTM (Merck, Whitehouse Station, NJ). Fractions were analyzed by Thin Layer Chromatography (TLC) using silica gel 60 F254 pre-coated plates with a solvent system of EtOAc–MeOH–H_2O (77:13:19). The TLC plates were visualized by spraying with reagents for primary separation and identification compounds. The spraying reagents used were vanillin–HCl (vanillin–HCl–MeOH, 1:1:20, v/v/v) and cleaning reagent (sodium dichromate $Na_2Cr_2O_7 \cdot 2H_2O$–H_2O–H_2SO_4, 1:5:8, w/v/v) at 110 °C for 10 min.

Commercial standards of (+)-catechin, (−)-epicatechin, (−)-gallocatechin, (−)-epigallocatechin, (−)-gallocatechin-3-*O*-gallate, (−)-epigallocatechin-3-*O*-gallate, tyrosol (**1**), salidroside (**3**), rosin, rosarin and rosavin (Chromadex, Laguna Hills, CA) were used to compare with compounds isolated from *Rhodiola* extracts.

3.2. Plant material

Fresh rhizomes of *R. heterodonta* (Hooker & Thomson, Boriss) (K. Tojboev s.n., 12 June 2004, ICBG Central Asia voucher UPL_00085, ILLS, MO) and *R. semenovii* (Regel & Herder, Boriss) (I. Sodombekov s.n., 28 July 2004, ICBG Central Asia voucher KPL_00142, ILLS, MO) were harvested in the mountainous regions of Uzbekistan and Kyrgyzstan, respectively, in 2004. Fresh rhizomes of golden root (*R. rosea*) harvested in May 2005 from coastal islands in central Norway were generously donated by Rosenrot Norge Ltd., Oslo, Norway. Rhizomes were immediately upon arrival cleaned thoroughly with water, cut into segments (2–5 g), and stored at −80 °C until extraction.

3.3. Extraction and isolation

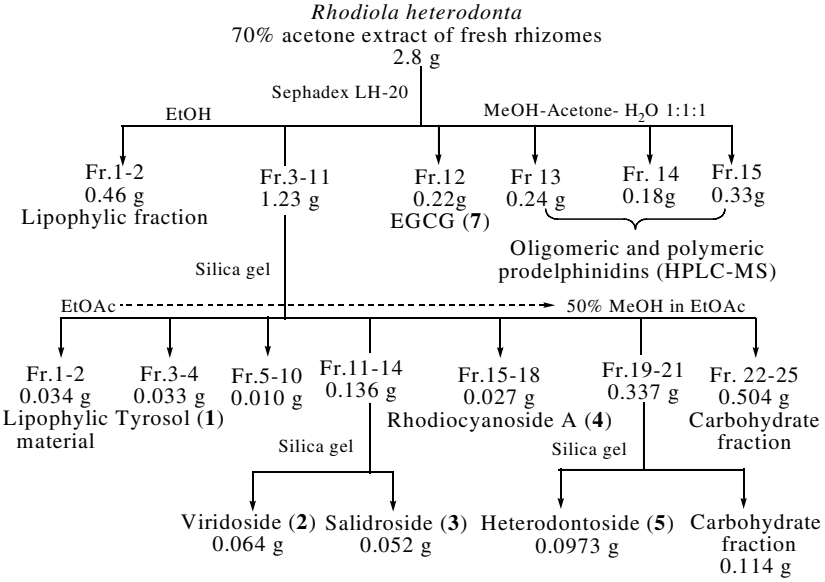
Frozen rhizome tissues (150 g) were exhaustively extracted with 70% aq-acetone (7 × 500 ml, 2 min each) at room temperature using a Turbo-twister blender (Hamilton Beach/Proctor-Silex Inc., Southern Pines, NC). The rhizome tissue was blended for 2 min at high speed and filtered first with cheesecloth, then through Whatman # 4 paper. The acetone and some of the water were removed under reduced pressure at 40 °C. The concentrate was then frozen at −20 °C and lyophilized. The average 70% acetone crude-extract dry mass was 17%, 10%, and 11% for *R. heterodonta*, *R. semenovii*, and *R. rosea*, respectively. This crude extract (10 g) was then fractionated via vacuum liquid chromatography (VLC) over silica gel (Type 60, 10–40 μm, $CaSO_4$ binder S-6503, Sigma Chemical Co., St. Louis, MO). Twenty-two fractions (100 ml each) of increasing polarity were obtained (Yousef et al., 2004). The elution system started with petroleum ether (100%), petroleum ether–EtOAc (1:1), EtOAc (100%), followed by 2%, 5%, 8%, 12%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 60%, 70%, 80%, 90%, 100% of MeOH–H_2O (1:1) in EtOAc. Solvents were removed using reduced pressure at 40 °C, and the dried fractions were stored at −20 °C. From each powdered dry fraction, 5 mg were dissolved in 1 ml 50% aq-MeOH, filtered though 0.45 nm nylon filters and 10 μl were injected for analysis by HPLC-ESI-MS.

3.4. Purification and characterization of phenolic and/or cyanogenic glycosides and proanthocyanidins

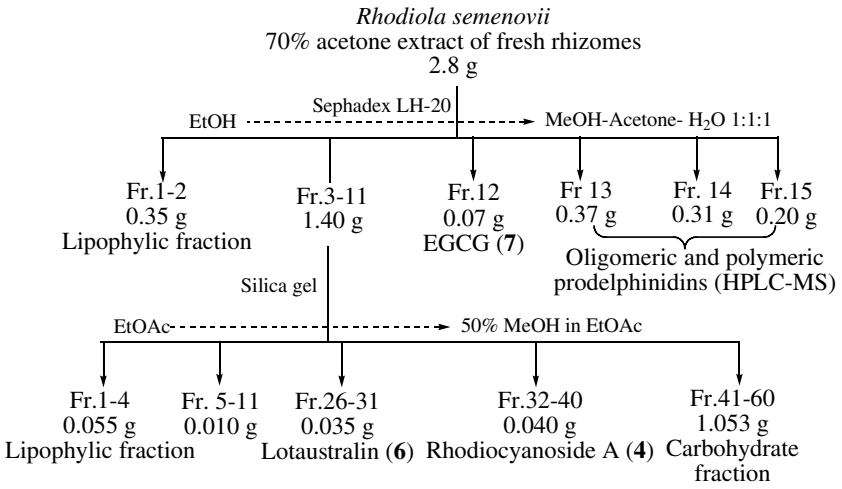
To separate phenolic and/or cyanogenic glycosides from proanthocyanidins, a portion of the 70% acetone crude extract (2.8 g) of each plant was applied to a Sephadex LH-20 column (2.5 × 30 cm) which had been equilibrated with EtOH, collecting a total of 15 50-ml fractions. Elution started with EtOH (Fr. 1–11) to collect phenolic and cyanogenic glycosides. Oligomeric and polymeric proanthocyanidins were recovered from the column using MeOH–acetone–H_2O (1:1:1) as the elution solvent (Fr. 12–15), Schemes 1–3.

3.4.1. R. heterodonta phytochemical constituents

Fractions 3–11 from the Sephadex LH-20 column were combined (1.23 g) and applied to a silica gel column



Scheme 1. Sequential fractionation of the 70% aq. acetone crude extract from *R. heterodonta*.



Scheme 2. Sequential fractionation of the 70% aq. acetone crude extract from *R. semenovii*.

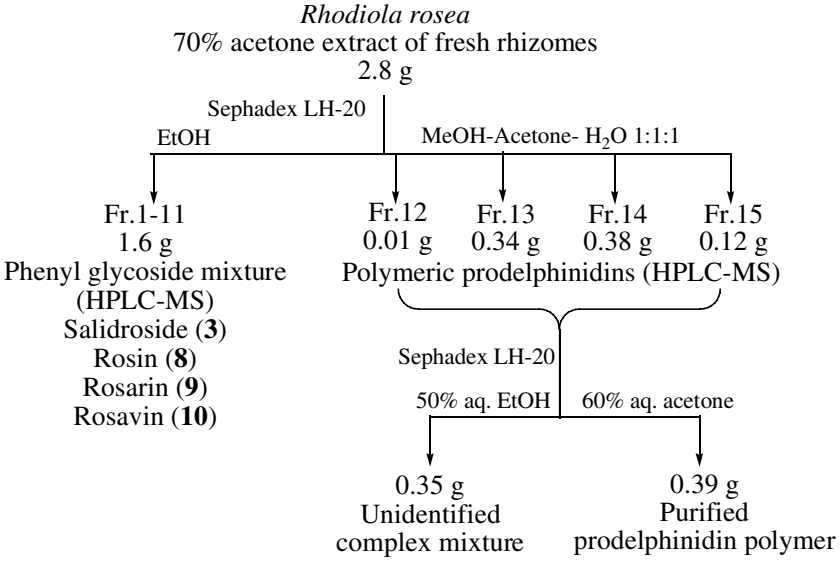
(2.5 cm × 35 cm). Elution started with EtOAc followed by an EtOAc–MeOH gradient (2–50%), collecting 50-ml fractions (Scheme 1). Compound **1** (tyrosol, 33 mg) was eluted in the early fractions (Fr. 3–4) with 100% EtOAc. Compounds **2** and **3** were obtained in a mixture in fractions 11–14 eluted with 10% MeOH in EtOAc. This mixture was further separated on a second silica gel column using EtOAc–MeOH (9:1, v/v) solvent where 64 mg and 52 mg of viridoside (**2**) and salidroside (**3**), respectively, were isolated. Compound **4** (rhodiocyanoside A, 27 mg) was obtained in the fractions 15–18 eluted with 16% MeOH in EtOAc. Fractions 19–20 eluted with 20% MeOH in EtOAc, were combined and further chromatographed on a silica gel column (EtOAc–MeOH–H_2O, 79:11:10) to give compound **5** (heterodontoside) (97.3 mg). Preliminary TLC for the fractions followed by NMR and HPLC-ESI-MS allowed the characterization of proanthocyanidins in the fractions 12–15.

3.4.2. *R. semenovii* phytochemical constituents

Fractions 3–11 from Sephadex LH-20 column were combined (1.4 g) and fractionated over a silica gel column similar to the above procedure with *R. heterodonta* where compounds **6** (lotaustralin, 35 mg) and **4** (rhodiocyanoside A, 40 mg) were isolated (Scheme 2). Proanthocyanidin fractions (12–15), containing monomeric and polymeric proanthocyanidins, were similarly characterized by TLC, NMR, and HPLC-MS.

3.4.3. *R. rosea* phytochemical constituents

Fractions 1–11 containing the ethanoid and propanoid glycosides were combined (1.6 g), and subjected to



Scheme 3. Sequential fractionation of the 70% aq. acetone crude extract from *R. rosea*.

HPLC-MS analysis. In a similar way to the above two species, fractions 12–15, containing oligomeric and polymeric proanthocyanidins, were further analyzed by HPLC-ESI-MS. For NMR analysis, the fractions from 12 to 15 (0.85 g) were combined and further purified on Sephadex LH-20 column (Scheme 3). The column was first washed with an excess of EtOH–H_2O (1:1, v/v) before eluting the proanthocyanidin polymer with 60% aq. acetone. The purified polymeric proanthocyanidin fraction was freeze-dried (0.394 g) before HPLC-ESI-MS, and ^1H and ^{13}C NMR analyses.

3.5. Thiolytic degradation of proanthocyanidin polymers

To estimate the degree of polymerization and identify the stereochemistry of the extension units of the proanthocyanidin polymeric fractions for the three *Rhodiola* genotypes, a thiolysis degradation method was used (Gu et al., 2002; Guyot et al., 1998, 2001). Based on the HPLC-ESI-MS profile for Sephadex LH-20 fractions, fraction 14 (highly polymerized) from each plant was used for this analysis. The thiolysis analysis was performed using an Agilent 1100 HPLC system (Agilent Technologies Inc., Wilmington, DE) with autosampler, diode array detector (DAD, 280 nm) and 24 mm × 4 mm × 5 μm Supelcosil LC-18 reversed-phase column (25 °C) (Sigma-Aldrich, Bellefonte, PA). The elution solvents consisted of 2% acetic acid in H_2O (A) and 100% MeOH (B). The linear gradient started at 15% B and increased to 80% B in 45 min and continued for 5 min at 80% B followed by 15 min of 15% B to equilibrate the column with a constant flow rate of 1 ml/min.

Duplicates of commercial standards (1.25 mg/ml 100% MeOH) including (+)-catechin, (−)-epicatechin, (−)-gallocatechin and (−)-epigallochatechin, (−)-gallocatechin-3-*O*-gallate, and (−)-epigallocatechin-3-*O*-gallate, and the *Rhodiola* samples both before and after thiolysis, were directly injected into the HPLC. Degree of polymerization was calculated by dividing the peak area of the extension units (benzylthioethers) by that of the terminal units for each sample (Guyot et al., 2001).

3.5.1. Isolation and purification of the thiolysis products

To separate the individual components from the thiolysis mixture, a larger concentration of the same fraction in MeOH (~100 mg/ml) was subjected to thiolysis the same way as above. The thiolysis mixture was freeze-dried and the obtained dry powder was chromatographed over Sephadex LH-20 column (EtOH) to afford two main compounds; (−)-epigallocatechin-3-*O*-gallate-(4*β*-*S*) benzylthioether (extension units, **7a**) and (−)-epigallocatechin-3-*O*-gallate (terminal units, **7**).

(−)-Epigallocatechin-3-*O*-gallate-(4*β*-*S*) benzylthioether (**7a**): pale brown amorphous powder, $[\alpha]_D^{25}$ – 108 (*c* 5.5, acetone), ESI-MS $[M+1]^+$ *m/z* 581, ^1H NMR (500 MHz, acetone-d_6): *δ* 7.21–7.52 (5H, m, benzene), 7.01 (1H, s, 2″, 6″ gallate), 6.6 (1H, s, 2′,6′), 6.03, 6.05 (1H each, d, $J = 2.2$ Hz, H-6 and 8), 5.50 (1H, s, H-2), 5.46 (1H, m, H-3), 4.25 (1H, d, $J = 2.2$ Hz, H-4), 4.11 and 4.20 (1H each, d, $J = 13.2$ Hz, Ph-CH_2-S); ^{13}C NMR (125 MHz) δ_c 36.3 (–CH_2S–), 40.0 (C4), 72.1 (C3), 73.6 (C2), 95.0 (C6), 96.4 (C8), 98.2 (C10), 106.0 (C2′,6′), 109 (C″,6″), 120.5 (C1″), 127.1 (C4 benzene), 128.6, 129.3 (C2,3,4,5 benzene), 132.6 (C1′), 138.4, 139.0 (C4′,C4″), 145.3, 145.7 (C3′,5′ and 3″,5″), 156.3, 157.5, 158.8 (C5,7,9), 165.6 (C=O).

3.6. Heterodontoside (**5**)

White powder; $[\alpha]_D^{25}$ – 28.5 (*c* 1.0, MeOH); UV (MeOH) λ_{max} (log *ε*) 225 (1.01), 277 (0.25), 280 (0.19) nm; ^1H NMR and ^{13}C NMR: see Table 1; HRESIMS *m/z* 447.1875 (Calc. for $C_{20}H_{31}O_{11}$ 477.1866 $[M+1]^+$).

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