Variability in Foliar Ellagitannins of *Hippophaë rhamnoides* L. and Identification of a New Ellagitannin, Hippophaenin C

Jussi Suvanto, Petri Tähtinen, Saku Valkamaa, Marica T. Engström, Maarit Karonen, Juha-Pekka Salminen*

Natural Chemistry Research Group, Department of Chemistry, University of Turku, FI-20014, Turku, Finland

*Corresponding author. Phone: +358 400 824452. E-mail: j-p.salminen@utu.fi.

Abstract

Berries of common sea-buckthorn (*Hippophaë rhamnoides* L.) are well known and used for their bioactive components and while there is a considerable amount of research on the leaves as well, their ellagitannins (ETs) have not been a prominent focus of research. We identified and quantified ten major hydrophilic polyphenols, all ETs, in *H. rhamnoides* leaves and compared their abundance between 58 plant individuals. Of these compounds, hippophaenin C was characterized as a new ellagitannin by various spectrometric methods. The total concentrations of ETs ranged from 42.5 mg g^{-1} dry weight (DW) to 109.1 mg g^{-1} DW between individual plants. Among the ETs, hippophaenin C, stachyurin, and casuarinin were on average the most abundant compounds. Sexes did not differ significantly, while cultivars showed variation in some ETs. These results suggest that *H. rhamnoides* leaves could be a potential and rich source of several ETs.

Keywords

common sea-buckthorn; electronic circular dichroism; ellagitannins; hippophaenin C; *Hippophaë rhamnoides* L.; HPLC-DAD; UHPLC-DAD–ESI-Orbitrap-MS; NMR

Introduction

Common sea-buckthorn (*Hippophaë rhamnoides* L.) is a deciduous and dioecious shrub or tree that can reach a height of up to 10 meters. Belonging to the family Elaeagnaceae, common sea-buckthorn is the most widespread species of its genus, *Hippophaë* L., and is native to several regions in Europe and Asia. Furthermore, *H. rhamnoides* has several subspecies, *Hippophaë rhamnoides* L. ssp. *rhamnoides* which is the subspecies found growing in coastal areas of Northern and Western Europe.[1–4] The identification of and particular information about the subspecies of *H. rhamnoides* used in studies is often omitted from publications; therefore the literary references are likely to contain subspecies other than ssp. *rhamnoides*.

The female plants produce yellow to orange drupes, and the leaves, which are green-grey in color on the upper surface and silver-grey on the lower surface, are narrow and lanceolate in shape.[1] There are some reports on the chemical profile of the leaves of *H. rhamnoides*, focusing most often on lipophilic compounds such as carotenoids[5,6] as well as tocopherols and plastochromanol-8.[5] However, there has also been research on some hydrophilic phenolics such as flavonoids[7–9] and ellagitannins.[10,11] The berries and berry juices of *H. rhamnoides* have been studied comprehensively and are known to contain high concentrations of fatty acids,[12,13] vitamins C[14,15] and E,[13,16] and carotenoids,[15] amongst others. The latter three contribute to the high antioxidative capacity of the berries. In addition to its nutritional use, *H. rhamnoides* has been used as a traditional medicinal herb in several regions in Asia for centuries.[17]

Although several plant parts of *H. rhamnoides*, such as berries, leaves, seeds, and bark, have been studied to investigate their polyphenolic compounds, the reports concerning bioactivities often omit the exact characterization of the studied material.[7,9,18,19] Moreover,

ellagitannins (ETs) are often disregarded completely. Furthermore, even though the method of reporting total ETs, total hydrolyzable tannins, total tannins or even total phenolics is still relatively common, it is far from ideal, since there can be considerable variation in the activities with even minor structural modifications in ETs.[20] These differences have been shown for e.g. their *in vitro* oxidative,[21] anthelmintic,[22] anti-methanogenic,[23] antimicrobial,[24] and antiviral activities[25] as well as protein affinities.[26] Altogether, ellagitannins have proved to be promising and potent compounds with multiple uses concerning human and animal health.

The main ETs and their combined total concentration found in the leaves of *H. rhamnoides* have been reported previously.[11,27,28] In this study, our aim was to reveal, for the first time, quantitative data on the ten main foliar ETs of *H. rhamnoides*, allowing more precise conclusions to be drawn on the possible variations of the foliar bioactivity between the two sexes and different cultivars. Thus future research will be able to avoid resorting only on the total phenolic or ET content of the leaves, and can use our data to plan their studies with either of the sexes or some selected cultivar. A total of 58 plant individuals were included from three different cultivars and both sexes. A comprehensive structural elucidation of hippophaenin C, using LC-MS^n and NMR and CD spectroscopic methods, is also presented.

Materials and Methods

Chemicals and Reagents

LC-MS grade acetonitrile was from Sigma-Aldrich GmbH (Steinheim, Germany) and formic acid (for LC-MS) and analytical grade acetone from VWR (Helsinki, Finland). LC grade acetonitrile was from Lab-Scan (Dublin, Ireland) and phosphoric acid from J.T. Baker (Deventer, Netherlands). Water was purified with either a Millipore Synergy UV (Merck KGaA, Darmstadt, Germany) or an Elgastat UHQ-PS (Elga, Kaarst, Germany) water

purification system. Acetone-d_6 (99.96 %) was from Euriso-top SAS (St-Aubin Cedex, France).

Plant Material

Sea buckthorn leaf samples were collected from Nivala, Northern Ostrobothnia, Finland in September 2006. The samples included both female and male individuals from cultivars K (13 female and male individuals), R (9 female and male individuals), and RUXRA (7 female and male individuals). The collected leaves were air dried at 40 °C. All of the plant cultivars were hybrids developed at Natural Resources Institute Finland in Ruukki, Siikajoki, Northern Ostrobothnia, Finland. Shortly after drying, the samples were homogenized into a powder using a water-cooled blade mill and stored at –20 °C until extracted and analysed within two months of sample collection.

HPLC-DAD and HPLC-ESI-MS

Dried and ground leaves (200 mg per sample) were extracted four times (4 × 1 hr) with 70% aqueous acetone (4 × 8 ml) on a planary shaker within two months of sample collection. After the evaporation of acetone *in vacuo* and lyophilization, the extract was dissolved in water (3 × 2 ml) and the supernatant of the centrifuged (10 min at 2000×g) sample was filtered through a 0.45 µm PTFE syringe filter and kept frozen at –20 °C until analyzed with HPLC-DAD. For quantification, HPLC-DAD analyses of the extracts were performed on a Merck-Hitachi LaChrom HPLC system, which consisted of a D-7000 interface, an L-7100 pump, an L-7200 autosampler and an L-7455 diode array detector (Merck-Hitachi, Tokyo, Japan). A LiChroCART Superspher 100 RP-18 column (75 × 4 mm i.d., 4 µm; Merck KGaA, Darmstadt, Germany) was used. The mobile phase consisted of acetonitrile (A) and 0.05 M phosphoric acid (B) and the elution profile was as follows: 0–3 min, 2% A in B; 3–22 min, 2–20% A in B (linear gradient); 22–30 min, 20–30% A in B (linear gradient); 30–35 min, 30–

45% A in B (linear gradient); 35–70 min, column wash and stabilization. The injection volume was 20 µl and flow rate 1.0 ml min^{-1}. UV spectra were acquired between 195 and 450 nm and the quantification for each compound was done by calculating the peak area at 280 nm. The concentrations are reported as pedunculagin equivalents. This approach may slightly under- or overestimate the contents of the other ETs except pedunculagin. However, we believe that this effect is a minor one due to minor structural differences between the studied ETs, and similarities in their UV spectra.

Selected extracts were also analysed by HPLC-ESI-MS analysis in 2006 using a Perkin-Elmer API Sciex triple quadrupole mass spectrometer (Sciex, Toronto, Canada) as in Salminen et al.[29,30] Nine of the ten *H. rhamnoides* ETs were thus characterized as shown in detail by Moilanen & Salminen.[21] Only the structure of hippophaenin C remained unresolved, since it gave *m/z* values of 1103 and 1085 that corresponded to $[M–H]^-$ and $[M–H_2O–H]^-$ of an ellagitannin with molecular mass of 1104. The molecular mass was the same as for hippophaenin B but it had not earlier been witnessed in *H. rhamnoides*. This specific structure thus required further studies.

UHPLC-DAD–ESI-Orbitrap-MS

To measure accurate masses for the correct identification of hippophaenin C and for the verification of all other compounds quantified by HPLC-DAD, selected samples were extracted for UHPLC-DAD–ESI-Orbitrap-MS analyses in 2015. Twenty mg of freeze-dried and ground plant leaf powder was extracted twice with 1.4 ml of acetone/water (4:1, v/v) on a planar shaker (280 min^{-1}) for 3 h and then centrifuged at 21913×g for 10 min. Before the first extraction the powder was let to macerate overnight at +4 °C in the first solvent batch. Supernatants from both extractions were combined, acetone was evaporated in an Eppendorf Concentrator plus (Eppendorf AG, Hamburg, Germany) and the volume was adjusted to 1 ml

with water. The samples were filtered using a PTFE syringe filter (4 mm, 0.2 µm, Thermo Fisher Scientific Inc., Waltham, MA, USA) and analyzed using an ultra-high performance liquid chromatograph coupled to a photodiode array detector (UHPLC-DAD, Acquity UPLC, Waters Corporation, Milford, MA, USA) and a hybrid quadrupole-Orbitrap mass spectrometer (Q Exactive™, Thermo Fisher Scientific GmbH, Bremen, Germany). The prolonged storage of the samples between 2006 and 2015 was ensured to have no effect on the ET composition, i.e. all the same compounds were detected as in 2006. The column used was Acquity UPLC® BEH Phenyl (100 × 2.1 mm i.d., 1.7 µm; Waters Corporation, Wexford, Ireland). The mobile phase consisted of acetonitrile (A) and water and formic acid (99.9:0.1, v/v) (B). The elution profile was as follows: 0–0.5 min, 0.1% A in B; 0.5–5.0 min, 0.1–30% A in B (linear gradient); 5.0–8.5 min, column wash and stabilization. The injection volume was 5 µl and flow rate 0.5 ml min^{-1}.

The heated ESI source (H-ESI II, Thermo Fisher Scientific GmbH, Bremen, Germany) was operated in negative ion mode. The parameters were set at as follows: spray voltage, –3.0 kV; sheath gas (N_2) flow rate, 60 (arbitrary units); aux gas (N_2) flow rate, 20 (arbitrary units); sweep gas flow rate, 0 (arbitrary units); capillary temperature, +380 °C. A resolution of 70,000 and an automatic gain of 3×10^6 was used in the Orbitrap mass analyzer. Pierce ESI Negative Ion Calibration Solution (Thermo Fischer Scientific Inc., Waltham, MA, USA) was used to calibrate the detector. Mass range was set to *m/z* 150–2000. The data was processed with Thermo Xcalibur Qual Browser software (Version 3.0.63, Thermo Fisher Scientific Inc., Waltham, MA, USA).

In addition, the same instrument with the same parameters was used to characterize a purified sample of hippophaenin C (**10**) injected through the UHPLC system to further ensure the purity of the compound. For fragmentation analyses, collision energy of 40 eV was used in the higher-energy collisional dissociation (HCD) cell.

Isolation and Purification of Hippophaenin C

For structural elucidations, compound **10** was isolated and purified from a crude *H. rhamnoides* leaf extract. 9.46 grams of the extract was dissolved in 40 ml of water, centrifuged and the supernatant was applied onto a column (Chromaflex, 320 × 55 mm; Kimble-Chase Kontes, Vineland, NJ, USA) packed with Sephadex LH-20 gel equilibrated in water. Fractionation was performed with 10–50% aqueous methanol and 20–80% aqueous acetone with compound **10** eluting using 40–50% methanol. Methanol was evaporated from the main fractions containing compound **10** followed by their lyophilization, yielding 407 mg of fractions with compound **10**. The purification was completed with reversed-phase high-performance liquid chromatograph (consisting of a Waters 2535 Quaternary Gradient Module, Waters 2998 Photodiode Array Detector, and a Waters Fraction Collector III; Waters Corporation, Milford, USA) equipped with a Gemini 10µ C18 110 Å (150 × 21.2 mm i.d., 10 µm, Phenomenex, Torrance, CA, USA) column using a flow rate of 8 ml min^{-1} and a gradient elution with acetonitrile and 0.1% aqueous formic acid as eluents. The total yield of purified compound **10** was 9.6 mg.

Nuclear Magnetic Resonance Spectroscopy (NMR)

NMR spectra for **9** and **10** in acetone-d_6 (*ca.* 0.015 M) were measured with a Bruker Avance III NMR spectrometer equipped with a Prodigy TCI CryoProbe (Fällanden, Switzerland) operating at 600.16 MHz for ^{1}H and 150.93 MHz for ^{13}C. The structure elucidations and complete assignments of ^{1}H and ^{13}C chemical shifts were done with the aid of DQF-COSY, multiplicity-edited HSQC, HMBC and band-selective CT-HMBC (optimized for 4 and 8 Hz long-range J_{CH} coupling constants), and selective 1D-ROESY (with 200 ms mixing time) experiments. The chemical shifts are reported with respect to the chemical shifts of the solvent signals: δ_H = 2.05 ppm and δ_C(Me) = 29.92 ppm.

Electronic Circular Dichroism Spectroscopy (ECD) and Polarimetry

ECD spectra for **6**, **9**, and **10** utilizing a 1 mm path-length cuvette at 298 K were measured with a Chirascan^{TM} circular dichroism spectrometer (Applied Photophysics, Leatherhead, UK). The spectra were scanned over the range of 190–450 nm, background subtracted and smoothed.

Optical rotation for compound **10** was recorded with an Anton Paar MCP200 polarimeter (Ostfildern-Scharnhausen, Germany) equipped with a 1 dm path-length cuvette.

Hippophaenin C (10)

$[\alpha]_D^{20}$ –217° (H_2O, 1.05mM); UV, λ_{max} (nm) 225, 265 sh (17/83 0.1% HCOOH in H_2O / CH_3CN (V/V)); Cotton effects (× 10^4 deg cm^2 mol^{-1}, H_2O, 0.1 mM): $[\Theta]_{195}$ –12.82, $[\Theta]_{224}$ +17.73, $[\Theta]_{253}$ –4.16, $[\Theta]$280 +4.50; UHPLC-DAD–ESI-Orbitrap-MS (negative, CE = 40 eV): *m/z* 249.04012 ($C_{12}H_9O_6^-$, error –1.4 ppm), 275.01982 ($C_{13}H_7O_7^-$, error 0.4 ppm), 300.99901 ([ellagic acid–H]$^-$, error 0.1 ppm), 529.04341 ([M–COOH–H]$^{2-}$, error –1.5 ppm), 551.03898 ([M–2H]$^{2-}$, error –0.3 ppm), 917.06864 ([M–H_2O–COOH]$^-$, error –0.5 ppm), 935.07965 ([M–gallic acid+H]$^-$, error 0.1 ppm), 1041.08612 ([M–H_2O–COOH]$^-$, error 1.0 ppm), 1059.09585 ([M–COOH]$^-$, error 0.2 ppm), 1085.07536 ([M–H_2O–H]$^-$, error 0.5 ppm), 1103.08521 ([M–H]$^-$, error –0.3 ppm); ^{1}H NMR (600.16 MHz, CD_3COCD_3, 298 K): δ 3.97 (d, 1, *J* = 13.2 Hz, H_{Glc}-6´), 4.77 (dd, 1, *J* = 3.5, 13.2 Hz, H_{Glc}-6), 4.84 (t, 1, *J* = 1.9 Hz, H_{Glc}-2), 4.96 (d, 1, *J* = 1.9 Hz, H_{Glc}-1), 4.98 (t, 1, *J* = 1.9, 2.7 Hz, H_{Glc}-3), 5.31 (dd, 1, *J* = 3.5, 9.0 Hz, H_{Glc}-5), 5.61 (dd, 1, *J* = 2.7, 9.0 Hz, H_{Glc}-4), 6.25 (s, 1, H_E-6), 6.47 (s, 1, H_B-6), 6.81 (s, 1, H_D-6), 7.07 (s, 2, H_C-2,6), 7.14 (s, 1, H_F-6); ^{13}C NMR (150.93 MHz, CD_3COCD_3, 298 K): δ 65.01 (C_{Glc}-6), 65.25 (C_{Glc}-1), 70.74 (C_{Glc}-5), 72.23 (C_{Glc}-3), 73.46 (C_{Glc}-4), 81.05 (C_{Glc}-2), 105.29 (C_E-6), 105.62 (C_B-6), 108.27 (C_D-6), 110.19 (C_F-6), 110.32 (C_C-2), 110.32 (C_C-6), 115.37 (C_F-1), 115.96 (C_A-2), 116.16 (C_B-2), 116.27 (C_D-2), 117.54 (C_E-2), 119.31 (C_A-6),

121.30 (C_C-1), 123.51 (C_A-1), 125.03 (C_D-1), 127.02 (C_E-1), 128.33 (C_B-1), 134.91 (C_B-4), 136.87 (C_D-4), 136.93 (C_E-4), 137.62 (C_A-4), 137.87 (C_F-4), 139.04 (C_C-4), 139.78 (C_F-2), 140.40 (C_F-3), 143.18 (C_F-5), 143.69 (C_B-5), 144.31 (C_A-5), 145.01 (C_D-5), 145.15 (C_E-3), 145.23 (C_D-3), 145.75 (C_B-3), 145.88 (C_C-3), 145.88 (C_C-5), 146.59 (C_A-3), 146.91 (C_E-5), 164.72 (C_A-7), 165.80 (C_C-7), 167.02 (C_F-7), 168.68 (C_D-7), 168.77 (C_E-7), 169.12 (C_B-7).

Statistical Analyses

Statistical analyses were performed in R[31] using RStudio integrated development environment.[32] Comparisons between sexes and cultivars were analyzed using one-way analysis of variance (ANOVA). Tukey's honest significant difference test (from R package agricolae)[33] was used to perform pairwise comparison of least squares means. Statistical significance was defined at $p < 0.01$.

Results and Discussion

Compound Identification

A total of ten ETs were detected as the main phenolic compounds in *Hippophaë rhamnoides* leaves, with nearly all of them appearing in quantifiable levels in all of the 58 individual plants. The ETs (Figure 1) were identified as castalagin (**1**),[11,34–36] vescalagin (**2**),[11,35–37] pedunculagin (**3**),[11,21,27,38,39] isostrictinin (**4**),[11,21,27,40] casuarinin (**5**),[11,21,27,35,38,39] stachyurin (**6**),[11,21,27,35,38,39] elaeagnatin A (**7**),[21,41] pterocarinin A (**8**),[21,42] hippophaenin B (**9**),[11,21,27,28,41] and hippophaenin C (**10**). First HPLC-ESI-MS and then UHPLC-DAD–ESI-Orbitrap-MS was used alongside literature to identify and to determine accurate masses (Table 1) for each of the ten ETs. A UV chromatogram at 280 nm of one of the quantified *H. rhamnoides* samples along with extracted ion chromatograms corresponding to each of the ten ETs from the UHPLC-DAD–ESI-Orbitrap-MS analyses are presented in Figure 2. The accurate

characterization of compound **10** has not been reported before, and it has only been included in one study, showing its high anthelmintic potential. The structure of compound **10** was now elucidated using mass spectrometry, and NMR and ECD spectroscopy.

All the determined ETs are monomeric, but they include compounds with both cyclic glucopyranose and acyclic *C*-glycosidic cores. Many of the compounds are similar in structure with relatively small structural modifications; three epimer pairs (compounds **1** and **2**, **5** and **6**, and **9** and **10**) and two pairs only differing in the presence or absence of one C-C bond (compounds **1** and **5**, and **2** and **6**) are included. Compound **8** is a lyxoside of compound **6**, and compound **7** has an additional gallic acid unit attached to the HHDP (hexahydroxydiphenoyl) group in comparison to compound **8**, thus forming a valoneoyl group. Similarly, compounds **9** and **10** have a valoneoyl group in place of an HHDP group as compared to compounds **5** and **6**.

The Structures of Hippophaenins B and C

Among the ten main ellagitannins, we identified a novel ellagitannin, earlier named hippophaenin C (**10**)[22] (Figure 3), bearing structural similarity with hippophaenin B (**9**). The structure has previously been reported, but was presented incorrectly along with compound **9** with regard to the orientation of the valoneoyl group, and in addition, compounds **9** and **10** were mixed with one another.[22] Furthermore, to our knowledge, only two previous papers report the structure of hippophaenin B correctly.[41,43] Therefore, also the correct structure of hippophaenin B required to be confirmed.

The UHPLC retention time difference between compounds **9** and **10** was similar to the ones between compounds **1** and **2**, and **5** and **6**, revealing the possibility of compound **10** being an epimer of compound **9**. The UV spectra of compounds **9** and **10** were virtually identical,

showing a maximum at 225 nm and a shoulder at 265 nm, suggesting the presence of both galloyl and HHDP/valoneoyl groups.[28]

The quasi-molecular ion of compound **10** was detected at *m/z* 1103, with a corresponding doubly charged ion at *m/z* 551. The MS^2 experiments on purified compound **10** showed a range of fragments further suggesting that it is an epimer of compound **9**. These include the elimination of water (*m/z* 1085), which is widely observed for *C*-glycosidic ellagitannins with a β-OH at C-1 (an α-OH does not typically produce the dehydration fragment),[28,44] the fragmentation of carboxylic acid and gallic acid from the valoneoyl moiety (*m/z* 1059 and 935, respectively), and combinations of these fragmentations. Also observed were ellagic acid, which is typical for all HHDP-containing ellagitannins (*m/z* 301), and two related fragmentation products at *m/z* 275 and 249; the first corresponds to a lactonized and decarboxylated HHDP group and the latter to a doubly decarboxylated HHDP group.

Resulting from the NMR studies, the HHDP, galloyl and valoneoyl groups in compounds **9** and **10** were found to be linked to an open-chain glucose as shown in Figure 1. The α configuration of C-1 in compound **9** and β in compound **10** was confirmed by the magnitude of the $^3J_{1,2}$ coupling constant in each case, which is typically large (5 Hz) for α-epimers and small (2 Hz) for β-epimers of *C*-glycosidic ellagitannins.[35] The position of the valoneoyl group in compound **10** (Figure 3) was confirmed by the observed NOEs between H_F-6 and H_E-6, and H_E-6 and H_C-2,6. Further proof of the indicated valoneoyl group position was obtained from the H_D-6 and H_E-6 chemical shifts (6.81 and 6.25 ppm, respectively) which have been shown to provide diagnostic information about the valoneoyl group orientation.[45] The latter is remarkably upfield shifted in comparison to the corresponding chemical shift in stachyurin (**6**) which has an HHDP group linked to glucose positions 4 and 6 instead of a valoneoyl group.[38,39] Thus, also this upfield shifted chemical shift value indicates that the *O*-

linked gallic acid group (F) is linked to the E ring in compound **10**. Similar upfield shifting was observed for compound **9**.

Finally, *S* configurations for the axially chiral HHDP and valoneoyl groups in compounds **9** and **10** were confirmed by comparing their ECD spectra to that of compound **6** (Figure 4), for which the absolute configurations of the HHDP groups are previously known to be *S*.[46] Neither the additional *O*-linked gallic acid group in compounds **9** and **10** in comparison to compound **6** nor the configuration of C-1 of the central glucose affect significantly to the ECD spectra, and as a result, the observed spectra are essentially similar.

The Biogenesis and Concentrations of the Ellagitannins in *H. rhamnoides*

The biosynthetic linkages within the hydrolysable tannin pathway have been revealed by enzyme studies from gallic acid to pentagalloyl glucose and further to the first ellagitannin of the pathway, i.e. tellimagrandin II.[47,48] The next steps of the pathway have been proposed by comparing the known structures of the ellagitannins and their seasonal variation in both *Betula pubescens* and *Quercus robur* foliage.[30,49] This way the linkages of the glucopyranose-based simple HHDP esters in the biogenesis of *H. rhamnoides* must be as shown in Figure 5. The formation of the *C*-glycosidic ellagitannins stachyurin and casuarinin takes place after ring opening of pedunculagin and its further galloylation to O-5 (Salminen et al. 2004, Fig. 1). The NHTP (nonahydroxytriphenoyl) -derivatives vescalagin and castalagin are formed from stachyurin and casuarinin via linking the 5-galloyl to the 2,3-HHDP to form the 2,3,5-NHTP group. The other ellagitannins in the biogenesis of *H. rhamnoides* do not have NHTP groups, meaning that they need to be produced from stachyurin and casuarinin, not from vescalagin and castalagin (Fig. 5).

285 The orientation of the valoneoyl groups of compounds **9** and **10** have been at times presented
286 incorrectly, possibly stemming from their difference when compared to e.g. castavaloninic
287 acid and vescavaloninic acid present in leaves of *Q. robur*. The latter two have the valoneoyl
288 group oriented so that the *O*-linked gallic acid in the valoneoyl group is bound to the
289 glucosidic C-4 side of the HHDP group (D ring), while the gallic acid is bound to the
290 glucosidic C-6 side in compounds **9** and **10**. Compounds **1** and **2** are apparently converted
291 further to other *C*-glycosidic ellagitannins differently depending on the plant species, such as
292 castavaloninic acid and vescavaloninic acid in several *Quercus* species,[34,37,51–53] and
293 salicarinins A, B, and C in *Lythrum salicaria*.[54] The aforementioned hippophaenin B (**9**) and
294 hippophaenin C (**10**) in *H. rhamnoides*, on the other hand, are not biosynthetic products of
295 compounds **1** and **2**, but presumably those of compounds **5** and **6**. We did not find a gallic
296 acid unit to be attached in *H. rhamnoides* to the C-4 or the C-6 side of the 4,6-HHDP group
297 found in castalagin (**1**) or vescalagin (**2**), or to the C-4 side of the 4,6-HHDP group of
298 casuarinin (**5**) or stachyurin (**6**). This suggests that the enzymes catalyzing the addition of the
299 gallic acid unit to the 4,6-HHDP must be species-specific and sensitive to the presence of the
300 2,3,5-NHTP group in compounds **1** and **2** (e.g. *Quercus*) vs. the corresponding 5-galloyl +
301 2,3-HHDP groups in compounds **5** and **6** (e.g. *Hippophaë*). In a similar fashion the
302 dimerization of compounds **1** and **2** to form the salicarinins in *Lythrum*, but not in *Quercus* or
303 *Hippophaë*, highlights the specific enzymatic differences between these three plant genera (or
304 species) that otherwise are able to produce the common *C*-glycosidic ellagitannins such as
305 vescalagin and castalagin.

306 The concentrations of each of the ten ETs in different cultivars and sexes are presented in
307 Figures 6 and 7. The total concentrations of all of the ten ETs in the samples ranged from
308 42.5 mg per dry weight gram to 109.1 mg g^{-1} with a mean of 71.6 mg g^{-1} and median of 67.4
309 mg g^{-1}.

Among the three cultivars, R was determined to contain the most ETs on average with 77.9 mg of ellagitannins per dry weight gram with a true standard deviation of 3.1 mg g^{-1}. K and RUXRA had total ET concentrations of 68.5 (3.3) mg g^{-1} and 69.3 (3.8) mg g^{-1}, respectively. Male plants had slightly higher concentrations than female plants with 74.3 (3.0) mg g^{-1} versus 68.9 (2.8) mg g^{-1}. However, no statistically significant difference on total ETs was found between the sexes or cultivars.

For most of the studied samples, casuarinin (**5**), stachyurin (**6**), and hippophaenin C (**10**) were the most abundant ETs, with their total concentrations accounting to 39.6–62.6% of the total ellagitannin concentration. These three individual compounds accounted to 11.6–24.8%, 7.7–24.8% and 12.7–22.7% of the total ET concentration, respectively. In most samples, castalagin (**1**) and vescalagin (**2**) were least abundant among the ten main compounds, and they were the only ETs not detected in quantifiable amounts in some individuals. This reflects the specific nature of the ET biosynthesis in *Hippophaë rhamnoides* leaves that favors the transformation of stachyurin (**6**) and casuarinin (**5**) to other than NHTP-containing *C*-glycosidic ETs (see Figure 5). On average, total concentrations of the stachyurin-type ETs were slightly over double compared to the casuarinin-type ETs, and this ratio was fairly consistent for all the samples. This highlights the higher biosynthetic flux towards the *β*-oriented *C*-glycosidic ETs from the glucopyranose-based monomers in both sexes and all cultivars. In general, the *β*-epimers are chemically more reactive than the corresponding *α*-epimers[50] and this was also highlighted by the lyxose-containing ETs (compounds **7** and **8**) being found only with the *β*-oriented ETs.

Average concentrations of individual ETs among the cultivars showed little variance (Figures 5 and 6) with statistically significant differences only showing in elaeagnatin A (**7**), hippophaenin B (**9**), and hippophaenin C (**10**). In dioecious plant species female plants seem to allocate more resources to their chemical defense than males, observable e.g. as higher

concentrations of secondary metabolites such as phenolics.[55] While this has been observed to be generally true for various lipophilic antioxidants in *H. rhamnoides* vegetative parts as well,[5,56] no significant differences between sexes were found in any individual compounds or total ETs. On the other hand, the differences in the concentrations of individual ETs in different individuals among the same cultivar or sex was fairly large at times, as shown in Figures 6 and 7, possibly eliminating statistically significant differences to be observed between sexes or cultivars.

Sea buckthorn leaves are known to be rich in ETs; they have been found to be one of the most ET-rich plant sources in Finland, but previous publications quantifying individual ETs in *H. rhamnoides* leaves have been approximate at best.[10,11] The substantial differences and the relative simplicity of the ET profile reported in sea buckthorn leaves by Tian et al.[10] when compared to our results might stem from e.g. the used analysis methods or extraction solvents, as the variation in our individuals and cultivars was seen to be relatively modest.

These results confirm and bring more detail into the structures and concentrations of ETs in the leaves of *Hippophaë rhamnoides*, substantial amounts of which are collected as by-products of harvesting berries. While the leaves are already widely being used for e.g. herbal infusion drinks, the results confirm and further explain their great potential in therapeutic and medicinal usage; the total concentration and diversity of ETs, together with the accumulation of rare *C*-glycosidic ETs such as hippophaenin C, make *H. rhamnoides* leaves a potential source of compounds possessing various known and perhaps even yet unknown bioactivities described earlier. Our findings suggest that foliage of both sexes and all tested cultivars are equally good sources of these compounds and they could be simultaneously taken into account in future studies that focus on e.g. bioactivities found in *H. rhamnoides* berries that are not known to be able to produce ellagitannins.

Abbreviations Used

1D-ROESY, one-dimensional rotating frame nuclear Overhauser effect spectroscopy; CT-HMBC, constant time heteronuclear multiple-bond correlation; ECD, electronic circular dichroism; ET, ellagitannin; DQF-COSY, double quantum filtered correlation spectroscopy; HCD, higher-energy collisional dissociation; HHDP, hexahydroxydiphenoyl; HPLC-DAD, high-performance liquid chromatography diode array detection; HSQC, heteronuclear single quantum coherence; LC-MS^n, liquid chromatography–tandem mass spectrometry; NHTP, nonahydroxytriphenoyl; NMR, nuclear magnetic resonance; UHPLC-DAD–ESI-Orbitrap-MS, ultra-high performance liquid chromatography diode array detection–electrospray ionization Orbitrap mass spectrometry

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Supporting Information

^1H, ^1H,^1H COSY, 1D-ROESY, ^{13}C, ^1H,^{13}C HSQC, ^1H,^{13}C HMBC, and selective ^1H,^{13}C CT-HMBC NMR spectra, and observed NOE's for compound **10**.

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Figure Captions

Figure 1. The ellagitannins quantified from *Hippophaë rhamnoides*.

Figure 2. UV chromatogram (λ = 280 nm) of a *H. rhamnoides* leaf extract (A) and extracted ion chromatograms of the *m/z* values corresponding to the studied ETs (B). For peak identification, see Table 1.

Figure 3. The key HMBC (black) correlations and NOE's (red) confirming the assignment of the chemical shifts and the deduced constitution of hippophaenin C (**10**).

Figure 4. The ECD spectra of stachyurin (**6**), and hippophaenins B (**9**) and C (**10**) in water.

Figure 5. The proposed biosynthetic pathway of the studied ellagitannins in *Hippophaë rhamnoides* leaves, including their common precursors pentagalloyl glucose, tellimagrandin II, and casuarictin.[49,50] Included are the three groups to which the studied ETs were grouped.

Figure 6. Concentrations (mg g^{-1} DW in pedunculagin equivalents) of individual ellagitannins organized by cultivars. Statistically significant ($p < 0.01$) differences in the concentrations between the cultivars are denoted by non-overlapping lettering. If no significant differences were found for a compound, the lettering is omitted.

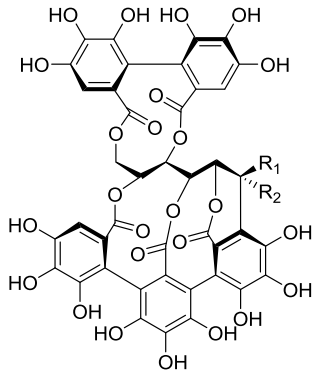
Figure 7. Concentrations (mg g^{-1} DW in pedunculagin equivalents) of individual ellagitannins organized by sexes. No statistically significant ($p < 0.01$) differences between the sexes were found in any compounds.

Table 1. Identification, retention times, molecular formulas, and mass spectral data of the ellagitannins quantified from *Hippophaë rhamnoides* leaves using UHPLC-DAD–ESI-Orbitrap-MS. For structures, see Figure 1.

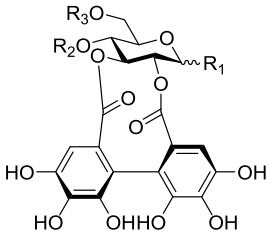
no.	compound identification	retention time (min)	molecular formula	$[M–H]^–$	other *m/z* values	exact mass, calculated	exact mass, measured[a]	error (ppm)	references[b]
1	castalagin	2.64	$C_{41}H_{26}O_{26}$	933.0641	466.0281 $[M–2H]^{2–}$	934.0712	934.0714	0.2	[11]
2	vescalagin	2.29	$C_{41}H_{26}O_{26}$	933.0641	466.0283 $[M–2H]^{2–}$	934.0712	934.0714	0.2	[11]
3	pedunculagin	2.56, 2.87	$C_{34}H_{24}O_{22}$	783.0687	391.0306 $[M–2H]^{2–}$, 1567.1429 $[2M–H]^–$	784.0759	784.0760	0.1	[11,21,27]
4	isostrictinin	3.14	$C_{27}H_{22}O_{18}$	633.0733	316.0327 $[M–2H]^{2–}$, 1267.1520 $[2M–H]^–$	634.0806	634.0806	–0.1	[11,21,27]
5	casuarinin	3.24	$C_{41}H_{28}O_{26}$	935.0795	467.0357 $[M–2H]^{2–}$	936.0869	936.0868	–0.2	[11,21,27]
6	stachyurin	3.10	$C_{41}H_{28}O_{26}$	935.0795	467.0358 $[M–2H]^{2–}$	936.0869	936.0868	–0.2	[11,21,27]
7	elaeagnatin A	3.01	$C_{53}H_{40}O_{35}$	1235.1265	617.0602 $[M–2H]^{2–}$	1236.1350	1236.1349	–0.1	[21]
8	pterocarinin A	3.04	$C_{46}H_{36}O_{30}$	1067.1216	533.0574 $[M–2H]^{2–}$	1068.1291	1068.1293	0.2	[21]
9	hippophaenin B	3.21	$C_{48}H_{32}O_{31}$	1103.0843	529.0433 $[M–H–COOH]^{2–}$, 551.0391 $[M–2H]^{2–}$	1104.0928	1104.0927	–0.1	[11,21,27,28]
10	hippophaenin C	3.05	$C_{48}H_{32}O_{31}$	1103.0852	529.0441 $[M–H–COOH]^2$, 551.0391 $[M–2H]^{2–}$, 1085.1038 $[M–H_2O–H]^–$	1104.0928	1104.0927	–0.1	

[a]The measured value was calculated using the $[M–H]^–$ ion for compounds under 1000 Da and the $[M–2H]^{2–}$ ion for compounds over 1000 Da.

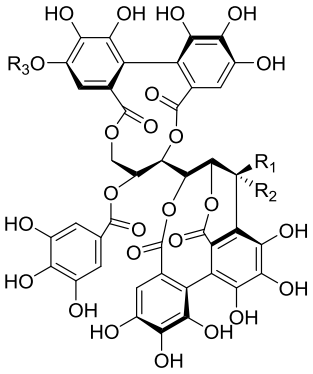
[b]Previous reports of the compounds found in *H. rhamnoides*.



1 Castalagin: R^1 = H, R^2 = OH
2 Vescalagin: R^1 = OH, R^2 = H



3 Pedunculagin: R^1 = OH, R^2~R^3 = (*S*)-HHDP
4 Isostrictinin: R^1 = β-OG, R^2 = R^3 = H



5 Casuarinin: R_1 = R_3 = H, R_2 = OH
6 Stachyurin: R_1 = OH, R_2 = R_3 = H
7 Elaeagnatin A: R_1 = L, R_2 = H, R_3 = A
8 Pterocarinin A: R_1 = L, R_2 = R_3 = H
9 Hippophaenin B: R_1 = H, R_2 = OH, R_3 = A
10 Hippophaenin C: R_1 = OH, R_2 = H, R_3 = A

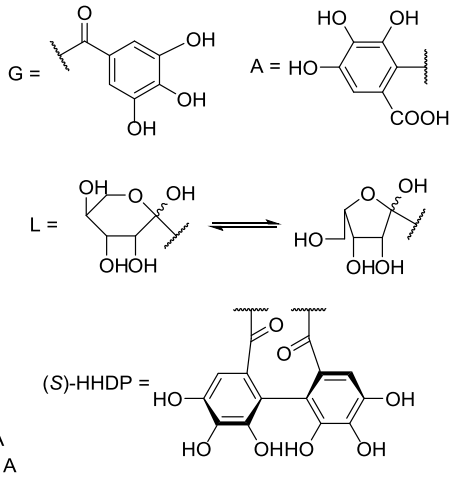


Figure 1.

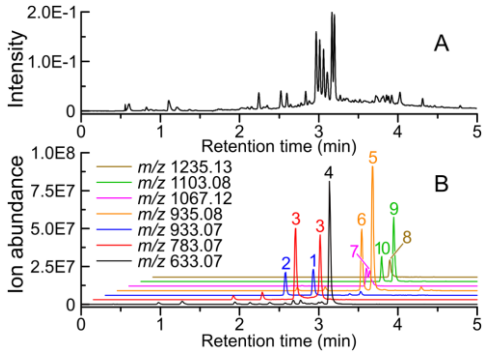

2.0E-1
1.0E-1
0
Intensity
A
0
1
2
3
4
5
Retention time (min)
1.0E8
7.5E7
5.0E7
2.5E7
0
Ion abundance
m/z 1235.13
m/z 1103.08
m/z 1067.12
m/z 935.08
m/z 933.07
m/z 783.07
m/z 633.07
B
1
2
3
3
4
5
6
7
8
9
10
Retention time (min)

Figure 2.

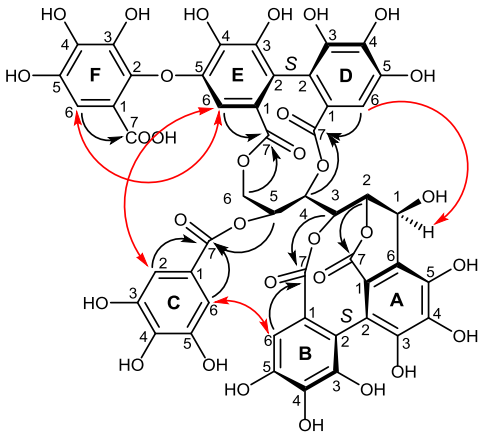


Figure 3.

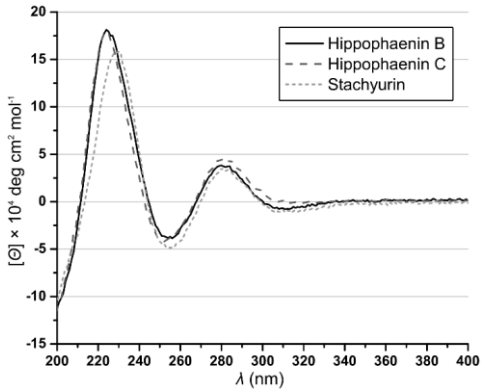

Hippophaenin B
Hippophaenin C
Stachyurin
[Θ] × 10⁴ deg cm² mol⁻¹
λ (nm)
20
15
10
5
0
-5
-10
-15
200
220
240
260
280
300
320
340
360
380
400

Figure 4.

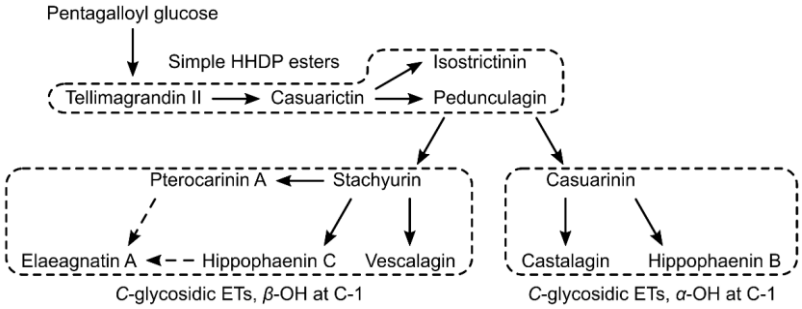

Pentagalloyl glucose
Simple HHDP esters
Isostrictinin
Tellimagrandin II
Casuarictin
Pedunculagin
Pterocarinin A
Stachyurin
Casuarinin
Elaeagnatin A
Hippophaenin C
Vescalagin
Castalagin
Hippophaenin B
C-glycosidic ETs, β-OH at C-1
C-glycosidic ETs, α-OH at C-1

Figure 5.

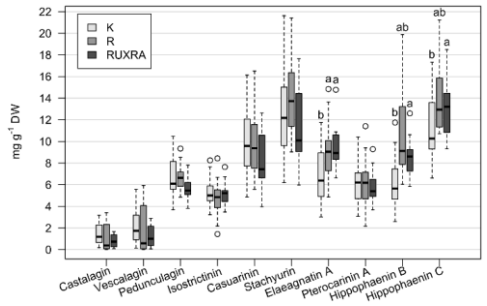

K
R
RUXRA
mg g-1 DW
Castalagin
Vescalagin
Pedunculagin
Isostrictinin
Casuarinin
Stachyurin
Elaeagnatin A
Pterocarinin A
Hippophaenin B
Hippophaenin C

Figure 6.

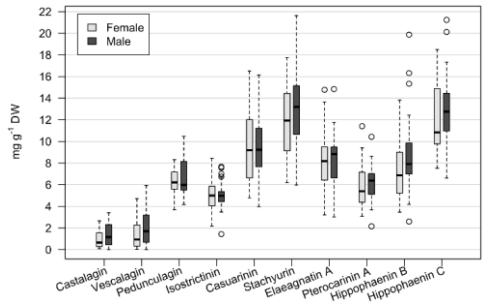


Figure 7.

Formatted: English (United States)

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