Phenolic compounds and antioxidant activities of tea-type infusions processed from sea buckthorn (*Hippophaë rhamnoides*) leaves

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Abstract

Sea buckthorn (*Hippophaë rhamnoides*, SB) leaves contain wide-ranging bioactive compounds. Processing of the leaves into beverages/food presents great potential for supporting human health. The research aimed to investigate the impact of different processing methods on phenolic compounds and antioxidant activities of tea-type infusions prepared from SB leaves. Leaves of two SB cultivars, 'Terhi' and 'Tytti', were processed with different methods commonly used for tea processing. Phenolic compounds in the infusions were analyzed with High-Performance Liquid Chromatography-Diode-Array Detection and High-Performance Liquid Chromatography Electrospray Ionization-Tandem Mass Spectrometry. Isorhamnetin-3-*O*-glucoside-7-*O*-rhamnoside, isorhamnetin-3-*O*-rutinoside and kaempferol-3-*O*-hexoside-7-*O*-rhamnoside were the three major flavonol glycosides, stachyurin and casuarinin were the most abundant ellagitannins. The infusions of 'Tytti' contained more total phenolics than those of 'Terhi' ($p < 0.05$). High temperature processing resulted in higher content of total phenolics and ellagitannins in the infusions compared with low temperatures ($p < 0.05$). Thermal processing decreased the antioxidant activities of the infusions.

Keywords

Antioxidative activities; Drying processes; Ellagitannins; Flavonol glycosides; *Hippophaë rhamnoides*; Sea buckthorn leaves; Tea-type infusions

1. Introduction

Sea buckthorn (*Hippophaë rhamnoides* L.) is hardy and a medium-sized deciduous tree widely distributed in Asia and Europe. The leaves of sea buckthorn (SB) have been reported to contain higher contents of phenolic compounds and antioxidant activities than the berries, due to the high content of nutrients and bioactive compounds such as minerals, vitamins, fatty acids, carotenoids and phenolic compounds (Hellström, Pihlava, Marnila, Mattila & Kauppinen, 2013, Pop, Weesepoel, Socaciu, Pintea, Vincken & Gruppen, 2014, Tian et al., 2017). In the recent years, various SB leaf extracts have been extensively studied, and a range of pharmacological activities such as anti-inflammatory, antioxidative, antibacterial, adaptogenic and tissue regenerative properties have been reported (Ganju et al., 2005, Saggu, Divekar, Gupta, Sawhney, Banerjee & Kumar, 2007, Tian, Puganen, Alakomi, Uusitupa, Saarela & Yang, 2018). In addition, the leaves of SB do not show cell cytotoxicity according to *in vitro* assays or any adverse effect in rats after oral administration (Upadhyay, Kumar, Siddiqui & Gupta, 2011). Despite these potential commercial interests and health benefits, the majority of SB leaves have not been utilized remaining as agricultural wastes after berry harvesting.

Sea buckthorn leaves are rich in flavonol glycosides (FGs), mainly isorhamnetin and quercetin derivatives, at levels up to 11 mg/g dry weight (DW) on average (Pop et al., 2013). FGs are of interest because of their health benefits, such as significant antioxidant activity, antitumor activity, and anti-inflammatory activity (Xiao, Capanoglu, Jassbi & Miron, 2016). Moreover, they have low sensory thresholds for astringent sensations (Scharbert & Hofmann, 2005). In addition to FGs, notable contents of ellagitannins (ETs) are also found in SB leaves, and the total content of ETs reaches more than 100 mg/g DW (Suvanto, Tähtinen, Valkamaa, Engström, Karonen & Salminen, 2018). ETs also possess a wide range of biological activities, such as antioxidative functions, anti-inflammatory activities, and prebiotic effects (Landete, 2011). Factors such as cultivar, harvesting time, and leaf position in the plant as well as

processing technologies are known to affect significantly the content and composition of phenolic compounds (Bakkalbaşi, Menteş & Artik, 2008, Mäkilä, Laaksonen, Kallio & Yang, 2017, Yang, Alanne, Liu, Kallio & Yang, 2015).

In recent years, the interest and consumption of herbal infusions (commonly called teas or tisanes) from a great diversity of edible plants has increased considerably due to their potential health benefits and attractive flavor and taste. Similarly, there has been an increasing interest in utilization of SB leaves and converting them into tea-type beverage products. However, most studies have focused on the fresh leaves of SB, and the emphases have been on organic solvent extractions aiming to identify new compounds or maximize the yields of phenolics (Pop et al., 2013, Suvanto et al., 2018, Tian et al., 2017). Little is known about the phenolic profiles and antioxidant activities of water extracts of SB leaves as tea-type infusions as beverages. In present study, SB leaves were processed using different drying methods (thermal and non-thermal). Drying process can cause changes in the texture and color of SB leaves (Kyriakopoulou, Pappa, Krokida, Detsi & Kefalas, 2013). Food processing often induces the degradation of phenolic compounds, thus reducing their amount in processed foods (Amarowicz et al., 2009, Bakkalbaşi et al., 2008). Along with the changes in phenolic compounds, processing conditions may have important effect on antioxidant activity (Bakkalbaşi et al., 2008, Donlao & Ogawa, 2018, Kyriakopoulou et al., 2013).

The objective of this work was to investigate the major phenolic compounds and to obtain quantitative data on the flavonols and ellagitannins in tea-type infusions prepared from SB leaves in the laboratory scale. The special focus was on the effect of different drying methods and SB cultivars on the phenolic contents and antioxidant activity of tea-type infusions brewed in the same way as tea brewing commonly used by consumers. In addition, the phenolic profiles were studied in the original fresh leaves and the leaf residues after infusions to monitor the

efficiency of hot water infusion typically utilized in tea preparation for extracting phenolic compounds.

2. Materials and methods

2.1. Plant material

The leaves of two sea buckthorn (ssp. *rhamnoides*) cultivars, 'Terhi' and 'Tytti', were harvested in August 2015 in Turku in Finland. The leaf samples were picked from random sites in 4–6 bushes and mixed for each cultivar and stored at −18 °C after picking until processing and analysis.

2.2. Drying processes

Sea buckthorn leaves were processed with five different drying methods: 1) Leaves were lyophilized for two days in a vacuum flask at 0.288 mbar and –40 °C in a freeze-dryer (Virtis Wizard 2.0, NY, USA)(FD); 2) leaves were first "fixed" by steaming at 95−100 °C for 30−40 s to inactivate enzymes and then dried at 80−90 °C for 2.5 h (S+HT); 3) leaves were first steamed as described above followed by a four-step heat drying at different temperatures: 70−80 °C for 35−40 min, 60−70 °C for 30−40 min, 80−90 °C for 15−20 min, and 60−75 °C for 30−40 min (S+DT); 4) leaves were dried in low temperature heating 60−70 °C for 3.5 h (LT); 5) leaves were air-dried in the laboratory for three days (room temperature 25 ± 2 °C and relative humidity of 27%) (AD). For each of the above drying methods, about 20 g of fresh leaves were used, and the leaves were spread out evenly on a Petri dish. After drying, the leaves were cooled in a desiccator, then put into a sealable plastic bag and stored at −18 °C. All drying processes of leaf samples were finished within one month.

For dry weight (DW) measurement, c.a. 3 g of leaves dried with each processing method and fresh leaves of both cultivars were weighed accurately, dried to a constant weight at 103−105 °C, cooled in a desiccator, and weighed.

2.3. Preparation of tea-type infusions

Prior to infusion, the processed SB leaves were milled into a powder and passed through a 14-mesh sieve to keep same size with commercial green tea (Vintage, Sri Lanka). An infusion was prepared by infusing 1.0 g of leaf powder for 5 min with 100 mL of freshly boiled carbon filtered water without agitation. The infusion was filtered through Whatman filter paper (Grade 0858, Whatman International, Ltd., Maidstone, U.K.), and cooled to room temperature. For each powdered leaf sample, duplicate infusions were prepared.

2.4. Preparation of samples for analysis

Prior to analysis, 20 mL of infusions were freeze-dried. The freeze-dried powder was dissolved in 2 mL of methanol and filtered through a 0.45 μm or 0.2 μm filter (VWR International, LLC, PA) for FG analysis of High-Performance Liquid Chromatography-Diode-Array Detection (HPLC-DAD) and High-Performance Liquid Chromatography Electrospray ionization-tandem Mass Spectrometry (HPLC-DAD-ESI-MS/MS). For ET analysis, tea infusions were filtered through a 0.45 μm or 0.2 μm filter prior to HPLC-DAD and HPLC-DAD-ESI-MS analysis. For each infusion, one analytical sample was prepared for FGs analysis, and two replicates of analytical samples were prepared for ETs.

After preparation of the infusion, an extraction method developed by our group was used to extract the phenolic compounds from the solid residues (Yang et al., 2015). The residues were extracted three times with 20 mL of 70 % aqueous acetone by sonicating for 20 min during each extraction, followed by centrifugation (4420×g) for 10 min. The supernatants were

combined. For FGs analysis, the extracts were dried by a vacuum rotary evaporator, re-dissolved in 2 mL of methanol, and filtered through a 0.45 μm or 0.2 μm filter for HPLC-DAD and HPLC-DAD-ESI-MS/MS analysis. For ET analysis, the extraction was performed as described above, the organic solvent was evaporated with a rotary evaporator and the remaining water phase was freeze-dried. The dried extract was dissolved in 10 mL of water and filtered as described above prior to HPLC-DAD and HPLC-DAD-ESI-MS analysis.

For analysis of phenolic compounds in freshly frozen leaves, 5 g of frozen leaves of each cultivar were milled into a fine powder with the aid of liquid nitrogen. An aliquot of 1 g of leaf powder was extracted with the same method as described above for the extraction of residues.

2.5. Identification of phenolic compounds in sea buckthorn leaf samples

The analysis was carried out with a Waters Acquity ultrahigh performance LC system (Waters Corp., Milford, MA) consisting of a sample manager, binary solvent delivery system, coupled with a Waters 2996 PDA detector and a Waters Quattro Premier Tandem quadrupole mass spectrometer (Waters Corp., Milford, MA) with an electrospray ionization (ESI). The chromatograph and mass spectrometer were operated using the MassLynx 4.1 software.

2.5.1. Analysis of flavonol glycosides

A Phenomenex Aeris peptide XB-C18 (3.6 μm, 150 × 4.60 mm, Torrance, CA) column combined with a Phenomenex Security Guard Cartridge Kit (Torrance, CA) was used for the analysis of samples at an oven temperature 40 ºC. The mobile phase consisted of a binary gradient eluting system as described previously (Yang et al., 2015). The analyses were carried out by a gradient elution with formic acid/water (0.1:99.9, v/v) as solvent A and formic acid/acetonitrile (0.1:99.9, v/v) as solvent B. The gradient program of solvent B in A (v/v) was 0–15 min with 15–20% B, 15–20 min with 20–25% B, 20–25 min with 25% B, 25–30 min

148 with 25–60% B, 30–35 min with 60–15% B, and 35–40 min with 15% B. The injection volume
149 was 10 μL for each sample. FGs were monitored at 360 nm. The whole flow of 0.5 mL/min
150 was led to the mass spectrometer. The mass spectrometer was operated in positive ion mode
151 and the ESI inlet conditions were the same as previously reported by (Yang et al., 2015).

152 Reference compounds of quercetin-3-*O*-rutinoside (Qu-3-R), isorhamnetin-3-*O*-glucoside
153 (Is-3-G) and isorhamnetin-3-*O*-rutinoside (Is-3-R) (≥ 99%) were purchased from
154 Extrasynthese (Genay, France). Two sets of reference compounds: isorhamnetin-3-*O*-
155 glucoside-7-*O*-rhamnoside (Is-3-G-7-Rh), isorhamnetin-3-*O*-sophoroside-7-*O*-rhamnoside
156 (Is-3-S-7-Rh), and quercetin-3-sophoroside-7-rhamnoside (Qu-3-S-7-Rh) isolated from SB
157 berries were kindly donated by Professor Zhang Hao at Sichuan University (China) and
158 Professor Lothar W. Kroh at Technische Universität Berlin (Germany). The samples spiked
159 with the reference compounds were analyzed by HPLC to compare the retention times of the
160 sample peaks with those of the reference compounds.

161 2.5.2. Analysis of ellagitannins

162 The same column and oven temperature were used as described above for FGs analysis. The
163 analyses were conducted with a gradient elution with formic acid/water (0.1:99.9, v/v) as
164 solvent A and acetonitrile/water (7:3, v/v) as solvent B. The eluting gradient program was: 0–
165 5 min, 3% B; 5–30min, 3–43% B; 30–35 min, 43–100% B; 35–40 min, 100% B; 40–43 min,
166 100–3% B; 43–50 min, 3% B. The flow rate was 1 mL/min, and the injection volume was 10
167 μL for the residues and fresh leaves and 30 μL for infusions. The detection wavelength was
168 280 nm.

169 The column flow was split with a ratio of 1:1 prior to the mass spectrometer. The mass
170 spectrometer was operated in the negative ion mode. The ESI conditions were the following:
171 capillary voltage 3 kV; cone voltage 35 V; source temperature 150 °C; desolvation temperature

500 °C; desolvation gas flow 700 L/h; cone gas flow 100 L/h. The mass range for full scan was *m/z* 290–2000. Characterization of ETs was based on UV-spectra, retention times and MS-spectra as described in Moilanen et al (2013).

2.6. Quantitative HPLC-DAD analysis of phenolic compounds

The HPLC-DAD instrument consisted of a Shimadzu (Shimadzu Corporation, Kyoto, Japan) SIL-30AC autosampler, a sample cooler, two LC-30AD pumps, a CTO-20AC column oven, an SPD-M20A diode array detector, and a CBM-20A central unit. The system was operated using the LabSolutions Workstation software.

2.6.1. Quantitative analysis of flavonol glycosides

The quantitative analysis of FGs in the extracts was carried out with HPLC-DAD using the same HPLC parameters as in the HPLC-DAD-ESI-MS analysis. The quantification was carried with an external standard method using calibration curves constructed with standard solutions of Is-3-G and Is-3-R in methanol in the concentration range of 0.001−0.2 mg/mL. All other FGs except Qu-3-R (**11**) were quantified as equivalents of Is-3-R. The isorhamnetin-hexoside-rhamnoside II (**12,** Is-He-Rh II) overlapped with Qu-3-R (**11**) in the HPLC-DAD chromatogram (Fig.1), and the content of these two compounds were calculated together as the content of Is-He-Rh II, since the HPLC-ESI-MS spectrum showed Is-He-Rh II to be the predominant (Supplementary Figure 1K). The correlation coefficients of the standard curves varied from 0.9947 to 0.9996. The injection volume was 10 µL for each solution. The content of FGs were expressed as mg/100 mL for the infusions and as mg/g DW for the residues and the fresh leaf samples.

2.6.2. Quantitative analysis of total phenolics and ellagitannins

194 The quantitative analysis of total phenolics (the sum of all the peak areas at 280 nm), total
195 ETs (the sum of 6 main ellagitannins) and 6 main ETs in the extracts was carried out with
196 HPLC-DAD using the same HPLC parameters as in the HPLC-DAD-ESI-MS analysis. Gallic
197 acid (≥ 99 %, 0.0005−0.003 mg/mL) (Sigma-Aldrich, Steinheim, Germany) was used as an
198 external standard. The content of total phenolics and ETs were expressed in the same units as
199 described above for the analysis of FGs.

200 2.7. Antioxidant activity measurements using 2-deoxyribose assay

201 The hydroxyl radical scavenging activity, pro-oxidant activity and the ability to chelate iron
202 ions of infusions were measured with the method previously described by Moilanen et al (2016)
203 with minor modifications. Briefly, for the hydroxyl radical scavenging activity measurement,
204 the following reagents were pipetted into an Eppendorf tube in the order listed below: 1240 µL
205 of 20 mM NaH_2PO_4 (Baker, Deventer, Holland) buffer (pH 7.4), 160 µL of 0.1 mM 2-
206 deoxyribose (2-DR, Sigma-Aldrich, Steinheim, Germany), 40 µL of 4.16 mM EDTA (Baker,
207 Deventer, Holland), 40 µL of 4 mM $FeCl_3$ (Sigma-Aldrich Steinheim, Germany), 160 µL of
208 different infusions, 40 µL of 4 mM ascorbic acid (Sigma-Aldrich, Steinheim, Germany) and
209 40 µL of 40 mM H_2O_2 (Baker, Deventer, Holland). In blanks, no 2-DR and sample solutions
210 were replaced with buffer. In control samples, 2-DR solution was included, but sample solution
211 was replaced with buffer. The tubes were incubated in a 37 °C water bath for 2 hours, after
212 which each sample was divided into two 300 µL sub-sample and the color reagents were added.
213 Thus, 300 µL of 1 % thiobarbituric acid (Merck, Darmstadt, Germany) in 50 mM NaOH
214 (Sigma-Aldrich, Steinheim, Germany) and 300 µL of 2.8 % trichloroacetic acid (Baker,
215 Deventer, Holland) were pipetted into the tubes, thereafter the tubes were incubated in an 80 °C
216 water bath for 30 min. Immediately after incubation, the tubes were cooled at room temperature
217 for 30 minutes. For the absorbance measurement, 300 µL of each sample, control and blank

218 solutions were pipetted into a 96-well plate, and the absorbance was measured with Hidex
219 Sense microplate reader (Hidex, Finland) at 520 nm.

220 For the metal chelation ability measurements, the infusions were diluted ten times (v/v) with
221 water based on the results obtained previously by Moilanen et al (2016), and EDTA solution
222 was replaced with buffer. The pro-oxidant activity measurements of infusions were performed
223 in the same way as described for hydroxyl radical scavenging activity measurements, except
224 that ascorbic acid was replaced with buffer. In each of the assays, three replicates were prepared
225 for each sample. These samples were divided into two sub-samples before the color reagents
226 were added and thus, the final number of replicates is 12 per processing method.

227 The results were calculated from the average absorbance values for each infusion by
228 subtracting the absorbance of the blank sample and then by using the following equation:

229 $$\text{Inhibition (\%)} = \frac{[\text{Abs (control)} - \text{Abs (sample)}]}{\text{Abs (control)}} \cdot 100$$

230 2.8. Qualitative sensory evaluation of tea-type infusions

231 Four tea-type infusion samples freshly prepared from sea buckthorn leaves processed using
232 FD, S+HT, S+DT and LT methods were evaluated by an untrained panel of 30 volunteers (age
233 22−48; students and staff of the university). An infusion from a commercial green tea was also
234 included in the evaluation as a reference. 10 mL of each sample was presented in 50 mL
235 transparent glass beakers with glass lids in randomized order during one session. The sensory
236 panel was asked to describe the color, aroma and flavor of the samples and leave out hedonic
237 descriptors. The panelists were instructed to first look at the sample, then lift the lid and smell
238 the sample and, finally, take a sip of sample in mouth, to swirl it around in the mouth briefly
239 and to write the perceived sensory attributes of the samples using their own words on a blank
240 paper sheet. The panelists were instructed to chew a cracker and rinse their mouths with water

between samples. Data was collected as frequencies of descriptors (values between 0 and 30) by the panel in each sample.

2.9. Statistical analysis

Statistical analyses were carried out with SPSS 22.0 (SPSS, Inc., Chicago, IL). The results were presented as the means ± SD (standard deviations). Differences in the phenolic compounds and antioxidant activities between the samples prepared from leaves dried with different methods were analyzed by a One-Way Analysis of Variance (ANOVA) together with the Tukey's HSD and the Tamhane tests. Independent Samples t-test was used for comparing the content means of the FGs, ETs and total phenolics as well as the means of the antioxidant activities in the extracts between two cultivars. Differences reaching a minimal confidence level of 95% ($p < 0.05$) were considered as being statistically significant.

Unsupervised classification with principal component analysis (PCA) was used to investigate variations in the compositional profiles (n=33) of the SB leaf infusions and the infusion residues. It was also applied to study the frequencies of color, aroma and flavor descriptors (frequencies) in sensory evaluations, as well as to investigate relationships between the compositional variables (X-data, n=33) and the bioactivities (Y-data, n=3) in the infusions within the two cultivars. Multivariate models were created with Unscrambler 10.3 (Camo Process AS, Oslo, Norway).

3. Results and discussion

3.1. Identification of phenolic compounds in sea buckthorn leaves

3.1.1. Identification of flavonol glycosides

Twenty-five FGs were identified or tentatively identified in the extracts of all the samples. As summarized in Table 1, identifications of the compounds were based on retention

characteristics, UV and mass spectra, as well as comparisons with the reference compounds and the literature (Ma et al., 2016, Pop et al., 2013, Rösch, Krumbein, Mügge & Kroh, 2004, Zheng, Kallio & Yang, 2016). The mass spectra of the identified FGs were presented in Supplementary Fig. 1.

Structures of six FGs, Qu-3-S-7-Rh (peak **2** in Fig. 1A), Is-3-S-7-Rh (**5**), Qu-3-R (**11**), Is-3-G-7-Rh (**14**), Is-3-R (**19**) and Is-3-G (**21**), were confirmed directly with the aid of reference compounds. As reported previously, peaks 1, 4, 9, 12 and 18 were preliminarily identified as glycosides of quercetin or isorhamnetin with different sugar moieties such as sophoroside, hexoside, rhamnose or rutinoside by comparing the fragmentation patterns in the mass spectra to those of reference compounds (Supplementary Fig. 1 A, B, E and K) (Ma et al., 2016, Zheng et al., 2016).

Peak 6 was tentatively identified as Qu-Rh based on the protonated molecular ion $[M+H]^+$ at *m/z* 449 (303 + 146) (Supplementary Fig. 1C). Similarly, peak 15 was tentatively identified as Is-Rh based on the protonated molecular ion $[M+H]^+$ at *m/z* 463 (317 + 146). Peaks 7, 8, 10 and 16 were tentatively identified as quercetin-hexoside-rhamnoside (Qu-He-Rh) I, II, III and IV, respectively, based on the protonated molecular ions $[M+H]^+$ at *m/z* 611 (317 + 146 + 162) and *m/z* 449 (303 + 146)(Supplementary Fig. 1D). MS-MS fragmentation of $[M+H]^+$ ion of *m/z* 595 (317 + 146 + 132) produced ions at *m/z* 463 (317 + 146) and *m/z* 317, which suggests the peak 17 to be isorhamnetin-pentoside-rhamnoside (Is-Pe-Rh) (Supplementary Fig. 1G) (Rösch et al., 2004). Peak 13 also lost a rhamnose moiety during MS-MS fragmentation, yielding a fragment ion at *m/z* 449 (Supplementary Fig. 1F). Based on a previous report in literature and the preferred linkage position of the rhamnose moiety, peak 13 was preliminarily identified as Ka-3-He-7-Rh, but an opposite assignment of the position of the sugar moieties could not be excluded (Rösch et al., 2004). Peak 20 was tentatively identified as Ka-He based on the fragments at *m/z* 287 and *m/z* 449 (287+162) (Supplementary Fig. 1H).

Peaks 22 and 23 displayed typical UV spectra of acylated FGs and increased retention compared to mostly FGs in the HPLC-DAD analysis, and they were tentatively identified as Ka-*p*-coumaroylhexoside I and Ka-*p*-coumaroylhexoside II based on the fragments at *m/z* 287 and *m/z* 595 (Lin, Chen & Harnly, 2008). The main characteristic of the *p*-coumaroylglycosylated flavonols is the shift of absorption band II from a λ_{max} of 350–360 nm backwards to a λ_{max} of 310–316 nm. Peaks 3, 24 and 25 had a major fragment ion at *m/z* 317 or 287 in the mass spectra and displayed typical UV spectra of FGs. However, no proper mass spectra were achieved for identification of the sugar moieties. Hence, the compounds were preliminarily identified as glycosides of isorhamnetin or kaempferol, respectively (Table 1).

3.1.2. Identification of ellagitannins

Six main ETs were tentatively identified in the extracts of all the samples based on their characteristic chromatographic behaviors, UV spectroscopic features and mass spectra. Peak 26 was identified as pedunculagin, a glucopyranose-based ET that contains two hexahydroxydiphenoyl (HHDP) groups attached to the glucose core (Okuda, Yoshida, Hatano & Ito, 2009). A characteristic feature for pedunculagin is that it produces two peaks with identical UV spectra in to the chromatogram (Moilanen, Sinkkonen & Salminen, 2013). Pedunculagin has also a unique UV spectrum, which makes its identification straightforward (Moilanen et al., 2013). The mass spectrometric data reinforced the characterization: a deprotonated molecular ion ($[M–H]^-$) at *m/z* 783 and a doubly charged molecular ion ($[M–2H]^{2-}$) at *m/z* 391 were observed (Supplementary Fig. 1I). An ion at *m/z* 481 indicated the fragmentation of an HHDP group, which is lactonized spontaneously to ellagic acid [M–H–EA]$^-$. In addition, an ion at *m/z* 301 was observed and can be interpreted as deprotonated ellagic acid ([EA–H]$^-$).

Peak 27 was tentatively identified as stachyurin, a C-glycosidic ET, which contains two HHDP groups and a galloyl group (Okuda et al., 2009). It is a *β*-isomer of casuarinin (peak 29). Both stachyurin and casuarinin have same molecular weight of 936 Da and identical UV spectra, but they can be distinguished from each other by comparing their retention times and mass spectra. Stachyurin has shorter retention time than casuarinin because of the *β*-configuration of the C–1–OH group (Moilanen et al., 2013). These two isomers are also distinguished from each other by their characteristic mass spectra. They both showed deprotonated molecular ion ([M–H]$^-$) at *m/z* 935 and a doubly charged molecular ion ([M–2H]$^{2-}$) at *m/z* 467 (Supplementary Fig. 1J). In addition, an ion at *m/z* 917, indicating the fragmentation of water [M–H–H_2O]$^-$, could be found for stachyurin, but not for casuarinin (Moilanen et al., 2013, Suvanto et al., 2018). Also, the characteristic fragmentation of an HHDP group (ion at *m/z* 301 [EA–H]$^-$) was detected in the spectra of both compounds.

Peak 28 was tentatively identified as hippophaenin C and peak 30 as hippophaenin B (Suvanto et al., 2018). Again, these two compounds are isomers of each other, and differ from stachyurin and casuarinin in that they have a valoneoyl group instead of an HHDP group at 4, 6-position of the glucose core (Moilanen et al., 2013, Suvanto et al., 2018). The two compounds have identical UV spectra, but can be distinguished from each other based on the retention times and the mass spectra. Hippophaenin C (*β*-isomer) elutes before hippophaenin B (*α*-isomer). For both of these compounds a deprotonated molecular ion ([M–H]$^-$) at *m/z* 1103 was detected. For hippophaenin C additional ions at *m/z* 1085 and at *m/z* 1041 were detected. These ions indicate loss of a water molecule ([M–H–H_2O]$^-$) and loss of a water molecule together with a carboxylic acid group [M–H_2O–COOH], respectively (Moilanen et al., 2013, Suvanto et al., 2018). In the case of hippophaenin B, fragmentation of water was not detected, but the fragmentation of a carboxylic acid ([M–COOH]$^-$) was detected at *m/z* 1059 (Moilanen et al., 2013, Suvanto et al., 2018, Quideau et al. 2005).

Peak 31 was tentatively identified as casuarictin, a glucopyranose-based ET, which consists of two HHDP groups and a galloyl group (Okuda et al., 2009). The identification was based on late retention time and typical UV spectrum (Moilanen et al., 2013). In the mass spectrum, a deprotonated molecular ion ($[M–H]^-$) at *m/z* 935 was detected together with a doubly charged molecular ion ($[M–2H]^{2-}$) at *m/z* 467. All the above-mentioned compounds have previously been characterized from SB leaves (Moilanen et al., 2013, Suvanto et al., 2018, Yoshida, Tanaka, Chen & Okuda, 1991).

3.2. Composition and contents of phenolic compounds

3.2.1. Flavonol glycosides in sea buckthorn leaf samples

As reported previously, isorhamnetin, quercetin and kaempferol derivatives as major compounds in the samples (Rösch et al., 2004, Zu, Li, Fu & Zhao, 2006). Glycosides of isorhamnetin (46.2–64.9 % of total FGs) represented the highest percentage and a clearly dominating majority among the diversity of flavonol compounds in all the leaf samples. Glycosides of quercetin (20.8–27.1%) also presented a large proportion of the total FGs. Compared with flavonol profiles of berry (Zheng et al., 2016), the presence of kaempferol derivatives (12.4–29.2%) was specific to leaves in ssp. *rhamnoides*, represented by four compounds, Ka-3-He-7-Rh (**12**), Ka-He (**20**), Ka-*p*-coumaroylhexoside I (**22**) and Ka-*p*-coumaroylhexoside II (**23**) (Table 1). Is-3-G-7-Rh (12.6−18.6% of total FGs), Is-3-R (5.2−13.7%), and Ka-3-He-7-Rh (5.0−15.8%) were the three most abundant FGs in all the samples (Table 2 and Table 3). Flavonol aglycones were not found in significant quantities in SB leaf samples, most likely, due to both the low concentration presented in the leaves and their poor solubility in water (Price, Rhodes & Barnes, 1998).

The concentration of the FGs ranged from 9.7 mg to 11.7 mg/100 mL in infusions (Table 2), which is higher than reported in black tea infusions (3.6–8.8 mg/100 mL) (Price et al., 1998).

The total FGs content varied in the range from 13.1 mg to 14.1 mg/g DW in the fresh leaves and 5.8 mg to 7.4 mg/g DW in the residues after the water infusion (Table 3). The total contents of FGs in the leaves of SB were higher than reported in the leaves of green tea, oolong tea and black tea (2.3–5.7 mg/g DW) (Jiang, Engelhardt, Thräne, Maiwald & Stark, 2015).

3.2.2. Ellagitannins in sea buckthorn leaf samples

The total contents of identified ETs were 9.2–20.1 mg/100 mL in tea infusions, 4.0–15.2 mg/g DW in tea residues and about 30 mg/g DW in the fresh leaves (Table 2 and Table 3). Average concentrations of total ETs in the residues were close to one-third of the levels in the fresh leaves (Table 3). This may be due to monomeric ETs are highly water soluble, i.e. most of the ETs are in the infusions (Tanaka, Zhang, Jiang & Kouno, 1997). Casuarinin (25.8–44.6 % of total ETs) and stachyurin (19.2–45.4 % of total ETs) were the most abundant compounds in all samples, whereas hippophaenin C (2.5–9.8 % of total ETs), hippophaenin B (2.3–14.0 % of total ETs) and pedunculagin (0–10.3 % of total ETs) were detected at significantly lower levels.

3.3. Comparison of the cultivars

The t-test showed no statistically significant difference ($p > 0.05$) in the content of total FGs, total ETs and total phenolics in the fresh leaves between ‘Terhi’ and ‘Tytti’. However, there were significant differences in the contents of individual ETs in the infusion samples between the two cultivars, except for stachyurin ($p < 0.05$, Table 2). On the other hand, little variance was seen between the two cultivars in individual ETs in the residue samples, with casuarinin being a clear exception. It was detected in significantly lower quantities in ‘Tytti’ than in ‘Terhi’ (Table 3). The total content of FGs and the content of some individual FGs were significantly higher in the residue samples of ‘Terhi’ than in those of ‘Tytti’ (Table 3, $p < 0.05$). The ratio

384 between isorhamnetin and quercetin glycosides in ‘Terhi’ was 2.3 and 1.9 in ‘Tytti’, and the
385 same trend was detected in the infusions and in the fresh leaves.

386 In order to further study the differences and similarities between the two cultivars,
387 unsupervised classification (PCA models) were applied to the infusion and residue samples
388 (Fig. 2A and 2B, respectively). The first two validated principal components shown in Fig. 2A
389 explained 57% of the variance of the data (contents of phenolic compounds, n=41, as X-data)
390 in the samples of tea infusions. Cultivars of ‘Terhi’ and ‘Tytti’ were separated from each other
391 due to different composition and content of phenolic compounds. The first component shows
392 clear classification of ‘Terhi’ on the right and ‘Tytti’ on the left due to different phenolic
393 profiles. Is-3-R was located on the extreme right and showed higher concentration in ‘Terhi’
394 than in ‘Tytti’. In addition, higher content of casuarinin, Ka-3-He-7-Rh, and acylated
395 kaempferol glycosides were shown in ‘Terhi’ than in ‘Tytti’. In contrast, the contents of total
396 ETs, total phenolics and main ETs were higher in ‘Tytti’ than in ‘Terhi’.

397 The residue samples of the two cultivars were also distinguishable from each other as shown
398 in Fig. 2B. The first two validated principal components (PCs), which explained 62 % of the
399 total variance, demonstrated the separation of ‘Terhi’ and ‘Tytti’ in scores plot. The sample
400 locations of ‘Terhi’ and ‘Tytti’ in the plot were similar as described above for the infusion
401 samples (Fig. 2A). However, compared to the infusions, the residues of ‘Terhi’ had higher
402 contents of individual ETs, Qu-3-S-7-Rh, Is-3-R, total FGs and total ETs than those of ‘Tytti’.
403 The residues of ‘Tytti’ contained significantly more Qu-He-Rh I and Is-Pe-Rh compared to the
404 residues of ‘Terhi’.

405 3.4. Comparison of different drying methods

406 In general, the content of ETs varied significantly among the different drying methods
407 studied, whereas the processing had less effect on the FGs content (Table 2 and Table 3). The

content of hippophaenin B varied among different drying methods by close to 4-fold (from 0.4 mg/100 mL in AD to 1.5 mg/100 mL in S+HT). Interestingly, the air-drying residue of 'Terhi' is located on the left in Fig. 2B, which is close to the AD residue of 'Tytti'. This may indicate that AD as a processing method had more profound impact than the genetic background on the composition and content of phenolic compounds. The processing of steam with high temperature (S+HT) led to the highest content of hippophaenin B, hippophaenin C and casuarictin in the infusions of 'Tytti' (Table 2). Moreover, the processing of S+HT resulted in the highest total content of ETs and total phenolics in the infusions. It is possible that some enzymes such as polyphenol oxidase are present in the fresh sea buckthorn leaves, which might have played a major role in degradation of tannins during the drying process (Yoruk & Marshall, 2003). Air-drying process was a slow process lasting three days, and the metabolic processes might have continued even longer, which may lead to degradation of some key compounds and quality loss of the plant materials and subsequently loss of bioactive ingredients in the extracts. Steaming combined with high temperature drying likely have led to deactivation of enzymes and resulted in better preservation of the tannin compounds in the leaves. The results suggest that it is possible to select production conditions that maximize levels of beneficial tea infusion ingredients (Donlao & Ogawa, 2018).

Processing conditions led to a thermally induced degradation of flavonol glycosides to the corresponding aglycone, where the kinetics of this reaction depended on processing time and temperature (Rohn, Buchner, Driemel, Rauser & Kroh, 2007). Flavonol aglycones were not found in significant quantities in SB leaf samples, and there were no significant differences in the contents of total FGs. This may indicate that the FGs in the leaves of SB are generally stable during different drying processes. This is consistent with previous reports that FGs are more resistant to heat processing than some other groups of phenolics, such as anthocyanins (Mäkilä et al., 2017).

The thermal process can convert the highly astringent vescalagin and castalagin into less astringent degradation products (Glabasnia & Hofmann, 2007). Due to the typical astringent and/or bitter taste elicited by ETs (Glabasnia & Hofmann, 2007, Hofmann, Scharbert & Stark, 2006), the processing method may play an important role in the sensory quality of SB leaf beverage by altering the content and profile of phenolic compounds in the products.

3.5. Qualitative sensory evaluation of tea-type infusions

The panelists described mainly the color of the samples as brown/dark yellow, whereas the commercial green tea was more often described as having greenish and lighter color (the PCA model in Supplementary Fig. 2A). The SB leaf infusions were generally more often described as having stronger berry, fishy and fermented aroma in comparison to the green tea samples (Supplementary Fig. 2B). The flavors of the SB infusions were described more often as sweet, mild and fishy, while lacking the astringent and bitter flavor of green tea (Supplementary Fig. 2C). Use these descriptors and the lack of negative descriptors indicate that tea-type infusions prepared from the SB leaves may be acceptable to consumers.

3.6. Antioxidant activity of sea buckthorn leaf tea-type infusions

3.6.1. Antioxidant activity measurements

Generally, the tea-type infusions in this study presented intense antioxidant activities (Fig. 3), and ‘Terhi’ samples showed higher activities than ‘Tytti’. The hydroxyl radical scavenging activities were about 40 % in both cultivars, and there were no significant differences between the infusion samples (Fig. 3A). In this study, the metal chelation abilities was around 5% in Terhi and 10% in Tytti as tested in the diluted samples of the infusion. The total concentration of ETs were approximately 0.01−0.02 mM in the diluted samples. In a previous research, metal chelating activities of pure compounds of ETs were reported to be 15−70% when the

concentration of pure ellagitannins used were 0.1 mM (Moilanen et al., 2016). Previously, non-linear relationship has been shown between metal chelating abilities and concentration of ETs (Karamać, 2009). The antioxidant activity results were higher than pure ETs. The infusions contained high concentration of phenolic compounds, which may have had synergistic and additive interactions, affecting the total antioxidant capacity of tea water extract (Colon & Nerín, 2016). There were no significant differences observed between different drying methods, nor between the cultivars (Fig. 3B). Moreover, both cultivars showed low pro-oxidant activities, and 'Tytti' exhibited more pro-oxidant activity than did those of 'Terhi' regardless of the drying method used (Fig. 3C). The leaves were treated with steam and thermal processing (S+HT or S+DT) led to the highest pro-oxidative activities and higher deviations in the infusions of both cultivars. These changes were suggested to be due to modifications in the total phenolic content and profile by phenolic oxidation or polymerization caused by thermal processing (Randhir, Kwon & Shetty, 2008). The deviations might be caused by some degradation products produced during heat treatment, for example ETs can be hydrolyzed partially into insoluble ellagic acid, although they were not detected in this study (Bakkalbaşi et al., 2008, Gancel, Feneuil, Acosta, Pérez & Vaillant, 2011).

The radical scavenging activities of two non-thermal drying methods (air- and freeze-drying) are similar and stable, appearing to preserve abilities of SB leaves without degrading their antioxidant content in the infusions (Fig. 3A) (Kyriakopoulou et al., 2013). The thermal drying methods (LT, S+HT and S+DT) present higher pro-oxidant and lower radical scavenging activities than the non-thermal ones in infusions. Thus, it seems that harsh drying methods increase the pro-oxidative effects of the infusions, and the antioxidant activity shows sensitivity to drying temperatures in tea infusions (Donlao & Ogawa, 2018). Pure ellagitannins present high pro-oxidant activities (Moilanen et al., 2016), however, the infusions show lower activities than pure ETs. It is possible that FGs act as antioxidants to reduce the pro-oxidative activities

of ETs in the infusions, due to their strong antioxidant ability, and synergistic and additive interactions among phenolic compounds (Colon & Nerín, 2016, Kyriakopoulou et al., 2013).

3.6.2. Correlations between phenolic compounds and antioxidant activities

PCA models were created to investigate the contributions of phenolic variables (X-variables, n=33) to antioxidant activity variables (Y-variables, n=3) in 10 infusions (Fig. 3D). The first two validated principal components (PCs), which explained 58 % of the total variance, demonstrated the correlations between FGs and ETs and antioxidant activities. The total ETs, total phenolics and all individual ETs, except casuarinin, were all located on the left side of the plot suggesting strong and positive correlation to the metal chelation ability and negative association with the pro-oxidant activity. Is-R and Is-3-G were located on the right side of the plot, which positively correlated with pro-oxidant activity. Some quercetin glycosides such as Qu-He-Rh III, Qu-He-Rh IV and Qu-3-S-7-Rh are closely associated with radical scavenging activities, whereas kaempferol glycosides and part of quercetin glycosides were located in the middle of the plot suggesting weak correlation with antioxidant activity. The results suggest that the antioxidative activities of ETs are more likely due to their capacity for chelating metal ions than the radical scavenging activities (Gyamfi & Aniya, 2002, Moilanen et al., 2016). Other studies published earlier reported strong correlation between phenolic contents of plant extracts and the antioxidant activity evaluated by different assays (Liaudanskas, Viskelis, Raudonis, Kviklys, Uselis & Janulis, 2014). However, the antioxidant activity of the extracts is affected by many factors, and no clear association was found between the antioxidant potential of extracts and the relative proportion of FGs and ETs despite the fact that these compounds are generally known to be potent antioxidants.

4. Conclusion

In this study, twenty-five flavonol glycosides and six main ellagitannins were investigated in tea-type beverages produced by different drying processes from sea buckthorn leaves. Although only two cultivars were used in this study, the results showed that the cultivar may be a significant factor in determination of the contents and profiles of phenolic compounds in the beverages. The results showed that ellagitannin contents varied significantly among the different processing methods studied, whereas the methods had less effect on the flavonol glycosides. The non-thermal drying methods had higher antioxidant activities than thermal one in the infusions. It is worth to note that the infusions of 'Terhi' showed higher antioxidant activity than those of 'Tytti'. All in all, the leaves of sea buckthorn, currently a side stream from berry production, may be utilized to produce herbal tea or tea-type beverages. The beverages are acceptable for consumers associated with high antioxidant activity. Moreover, utilization of typical processes used in tea manufacturing results in notable contents of various phenolic compounds in the leaf residues after hot water extraction thus they may be further utilized in different solutions. This study provides guidance for the utilization of sea buckthorn leaves as a raw material for tea-type beverages, nutraceuticals and functional products.

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Conflict of interest statement

There are no conflicts of interest.

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645

646 **Figure captions**

647 Fig. 1. Phenolic profiles of 'Tytti' fresh leaf sample collected from Finland in 2015 at 360 nm
648 (A) and 280 nm (B). The numbering and identification of the peaks refer to Table 1.

649 Fig. 2. PCA models for variations in sea buckthorn (*Hippophaë rhamnoides* ssp. *rhamnoides*)
650 samples: (A) scores and loadings plots for the infusions (n = 18 × 2) of the two cultivars (▪
651 Terhi, Te; ● Tytti, Ty); (B) scores and loadings plots for the residues after preparation of
652 infusion (n = 18 × 2) of the two cultivars (▪ Terhi, Te; ● Tytti, Ty). Abbreviations of the
653 compounds refer to Table 1.

654 Fig. 3. Radical scavenging activities (A), metal chelating abilities (B), pro-oxidant activities
655 (C) of sea buckthorn leaf infusions, and PCA models showing the correlations between
656 chemical variables and bioactivities in sea buckthorn (ssp. *rhamnoides*) leaf infusions (D).
657 Negative inhibition values in C indicate pro-oxidant activity. In the PCA models chemical
658 variables is set as X-variables (n=33; black font) and bioactivity variables as Y-variables (n=3;
659 red font) in 10 samples of two cultivars (▪ Terhi, Te; ● Tytti, Ty). Abbreviations of the
660 compounds refer to Table 1.

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Table 1. Flavonol glycosides and ellagitannins detected by HPLC-DAD-ESI-MS/MS from the extracts of all leaf samples.

Peak No. [a]	Rt (min) [a]	λ max (nm)	[M+H]+ (*m/z*)	[M–H]- (*m/z*)	other ions in MS (*m/z*) [b]	MS/MS[c]	Tentative identification [d]
1	4.02	254,345	773		303, 465, 627	465, 611, 773	Qu-S-Rh
2	4.67	256, 355	773		303, 465, 611, 612		Qu-3-S-7-Rh*
3	5.20	257, 346			317, 287, 479, 579		Is Gly I
4	5.66	254, 351	787		317, 479	479, 641	Is-S-Rh
5	6.30	256, 356	787		317, 463, 464, 625		Is-3-S-7-Rh *
6	7.95	250, 350	449		303, 317	449	Qu-Rh
7	8.65	255, 351	611		303, 449	449, 611	Qu-He-Rh I
8	10.20	252, 350	611		303, 449, 463	449, 611	Qu-He-Rh II
9	11.29	255, 345	625		317, 463	463, 625	Is-He-Rh I
10	11.75	253, 332	611		303, 449	449, 465, 611	Qu-He-Rh III
11	12.56	254, 350	611		303, 449	449, 611	Qu-3-R*
12	12.72	254, 351	625		317, 463	463, 625	Is-He-Rh II
13	12.88	252, 364	595		287, 433	449,595	Ka-3-He-7-Rh
14	13.84	254, 352	625		317, 463		Is-3-G-7-Rh *
15	14.74	253, 344	463		317	463	Is-Rh
16	15.52	252, 330	611		303, 317 , 463	465, 611	Qu-He-Rh IV
17	16.04	254, 337	595		317	463, 595	Is-Pe-Rh
18	18.15	253, 346	625		287, 317, 479	625	Is-R
19	18.55	254, 353	625		317, 479		Is-3-R *
20	18.92	347	449		287	449	Ka-He
21	19.48	253, 350	479		317		Is-3-G *
22	29.45	266, 313			287,303, 313, 595, 617	595	ka-*p*-coumaroylhe I
23	29.96	266, 312			287,303, 595, 618		ka-*p*-coumaroylhe II
24	30.40	336			287,317, 454, 595, 617	595	Ka Gly I
25	30.89	253, 353			317, 287, 611, 629, 791	629, 791	Is Gly II
26	8.51; 11.83			783	301, 391, 481		Pedunculagin
27	14.31			935	458, 467, 917		Stachyurin
28	14.68			1103	520, 529,1041, 1085		Hippophaenin C
29	14.83			935	467		Casuarinin
30	15.23			1103	529, 1059		Hippophaenin B
31	18.05			935	467		Casuarictin

[a] The compound numbers and the retention time correspond to the peaks and numbering in the HPLC-DAD chromatograms shown in Figure 1.
[b] Major fragments in the fragmentation process are underlined.
[c] The MS/MS data were obtained by scanning the parents ion of *m/z* 287, 303 and 317.
[d] Compounds with * in the column were identified with reference compounds; the others were identified based on UV and mass spectra. G, glucoside; gly, glycoside; Is, isorhamnetin; Qu, quercetin; ka, kaempferol; Rh, rhamnoside; R, rutinoside; S, sophoroside; He, hexoside; Pe, pentoside.

675 Table 2. Contents of flavonol glycoside and ellagitannins (mg/100 mL) in sea buckthorn leaf infusions of two cultivars. [#]

Compounds[&]	Terhi						Tytti					
	FD	S+DT	S+HT	LT	AD	Means	FD	S+DT	S+HT	LT	AD	Means
Qu-3-S-7-Rh	0.27±0.02^{ab}	0.26±0.01^{ab}	0.28±0.01^{bc}	0.25±0.01^{a}	0.30±0.01^{c}	0.27±0.02	0.27±0.01	0.25±0.01	0.26±0.01	0.25±0.01	0.26±0.01	0.26±0.01
Is-S-Rh	0.58±0.03^{a}	0.57±0.02^{a}	0.61±0.01^{a}	0.56±0.01^{a}	0.70±0.02^{b}	0.60±0.05^{X}	0.76±0.01^{b}	0.56±0.03^{a}	0.65±0.08^{ab}	0.71±0.04^{ab}	0.73±0.06^{ab}	0.68±0.09^{Y}
Is-3-S-7-Rh	0.43±0.03^{b}	0.39±0.01^{ab}	0.43±0.01^{b}	0.38±0.01^{a}	0.36±0.01^{a}	0.40±0.03	0.43±0.02^{ab}	0.33±0.06^{a}	0.46±0.01^{b}	0.38±0.03^{ab}	0.33±0.01^{a}	0.39±0.06
Qu-He-Rh I	0.41±0.01	0.43±0.06	0.43±0.01	0.40±0.02	0.47±0.01	0.43±0.03^{X}	0.55±0.01	0.50±0.01	0.52±0.06	0.48±0.01	0.51±0.04	0.51±0.04^{Y}
Qu-He-Rh II	0.47±0.25	0.25±0.03	0.54±0.08	0.39±0.02	0.21±0.03	0.37±0.16	0.51±0.01	0.32±0.13	0.47±0.16	0.42±0.04	0.43±0.05	0.41±0.10
Is-He-Rh II	0.43±0.12^{ab}	0.49±0.01^{ab}	0.31±0.05^{a}	0.30±0.01^{a}	0.64±0.02^{b}	0.43±0.14^{X}	0.97±0.05	0.72±0.10	0.79±0.14	0.81±0.07	0.81±0.05	0.82±0.11^{Y}
Ka-3-He-7-Rh	0.71±0.14^{ab}	1.09±0.25^{ab}	1.58±0.21^{b}	1.0±0.08^{ab}	0.41±0.01^{a}	0.96±0.46	0.82±0.06^{ab}	0.73±0.26^{ab}	0.97±0.24^{b}	0.91±0.13^{ab}	0.28±0.02^{a}	0.74±0.29
Is-3-G-7-Rh	1.45±0.27	1.56±0.41	1.46±0.15	1.55±0.42	1.83±0.05	1.57±0.26	2.02±0.53	1.81±0.45	1.52±0.32	1.44±0.01	1.77±0.14	1.71±0.34
Is-Pe-Rh	0.29±0.02	0.29±0.02	0.29±0.01	0.27±0.03	0.32±0.01	0.29±0.02^{X}	0.38±0.01	0.30±0.02	0.33±0.05	0.35±0.01	0.32±0.01	0.34±0.03^{Y}
Is-3-R	1.40±0.01^{ab}	1.41±0.06^{ab}	1.51±0.04^{b}	1.39±0.07^{ab}	1.24±0.02^{a}	1.39±0.10^{X}	0.61±0.01	0.55±0.05	0.59±0.08	0.68±0.02	0.68±0.05	0.62±0.07^{Y}
Is-3-G	0.22±0.01^{a}	0.23±0.01^{a}	0.24±0.01^{a}	0.22±0.03^{a}	0.32±0.02^{b}	0.25±0.04^{X}	0.20±0.01	0.15±0.01	0.20±0.05	0.22±0.01	0.22±0.01	0.20±0.03^{Y}
Total FGs[§]	10.42±0.43	10.83±0.27	11.57±0.87	10.16±0.92	10.75±0.02	10.75±0.68	11.72±0.80	9.73±0.62	10.57±1.46	10.4±0.07	10.14±0.61	10.51±0.94
Pedunculagin	0.80±0.02^{a}	1.00±0.06^{b}	1.40±0.11^{c}	0.78±0.11^{a}	0.86±0.05^{a}	0.97±0.25^{X}	1.36±0.11^{bc}	1.75±0.22^{d}	1.59±0.16^{cd}	1.14±0.08^{b}	0.6±0.09^{a}	1.29±0.43^{Y}
Stachyurin	4.79±0.08^{b}	5.91±0.20^{c}	9.07±0.56^{d}	3.77±0.56^{a}	4.25±0.33^{ab}	5.56±1.96	6.18±0.48^{c}	7.91±0.95^{d}	7.92±0.57^{d}	4.93±0.23^{b}	2.74±0.81^{a}	5.94±2.08
Hippophaenin C	0.37±0.02^{b}	0.47±0.03^{c}	0.59±0.05^{d}	0.28±0.04^{a}	0.63±0.06^{d}	0.47±0.14^{X}	0.80±0.15^{bc}	0.98±0.21^{c}	1.51±0.27^{d}	0.54±0.08^{ab}	0.37±0.17^{a}	0.84±0.44^{Y}
Casuarinin	4.37±0.25^{ab}	4.94±0.12^{c}	6.73±0.28^{d}	4.66±0.46^{bc}	4.09±0.31^{a}	4.96±0.99^{X}	4.23±0.24^{b}	4.94±0.49^{c}	5.36±0.23^{c}	3.60±0.10^{a}	3.14±0.32^{b}	4.25±0.88^{Y}
Hippophaenin B	0.30±0.03^{a}	0.39±0.01^{b}	0.45±0.04^{c}	0.57±0.05^{d}	0.60±0.05^{d}	0.46±0.12^{X}	0.74±0.12^{bc}	0.92±0.19^{c}	1.46±0.25^{d}	0.55±0.07^{ab}	0.40±0.18^{a}	0.81±0.40^{Y}
Casuarictin	0.84±0.13^{a}	1.33±0.05^{b}	1.74±0.11^{c}	0.99±0.13^{a}	1.79±0.16^{c}	1.34±0.40^{X}	2.04±0.17^{bc}	2.62±0.30^{d}	2.26±0.15^{c}	1.71±0.11^{a}	1.92±0.12^{ab}	2.11±0.36^{Y}
Total ETs[*]	11.47±0.3^{a}	14.03±0.45^{b}	19.99±1.13^{c}	11.05±1.35^{a}	12.22±0.95^{a}	13.75±3.45	15.35±1.06^{c}	19.12±2.29^{d}	20.1±1.53^{d}	12.46±0.57^{b}	9.18±1.66^{a}	15.24±4.39
Total phenolics	41.15±0.97^{ab}	43.71±1.9^{b}	58.62±2.63^{d}	39.26±3.10^{a}	50.94±3.10^{c}	46.74±7.62^{X}	55.12±4.54^{bc}	60.22±7.04^{cd}	64.34±4.16^{d}	46.67±2.81^{a}	49.07±2.01^{ab}	55.08±7.90^{Y}

676 [#] Values are presented as means ± standard deviation. Significant differences ($p < 0.05$) between drying method in each cultivar are marked with a-d. Differences ($p < 0.05$) between infusion means of two cultivars are
677 marked with X–Y.
678 [&] Abbreviations of the compounds refer to Table 1.
679 [§] Sum of 24 flavonol glycosides identified in this study.
680 [*] Sum of 6 ellagitannins identified in this study.

Table 3. Flavonol glycoside and ellagitannin contents (mg/g dry weight) in infusion residues from leaves prepared with different drying methods and in fresh leaves of two cultivars. [#]

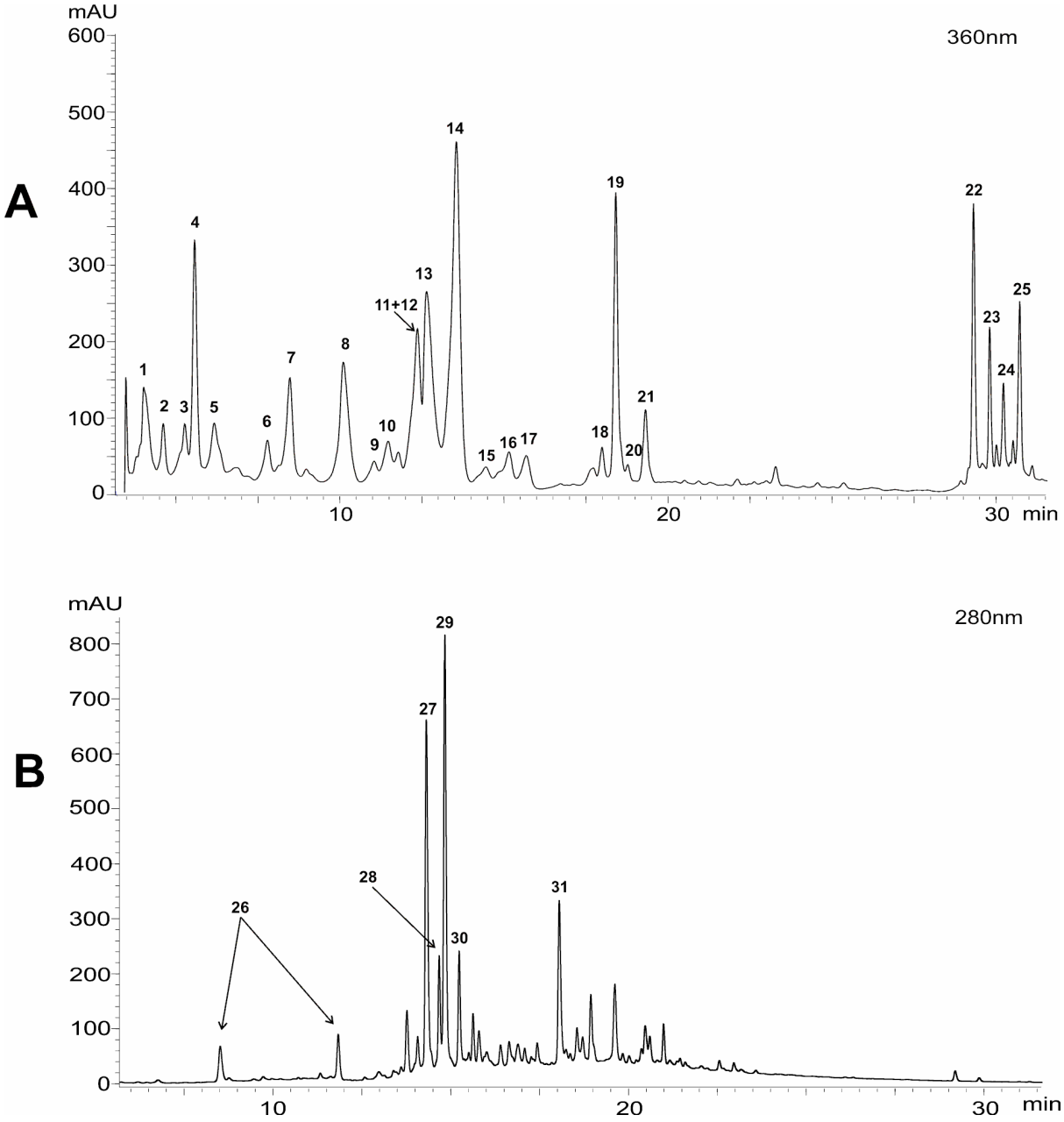
Compounds[&]	Terhi							Tytti						
	FD	S+DT	S+HT	LT	AD	Means	Fresh leaves	FD	S+DT	S+HT	LT	AD	Means	Fresh leaves
Qu-3-S-7-Rh	0.23±0.01^{b}	0.25±0.02^{b}	0.26±0.01^{b}	0.24±0.01^{b}	0.19±0.01^{a}	0.23±0.03^{A}	0.44±0.01	0.19±0.01	0.18±0.03	0.17±0.01	0.18±0.01	0.17±0.01	0.18±0.01^{B}	0.34±0.04
Is-S-Rh	0.34±0.01	0.30±0.03	0.26±0.03	0.26±0.01	0.28±0.06	0.29±0.04	0.79±0.01	0.42±0.01^{b}	0.28±0.06^{a}	0.27±0.03^{a}	0.32±0.03^{a}	0.31±0.01^{a}	0.32±0.06	0.77±0.09
Is-3-S-7-Rh	0.20±0.05	0.19±0.05	0.17±0.01	0.15±0.01	0.16±0.01	0.17±0.03	0.46±0.01	0.22±0.06	0.15±0.02	0.14±0.01	0.14±0.02	0.14±0.01	0.15±0.04	0.46±0.01
Qu-He-Rh I	0.27±0.01	0.27±0.01	0.26±0.02	0.25±0.01	0.25±0.02	0.26±0.01^{A}	0.54±0.01	0.37±0.01^{b}	0.30±0.03^{ab}	0.28±0.02^{a}	0.29±0.01^{a}	0.33±0.01^{ab}	0.31±0.04^{B}	0.65±0.07
Qu-He-Rh II	0.45±0.03	0.40±0.07	0.42±0.02	0.33±0.01	0.39±0.06	0.39±0.06	0.80±0.02	0.47±0.09	0.37±0.02	0.33±0.01	0.32±0.01	0.4±0.03	0.38±0.07	0.79±0.09
Is-He-Rh II	0.49±0.30	0.97±0.16	1.05±0.03	0.74±0.07	0.24±0.02	0.66±0.31	0.66±0.01	0.44±0.22	0.58±0.13	0.33±0.02	0.39±0.05	0.45±0.01	0.43±0.12	1.19±0.23
Ka-3-He-7-Rh	0.64±0.13	0.59±0.01	0.79±0.11	0.96±0.07	0.88±0.09	0.79±0.19	1.3±0.01^{X}	0.69±0.35	0.86±0.19	0.90±0.07	0.85±0.25	0.63±0.06	0.82±0.20	0.65±0.06^{Y}
Is-3-G-7-Rh	0.88±0.14	0.87±0.12	0.70±0.11	0.77±0.01	0.81±0.16	0.85±0.12	2.02±0.07	1.11±0.14^{b}	0.68±0.01^{a}	0.74±0.08^{a}	0.97±0.28^{a}	0.95±0.03^{ab}	0.86±0.17	2.04±0.23
Is-Pe-Rh	0.11±0.01	0.12±0.01	0.10±0.01	0.10±0.01	0.11±0.01	0.11±0.01^{A}	0.27±0.01	0.17±0.01	0.15±0.02	0.13±0.01	0.15±0.01	0.16±0.01	0.15±0.01^{B}	0.36±0.03
Is-3-R	0.73±0.01	0.75±0.04	0.68±0.08	0.71±0.02	0.69±0.12	0.72±0.05^{A}	1.80±0.02^{X}	0.33±0.01	0.31±0.06	0.28±0.02	0.34±0.03	0.37±0.03	0.32±0.04^{B}	0.73±0.07^{Y}
Is-3-G	0.17±0.01	0.18±0.01	0.16±0.01	0.18±0.01	0.21±0.04	0.18±0.02	0.42±0.01^{X}	0.16±0.01	0.14±0.03	0.14±0.01	0.18±0.03	0.20±0.04	0.16±0.03	0.29±0.03^{Y}
Total FGs[§]	6.81±0.01	7.42±0.59	7.08±0.56	6.82±0.06	6.40±0.35	6.9±0.48^{A}	14.1±0.24	7.15±0.15	6.26±0.52	5.83±0.35	6.11±0.34	6.56±0.29	6.42±0.52^{B}	13.06±1.22
Pedunculagin	0.51±0.01^{b}	0.64±0.22^{b}	0.64±0.01^{b}	0.39±0.05^{ab}	0.01±0.01^{a}	0.44±0.26	3.00±0.67	0.22±0.04^{b}	0.44±0.04^{c}	0.32±0.01^{b}	0.22±0.02^{b}	ND^{a}	0.24±0.16	2.47±0.40
Stachyurin	4.33±0.09^{ab}	5.22±2.09^{b}	5.17±0.42^{b}	3.56±0.17^{ab}	1.03±0.12^{a}	3.86±1.78	7.87±2.91	2.40±0.34^{b}	3.90±0.55^{c}	3.15±0.13^{bc}	2.63±0.02^{b}	0.94±0.15^{a}	2.56±1.06	8.47±0.03
Hippophaenin C	0.70±0.03^{ab}	0.79±0.28^{b}	0.88±0.14^{b}	0.54±0.04^{ab}	0.18±0.02^{a}	0.62±0.28	1.25±0.05	0.74±0.09^{b}	1.22±0.23^{c}	1.09±0.04^{bc}	0.80±0.07b^{c}	0.24±0.05^{a}	0.82±0.37	2.03±0.67
Casuarinin	4.63±0.04^{b}	5.00±1.74^{b}	5.11±0.40^{b}	4.11±0.07^{ab}	1.49±0.20^{a}	4.07±1.53^{A}	13.01±1.94	2.82±0.29^{ab}	3.60±0.56^{b}	2.89±0.02^{b}	2.68±0.09a^{b}	1.79±0.01^{a}	2.76±0.65^{B}	11.48±1.85
Hippophaenin B	0.93±0.01	1.06±0.45	1.10±0.18	0.75±0.06	0.34±0.04	0.83±0.34	0.90±0.08^{X}	1.00±0.09^{ab}	1.52±0.29^{b}	1.56±0.02^{b}	1.07±0.08^{b}	0.50±0.07^{a}	1.13±0.42	3.36±0.32^{Y}
Casuarictin	1.50±0.01^{ab}	2.32±0.71^{b}	2.30±0.23^{b}	1.81±0.03a^{b}	0.93±0.09^{a}	1.77±0.56	3.17±0.26^{X}	2.23±0.24^{bc}	2.97±0.36^{c}	2.11±0.03^{ab}	2.09±0.07^{ab}	1.42±0.03^{a}	2.16±0.54	4.70±0.01^{Y}
Total ETs[*]	12.60±0.09^{ab}	15.04±5.51^{b}	15.19±1.37^{b}	11.15±0.45^{ab}	3.97±0.50^{a}	11.50±4.73	29.20±5.66	9.41±1.07^{b}	13.65±2.03^{b}	11.12±0.14^{b}	9.49±0.32^{b}	4.89±0.23^{a}	9.71±3.11	32.51±1.86
Total phenolics	33.82±0.69^{ab}	37.84±12.19^{b}	37.86±1.02^{b}	31.45±1.22^{ab}	12.91±1.27^{a}	30.77±10.59	98.68±0.13	30.02±2.58^{b}	35.4±3.15^{b}	31.39±0.64^{b}	27.89±0.86^{ab}	22.33±0.89^{a}	29.41±4.76	98.80±5.34

[#] Values are presented as means ± standard deviation. ND, not detected. Significant differences ($p < 0.05$) between drying method in each cultivar are marked with different letters a-c. Differences ($p < 0.05$) between residue means and between fresh leaves of two cultivars are marked with different letters A–B and X–Y, respectively.

[&] Abbreviations of the compounds refer to Table 1.

[§],[*] refer to Table 2.

687 **Fig. 1**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8
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13
14
15
16
17
18
19
20
21
22
23
24
25
B
mAU
280nm
800
700
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200
100
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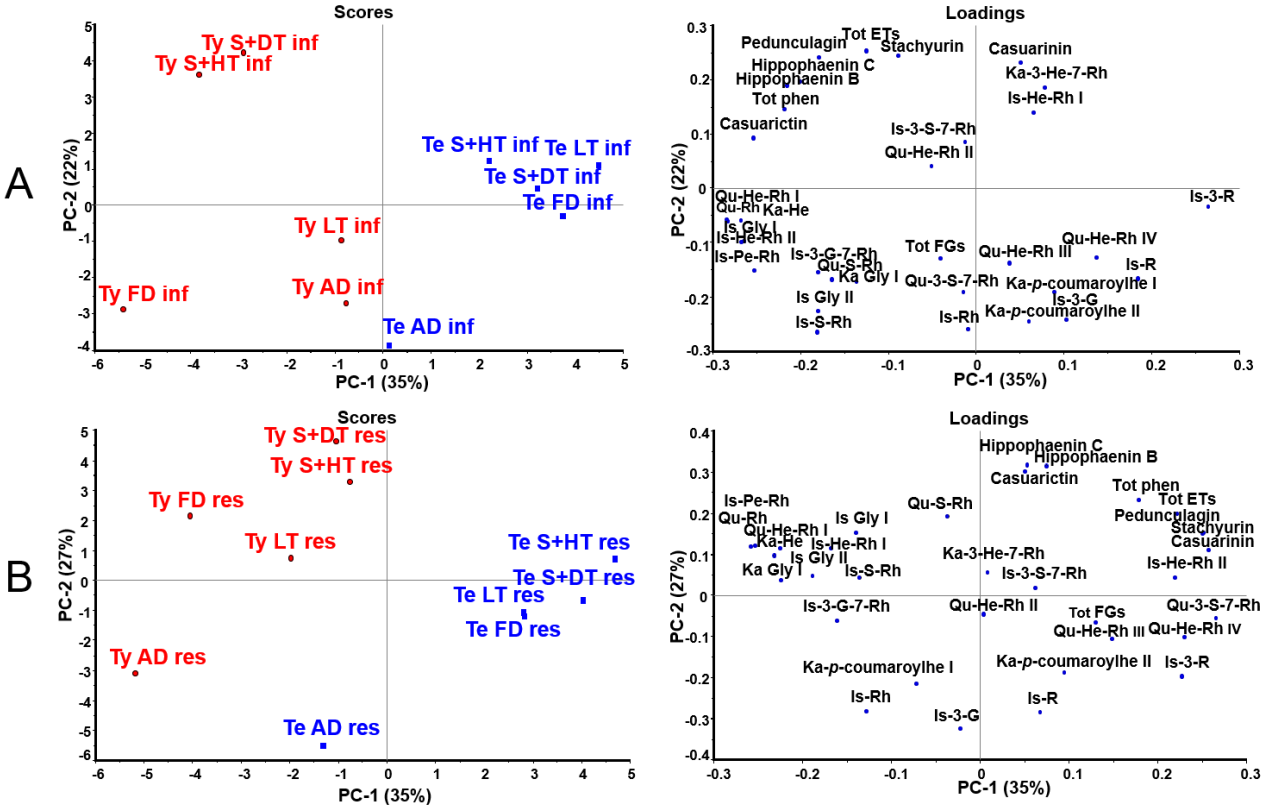
10
20
30
min
26
27
28
29
30
31

688

Fig. 2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Te S+HT inf
Te LT inf
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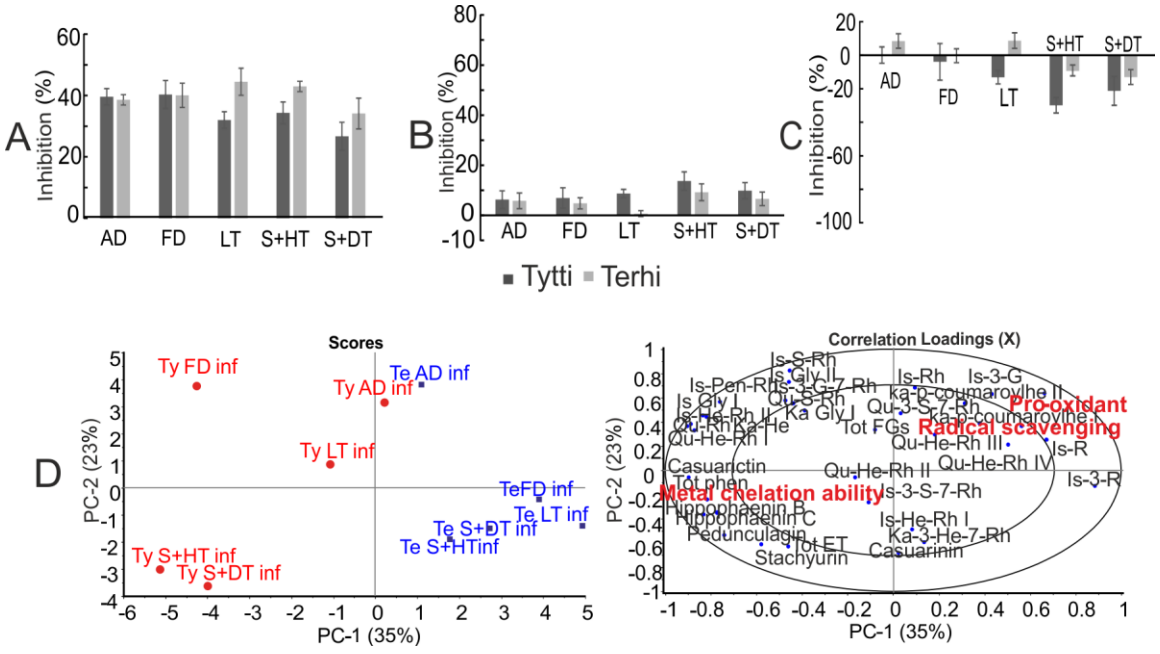
Te FD inf
Ty LT inf
Ty FD inf
Ty AD inf
Te AD inf
PC-2 (22%)
PC-1 (35%)
Loadings
Pedunculagin
Tot ETs
Stachyurin
Casuarinin
Hippophaenin C
Hippophaenin B
Ka-3-He-7-Rh
Tot phen
Is-He-Rh I
Casuarictin
Is-3-S-7-Rh
Qu-He-Rh II
Qu-He-Rh I
Is-3-R
Qu-Rh
Ka-He
Is Gly I
Is-He-Rh II
Qu-He-Rh IV
Tot FGs
Is-Pe-Rh
Is-3-G-7-Rh
Qu-He-Rh III
Qu-S-Rh
Is-R
Ka Gly I
Qu-3-S-7-Rh
Ka-p-coumaroylhe I
Is Gly II
Is-3-G
Is-S-Rh
Is-Rh
Ka-p-coumaroylhe II
B
Scores
Ty S+DT res
Ty S+HT res
Ty FD res
Ty LT res
Te S+HT res
Te S+DT res
Te LT res
Te FD res
Ty AD res
Te AD res
PC-2 (27%)
PC-1 (35%)
Loadings
Hippophaenin C
Hippophaenin B
Casuarictin
Tot phen
Is-Pe-Rh
Tot ETs
Qu-S-Rh
Pedunculagin
Qu-Rh
Is Gly I
Stachyurin
Qu-He-Rh I
Casuarinin
Ka-He
Is-He-Rh I
Ka-3-He-7-Rh
Is Gly II
Is-He-Rh II
Ka Gly I
Is-S-Rh
Is-3-S-7-Rh
Is-3-G-7-Rh
Qu-He-Rh II
Qu-3-S-7-Rh
Tot FGs
Qu-He-Rh III
Qu-He-Rh IV
Ka-p-coumaroylhe I
Ka-p-coumaroylhe II
Is-3-R
Is-Rh
Is-R
Is-3-G
PC-2 (27%)
PC-1 (35%)

691 **Fig. 3**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FD
LT
S+HT
S+DT
B
C
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Terhi
D
Scores
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Ty LT inf
TeFD inf
Te LT inf
Te S+DT inf
Te S+HTinf
Ty S+HT inf
Ty S+DT inf
PC-2 (23%)
PC-1 (35%)
Correlation Loadings (X)
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Is-Gly-II
Is-Pen-Rhs-3-Gz-7-Rh
Is-Rh
Is-3-G
ka-p-coumaroylhe II
Is Gly I
Qu-3-Rh
Qu-3-Sr-7-Rh
Pro-oxidant
Qu-He-Rh II
Ka Gly I
Tot FGs
Radical scavenging
Qu-He-Rh I
Qu-He-Rh III
Is-R
Casuarictin
Qu-He-Rh IV
Tot phen
Is-3-R
Metal chelation ability
Is-3-S-7-Rh
Nippophaenin B
Nippophaenin C
Is-He-Rh I
Pedunculagin
Ka-3-He-7-Rh
Tot ET
Casuarinin
Stachyurin

692