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Sephadex LH-20 fractionation and bioactivities of phenolic compounds from extracts of Finnish berry plants

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Abstract

In order to assist developing a natural, safe food-preservative, aqueous ethanolic extracts of leaves and berries of eight Finnish berry plants were fractionated with Sephadex LH-20 column chromatography. For each fraction, phenolic compounds were analyzed with NMR, UPLC-DAD-ESI-MS and HPLC-DAD. The antioxidant activities of the fractions were investigated using oxygen radical absorbance capacity (ORAC) assay, and the antibacterial activities were evaluated against foodborne pathogens *Staphylococcus aureus* and *Escherichia coli*. Antioxidant activities of the fractions correlated highly with both the total concentration and structural feature of phenolic compounds, including both flavonoids and non-flavonoid phenolics. ORAC value correlated strongly with the concentration of (+)-catechin, (-)-epicatecin, quercetin glycosides, and anthocyanins. Increase in size and number of sugar moieties may reduce the antioxidative activities of quercetin glycosides. Type of sugar moieties may have a significant role in influencing peroxyl-radicals scavenging ability of quercetin glycosides with monosaccharides as a single sugar moieties. Most of the fractions inhibited the target microbes. *S. aureus* strains expressed a higher sensitivity to phenolic compounds than *E. coli* strains.

Keywords: Antioxidant, anti-bacteria, berry, leaf, phenolic compounds, Sephadex LH-20

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1. Introduction

It is well-known that berry plants contain high concentrations of phenolic compounds. Synthesized from the shikimate pathway and the acetate pathway of plants, phenolic compounds are mainly classified into flavonoids and non-flavonoid phenolics based on the structures of carbon skeletons (Shahidi & Ambigaipalan, 2015; Skrovankova, Sumczynski, Mlcek, Jurikova, & Sochor, 2015). Flavonoids are present ubiquitously in the leaves and fruits of berry plants, primarily consisting of flavan-3-ols, proanthocyanidins, flavonols, flavones, flavanones and anthocyanins (Lätti, Riihinen, & Jaakola, 2011; Kallio, Yang, Liu, & Yang, 2014; Vagiri et al., 2015). Non-flavonoid phenolic compounds also display a diversity of structures from simple phenolic acids to hydrolysable tannins, as parts of self-protection mechanism of plants against pathogens and insects (Kähkönen, Kylli, Ollilainen, Salminen, & Heinonen, 2012; Lattanzio, Lattanzio, & Cardinali, 2006).

A number of previous researches have reported that phenolic compounds are responsible for antioxidative and antimicrobial effects of berry plant extracts. In bilberry (*Vaccinium myrtillus* L.), the phenolic content in the leaf and stem extracts correlated strongly with the ability of scavenging DPPH radicals. Higher antioxidant capacity was observed in extracts of colored samples of bilberry than non-colored samples in DPPH, FRAP (Ferric ion reducing antioxidant power), and ORAC (oxygen radical absorbance capacity) assays probably due to

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the presence of anthocyanins (Bujor, Le Bourvellec, Volf, Popa, & Dufour, 2016; Colak et al., 2017). Compared to ascorbic acid, flavonoids were more important contributors to antioxidant activity (TEAC assay) in the extracts of blueberry (*Vaccinium corymbosum*), cranberry (*Vaccinium oxycoccus*), raspberry (*Rubus idaeus*), black currant (*Ribes nigrum*), and red currant (*Ribes rubrum*) (Borges, Degeneve, Mullen, & Crozier, 2010). Many berry extracts are also known for the potential activities against foodborne pathogens. The extracts of cranberry, blueberry and strawberry (*Fragaria* × *ananassa*) inhibited the growth of both Gram-positive (*Listeria*, *Staphylococcus aureus*, and *Clostridium perfringens*) and Gram-negative bacteria (*Salmonella enterica*, *E. coli* and *Campylobacter* spp.) (Das, Islam, Marcone, Warriner, & Diarra, 2017). Raspberry pomace extracts showed strong growth inhibition against *Salmonella typhymurium*, *Escherichia coli*, and *Pseudomonas aeruginosa* (Cetojević-Simin et al., 2015). *Staphylococcus aureus* was sensitive to the leaf and berry extracts of blueberry (Silva et al., 2015).

The *in vitro* assays of antioxidative activities have been a subject of debate. There is a lack of evidence proving a direct link between antioxidative activities and the health promoting mechanisms (Balentine et al., 2015). Strong *in vitro* activities do not necessarily correspond to *in vivo* effect, considering bioavailability and physicochemical properties of different compounds involved in *in vivo* antioxidative assays (Holst, & Williamson, 2008; Del Rio,

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Rodriguez-Mateos, Spencer, Tognolini, Borges, & Crozier, 2013). Nevertheless, *in vitro* antioxidative assays are fast methods for screening potential natural food preservatives, For example, the extracts of bearberry (*Arctostaphylos* sp.), blackberry (*Rubus* sp.), blackcurrant, cranberry, cloudberry (*Rubus chamaemorus*), and strawberry have shown potential in meat production and processing as natural antioxidants, decreasing the usage of synthetic antioxidants (Lorenzo et al., 2017).

In our previous studies, we compared the composition and bioactivities of twenty-nine extracts obtained from berries and leaves of different berry species (Tian et al., 2017). Clear correlations were detected between antioxidative and antimicrobial activities and specific groups of phenolic compounds (Tian, Puganen, Alakomi, Uusitupa, Saarela, & Yang, 2018). Nevertheless, some aromatic compounds have been found in the certain leaves (for example, prunasin in saskatoon leaf, and tyramine in white currant leaf) and they may cause the issue of food safety. It is necessary to fractionate the raw materials and fully evaluate the efficacies of the fractions against free radicals and foodborne pathogens. In the present study, eight aqueous ethanol extracts of leaves and berries were selected based on their strong antioxidative and antimicrobial activities. In order to assist the development of a natural, safe food-preservative, we fractionated each extract into eleven fractions using a Sephadex LH-20 column. The composition of the extracts was analyzed with HPLC-DAD, HPLC-MS, and ^{1}H

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NMR. The anti-oxidative and antimicrobial activities of the fractions were investigated *in vitro*. Bivariate and multivariate correlation between phenolic composition and bioactivities was performed to determine the main compounds responsible for the oxygen radical-scavenging capacity and anti-bacterial activities. In our previous research, we have reported association between different groups of phenolic compounds with antioxidative and microbial activities of the crude aqueous-ethanolic extracts. However, it was not possible to define the importance of individual compounds to the bioactivities, where many compounds were present and might have contributed to the activities observed. The aim of this study was also to go one step further in the investigation of the structure-activity relationship of phenolic compounds based on detailed studies on the composition and activities of the fractions, which were enriched with selected compounds.

2. Materials and Method

2.1 Plant materials and extracts

Based on the high antimicrobial and antioxidant activities reported in previous research (Tian et al., 2017), eight phenolic extracts from berries and leaves of Finnish berry plants were selected. The plant materials were collected in the summer of year 2013. In order to represent the average level of phenolic profiles, the berries were harvested optimally ripe based on color, flavor, and structure. Leaves were randomly collected from different sides of the

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bushes (or trees) and then pooled. After that, representative samples were taken from the pooled samples. All the samples of berries and leaves were stored in a freezer at -20 °C right after collection. The storage temperature was chosen based on the practicality of food industry. The materials were extracted with acidic aqueous ethanol (Tian et al., 2017). The raw extracts were fractionated in this study. All information of the samples is shown in **Supplemental Table 1**.

2.2 Chemicals

Reference compounds of flavan-3-ols (catechin and epicatechin), flavonols (3-*O*-rutinosides and 3-*O*-glucosides of quercetin, myricetin, kaempferol, isorhamnetin, and syringetin), flavone (apigenin 8-*C*-glucoside) and anthocyanins (3-*O*-glucosides of cyanidin, delphinidin and malvidin) were purchased from Extrasynthese (Genay, France). Caffeoylquinic acid (5-*O*-, 4-*O*-, and 3-*O*-), 6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox), 2, 2'-azobis(2-amidinopropane) dihydrochloride (AAPH), and fluorescein (98%) were purchased from Sigma-Aldrich Co. (St. Louis, USA). Monobasic potassium phosphate (KH_2PO_4), and dibasic potassium phosphate (K_2HPO_4) were from Merck Co. (Darmstadt, Germany). A reference compound of B-type procyanidin dimer was prepared by the Department of Chemistry, University of Turku. Deuterium oxide (D_2O, 99.96 % D) and

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acetone-d_6 ($(CD_3)_2CO$, 99.80 % D) were from VWR International BVBA (Leuven, Belgium). Other HPLC and MS grade chemicals were purchased from VWR International Oy (Espoo, Finland). The bacterial strains *Staphylococcus aureus* VTT E-70045 and *Escherichia coli* VTT E-94564 were supplied by VTT Technical Research Centre of Finland Ltd (Helsinki, Finland).

2.3 Extraction and fractionation of raw materials

The extraction was conducted according to our previous method, using 70% aqueous ethanol acidified with 1% acetic acid (the sample/solvent ratio of 1:10, w/v, fresh weight basis) (Tian et al., 2017). Each of selected extracts (6 mL) was centrifuged (4420× g for 15 min). After collection of supernatant, the subsidence was dissolved in aqueous ethanol (0.5 mL, 90%) and then centrifuged again (4420 × g for 15 min). The supernatants from the two times of centrifugation were combined. For fractionation by column chromatography, the combined supernatants were applied to a Sephadex LH-20 column (2.5 cm × 150 cm) and eluted successively with MQ water (50 mL), aqueous ethanol (20, 40, 70 and 90% ethanol, 50 mL for each) and aqueous acetone (50% and 90% acetone, 50 mL for each) at room temperature. The scheme of elusion was selected according to the previous study with some modification (Salminen, Karonen, 2011). Each extract yielded 11 fractions, which were all lyophilized after evaporation of organic solvents. The fractionation was carried out for identification and

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quantification, separately; all fractions were weighed and stored at -20 °C for further analysis (**Supplemental Table 2**).

2.4 Identification and quantification of phenolic compounds in fractions

Phenolic compounds in each fraction were analyzed using the method described in our previous publication (Tian et al., 2017). Identification was performed using NMR and UPLC-DAD-ESI-MS. NMR analyses were performed on a Bruker Avance 600 spectrometers (operating at 600.13 MHz for ^{1}H) equipped with a broadband inverse autotune BBI-5 mm-Zgrad-ATM probe (Bruker Corp., Billerica, MA, USA). Other one and two-dimensional NMR experiments (^{13}C, 1D TOCSY, DQF-COSY, HSQC and HMBC) was also applied for selected fractions to study their components in more detail. The lyophilized fractions in NMR analyses were dissolved into acetone-d_6:D_2O (8:2, v:v) while fraction 1.1 of each sample were in deuterium oxide. The spectra of NMR were processed with TopSpin 3.2 software (Bruker Corp., Billerica, MA, USA). The chemical shifts were referenced to an acetone resonance at 2.05 ppm; phase and baseline were manually corrected.

After the NMR analysis, 100 µL of each fraction were taken, and the deuterated solvent was evaporated under nitrogen flow. Each of the dried samples was mixed with 200 µL of water and was left to stand several hours in a fridge to ensure proper proton-deuterium exchange.

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Thereafter, the samples were lyophilized and dissolved into 200 μL of extraction solvent (70% aqueous ethanol with 1% acetic acid), filtered through 0.45 μm PTFE filters and analyzed with UPLC-DAD-ESI-MS (Waters Corp., Milford, MA, USA). A Phenomenex Aeris peptide XB-C18 column (150 × 4.60 mm, 3.6 μm, Torrance, CA) was used for liquid chromatographic separation at a total flow rate of 1 mL/min. The injection volume was 10 μL and the temperature was 25 °C. The mobile phase was a combination of Milli-Q water (A) and acetonitrile (B), both consisting of 5.0% (v/v, for the fractions containing anthocyanins) and 0.1% (for other fractions) of formic acid. LC gradients were reported in our previous study (Tian et al., 2017). For ESI-MS system, the source temperature and the desolvation temperature were set to 120 °C and 300 °C, respectively. For negative ion mode, capillary voltage, cone voltage and extractor voltage were 3.5 kV, 35 V, and 7 V, respectively; and 4.0 kV, 22 V, and 3 V for positive ion mode. The MS data analysis was performed with Masslynx 4.1 software (Waters Corp., Milford, MA).

For quantification of phenolic compounds with HPLC-DAD (Shimadzu Corp., Kyoto, Japan), each lyophilized fraction was dissolved in the extraction solvent (2 mL) and filtered through a 0.45 μm PTFE filter. The chromatographic conditions were same as described for the UPLC-DAD-ESI-MS analysis. The quantitative analysis was performed in quadruplicates. The calibration curves were constructed with external standards and applied in the quantitative

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analysis. Reference compounds were analyzed for constructing standard curves. For those compounds, of which we do not have reference compounds, the quantification was carried out using calibration curves constructed with reference compounds of similar chemical structures. For construction of calibration curve, 1 mg of each reference standard was dissolved in 10 mL methanol, and then diluted into four different concentrations. After analysis of the standards, the regression equations of calibration curves were established between the concentration and the peak area in the HPLC-DAD chromatograms.

2.5 Oxygen radical absorbance capacity (ORAC) assay

Based on modification of a previous method reported by Ou and co-workers (Ou, Hampsch-Woodill, & Prior, 2001; Prior et al., 2003), the ORAC assay was carried out on a Hidex Sense microplate reader (Hidex Oy, Turku, Finland) with black 96-well microplates (Greiner Bio-One GmbH, Frickenhausen, Germany). Briefly, 20 μL of fractions (or blank buffer,), 60 μL of potassium phosphate buffer (K_2HPO_4-KH_2PO_4, pH 7.4, 75 mM), and 100 μL of fluorescein solution (0.09 μM, in phosphate buffer) were added to each well of a microplate and incubated at 37 °C for approximately 3 min (the parameter was set to 5 cycles in microplate reader). After addition of AAPH solution (70 μL, 300 mM, in phosphate buffer) to each well, fluorescence (ex. 485 nm/em. 535 nm, 100 flashes) was recorded at 37 °C for 30 min (120 cycles with 15 s of interval). The final results were expressed as Trolox equivalent

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(μmol/mL). Trolox working solutions (100 μM, 50 μM, 25 μM, 12.5 μM) were used for the calibration curve. The ORAC values were calculated by using a regression equation (y = a + bx) between Trolox concentration (y) and the net area under the fluorescence decay curve (x). The area under curve (AUC) was calculated with OriginLab 8.0 software (OriginLab Corporation, Northhampton, MA) and the net AUC was obtained as follow: Net AUC = AUC sample/standard − AUC blank (Puganen, Kallio, Schaich, Suomela, & Yang, 2018). Fractions were tested at different dilutions to find the optimized concentration (the net AUC value is on the midrange of Trolox standard curve).

2.6 Measurement of antibacterial activities of fractions

Staphylococcus aureus (VTT E-70045) and *Escherichia coli* (VTT E-94564) were selected as target microbes for estimating the antibacterial activities of the fractions. A Bioscreen™ (Thermo Fisher Scientific Inc., Massachusetts, USA) automated turbidometer was applied for monitoring the growth of target microbes. The evaluation was performed by the following method previously published by Hanna-leena Alakomi (Alakomi et al., 2007). Each fraction (1 mL) was evaporated to completely remove the solvent, and the residue was restored into 1 mL of sterilized Milli Q-water. For each fraction, the test was carried out at two different dosage levels corresponding to 10 and 20 μL of fraction solution, respectively, added to each well (the total volume in each well: 300 μL). The control wells contained no addition of the

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fractions. The result was expressed by the percentage of growth inhibition of the bacteria.

2.7 Statistical Analyses

The data processing of this research was conducted with Microsoft Excel 2010 (Microsoft Corp., WA, US) and OriginLab 8.0 software (OriginLab Corporation, Northhampton, MA). The results were expressed as mean ± standard deviation (SD). To determine the relationship between the concentration of phenolics and bioactivities of the fractions, bivariate Pearson correlation analysis with two-tailed test was applied using IBM SPSS Statistics 24 for Windows (SPSS Inc., NY, US). For multivariate correlation, partial least squares regression (PLS) was performed using Unscrambler 10.1 (Camo Process AS, Oslo, Norway). PLS models were established with the concentration of phenolic compounds as the predictors (variable X), and the bioactivities as the responses (variable Y).

3. Results and Discussion

3.1 Distribution of phenolic compounds in different fractions

Phenolic composition in the fractions was studied with ^{1}H NMR and MS analyses, in order to further evaluate the contribution of phenolic compounds with different structural features to the antioxidant and antibacterial activities. In general, the fraction 1.1 of all the extracts contained sugars and some small aliphatic compounds. Different derivatives of

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hydroxycinnamic acids were eluted to fractions 1.2–4.2. Fractions 2.2–4.2 comprised a variety of glycosylated flavonoids, flavones and flavanones, as well as anthocyanins from the berry extracts. In fractions 5–7, typical broad resonance humps of tannins in ^{1}H NMR and negative ions at *m/z* 289 (catechin fragment) and 301 (ellagic acid fragment) were observed in MS spectra, suggesting proanthocyanindins and ellagitannins to be the main compounds (Liu, Kallio, & Yang, 2011; Suvanto, Tähtinen, Valkamaa, Engström, Karonen, & Salminen, 2018). Unfortunately, these tannin compounds could not be characterized and quantified individually owing to complicated compositions and insufficient separations with the method applied in our study. Therefore, the phenolic profiles in fractions 5–7 of all extracts were not present in this paper.

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3.1.1 Chokeberry fractions

Chokeberry mainly contained hydroxycinnamic acid derivatives, anthocyanins, flavanone and flavonol glycosides (**Fig. 1a** and **Supplemental Table 3a**). The main derivatives of hydroxycinnamic acid were caffeoylquinic acids, and the ^{1}H NMR spectra of fractions 1.2-2.2 showed typical resonances of caffeoyl moiety with a resonance of quinic acid substituent at 5.30 ppm (**Supplemental Fig. 1a**). Due to the low molecular weight (molecular weight <1000), the separation of caffeoylquinic acid in Sephadex LH-20 was mainly based on the

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interaction with the stationary and mobile phase, compared to the molecular size. The polarity of the isomers varies with the position of the ester bond between caffeic acid and quinic acid, resulting in difference of the strength of retention by the stationary phase of the column. As primary isomers, 5-*O*-caffeoylquinic acid was enriched in fractions 1.2-2.1 at a level of 13-33 mg/100 mL, whereas 3-*O*-caffeoylquinic acid was the major compound in fraction 2.2 (35 mg/100 mL). In chokeberry, cyanidin was the main anthocyanidin based on the MS spectra and confirmed by the backbone structure with the characteristic singlet signal of H3 in ^{1}H NMR spectra. A majority of anthocyanins eluted in the fraction 3.2. Four 3-*O*-glycosides of cyanidin, confirmed as galactoside, glucoside, arabinoside and xyloside, respectively, altogether accounted for 0.5% of the total content of phenolics (Tot-Ph) in fraction 2.2 (the content of anthocyanins was 0.3 mg/100 mL), 40% in fraction 3.1 (17), 87% in fraction 3.2 (151), 73% in fraction 4.1 (27), and 11% in fraction 4.2 (2). The only group of flavanone, consisted of eriodictyol derivatives, was mostly presented as a conjugation of methyl-hexoside in fraction 2.2 (32% of Tot-Ph) and fraction 3.1 (34%). Flavonol glycosides (fractions 3.2, 4.1, and 4.2) consisted of derivatives of quercetin, kaempferol and isorhamnetin with quercetin glycosides being the dominant compounds.

3.1.2 Crowberry fractions

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The primary phenolic compounds in fractions of crowberry were identified as hydroxycinnamic acids, anthocyanins and flavonol glycosides (**Fig. 1b**). As presented in **Supplemental Fig. 1b** (fractions 1.2-2.1), *trans*- and *cis*-coumaroyl derivatives were indicated by the resonances of two coumaroyl derivatives, coupled with the signals of *para*-substituted aromatic ring (two-fold doublets) and the pair of coupled methine protons in the NMR spectra. Another substituent of these derivatives was suggested to be quinic acid as shown in the mass spectra. As the main phenolic compounds, the concentration of coumaroyl quinic acids was 30 mg/100 mL in fraction 1.2 and 12 mg/100 mL in fraction 2.1 (**Fig. 1b**, **Supplemental Table 3b**). Although anthocyanins were enriched in fractions 2.2-3.2, the individual forms could not be identified with solely ^{1}H NMR. The mass spectra of corresponding fractions showed 3-*O*-glycosides of delphinidin, cyanidin, petunidin, peonidin, and malvidin, which is in accordance with previous reports of crowberry (Ogawa et al., 2008; Jurikova et al., 2016). The total content of anthocyanins represented 92% (fraction 2.2), 96% (fraction 3.1), and 93% of Tot-Ph (fraction 3.2), due to the presence of delphinidin, cyanidin, and malvidin derivatives. As shown in **Supplemental Table 3b**, delphinidins (185 mg/100 mL) and cyanidins (119) concentrated in fraction 3.1, while malvidins enriched in both fraction 2.2 (155) and 3.1 (87). Anthocyanins in berry fractions were all eluted with 40% of ethanol. For different groups of anthocyanins, the retention time was affected by their chemical structures, which caused the maximum concentration of anthocyanins observed in

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different fractions. In addition, Sephadex LH-20 column is not precise as HPLC column, which can be influenced by the composition of the compounds. It may be the reason resulting in the inconsistency between chokeberry and crowberry fractions. Eluted mainly in the fractions 4.1-4.2, glycosylated flavonols had primarily myricetin, quercetin, and laricitrin as the aglycones. Often 3-*O*-galactosides of these flavonols are the dominating compounds. Furthermore, epigallocatechin, catechin and epicatechin, as isomers of flavan-3-ols, were detected in fraction 4.1 (14 mg/100 mL in total), as well as a trace amount of A-type procyanidins in fraction 4.2.

3.1.3 Fractions of sea buckthorn berry and leaf extracts

Berries of sea buckthorn contained mainly glycosides of isorhamentin and quercetin as the major phenolic compounds (Chen, Zhang, Xiao, Yong, & Bai, 2007). ^{1}H NMR data of fractions 3.1–4.1 showed the presence of three main derivatives of isorhamnetin based on the methoxy resonance (-OCH_3) at approximately 3.9 ppm in the NMR spectra (**Supplemental Fig. 1c**). The results of mass spectra suggested that isorhamnetin and quercetin were the main groups of flavonols conjugated with mono-, di-, and even tri-saccharides as the sugar moieties (**Supplemental Table 3c**). Aside from flavonols, malic acid was identified in fraction 1.2 by comparison of the coupling constants and chemical shifts with those of the

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reference compound. Fraction 4.2 contained two different compounds of flavan-3-ols. Catechin was characterized by ^{1}H NMR based on the presence of 1',3',4'-trisubstituted aromatic ring (B-ring) together with H5 and H7 of A-ring doublets. Gallocatechin showed similar proton chemical shifts and coupling constants as catechin; however, the B-ring protons were observed as two-fold singlet at 6.41 ppm, indicating the symmetrical 1',3',4',5'-tetrasubstituted structure of B-ring. Even though sea buckthorn berry is a good source of flavonols, the Tot-Ph was still lower than the dark-skin berries owing to the absence of anthocyanins (**Fig. 1c**). **Supplemental Table 3c** showed that higher level of Tot-Ph was obtained in fraction 3.2 (48 mg/100 mL) and fraction 4.1 (31 mg/100 mL) than in other fractions.

Sea buckthorn leaves showed similar profiles of flavonoids as the berries. Isorhamnetin derivatives were present in large quantities from fractions 2.1–3.2, followed by quercetin glycosides. Catechin and gallocatechin were mainly eluted to fractions 4.1- 4.2 (**Fig. 1d** and **Supplemental Table 3d**). In contrast to the berry extract, the sea buckthorn leaf extract contained ellagic acid derivatives and hydrolysable tannins. Based on ^{1}H NMR, ellagic acid was characterized by one singlet resonance at 7.48 ppm in fractions 4.1&4.2. When one of the free hydroxyl groups was substituted, ellagic acid lost its symmetry and displayed two

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singlet resonances at 7.51 and 7.71 ppm in fractions 2.2-3.1 (**Supplemental Fig. 1d**). The substituents were further identified as deoxyhexose-hexoside, hexoside and pentosides with the aid of mass spectra. The total amount of ellagic acid and its derivatives ranged from 4 to 12 mg/100 mL in fractions 2.2-4.2 (**Supplemental Table 3d**). Hydrolysable tannins in sea buckthorn leaves were mainly as digalloyl-hexoside, galloyl-HHDP-hexoside and galloyl-bis(HHDP)-hexoside, accounting for 76% of Tot-Ph in fraction 3.2, 84% in fraction 4.1 and 83% in fraction 4.2.

3.1.4 Saskatoon leaf fractions

Derivatives of hydroxycinnamic acids are abundant in saskatoon leaf extract, primarily as caffeoylquinic acids (Lavola, Karjalainen, & Julkunen-Tiitto, 2012). The total content of caffeoylquinic acid derivatives was in the range of 0.4-169 mg/100 mL in fractions 1.2-4.2. Mono-caffeoylquinic acids (5-*O*-, 4-*O*-, 3-*O*-, and others) were concentrated in fractions 1.2-3.1, whereas di-caffeoylquinic acids were only present in fractions 4.1&4.2 (**Supplemental Table 3e**). The hydrophobicity of these compounds increased due to the presence of the second caffeoyl group. Caffeoylmalic acid and caffeoylglyceric acid, as other main derivatives of hydroxycinnamic acid appeared mostly in the fraction 1.2 (26 and 44 mg/100 mL, respectively). In the ^{1}H NMR spectra of fractions 3.2-4.2, flavonols displayed typical

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proton resonances with 1’,3’,4’-trisubstituted B-ring (**Supplemental Fig. 1e**), which were characterized mainly as quercetin derivatives (negative ion at 301 *m/z* in the MS spectra). As shown in **Supplemental Table 3e**, quercetin glycosides corresponded to 55% (fraction 3.1), 84% (fraction 3.2), 69% (fraction 4.1), and 34% of Tot-Ph (fraction 4.2) in different fractions with the majorities being arabinoglucoside, hexoside-deoxyhexoside, 3-*O*-rutinoside, and 3-*O*-galactoside, respectively. Eriodictyol 7-*O*-glucoside (30 mg/100 mL) was present in fraction 3.2 as the only form of flavanones; catechin, epicatechin, and B-type procyanidins were eluted mostly in fraction 4.1 & 4.2 (**Fig. 1e**). In addition, fraction 1.2 contained a cyanogenic glycoside, prunasin, ^{1}H and ^{13}C resonances of which were previously assigned in our laboratory (Tian et al., 2017).

3.1.5 White currant leaf fractions

Flavonol glycosides (14-241 mg/100 mL) were the major phenolic compounds, presented in all analyzed fractions of white currant leaf (**Fig. 1f**). In **Supplemental Fig. 1f**, ^{1}H NMR spectra of fractions 2.2-3.1 exhibited the resonances of two flavonols, the B-ring chemical shifts of which referred to quercetin (1’,3’,4’-trisubstituted aromatic ring) and kaempferol derivatives (1’,4’-disubstituted aromatic ring), respectively. The major derivatives were identified based on the mass spectra with oligo-saccharides being sugar moieties. Quercetin

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glycosides accounted for 49-93% of total content of flavonols in all fractions. Trace amounts of myricetin glycosides were also quantified from fractions 3.1-4.2. For phenolic acids, hydroxybenzoic acids (mostly as vanillic acid-hexoside) were distributed mainly into fractions 1.2 & 2.1, with concentrations ranging from 7 to 17 mg/100 mL; whereas hydroxycinnamic acids were primarily enriched in the fraction 2.1 (8 mg/100 mL). Flavan-3-ols and flavones were mainly identified from fractions 4.1 & 4.2. Furthermore, a phenolic compound, 4-(2-hydroxyethyl) phenol-hexoside, was found at a concentration of 22 and 15 mg/100 mL in fractions 1.2 & 2.1, respectively (**Supplemental Table 3f**).

3.1.6 Lingonberry leaf fractions

For lingonberry leaf, the fractions 1.2, 3.2, 4.1, and 4.2 contained large quantities of phenolic compounds, due to the presence of arbutin derivatives, quercetin glycosides, flavan-3-ols, and proanthocyanidins (**Fig. 1g**, **Supplemental Table 3g**). *β*-*p*-Arbutin has been previously characterized from the extract of lingonberry leaves (Tian et al., 2017). In the present study, *β*-*p*-arbutin and its isomers were present at the highest concentration in fraction 1.2 (464 mg/100 mL). 2-*O*-Caffeoyl-*β*-*p*-arbutin was found in fraction 3.2 & 4.1, according to the ^{1}H NMR resonances which were in agreement with previous research (Liu, Lindstedt, Markkinen, Sinkkonen, Suomela, & Yang, 2014). Still in the fraction 3.2, the resonances of

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two para-substituted aromatic rings and sugar moiety indicated another derivative of arbutin, which was identified as lanceoloside A based on the resonance data reported in literature (Pegnyemb, Messanga, Ghogomu, Sondengam, Martin, & Bodo, 1998) (**Supplemental Fig. 1g**). Enriched in fractions 3.2-4.2, quercetin 3-*O*-glycosides accounted for 43%, 54% and 58% of Tot-Ph, respectively. Flavan-3-ols (catechin and epicatechin) were found in fraction 4.1 & 4.2 at total concentration of 178, and 55 mg/100 mL, respectively. Proanthocyanidins in fraction 4.2 were mostly B-type procyanidin dimers, and the total content was around 46 mg/100 mL. Moreover, phenolic acids were present in lingonberry leaf at low concentration, mostly as caffeoylquinic acids and coumaroyl derivatives (Hokkanen, Mattila, Jaakola, Pirttilä, & Tolonen, 2009).

3.1.7 Hawthorn leaf fractions

Fig. 1h shows that the derivatives of hydroxycinnamic acid were distributed to the fractions 1.2-3.1 of the hawthorn leaf extract, the total content of which was in the range from 20 to 74 mg/100 mL. The dominant compounds belonged to caffeoylquinic acids, representing 24-95% of total phenolic acids (**Supplemental Table 3h**). Flavanones were found in the fractions 2.2 & 3.1 at concentrations of 26 and 81 mg/100 mL, respectively. LC-DAD-MS results showed UV absorption maxima at 280 and 330 nm and negative fragment at 287

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(*m/z*), indicating the presence of eriodictyol. The difference (176 *m/z*) between fragments of $[M\text{-}H]^-$ and $[M\text{-}287\text{-}H]^-$ indicated the compound to be eriodictyol methyl-hexoside or glucuronide, although more specific identification of the sugar moiety and its location in the molecule could not be done with the data obtained in this study. Flavonols were mostly present in the fractions 3.2-4.2, where glycosides of quercetin and kaempferol were the major compounds (Sirat, Rezali, & Ujang, 2010). Quercetin derivatives corresponded to 39% of Tot-Ph in fraction 3.2, 53% in fraction 4.1, and 66% in fraction 4.2; however, the proportion of kaempferol glycosides was decreased from 42% in fraction 3.2 to 8% in fraction 4.2. Most of flavan-3-ols (27 mg/100 mL) in the hawthorn leaf extract was recovered in the fraction 4.1. As the only monomer of flavan-3-ol in hawthorn leaf, (-)-epicatechin was distinguished from (+)-catechin based on the coupling constant between H2 and H3 (broad singlet) in ^{1}H NMR (**Supplemental Fig. 1h**). High amounts of B-type procyanidin dimers and timers were found in the fractions 4.1&4.2.

3.2 Antioxidant activities of fractions and contribution of phenolic compounds

3.2.1 Peroxyl-radicals scavenging ability (ORAC)

ORAC assay is applied to estimate the antioxidant properties of all the fractions. The mechanism of ORAC assay is based on the hydrogen atom transferring (HAT) capacity of compounds to inhibit peroxyl-radicals induced by 2, 2’-azobis(2-amidinopropane)

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dihydrochloride (AAPH). The antioxidant activities based on 1 mL of fraction solutions of berry plants are listed in **Table 1**.

As the dominant phenolic compounds in dark-skinned fruits, anthocyanins might be potent inhibitors against peroxyl radicals. Among all fractions of chokeberry, the highest antioxidant activity (17 TE μmol/mL) was found in the fraction 3.2, which has the highest the total concentration of anthocyanins. The ORAC values of the fractions 2.2-4.1 of crowberry were in proportion to the concentration of anthocyanins. In contrast, sea buckthorn berry fractions exhibited limited antioxidantive effects due to low Tot-Ph and the absence of anthocyanins. The antioxidant capacity of the leaf fractions of sea buckthorn was generally in the proportion to the Tot-Ph. For the hawthorn leaf extract, stronger hydrogen-transferring ability was present in the fraction 4.1, which contained higher levels of flavonol glycosides and flavan-3-ols than other fractions. Fraction 3.1 consisted of a large quantity of eriodictyol glycosides but represented lower ORAC result, suggesting eriodictyols to be a weak hydrogen donor. In the lingonberry leaf fractions, the abundance of *β*-*p*-arbutin resulted in the highest antioxidant capacity (226 TE μmol/mL) in fraction 1.2; fraction 4.1 showed the second strongest activity owing to the presence of glycosylated quercetins, catechin, and 2-*O*-caffeoyl-*β*-*p*-arbutin. Quercetin glycosides were also associated with strong activities in the fraction 3.2 of white currant leaf. Additionally, for both saskatoon leaf and sea buckthorn leaf, fraction 6

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represented better abilities of donating hydrogen atom, which was likely due to the presence of unidentified tannins.

3.2.2 The correlation between phenolic composition and antioxidative activity

3.2.2.1 Multivariate correlation

PLS regression models were applied to find the main compounds corresponding to the ability of scavenging peroxyl radicals (**Fig. 2**). Total content of phenolic compounds correlated strongly with ORAC assay among all fractions studied. For berry fractions, flavonoids were major contributors to ORAC values among the phenolic compounds. The PLS plot of chokeberry is shown in **Fig. 2a**, where 73% of the chemical variables explained 97% of the variation among ORAC data in three factors. The major contributors were cyanidin 3-*O*-galactoside (Cy-Gal) and cyanidin 3-*O*-arabinoside (Cy-Ara). Strong correlation was also found between ORAC value and some flavonols connected with di-saccharides, such as quercetin-hexoside-pentoside (Q-HexDeox), and quercetin 3-*O*-rutinoside (Q-Rut). In the PLS model of crowberry fractions, 66% of the phenolic variables explained 100% of the variation in antioxidant results in three factors (**Fig. 2b**). The antioxidative capacity was primarily attributed to 3-*O*-galactoside (Gal) of cyanidin (Cy), delphinidin (De), petunidin (Pt), and peonidin (Po). Compared to anthocyanins, the fractions containing flavonol mono-glycosides (Fraction 4.1&4.2) showed lower antioxidant activity, as indicated by less

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correlation of these compounds with ORAC values of these fractions. **Fig. 2c** showed the total content of isorhamnetin (I) in berry fractions of sea buckthorn highly correlated to ORAC values (45% of the phenolic variables responsible for 96% of the ORAC results in two factors). Weak correlation was found in isorhamnetin bound with tri-saccharides, as well as in quercetin (Q) and kaempferol (K) tri-saccharides, suggesting glycosylation of flavonols affected the ability of scavenging free radicals. Some moderate contributions were found in gallocatechin (G-Cat), (+)-catechin ((+)-Cat), B-type procyanidin dimers (B-PC di), and isorhamnetin 3-*O*-glucoside (I-Glu). For sea buckthorn leaves, both flavonoids and non-flavonoid phenolics correlated strongly with peroxyl-radicals scavenging capacities of the fractions (**Fig. 2d**, where 92% of the phenolic variables explained 95% of the ORAC results in five factors). As the main contributors, flavonoids represented mostly as gallocatechin, (+)-catechin, and kaempferol-hexoside-deoxyhexoside (K-HexDeox); while non-flavonoid phenolics as galloyl-bis(hexahydroxydiphenoyl)-hexoside (G-bisHHDP-Hex), galloyl-hexahydroxydiphenoyl-hexoside (G-HHDP-Hex), and digalloyl-hexoside (diG-Hex). In the model of leaf fractions of saskatoon (**Fig. 2e**), 85% of the chemical variables correspond to 99% of the variation among the antioxidant activity with four factors. The highest correlation was with (-)-epicatechin ((-)-Epic), quercetin 3-*O*-galactoside (Q-Gal) and quercetin 3-*O*-glucoside (Q-Glu). Some quercetin di-glycosides contributed strongly to ORAC values, such as 3-*O*-rutinoside (Rut), arabinoglucoside (AraGlu), and hexoside-deoxyhexoside

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(HexDeox). **Fig. 2f** showed quercetin 3-*O*-rhamnoside-rhamnoside-glucoside (Q-RhaRhaGlu), quercetin-hexoside-pentoside-deoxyhexoside (Q-HexPentDeox), and quercetin 3-*O*-rutinoside (Q-Rut) were the primary phenolic compounds responsible for antioxidative property of white currant leaves, where 58% of the chemical variables explained 83% of the variation of antioxidant data with three factors. *β*-*p*-Arbutin and its isomers contributed to the most of antioxidant effect of lingonberry leaves, and the moderate contribution was mainly from 2-*O*-caffeoyl-*β*-*p*-arbutin (CaA-Arb), (+)-catechin, quercetin 3-*O*-rutinoside, quercetin 3-*O*-galactoside, quercetin 3-*O*-rhamnoside (Q-Rha), and quercetin 3-*O*-4''-(3-hydroxy-3-methylglutaroyl)-rhamnoside (Q-hmgRha) (**Fig. 2g**, 84% of the chemical variables for 100% of the antioxidative variation in three factors). Additionally, the antioxidant activities of hawthorn leaf fractions was associated mostly with (-)-epicatechin and quercetin 3-*O*-galactoside (**Fig. 2h**, 57% of the chemical variables for 85% of the antioxidative variation in three factors).

3.2.2.2 Bivariate Pearson's correlation

Bivariate correlation of antioxidant activities with main groups of phenolic compounds was expressed by Pearson's correlation coefficients (**Supplemental Fig. 2**). A positive correlation between ORAC assay and total content of phenolics in fractions (R = 0.921, $p = 0.01$, n = 224), suggesting antioxidant effect of fractions mostly contributed by phenolic compounds,

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which is in agreement with findings of multivariate correlation analysis (**Supplemental Fig. 2a**). Non-flavonoid phenolic compounds, consisting of phenolic acid derivatives, ellagitannins, and other phenolics, correlated strongly with ORAC values (R = 0.839, p = 0.01, n = 168), so did flavonoids (R = 0.856, p = 0.01, n = 180) (**Supplemental Fig. 2b&c**). Among flavonoids, both flavan-3-ols (R = 0.878, p = 0.01, n = 64) and flavonol glycosides (R = 0.805, p = 0.01, n = 168) strongly contributed to antioxidative activities, followed by the glycosides of flavone (R = 0.425, p = 0.01, n = 36) and flavanone (R = 0.650, p = 0.01, n = 24). The highest correlation coefficient (R = 0.965, p = 0.01, n = 36) was found in anthocyanins, indicating these compounds were the strongest inhibitors against peroxyl radicals in berry extracts (**Supplemental Fig. 2d-h**). Quercetin glycosides, as the main group of flavonols, are distributed widely in nature. In the fractions studied, quercetin was identified as conjugates of mono-, di-, and tri-saccharides. Compared to the quercetin aglycone, quercetin derivatives show lower antioxidative activities, especially when the sugar moiety was connected at 3-hydroxyl group (-OH) of C-ring (Fernandez-Panchon, Villano, Troncoso, & Garcia-Parrilla, 2008; Heijnen, Haenen, van Acker, van der Vijgh, & Bast, 2001; López, Martínez, Del Valle, Ferrit, & Luque, 2003). Aside from the site of glycosylation, the number of sugar moieties also resulted in the variation in antioxidative activities of quercetin glycosides. According to our results, mono-glycosides of quercetin generally showed higher correlation coefficient value (R = 0.898, p = 0.01, n = 88) than its di-glycosides (R = 0.548, p

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= 0.01, n = 104) and tri-glycosides (R = 0.620, p = 0.01, n = 60) (**Supplemental Fig. 2i-l**).

ORAC results of some fractions were re-calculated based on 1 mg of fraction powder (dry weight) in order to investigate preliminarily the pattern of phenolic composition contributing to antioxidant activity (**Fig. 3**). Since each fraction was a mixer of various phenolic compounds, bivariate Pearson's correlation coefficients between ORAC values and concentration of individual phenolic compounds to determine their significance to ORAC values (**Fig. 4**).

As common monomers of flavan-3-ols, (+)-catechin might be the strongest antioxidant among phenolic compounds identified, as suggested by the highest ORAC values in fraction 4.2 and 5 of the sea buckthorn berry extract (**Fig. 3a**). According to bivariate correlation coefficients, (+)-catechin (R = 0.825, p = 0.01, n = 48) had higher correlation with ORAC than (-)-epicatechin (R = 0.704, p = 0.01, n = 36) (**Fig. 4a**&**b**). Villaño and co-workers reported that the antioxidative capacity of catechin was stronger than epicatechin based on the ORAC data (Villaño, Fernández-Pachón, Troncoso, & García-Parrilla, 2005). Such results were attributed to structural isomerism among hydroxyl groups in B-ring and C-ring. For flavonoids, substitution at hydroxyl groups is a crucial factor diminishing the hydrogen-donating ability of the compounds. Compared to quercetin 3-*O*-rhamnoside-rhamnoside-

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glucoside, the coefficient value of quercetin 3-*O*-sophoroside-7-*O*-rhamnoside decreased significantly after the substitution at 7-OH by sugar moiety (**Fig. 4c&d**). The effect of glycosylation on antioxidative activity was also found in isorhamnetin derivatives. In the fractions of sea buckthorn berries (**Fig. 3a**), the primary phenolics identified in fraction 2.1, 3.1, and 4.1 were isorhamnetin 3-*O*-sophoroside-7-*O*-rhamnoside (55% of fraction weight), isorhamnetin 3-*O*-glucoside-7-*O*-rhamnoside (58%) and isorhamnetin 3-*O*-glucoside (68%), respectively. Fraction 2.1 had lower activity (5 TE μmol/mg) than fraction 3.1 (12), indicating the substituent of di-glucosides at 3-OH of flavonols might cause significant decrease in antioxidant compared to mono-glucoside. In contrast, the glycosylation at 7-OH seemed to have less impact, and the ORAC value of fraction 4.1 was closed to that of fraction 3.1, although the content of isorhamnetin 3-*O*-glucoside-7-*O*-rhamnoside and isorhamnetin 3-*O*-glucoside differed clearly between these two fractions. The structure-activity relationship (SAR) of phenolic compounds has been discussed in our previous study (Tian, Puganen, Alakomi, Uusitupa, Saarela, & Yang, 2018); however, in the present research, we noticed the contribution of different flavonoids did not simply follow their inherent antioxidant abilities. Certain quercetin glycosides correlated to ORAC assay more strongly than (+)-catechin or (-)-epicatechin, although flavan-3-ols are more potent hydrogen donators. Among the derivatives of quercetin, the highest correlation was found in quercetin 3-*O*-rhamnoside (R = 0.963, p = 0.01, n = 20), which was followed by quercetin 3-*O*-arabinoside, quercetin 3-*O*-

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rutinoside, and quercetin 3-*O*-galactoside. Quercetin 3-*O*-glucoside (R = 0.664, $p = 0.01$, n = 76) and quercetin 3-*O*-arabinofuranoside (R = 0.514, $p = 0.01$, n = 36) showed lower correlation coefficient values with ORAC (**Fig. 4e-j**). The explanation might be the abundance of quercetins in the extracts of berry plants, but this result also indicated that sugar moieties may play different roles in determining the antioxidative effects. Anthocyanins have been known as main inhibitors against free radicals. In crowberry, anthocyanins were enriched in fraction 2.2, 3.1 and 3.1; the antioxidant activities of which were proportionated negatively to the percentage of malvidin glycosides, but positively to the derivatives of delpinidin and cyanidin. This may indicate that the inhibitory effect against free radicals might be more associated with delpinidins and cyanidins than with malvidins (**Fig. 3b**). Noda and coworkers reported that delphinidins in pomegranate (*Punica granatum* L.) extracts exhibited stronger activities of scavenging hydroxyl (•OH) and superoxide ($O_2^{\bullet -}$) radicals than cyanidins and pelargonidins due to the hydroxyl groups at 3’, 4’, and 5’ positions of the B-ring (Noda, Kaneyuki, Mori, & Packer, 2002). For quenching peroxyl-radicals as measured in this study, the correlation coefficients of 3-*O*-galactoside of anthocyanidins with ORAC values could be generally ranked in the following order: peonidin > petunidin > delphinidin > cyanidin; but there was no significant deviation between 3-*O*-galactoside and 3-*O*-glucoside of cyanidin (**Fig. 4k-o**). It should be considered that the antioxidant capacity can be interfered by the structure rearrangement of anthocyanins. As highly pH-sensitive compounds, over

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80% of anthocyanins have been proved to rearrange structure into carbinol pseudo-base at neutral media (Clifford, 2000).

Additionally, some phenolic compounds were not investigated successfully by bivariate Pearson's correlation, but the contribution of which might be suggested in certain fractions. In the chokeberry (**Fig. 3c**), fraction 2.1 only contained the derivatives of phenolic acids, whereas fraction 2.2 consisted of phenolic acids (68% of fraction weight) and flavanones (mainly eriodictyol-methyl-hexoside, 32%). A slight increase of antioxidant activity was present from 12 in fraction 2.1 to 15 TE μmol/mg in fraction 2.2, suggesting eriodictyol-methyl-hexoside may have stronger ability of scavenging peroxyl radicals. Fraction 3.1 contained less flavanone (34%, mainly as eriodictyol-methyl-hexoside) and more anthocyanins (39%, cyanidin 3-*O*-galactoside) in the phenolic compounds. The higher ORAC value (36 TE μmol/mg) in fraction 3.1 indicated anthocyanins to be more potent donators of hydrogen than flavanones. In the leaf fractions of sea buckthorn, the percentage of isorhamnetin glycosides was associated with the increase in antioxidative capacities from 2.1 to 3.1. Ellagitannins were enriched in fraction 3.2, 4.1 and 4.2. The antioxidative values of these fractions were positively correlated with the percentage of galloyl-bis(hexahydroxydiphenoyl)-hexoside, but negatively with that of galloyl-hexahydroxydiphenoyl-hexoside, suggesting that an additional hexahydroxydiphenoyl

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(HHDP) group may enhance the hydrogen-donating ability of the molecule (**Fig. 3d**). Fernandez-Panchon et. al summarized the antioxidant activity of pure phenolic compounds measured by ORAC assays, such as (+)-catechin, (−)-epicatechin, and certain flavonols (Fernandez-Panchon, Villano, Troncoso, & Garcia-Parrilla, 2008); however, the data of most of phenolic compounds is missing in the previous studies. It is difficult to define the contribution of a certain compound to antioxidative activities of fractions by using its own ORAC value multiplying the concentration presented in the fraction. Again, it is important to notice that most of the fractions contained mixtures of different phenolic compounds. Interaction and synergy among different compounds may have played significant role in the bioactivities.

3.3 Antibacterial activities of fractions and contribution of phenolic compounds

Two foodborne pathogens, *Staphylococcus aureus* and *Escherichia coli*, were applied as targets to evaluate the anti-bacterial activities of the fractions. **Table 2** shows the inhibitory effects of the fractions against the selected strains. Most of the fractions could inhibit the growth of both Gram-negative and Gram-positive bacteria at low dose (10 μL in 300 μL of media), and the effect was enhanced with increasing doses. A clear growth inhibition on *S. aureus* was observed by the fraction 6 of the chokeberry extract (87%) and the sea buckthorn berry extract (88%), when only 10 μL of the fractions was added to the growth media. This

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might have been associated with the anti-bacterial activities of some unidentified tannins being the major phenolic compounds in these fractions. Although most of the fractions had a stronger ability against *S. aureus* at 20 μL of addition, the strain showed high resistance to phenolic compounds in the berry fraction 4.1 and the leaf fraction 7 of sea buckthorn; the growth percentage of the strain was over 80% with the presence of 20 μL of these fractions compared with the control. As Gram-negative bacteria, *E. coli* was less sensitive to phenolic fractions at the low dose (10 μL), and the inhibition percentage was generally lower than that found in *S. aureus*. This was in the agreement with previous report that phenolic extracts are more efficient inhibitors against Gram-positive bacteria (Klančnik, Guzej, Hadolin-Kolar, Abramovič, & Smole Možina, 2009; Milenković-Andjelković, Andjelković, Radovanović, Radovanović, & Randjelović, 2016). The difference in sensitivity to phenolic compounds is due to the outer membrane in Gram-negative organisms restricting the diffusion of hydrophobic compounds (Nohynek et al., 2006). For both saskatoon leaf and white currant leaf, no effect was found after addition of the fraction 6 at either dosage level, and the fraction 7 inhibited the growth of both strains by only 10-20%.

With several mechanisms involved, the structure and content of phenolic compounds play a major role in capacity against bacteria. In a study on antimicrobial effects of pure compounds of phenolics, Jussi-Pekka Rauha and co-workers confirmed that flavanone was more active

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against *S. aureus* than other flavonoids. Glycosides of flavonols may have lower anti-bacterial activity compared to the corresponding aglycones (Rauha et al., 2000). Since most of the fractions contained mixture of different compounds, we are not able to interpret the contribution of different types of phenolic compounds to anti-bacterial activity based on the data in the current study. The concentration of phenolic compounds might also have lowered the pH of the fractions and contributed to anti-bacterial effects.

4. Conclusions

Phenolic extracts from leaves and berries of Finnish berry plants was fractioned using Sephadex LH-20 gel column. The phenolic compounds present in the raw extracts were selectively eluted in different fractions, resulting in fractions with simplified phenolic profile and enriched with specific compounds. The compositional analysis combined with assessment of the antioxidative activities and antibacterial efficacies produced more targeted information about the significance of different phenolic compounds and structural features of phenolic compounds that are important for antioxidative and antimicrobial activities. These findings could assist the development of functional food ingredients and natural preservatives of foods and health care products based on extracts from the berry species studied.

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Conflict of interest

The authors in this manuscript have no conflict of interest.

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Appendix A. Abbreviations used

All abbreviations of phenolic compounds used in this study are listed as below:

gallocatechin (**G-Cat**), epigallocatechin (**EpiG-Cat**), (+)-catechin (**(+)-Cat**), (-)-epicatechin (**(-)-Epic**), A/B-type procyanidin dimers/trimers (**A/B-PC di/tri**), bis(hexahydroxydiphenoyl)-hexoside (**bisHHDP-Hex**), ellagitannin (**Et**), galloyl-bis(hexahydroxydiphenoyl)-hexoside (**G-bisHHDP-Hex**), 4-(2-hydroxyethyl)phenol-hexoside (**HP-Hex**), vanillic acid-hexoside (**VA-Hex**), coumaric acid-hexoside (**CoA-Hex**),

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caffeic acid-hexoside (**CaA-Hex**), coumaroylquinic acid (**CoQA**), ferulic acid-hexoside (**FA-Hex**), cafferol-hexose-hydrophenol (**Ca-Hex-H**), caffeic acid (**CaA**), *p*-coumaric acid (***p*-CoA**), coumaroyl iridoid (**CoI**), 5/3/4-*O*-caffeoylquinic acid (**5/3/4-CQA**), dicaffeoylquinic acid (**diCQA**), caffeoylmalic acid (**CaMA**), caffeoylglyceric acid (**CaGA**), ellagic acid (**EA**), 1-*O*-benzoyl-*β*-glucose (**BA-Glu**), quercetin (**Q**), myricetin (**M**), isorhamnetin (**I**), kaempferol (**K**), laricitrin (**La**), syringetin (**S**), apigenin (**A**), eriodictyol (**E**), cyanidin (**Cy**), delphinidin (**De**), petunidin (**Pt**), peonidin (**Po**), malvidin (**Ma**), rutinoside (**Rut**), galactoside (**Gal**), glucoside (**Glu**), hexoside (**Hex**), rhamnoside (**Rha**), deoxyhexoside (**Deox**), xyloside (**Xyl**), arabinoside (**Ara**), arabinofuranoside (**Araf**), pentoside (**Pent**), glucuronide (**Gluc**), coumaroyl-glucoside (**coGlu**), hydroxy-methylglutaroyl-galactoside (**hmgGal**), hydroxy-methylglutaroyl-galactoside (**hmgRha**), benzoyl-galactoside/glucoside (**beGal/Glu**), malonyl-galactoside/glucoside (**maGal/Glu**), feruloyl-glucoside (**feGlu**), acetyl-glucoside (**acGlu**), methyl-hexoside (**meHex**), dihexoside (**diHex**), sophoroside (**Sop**), *β*-*p*-arbutin (**Arb**), and lanceoloside A (**Lan A**).

Appendix B. Supporting information description

The supporting information is provided: (1) ^{1}H NMR spectra of different fractions studied (**Supplemental Fig. 1**). (2) Pearson's correlation between main groups of phenolic compounds and ORAC values of all fractions (**Supplemental Fig. 2**). (3) Information of

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plant materials studied (**Supplemental Table 1**). (4) Sephadex LH-20 fractionation and weight of each fraction (**Supplemental Table 2**). (5) Concentration of phenolic compounds indifferent fractions (**Supplemental Table 3**).

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Figure captions

Fig. 1: Concentration (mg/100 mL of fraction) of phenolic composition in different fractions of berry plants: a. chokeberry; b. crowberry; c. sea buckthorn; d. sea buckthorn leaf; e. saskatoon leaf; f. white currant leaf; g. lingonberry leaf; h. hawthorn leaf. (Phenolic compounds in fraction 5-7 were not able to be identified)

Fig. 2: PLS plots of the correlation between ORAC values and phenolic composition of different fractions of berry and leaf extracts: a. chokeberry; b. crowberry; c. sea buckthorn; d. sea buckthorn leaf; e. saskatoon leaf; f. white currant leaf; g. lingonberry leaf; h. hawthorn leaf. The ORAC values are in red bold font. The fractions are in green italic bold font. The main groups of phenolic compounds are in blue bold font and individual phenolics are in blue font with a smaller letter size (**The color should be used in print**). Abbreviations of phenolic compounds refer to Appendix A.

Fig. 3: Antioxidative activity (TE μmol/mg of fraction) of different fractions of berry plants measured by ORAC assay: a. sea buckthorn; b. crowberry; c. chokeberry; d. sea buckthorn leaf; e. saskatoon leaf; f. white currant leaf; g. lingonberry leaf; h. hawthorn leaf.

Fig. 4: Pearson's correlation between phenolic compounds and ORAC values of all fractions.

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Table 1 Antioxidant activity (TE µmol/mL) of different fractions of extracts from berries and leaves measured by ORAC assay

Fraction No.	**ORAC** (TE µmol/mL)							
	Choke berry	**Crow berry**	**Sea buckthorn berry**	**Hawthorn leaf**	**Lingon berry leaf**	**Saskatoon leaf**	**Sea buckthorn leaf**	**White currant leaf**
1.2	8.3±2.0	13.4±1.4	5.1±1.5	29.9±2.1	226.3±4.2	42.6±1.7	20.7±2.5	23.8±1.6
2.1	12.5±2.7	8.1±1.5	4.6±1.3	22.2±1.5	22.7±1.0	27.4±1.8	17.8±2.8	12.0±0.8
2.2	9.3±2.1	18.1±2.4	3.3±1.3	21.6±1.8	27.7±3.5	36.7±2.5	15.8±2.2	14.7±1.1
3.1	6.6±2.1	35.8±4.5	4.0±1.0	18.1±0.6	14.6±0.6	38.8±0.8	17.6±0.8	25.5±0.7
3.2	17.3±2.1	11.9±2.5	10.0±1.9	24.1±1.9	39.0±2.4	53.6±3.9	22.1±3.6	32.3±1.0
4.1	6.6±1.6	10.8±2.2	9.3±1.3	39.1±2.4	111.3±5.1	68.0±1.2	15.3±2.3	24.5±0.9
4.2	6.4±1.0	13.9±2.2	9.5±1.6	34.9±2.2	54.4±4.0	38.6±2.2	29.6±7.7	14.3±1.2
5	4.1±1.6	15.1±2.6	6.6±1.4	19.1±1.8	56.4±4.4	29.3±2.6	24.7±5.1	7.6±0.8
6	13.2±2.5	27.2±2.3	18.5±2.3	20.0±1.7	87.7±4.3	73.9±2.8	79.3±12.2	31.9±0.9
7	4.6±0.9	5.2±1.0	6.4±1.3	5.6±0.8	13.6±1.0	14.7±1.5	48.2±2.8	9.7±0.2

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Table 2 Growth inhibition (%) of *Staphylococcus aureus* and *Escherichia coli* induced by different fractions of extracts from berries and leaves of different species

Fraction No.	Amount(μL/300 μL)	Growth inhibition					
		Choke berry	Crowberry	Sea buckthorn berry	Hawthorn leaf	Lingonberry leaf	Saskatoon leaf
***Staphylococcus aureus* (E-70045)**							
1.2	10	61±5	48±5	72±1	56±2	46±6	54±2
	20	88±2	73±1	89±0	73±10	85±0	73±3
2.1	10	49±3	43±2	38±0	67±6	53±5	34±0
	20	64±2	77±1	70±0	84±1	75±5	67±4
2.2	10	46±1	38±3	30±6	51±2	43±5	28±3
	20	67±1	66±6	75±4	67±19	77±1	78±12
3.1	10	56±0	47±9	44±3	63±16	49±5	57±1
	20	67±2	70±6	74±1	71±2	69±3	74±2
3.2	10	52±5	62±0	50±1	56±3	38±0	49±3
	20	64±12	70±11	81±9	79±1	72±2	70±24
4.1	10	57±1	64±5	13±4	68±1	60±1	71±1
	20	72±1	75±0	15±4	80±0	88±6	89±3
4.2	10	56±2	60±4	59±1	46±3	65±6	52±5
	20	74±1	67±0	79±5	76±4	84±3	88±6
5	10	54±0	67±6	48±13	67±3	68±2	41±17
	20	66±0	80±0	88±0	77±1	73±1	84±5
6	10	87±2	36	88±0	61±6	37±9	56±7
	20	85±3	65±12	85±2	75±1	37	98±3
7	10	55±6	59±2	66±2	63±10	63±4	26±3
	20	77±1	68±3	76±0	72±0	70±0	67±5
***Escherichia coli* (E-94564)**							
1.2	10	53±1	41±19	66±0	46±0	46±3	46±3
	20	93±9	89±8	99±0	75±12	78±3	79±4
2.1	10	45±4	49±4	34±3	41±2	40±2	35±0
	20	83±5	88±0	55±2	50±23	67±1	76±2
2.2	10	49±3	40±2	41±2	42±2	40±2	31±2
	20	88±3	68±4	74±0	81±6	70±0	77±16
3.1	10	48±3	36±5	39±7	35±3	21±15	39±7
	20	91±8	55±7	70±1	67±4	92±6	66±9
3.2	10	48±1	32±10	42±4	33±2	37±1	34±0
	20	76±9	80±5	82±8	63±8	65±1	55±1
4.1	10	42±4	34±1	45±6	33±8	38±1	32±1
	20	84±1	67±8	52±29	55±4	70±3	39±4
4.2	10	40±0	37±6	47±2	37±2	42±2	31±5
	20	79±3	77±7	98±2	60±3	89±2	57±6
5	10	49±7	31±1	46±1	35±6	54±6	25±0
	20	88±14	66±3	74±9	72±4	66±0	61±0
6	10	47±2	12±4	32±11	31±0	40±27	0±0
	20	63±4	22±13	74±8	56±8	60±4	0±0
7	10	34±2	19±10	41±8	33±3	35±0	10±1
	20	71±11	49±2	68±7	73±22	77±10	21±12

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Highlights

- Food-grade extracts of berries and leaves were fractionated by column chromatography
- Phenolic compounds were identified and quantified thoroughly in each fraction
- Both antioxidative and antibacterial activities of fractions were investigated
- The contribution to bioactivities were compared among various phenolic compounds
- Results of the study will assist developing natural food preservatives

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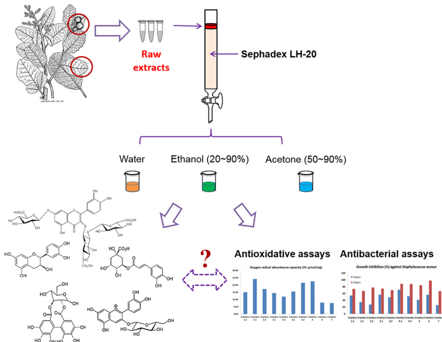


Graphics Abstract

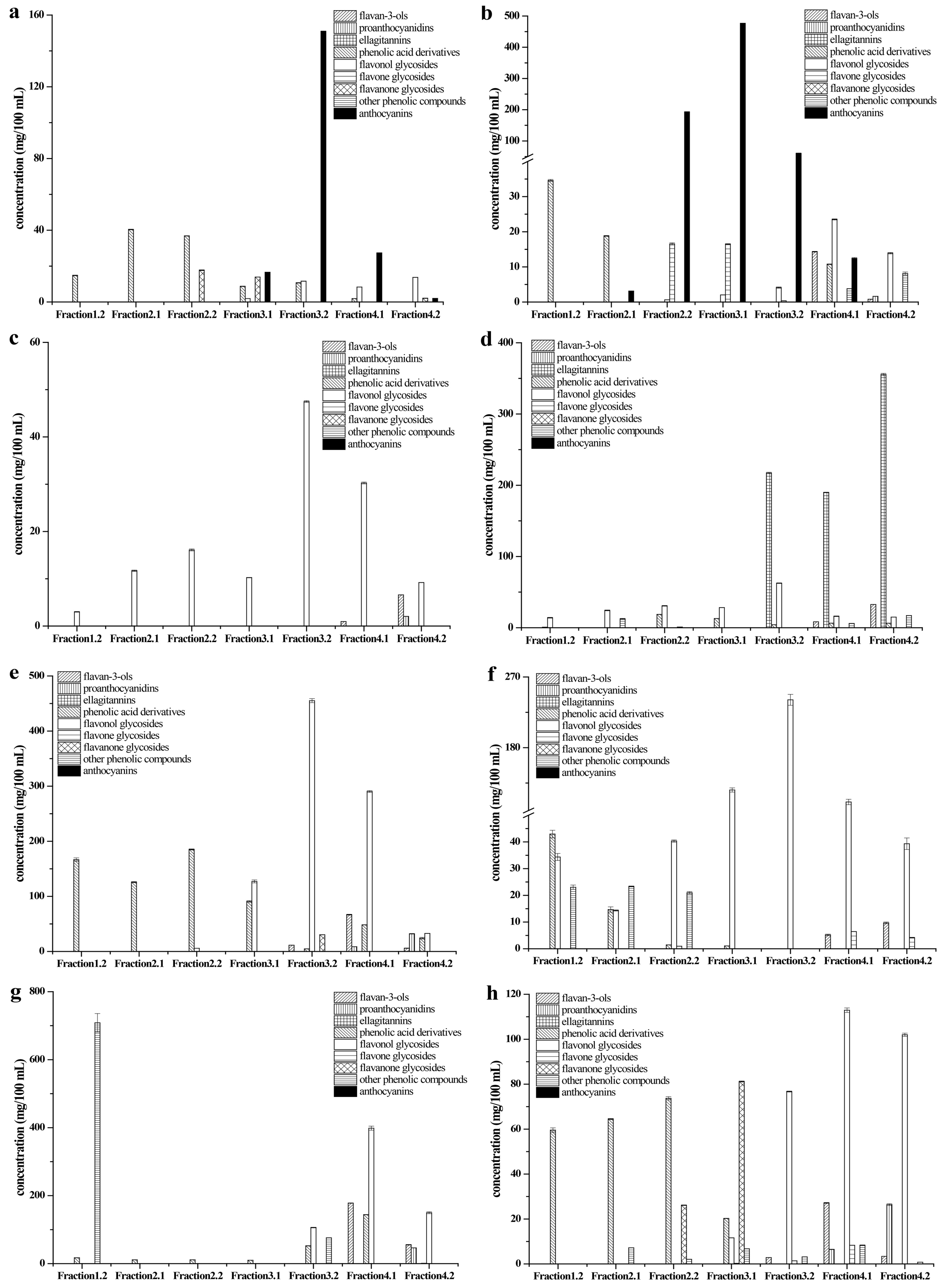


Figure 1

Correlation Loadings (X and Y)

a

Non-flavonoids
Phenolic acid derivatives
Fraction 2.1
CaQA derivatives
(4-CaQA, 5-CaQA, CaQA)
3-CaQA
Fraction 2.2
total phenolics
E
Flavanone glycosides
FA-Hex
E-meHex
VA-Hex
ORAC
Fraction 1.2
CoQAs
CoA-Hex
Fraction 3.2
other acid derivatives
Fraction 3.1
Q-maGlu
I di-saccharides
(I-Rut, I-HexDeox, I-HexPent)
diCaQA
I
Fraction 4.2
Cy (Cy-Gal, Cy-Glu, Cy-Ara)
Flavonoids
K
Q-Ara
E aglycone
Q pentosides
K di-saccharides
(K-Rut, K-HexPent)
Cy-Ara
Q-Gal
Q hexosides
Anthocyanins
Q-Glu
I mono-saccharides
Q di-saccharides
Q mono-saccharides
K mono-saccharides
(Q-Rut, Q-diHex,
Q-HexDeox, Q-HexPent)
Q
Fraction 4.1
Flavonol glycosides
Cy-Xyl

Factor-2 (23%, 11%)
Factor-1 (40%, 83%)

Correlation Loadings (X and Y)

b

Fraction 4.2
M Q
other phenolics
Flavonol glycosides
Q mono-saccharides
M mono-saccharides
(Q-Gal, Q-Glu, Q-beGal,
I-Gal
(M-Gal, M-Gul, M-Ara)
Q-Ara, Q-Araf)
S-Pent
Fraction 3.1
La (La-Gal, La-Araf)
I-Glu
Q-HexDeox
Po (Po-Gal, Po-Ara)
Pt (Pt-Gal, Pt-Ara)
Fraction 4.1
(+)-Cat
I
De (De-Gal)
Cy (Cy-Gal, Cy-Ara)
ORAC
EpiG-Cat
I mono-saccharides
S
I di-saccharides (I-Rut)
Flavonoids
S-Rut
S-Gal
Q di-saccharides
Anthocyanins
total phenolics
Ma-Ara
Q-HexPent
Q-Gluc
La-HexPent
Flavone glycosides
De-Ara
Fraction 2.2
S-Glu
Ma-Gal
Ma
Fraction 1.2
Ma-Glu
Non-flavonoids
Fraction 3.2
CoA
Q-Rut
La unknown
other acid derivatives
Cy-Glu
Phenolic acid derivatives
3-CaQA
CoA-Hex
CoQAs
5-CaQA
Fraction 2.1
CaQA derivatives

Factor-2 (26%, 10%)
Factor-1 (32%, 87%)

Figure 2A

Correlation Loadings (X and Y)

c

Fraction 4.2
Proanthocyanidins
(B-PC di)
I-Pent
Flavan-3-ols
(G-Cat, (+)-Cat)
Q-Glu
Q tri-saccharides
(Q-SopRha,
Q-HexHexDeox)
Fraction 2.2
K tri-saccharides
(K-SopRha)
I-GluGlu
Q-mlDeoxDeoxDeox
K
I-siHexHexDeox
I tri-saccharides
(I-siHexPentDeox,
I-mlDeoxDeoxDeox)
I-SopRha
I-siGluGluRha
I-RhaGluGlu
Fraction 1.2
Fraction 2.1
ORAC
Q hexosides
Q
Q mono-saccharides
K-Rut
I-HexDeox
I di-saccharides
Fraction 3.2
K di-saccharides
total phenolics
Flavonoids
Flavonol glycosides
I
I-Gal
I-Glu
I hexosides
I mono-saccharides
Fraction 4.1
M
M-Rut
Q di-saccharides
Q-Gal
M di-saccharides
Q-Rut
I unknown
I-HexDeoxDeox
I-RhaRhaGlu
I-coDeoxHexDeox
Fraction 3.1
K-HexDeox
K-RhaRhaGlu
Q-RhaRhaGlu

Factor-2 (13%, 6%)
Factor-1 (32%, 90%)

Correlation Loadings (X and Y)

d

Fraction 3.2
Q tri-saccharides
Flavonol glycosides
I
Q
EA-HexDeox
Q-feGluGluRha
Q-HexDeox
I-GluRha
Q-GluRha
I-PentRha
I di-saccharides
I-HexDeox
Q-GluRhaGlu
Q di-saccharides
I-Rut
I tri-saccharides
Fraction 1.2
Fraction 2.1
CoA-Hex
EA-Pent
I-coHexHexHex
I-feGluGluRha
I-feHexHexDeox
Fraction 3.1
other acid derivatives
Q unknown
I unknown
unkown phenolic compounds
I-GluGlu
phenolic acid derivatives
Fraction 2.2
Flavonoids
ORAC
Fraction 4.2
total phenolics
G-HHDP-Hex
Non-flavonoids
Ellagitannins
K mono-saccharides (K-Glu)
K
K di-saccharides
(K-HexDeox)
G-bisHHDP-Hex
Flavan-3-ols
(G-Cat, (+)-Cat)
diG-Hex
Q mono-saccharides
(Q-Glu)
EA
I mono-saccharides
(I-Glu)
Q-Rut
Fraction 4.1

Factor-2 (15%, 17%)
Factor-1 (34%, 72%)

Figure 2B

Correlation Loadings (X and Y)

e

Fraction 4.1
Flavan-3-ols
((+)-Cat, (-)-Epic)
diCaQA
Q mono-saccharides
(Q-Gal, Q-Glu. Q-Xyl)
ORAC
K mono-saccharides
(K-Gal, K-Glu)
Fraction 1.2
Non-flavonoids
Phenolic acid derivatives
unkown phenolic acids
CaMA
CoQAs
other acid derivatives
CaQA derivatives
B-PC di
Q-Araf
Proanthocyanidins
Fraction 2.2
CaA-Hex
3-CaQA
FA-Hex
I-HexPent
Fraction 2.1
4-CaQA
Fraction 4.2
Q deoxyhexosides
Fraction 3.1
Q-Rut
total phenolics
Fraction 3.2
Flavonoids
Flavonol glycosides
Q
K
CaSA
Flavanone glycosides
(E, E-Glu)
I di-saccharides
(I-Rut)
Q-maGlu
Q di-saccharides
(Q-diHex, Q-AraGlu,
Q-HexDeox)
I
K-maGal
Q-maGal
K-maGlu
K di-saccharides
(K-Rut, K-HexPent, K-HexDeox)

Factor-2 (26%, 11%)
Factor-1 (37%, 80%)

Correlation Loadings (X and Y)

f

Fraction 1.2
other acid derivatives
K-HexHexDeoxDeox
VA-Hex
Phenolic acid derivatives
Q tetra-saccharides
(Q-HexHexDeoxDeox, Q-HexPentHexDeox)
HP-Hex
Non-flavonoids
K-SopRha
other phenolics
Q-SopRha
ORAC
Fraction 3.2
total phenolics
Fraction 4.1
K-Rut
Flavonol glycosides
Flavonoids
K di-saccharides
K mono-saccharides
M
Q
Fraction 2.1
CaQA derivatives
Flavan-3-ols
Fraction 3.1
Q di-saccharides
(Q-Rut, Q-HexPent)
K
G-Cat
M di-saccharides
(M-Rut)
Q-HexHexDeox
K-HexDeox
Q-HexDeox
M tri-saccharides
(M-HexDeoxDeox,
M-HexPentDeox)
unkown phenolic compounds
Fraction 2.2
K-RhaGluRha
K tri-saccharides
Fraction 4.2
Q-diHex
CaA-Hex
Q tri-saccharides
(Q-RhaRhaGlu,
Q-HexPentDeox)

Factor-2 (16%, 15%)
Factor-1 (30%, 82%)

Figure 2C

Correlation Loadings (X and Y)

g

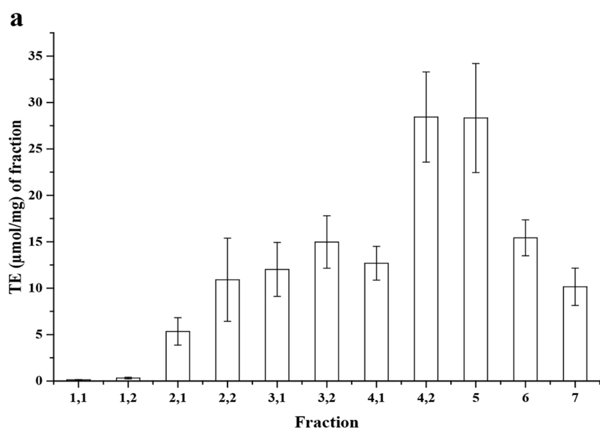
CoA-Hex
CaQA
CoQAs
4-CaQA
Non-flavonoids
other phenolics
(Arb)
ORAC
total phenolics
CaQA derivatives
Fraction 2.2
Fraction 3.1
3-CaQA
CaSA
Col
B-PC di
K-Pent
Lan A
K-Rut
Proanthocyanidins
K pentosides
Fraction 2.1
Phenolic acid derivatives
Fraction 1.2
Fraction 4.2
other acid derivatives
K di-saccharides
Fraction 3.2
Q-Araf
Q pentosides
Q-Xyl
(-)-Epic
Fraction 4.1
Q mono-saccharides
(Q-Gal, Q-Glu, Q-acGal, Q-acGlu,
Q-Rha, Q-hmgRha, Q-Ara)
Flavonol glycosides
Flavonoids
K mono-saccharides (K-Rha, K-hmgRha)
Q di-saccharides (Q-Rut)
Flavan-3-ols ((+)-Cat)
K
Q
CaA-Arb
Factor-1 (20%, 97%)
Factor-2 (45%, 2%)

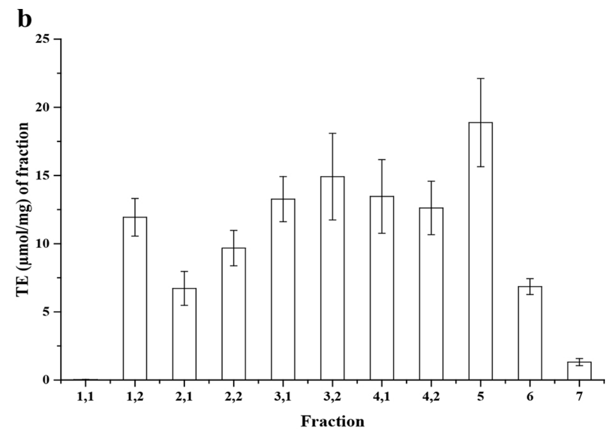
Correlation Loadings (X and Y)

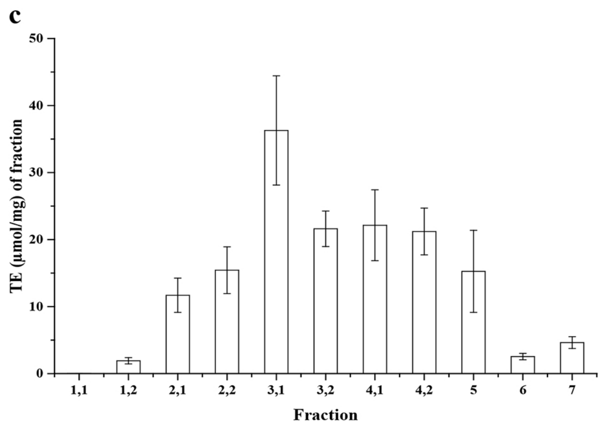
h

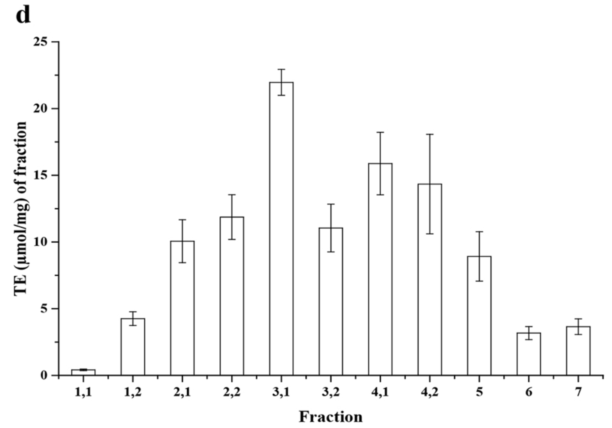
Fraction 1.2
unkown phenolic acids
other acid derivatives
(CaGA, CoA-Hex)
CaQA
Phenolic acid derivatives
Non-flavonoids
Fraction 2.1
4-CaQA
CoQAs
FQA
5-CaQA
CaQA derivatives
3-CaQA
FA-Hex
Fraction 2.2
CaA-Hex
Fraction 4.2
B-PC tri
K-meHexDeox
K di-saccharides
Proanthocyanidins
(B-PC di)
ORAC
I mono-saccharides
(I-Gal, I-acHex, I-Pent)
Q mono-saccharides
(Q-Gal, Q-Glu, Q-acGal, Q-acGlu,
Q-Rha, Q-Xyl, Q-Ara, Q-Araf)
K-Pent
K-Glu
Q
Flavonol glycosides
K-Rha
Flavan-3-ols ((-)-Epic)
I
K-Hex
K-Gal
total phenolics
I-HexPent
Flavonoids
Flavone glycosides
(A, A-Glu)
K
Fraction 4.1
K mono-saccharides
Fraction 3.2
I-HexDeox
I-Rut
Q-HexDeox
Q-HexPent
Q-Rut
I di-saccharides
K hexosides
Fraction 3.1
K-meHex
A-meHex
Flavanone glycosides
(E, E-meHex)
unknown flavonol glycosides
Factor-1 (45%, 82%)
Factor-2 (14%, 13%)

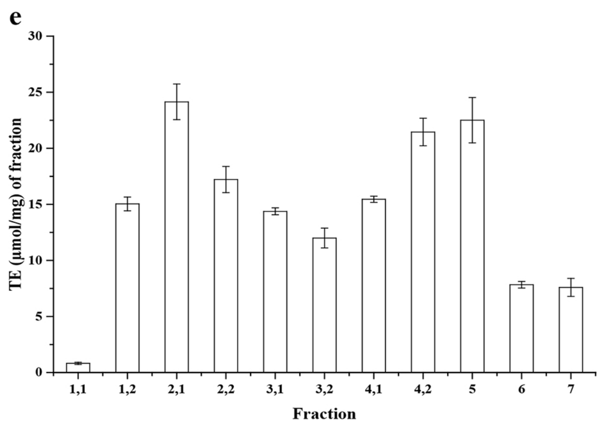
Figure 2D

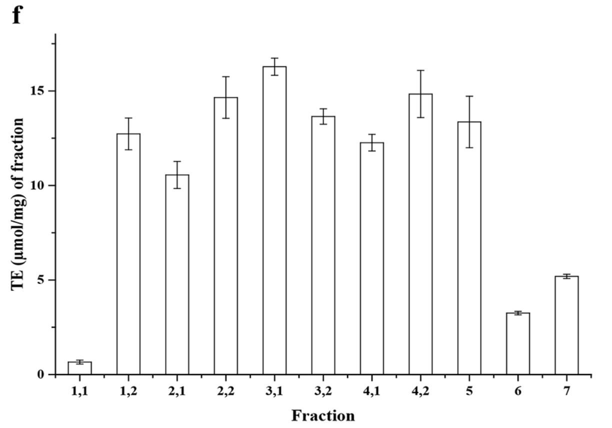


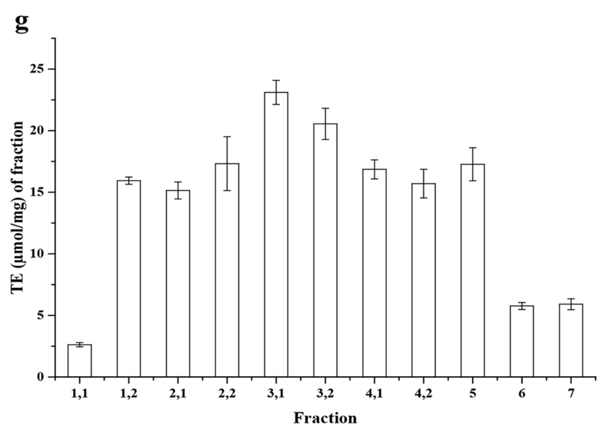












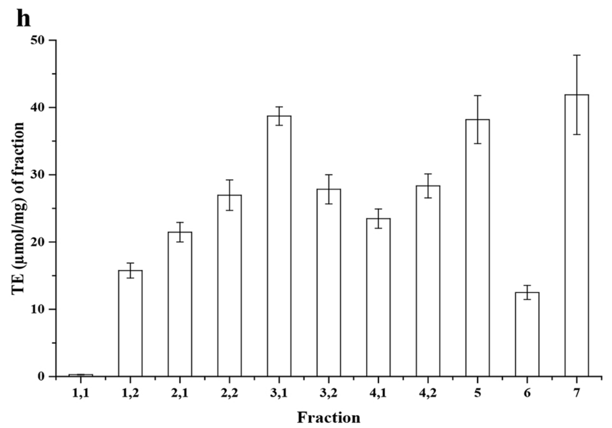


Figure 3

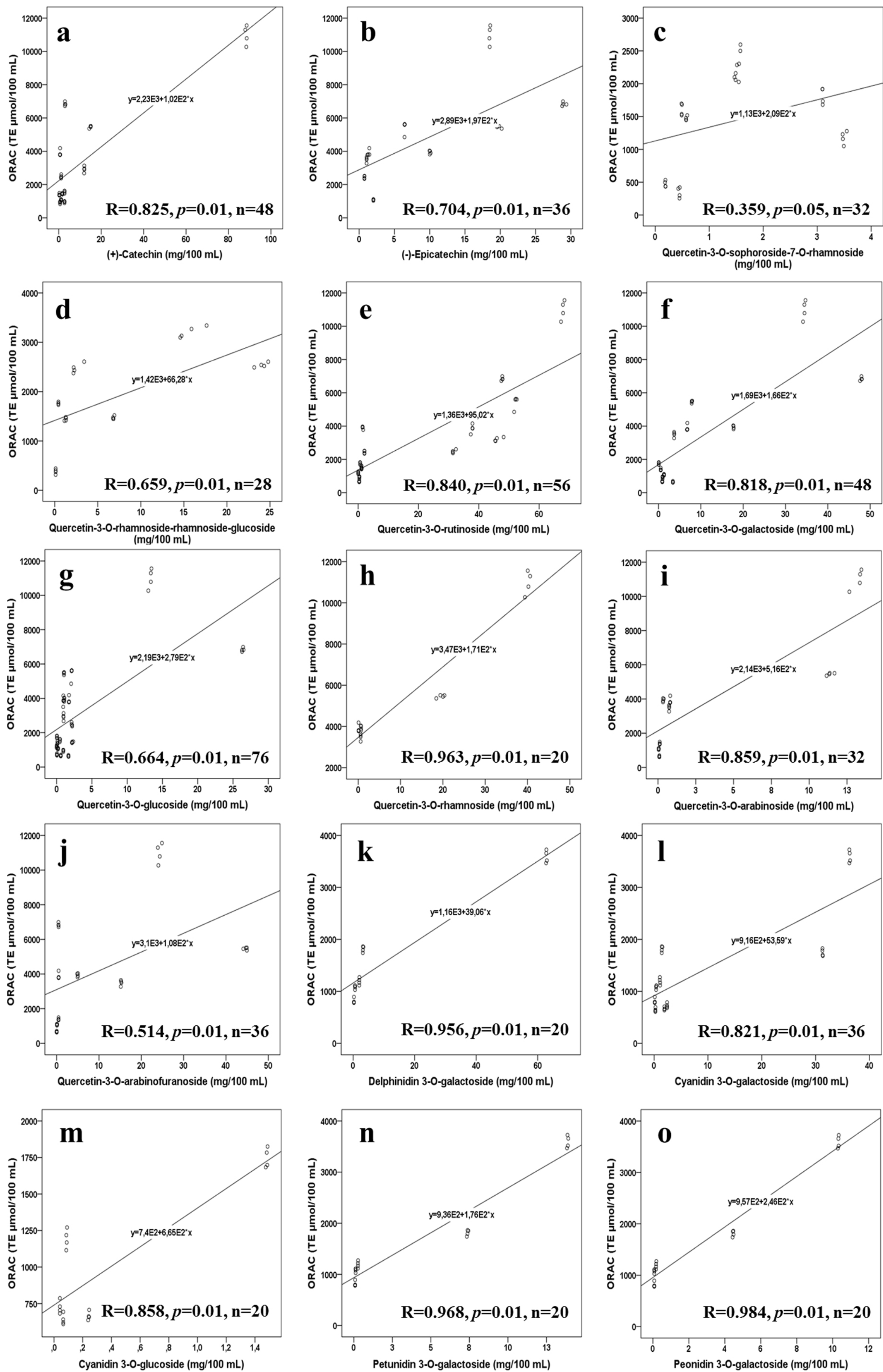


Figure 4