Food Chemistry: X 15 (2022) 100397

Contents lists available at ScienceDirect

Food Chemistry: X

journal homepage: www.sciencedirect.com/journal/food-chemistry-x

Phenolic compositions and antioxidant activities of *Hippophae tibetana* and *H. rhamnoides* ssp. *sinensis* berries produced in Qinghai-Tibet Plateau

Juan Wei [a], Sha Li [a], Tingting Su [a], Jinmei Zhao [a], Yumei Jiang [a], Yury A. Zubarev [b], Yang Bi [a,*]

[a] College of Food Science and Engineering, Gansu Agricultural University, Lanzhou 730070, China
[b] Department of Federal Altai Scientific Center of Agro-Biotechnologies, 35 Nauchniy Gorodok, Barnaul, 656910, Russian Federation

ARTICLE INFO

Keywords:
Hippophae tibetana
H. rhamnoides ssp. *sinensis*
Antioxidant activity
Phenolic compounds
Sea buckthorn
Flavonoids

ABSTRACT

Phenolic ingredients of *Hippophae tibetana* (Tib) and *H. rhamnoides* ssp. *sinensis* (Rha) berry from Qinghai-Tibet Plateau were identified by Ultra Performance Liquid Chromatography-triple Quadrupole Tandem Mass Spectrometry. Results demonstrated that both of them possessed high levels of total phenolic and flavonoid, and compared to Tib, Rha berry exhibited higher contents. Moreover, flavonols was the most predominant subclass in Rha berry, flavonols and flavanols were the two most abundant subclasses in Tib berry. Among them, rutin and narcissin were present in the most abundant amounts in Rha berry, while (−)-epigallocatechin was the richest substance in Tib berry. Furthermore, both phenolic extracts of sea buckthorn berry exhibited strong *in vitro* and cellular antioxidant properties. Rha berry extract exhibited much stronger effects because of its higher levels of phenolic and flavonoid profiles. This finding proved that the Rha berry could serve as a food source for better health with great potential antioxidant activity.

Introduction

Sea buckthorn (*Hippophae*) (SBT) refers a cold resistant deciduous shrub of the Elaeagnaceae family, which is native to Northern Europe, Northern Himalayas, western and northern China (Li et al., 2020). There are seven species and eleven subspecies of SBT have been recognized worldwide until now (Liu et al., 2017). Qinghai-Tibet Plateau is the main original and distribution area of *H. tibetana* (Tib) and *H. rhamnoides* ssp. *sinensis* (Rha) (Lian & Chen, 1996; Stobdan, Korekar, & Srivastava, 2013). Typically being used as an ethnic medicine and edible plant, SBT berries rich in bioactive substances, including vitamins, amino acids, organic acids, phenolics, carotenoids, etc. (Tiitinen, Hakala, & Kallio, 2005), which exert various kinds of healthy effects, like anti-oxidant, anti-diabetic, and anti-inflammatory activities (Ji, Gong, Li, Wang, & Li, 2020; Jiang et al., 2017; Olas, 2018).

In particularly, SBT berries are regarded as good resources of phenolic compounds, which are mainly composed of flavonoids and phenolic acids (Sytarova et al., 2020; Wang, Xu, & Liao, 2021). Flavonoid was the most widely distributed class of phenols in SBT berries, including flavanols, flavonols, and their glycosides. Specifically, isorhamnetin-3-rutinoside, isorhamnetin-3-glucoside-7-rhamnoside, and epigallocatechin present high content (Guo, Guo, Li, Fu, & Liu, 2017; Sytarova et al., 2020; Tian et al., 2017). Meanwhile, in SBT berries, twenty-one phenolic acid derivatives were detected, of which chlorogenic acid, ellagic acid, and salicylic acid were perceived as the abundant phenolic acid constituents (Sytarova et al., 2020; Zadernowski, Naczk, Czaplicki, Rubinskiene, & Szałkiewicz, 2005). The rich content of phenolic compounds made great contributions to the antioxidant activities of SBT berries (Kim, Kwon, Sa, & Kim, 2011). Aqueous ethanol extracts of SBT berries showed a high value of oxygen radical absorbance capacity (ORAC), which was 101–130 trolox equivalents (TE) mg/100 mL (Tian et al., 2018). Additionally, Sytarova et al. (2020) indicated that SBT berries displayed increased antioxidant activities both in water-soluble compounds and lipid-soluble compounds. More attractively, SBT berries exhibited great cellular antioxidant impacts on the HepG2 cells (Guo et al., 2017). Furthermore, the present researches suggested that SBT berry had no toxicity as a dietary supplement or just for potential dietary consumption (Wang et al., 2021).

Some phenolic compounds of SBT berries were investigated in previous studies. In addition, HepG2 cells were implemented to assess the cellular antioxidant activities of SBT berries. However, seldom research is reported on the comprehensive phenolic profiles and cellular antioxidant activities of SBT berry extracts in Caco-2 cells, which is a more appropriate cell line for the dietary phenolic study. Besides, no study has

* Corresponding author.
E-mail address: biyang@gsau.edu.cn (Y. Bi).

https://doi.org/10.1016/j.fochx.2022.100397
Received 25 March 2022; Received in revised form 12 July 2022; Accepted 15 July 2022
Available online 20 July 2022

been reported on the phenolic composition comparison and the antioxidant activities of Tib and Rha berries originated from Qinghai-Tibet Plateau. Therefore, this study aims to comprehensively quantify and qualify 127 pure phenolic compounds in these sea buckthorn berries using a targeted metabolomics approach– Ultra Performance Liquid Chromatography-triple Quadrupole Tandem Mass Spectrometry. Moreover, the antioxidant activities of phenolic extracts from SBT berries were examined with the extracellular assays and cellular method with Caco-2 cells. Subsequently, the correlation relationship between the polyphenols and antioxidant activities were discussed.

Materials and methods

Collection of plant material

Berries with branches of *H. tibetana* (Tib) and *H. rhamnoides* ssp. *sinensis* (Rha) were randomly collected from 12 bushes in September and October 2020 at their peak of ripeness, located in the city of Hezuo (Gansu Tibetan Autonomous Prefecture) (102.928448E, 34.906317 N). After being washed using running tap water, berries with branches were kept at −20 °C, then berries were collected and mixed, stored at −20 °C.

Extraction of phenolic compounds

The process of extraction was performed based on the approaches reported by Tian et al. (2017). 10 g berry was extracted in 200 mL aqueous ethanol (the ratio of ethanol and water is 2:1 (v/v)) by ultra sonication in cold water at 40 KHz, 360 W for 30 min. Afterwards the extracted mixture was centrifuged for 10 min at a speed of 13000 rpm, and the supernatant was harvested. In order to extract all phenolic compounds as much as possible, the above procedures were repeatedly twice. All supernatants of each time which contained phenolic compounds were merged, freeze-dried, and kept at a freezer (-20 °C) until analysis.

Measurement the contents of total phenolic and total flavonoid

Total phenolic content (TPC) and total flavonoid content (TFC) were quantified by Folin-Ciocaltue reagent and $NaNO_2$-$AlCl_3$-NaOH protocol using approaches illustrated by Everette et al. (2010) and Zou, Lu, and Wei (2004) with minor changes. The freeze-dried sample extracted from 10 g berry was dissolved in 60 mL ultrapure water. For analysis of TPC, 300 μL sample was mixed with 300 μL of Folin-Ciocaltue for 6 min, then 3 mL of 7 % sodium carbonate was supplemented, and kept in a dark place under an ambient condition for 1.5 h. For analysis of TFC, 1 mL sample reacted with 0.3 mL $NaNO_2$ (5 %) for 6 min, and was mixed with $AlCl_3$ (10 %) for another 6 min. Then, the mixture was added and reacted with 2 mL NaOH (8 %) for 15 min. The absorbance value was determined at the wavelength of 765 nm for TPC and 510 nm for TFC. Gallic acid and rutin was used to make standard curve for TPC and TFC analysis, separately. The values of TPC and TFC were presented as mg of gallic acid and rutin equivalents/g of fresh weight (namely, mg GA equiv./g FW and mg RT equiv./g FW), respectively.

Identification and quantification of phenolic compounds by Ultra Performance Liquid Chromatography-triple Quadrupole Tandem Mass Spectrometry with electrospray ionization interface (UPLC-ESI-MS/MS)

UPLC-ESI-MS/MS analysis was utilized for identification and qualification of phenolic compounds followed the method of Wei et al. (2022). The AB Sciex ExionLC UHPLC system (AB Sciex LLC, Framingham, MA, USA) with a T3 column (Waters Corp., Milford, MA) was used in liquid chromatography, conducted under the following conditions: 0.1 % of formic acid in water and acetonitrile set as the mobile phase A and B, individually, 5 μL sample injected, and a flow rate stabilized at 0.3 mL/min. Meanwhile, AB SCIEX API 6500 + Qtrap System (AB Sciex, MA, USA) with an ESI source was used in the ESI-MS/MS system, using the positive and negative ion multiple reaction detection mode.

The standard compounds published in supplementary table 1 of Wei et al. (2022) were employed in the analysis of UPLC-ESI-MS/MS. A mixed standard solution was serially diluted from 200 to 0.02 ng/mL.

The phenolic extracts was mixed with water-methanol at the ratio of 18:7 (v:v), filtered, and then placed into a brown LC injection vial. The vial was stored at a freezer (-80 °C) before use. The content of phenolic compound was estimated as μg in per gram of fresh berry (μg/g FW).

Determination of antioxidant activity in vitro

Based on the approach published by Wei et al. (2022), the ABTS, DPPH and FRAP assays were carried on following the protocols of the detection kits purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, Jiangsu, China). All results were calculated as mg of trolox equivalents/g of fresh berry weight (mg TE./g FW) in triplicate.

The examination of oxygen radical absorbance capacity (ORAC) was carried out in terms of the study of Xiang, Apea-Bah, Ndolo, Katundu, and Beta (2019). 20 μL of SBT extract or trolox standard was added into the wells of a black-walled microplate. 200 μL fluorescein solution was pipetted into per well. Then, the plate was maintained for 20 min at 37 °C. Afterward, 20 μL AAPH solution was applied as hydrophilic initiator. The wells with only fluorescein solution and AAPH were as control group, that with only fluorescein solution were as blank group. Fluorescence detection was measured every 5 min within 150 min at the emission and excitation wavelengths of 538 and 485 nm. The ORAC value of SBT berry extracts were represented as mg of trolox equivalents/g of fresh berry weight (mg TE./g FW) in triplicate.

Cytotoxicity tests

Cytotoxicity could cause cell death and other adverse reactions, resulting in the failure of drug efficacy test. Therefore, it is necessary to evaluate the concentrations above which the test compound became cytotoxic before analyzing the cellular antioxidant activity (Treml, Večeřová, Herczogová, & Šmejkal, 2021). So, the cytotoxicity activities of phenolic extracts were analyzed using MTS agent according to the approach of Wolfe and Liu (2007). Briefly, Caco-2 cells in log phase with a concentration of 1×10^5 were planted into a 96-well plate and cultivated in 5 % CO_2 for 24 h at 37 °C. Then, different levels of phenolic extracts were added and cultured with the cells for 24 h, respectively. The MTS agent was supplemented and cultivated for an additional 3 h, the absorbance value was analyzed at the wavelength of 490 nm. The cytotoxicity activities were evaluated by the equation below:

$$Cytotoxicity(\%) = (1 - A_1/A0) \times 100\%$$

where A_0 represents the absorbance value of the control cells; A_1 represents the absorbance value of the treated cells.

Cellular determination of antioxidant activity

The cellular antioxidant activity was tested using the intracellular antioxidant activity (CAA) assay (Kellett, Greenspan, & Pegg, 2018). The standard was quercetin. 1×10^5 cells/well of Caco-2 cells were planted in 96-well plate and cultured for 24 h. 100 μL medium added with various levels of quercetin and phenolic extract supplemented with 25 μmol/L DCFH-DA were transferred in per well. After being incubated for 1 h, the treatment medium was removed. Then, the interference of extracellular antioxidants was removed by washing cells with or without PBS. At last, 100 μL Hanks' balance salt solution (HBSS) with ABAP (600 μmol/L) was used and the absorbance value was measured every 10 min within 1 h at 538 and 485 nm as the emission wavelength and excitation wavelength. The CAA value was computed via the equation as follows:

$$\text{CAA unit} = 100 - \left(\int \text{SA} / \int \text{CA} \right) \times 100.$$

where ∫SA refers the whole area below the curve of the fluorescence of SBT sample versus time; ∫CA refers the entire area of control group.

The antioxidant activities in the cellular were displayed as micromoles of quercetin equivalent per 100 g fresh berry (μmol QE equiv./100 g FW).

Statistical analysis

All data were showed as the mean value ± standard deviation of at least three independent experimental data. The data was statistically analyzed by the software of IBM SPSS Statistics 25.0 (IBM, Armonk, NY). Duncan's multiple range tests were applied to analyze the differences among groups. A statistically significant difference was confirmed when the value of p was lower than 0.05.

Results and discussion

The levels of total phenolic and flavonoid in SBT berries

TPC and TFC of SBT berries were showed in Fig. 1. Significant differences were observed between Rha and Tib berry. The TPC values of Rha berry was 4.25 ± 0.08 mg GA equiv./g FW, which was 1.58-fold than that of Tib berry. Similarly, the variation of TPC value from 0.70 ± 0.01 to 3.62 ± 0.05 mg GA equiv./g FW were also recorded among nine SBT cultivars originated from Finland, Germany, Slovakia and Russia (Sytarova et al., 2020). Notably, Rha berries in our research showed higher TPC content than all of the above nine SBT cultivars. Similar variation trend of TFC were also identified, highest value of TFC was also found in the extracts of Rha berry, which was 1.98 times than that in Tib berry (Fig. 1). Our results were consistent with the TFC values of nine SBT cultivar berries mentioned above (Sytarova et al., 2020). Generally, Rha berry contains richer TPC and TFC content than Tib berry. Genetic variation maybe the first reason accounts for the difference, for these two SBT berries were harvest in the same place. As important secondary metabolites of plant, phenolic compounds are synthesized through the phenylpropanoid pathway, in which chalcone synthase, dihydroflavonol 4-reductase, leucoanthocyanidin dioxygenase, and flavonoid 3′-monooxygenase were indicated to display primary effects on the phenolic synthesis in SBT berry (Boudet, 2007; Fatima et al., 2015). Considering that the regulatory genes involved in phenylpropanoid pathway and its downstream branched metabolic pathways were varied with SBT genotype (Fatima et al., 2015), such significant differences of TPC and TFC in SBT berries are not surprising.

Comparison of phenolic types and contents in SBT berries

Totally, 127 phenolic substances were examined using the UPLC-ESI-MS/MS, and the ion chromatogram of phenolic compound were demonstrated in Fig. 2. 14 subclasses of phenolic profiles containing 49 compounds were observed and quantified in SBT berries, including flavonols, flavanols, anthocyanins, benzoic acid derivatives, proanthocyanidins, phenylpropanoids, benzaldehydes, flavanonols, dihydrochalcones, stilbenes, coumarins, isoflavones, benzyl alcohols and flavones (Table 1). The compositions of phenolic substances were varied, there were 46 and 44 phenolic substances been identified in Tib and Rha berry, individually. Among them, 41 compounds were detected in all SBT berries.

Composition and contents of flavonols

Flavonols was the predominant subclass of phenolic compounds, its content was 42.198 ± 3.529 and 395.171 ± 19.667 μg/g, comprising 48.17 % and 93.86 % of the total identified phenolic compounds in Tib and Rha berries, individually (listed in Table 1). 13 derivatives of flavonols were observed, including astragalin, isorhamnetin-3-o-glucoside, isorhamnetin, kaempferol, morin, myricetin 3-galactoside, myricetin, nicotiflorin, narcissin, prunin, quercetin 3-galactoside, quercetin and rutin. Among them, rutin and narcissin (isorhamnetin-3-rutinoside) were present in the predominant level, which were in accordance with other cultivars or species of SBT berry as reported previously (Jia et al., 2020; Ma, Yang, Marsol-Vall, Laaksonen, & Yang, 2020). Rutin showed wide range of medical benefits, such as antiallergic, antifungal and anticancer activities, which mainly due to its highly antioxidant effects (Sharma, Ali, Ali, Sahni, & Baboota, 2013). Rutin showed the highest

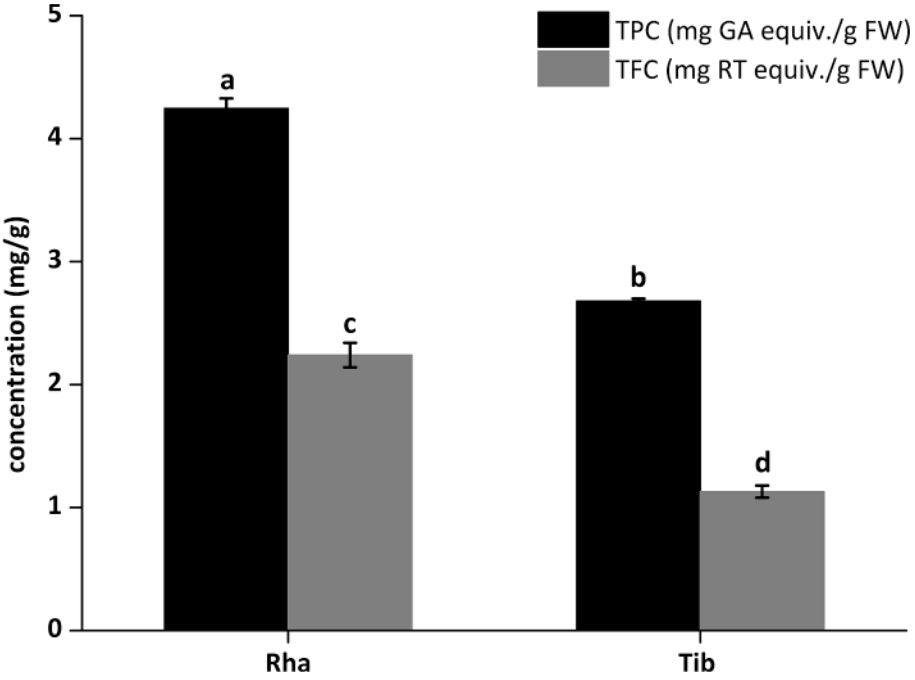

Fig. 1. Total phenolic content (TPC) and total flavonoid content (TFC) of Rha and Tib berry. Bars with no letters in common are significantly different ($P < 0.05$). Data are presented as mean ± SD, calculated from three replicates.

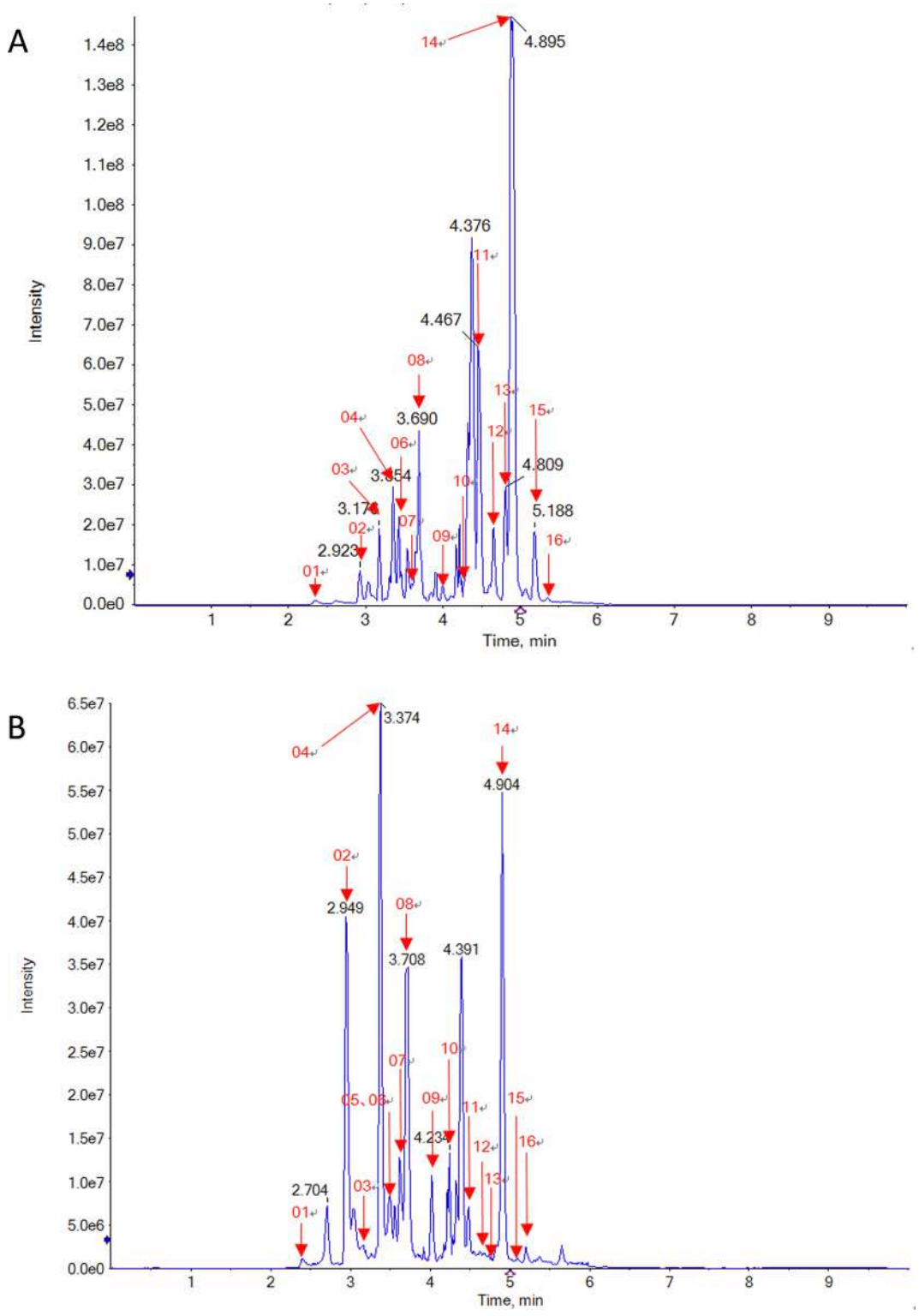

Fig. 2. The total ion chromatogram of Rha (A) and Tib (B) berry phenolic extracts identified by UPLC-ESI-MS/MS. Peak: 01) gallic acid, 02) gallocatechin, 03) delphinidin 3-glucoside, 04) (−)-epigallocatechin, 05) cyanidin 3-o-rutinoside chloride, 06) procyanidin b3, 07) catechin, 08) procyanidin b2, 09) epicatechin, 10) myricetin 3-galactoside, 11) rutin, 12) quercetin 3-galactoside, 13) nicotiflorin, 14) narcissin, 15) astragalin, 16) isorhamnetin-3-o-glucoside.

content in Rha berry at 166.032 μg/g FW, while it served as the third abundant phenolic compound in Tib berry at 12.570 μg/g FW. A previous research identified rutin in the content changing from 1.00 to 25.5 μg/g FW in water-methanol extracts from nine cultivars of SBT berries (Sytarova et al., 2020), which was in accordance with the content in Tib berry but significantly lower than that in Rha berry in our results.

Meanwhile, narcissin was the second abundant phenolic compound both in Rha and Tib berry in the amount of 158.348 and 26.550 μg/g FW. Its content in Rha berry was coincident with a previous report that narcissin content were in the range from 75.0 to 563.0 μg/g based on fresh berry weight of five sea buckthorn cultivars from Finland and Estonia (Ma et al., 2020). Additionally, quercetin 3-galactoside and

Table 1
The content of phenolic compounds in Rha and Tib berry. [a]

Component	Content (μg/g)		*P-value*	log2 (FC)
	Rha	Tib		
Anthocyanins				
Delphinidin 3-glucoside	4.652 ± 0.646	0.066 ± 0.002	0.2521	−0.15
Cyanidin 3-O-rutinoside chloride	0.000 ± 0.000	1.628 ± 0.149	0.0000	35.77
Sum	4.652 ± 0.646	1.694 ± 0.152		
Aromatic aldehydes				
Syringaldehyde	0.109 ± 0.024	0.089 ± 0.003	0.0000	−3.81
Vanillin	0.201 ± 0.008	0.079 ± 0.007	0.0289	−0.60
3,4-Dihydroxybenzaldehyde	0.038 ± 0.001	0.044 ± 0.004	0.0000	25.76
Coniferaldehyde	0.009 ± 0.001	0.004 ± 0.000	0.0000	24.95
Sum	0.357 ± 0.0034	0.216 ± 0.015		
Benzoic acid derivatives				
Gallic acid	1.402 ± 0.486	0.514 ± 0.033	0.0001	2.42
4-Hydroxybenzoic acid	0.063 ± 0.007	0.050 ± 0.006	0.0000	−2.99
Ellagic acid	0.202 ± 0.009	0.000 ± 0.000	0.0000	−1.61
Methyl gallate	0.011 ± 0.001	0.001 ± 0.000	0.0001	2.70
Protocatechuic acid	0.340 ± 0.110	0.215 ± 0.032	0.0000	3.55
Salicylic acid	0.046 ± 0.007	0.025 ± 0.004	0.0100	1.80
Syringic acid	0.013 ± 0.005	0.010 ± 0.004	0.0055	−0.68
Vanillic acid	0.013 ± 0.001	0.013 ± 0.002	0.0040	−0.63
Sum	2.090 ± 0.709	0.828 ± 0.081		
Benzyl alcohols				
Salicin	0.000 ± 0.000	0.003 ± 0.001	0.0000	0.00
Coumarins				
Aesculin	0.047 ± 0.001	0.025 ± 0.003	0.0709	1.92
Dihydrochalcones				
Phlorizin	0.013 ± 0.002	0.358 ± 0.041	0.0000	−2.51
Trilobatin	0.005 ± 0.001	0.012 ± 0.002	0.0001	−0.93
Sum	0.018 ± 0.003	0.370 ± 0.043		
Flavanols				
(−)-Epigallocatechin	15.007 ± 2.378	32.798 ± 1.656	0.0002	−2.20
Catechin	0.583 ± 0.170	1.594 ± 0.225	0.0001	−0.93
Epicatechin	0.738 ± 0.126	1.460 ± 0.148	0.0795	−0.95
Gallocatechin	0.916 ± 0.089	3.742 ± 0.414	0.0000	−24.70
Sum	17.244 ± 2.763	39.594 ± 2.443		
Flavones				
Vitexin	0.001 ± 0.000	0.001 ± 0.000	0.3565	−0.18
Flavonols				
Astragalin	1.858 ± 0.106	0.200 ± 0.069	0.0000	1.04
Isorhamnetin	0.007 ± 0.000	0.018 ± 0.008	0.0000	−0.85
Isorhamnetin-3-O-glucoside	15.719 ± 2.189	0.970 ± 0.112	0.0000	0.95
Kaempferol	0.000 ± 0.000	0.001 ± 0.000	0.0006	−0.73
Morin	0.005 ± 0.000	0.008 ± 0.003	0.0000	−0.79
Myricetin 3-galactoside	0.985 ± 0.117	0.187 ± 0.030	0.0000	−26.00
Myricetin	0.006 ± 0.000	0.011 ± 0.003	0.0000	2.55
Narcissin	158.348 ± 3.791	26.550 ± 1.953	0.3678	−0.11
Nicotiflorin	31.202 ± 2.975	1.024 ± 0.156	0.0029	1.15
Prunin	0.056 ± 0.006	0.037 ± 0.006	0.0038	1.19
Quercetin 3-galactoside	20.952 ± 3.212	0.615 ± 0.112	0.0008	−0.78
Quercetin	0.000 ± 0.000	0.006 ± 0.005	0.3249	−0.12
Rutin	166.032 ± 7.271	12.570 ± 1.071	0.0006	1.81
Sum	395.171 ± 19.667	42.198 ± 3.529		
Flavanonols				
Taxifolin	0.018 ± 0.006	0.023 ± 0.009	0.0012	−1.04
trans-3,3′,4′,5,5′,7-Hexahydroxyflavanone	0.185 ± 0.047	0.104 ± 0.024	0.0000	−2.66
Sum	0.203 ± 0.053	0.127 ± 0.033		
Isoflavones				
Daidzein	0.014 ± 0.002	0.000 ± 0.000		
Phenylpropanoids				
4-Hydroxycinnamic acid	0.077 ± 0.028	0.098 ± 0.012	0.0000	−2.67
Ferulic acid	0.008 ± 0.003	0.009 ± 0.002	0.0000	−2.26
Sinapic acid	0.014 ± 0.005	0.269 ± 0.024	0.0000	−2.86
trans-Cinnamic acid	0.065 ± 0.008	0.065 ± 0.006	0.0000	−4.79
1,5-Dicaffeoylquinic acid	0.000 ± 0.000	0.003 ± 0.001	0.0000	−3.32
Chlorogenic acid	0.154 ± 0.018	0.015 ± 0.002	0.0000	−1.52
Sum	0.317 ± 0.063	0.460 ± 0.048		
Proanthocyanidins				
Procyanidin B3	0.152 ± 0.058	0.000 ± 0.000	0.0000	−1.07
Procyanidin B1	0.447 ± 0.169	0.806 ± 0.149	0.0000	30.47
Procyanidin B2	0.257 ± 0.132	1.210 ± 0.213	0.0001	29.20
Sum	0.856 ± 0.359	2.016 ± 0.362		

(*continued on next page*)

Table 1 (*continued*)

Component	Content (μg/g)		*P-value*	log2 (FC)
	Rha	Tib		
Stilbenes				
trans-Piceid	0.039 ± 0.007	0.066 ± 0.00	0.0000	−1.22

[a] Data are presented as mean ± SD, calculated from three biological replicates. Components with $P < 0.05$ calculated from T-test and absolute log2 (FC) > 1 was considered differential metabolites.

kaempferol 3-rutinoside (Nicotiflorin) were both abundant in Rha berry, at the concentration of 20.952 and 31.202 μg/g FW. But these two phenolic compounds were only in the amount of 0.615 and 1.024 μg/g FW in Tib berry. What is more, astragalin was established from 0.20 (Tib) to 1.858 μg/g FW (Rha). Astragalin, quercetin 3-galactoside, and kaempferol 3-rutinoside were detected in sea buckthorn leaf in previous study (Heinaaho, Pusenius, & Julkunen-Tiitto, 2006; Kumar, Dutta, Prasad, & Misra, 2011; Pop et al., 2013), but they have not been observed in sea buckthorn berry. This was the first time to identify these three flavanols glycosides in sea buckthorn berries. Furthermore, isorhamnetin-3-O-glucoside also was recorded in rich amount reaching from 0.97 (Tib) to 15.719 μg/g FW (Rha). The contents were comparatively lower than previous research, which determined isorhamnetin-3-O-glucoside at concentrations between 49.0 and 246.0 μg/g in fresh berries of five SBT cultivars, including 'Raisa' and 'Pertsik' from Finland, as well as 'Vorobyevskaya', 'Botanitsheskaja ljubitel-skaja (Bot-lju)' and 'Askola' from Estonia (Ma et al., 2020). What is more, myricetin 3-galactoside was firstly identified in SBT berry at content of 0.985 (Rha) and 0.187 μg/g (Tib). Additionally, prunin and morin were also firstly discriminated in SBT berry, but the amounts were very low. Similarly, isorhamnetin was determined in nearly negligible amounts from 0.007 (Rha) to 0.018 μg/g FW (Tib), this value is significantly lower than the concentration of isorhamnetin at 58.3 μg/g FW in *H. rhamnoides* L. subsp. *Sinensis* berry (Guo et al., 2017). Extraction method maybe the key reason causing this difference, in our research the phenolics were extracted by aqueous ethanol, which is a food-grade method for extraction (Tian et al., 2017), but 80 % chilled acetone were used for extraction in the method of Guo et al. (2017). Meanwhile, myricetin was observed at nearly negligible concentration in Rha and Tib berry, which confirms the result of Hakkinen et al., 1999 who found that myricetin was not existed in SBT berry. Notably, kaempferol and quercetin were also observed in negligible level in the Tib berry, as well as were not presented in Rha berry in our results, which confirmed a published data concerning the absence of kaempferol and quercetin in nine SBT cultivar berries originated from Finland, Germany, Slovakia and Russia (Sytarova et al., 2020).

Generally speaking, total amount of flavonols in Rha berry was 9.36 times higher than that in Tib berry. According to the analysis of significant difference, it was found that the abundant compounds, including isorhamnetin-3-o-glucoside, rutin, narcissin, nicotiflorin, myricetin 3-galactoside, astragalin, and quercetin 3-galactoside, all exhibited higher levels in Rha berry, which were 16.20-,13.21-, 5.96-, 30.46-, 5.27-, 9.30-, 34.05- times higher than that in Tib berry (Table 1 and Fig. 3), separately. So, Rha berry is regarded as a better resource of flavonols, particularly rutin and narcissin.

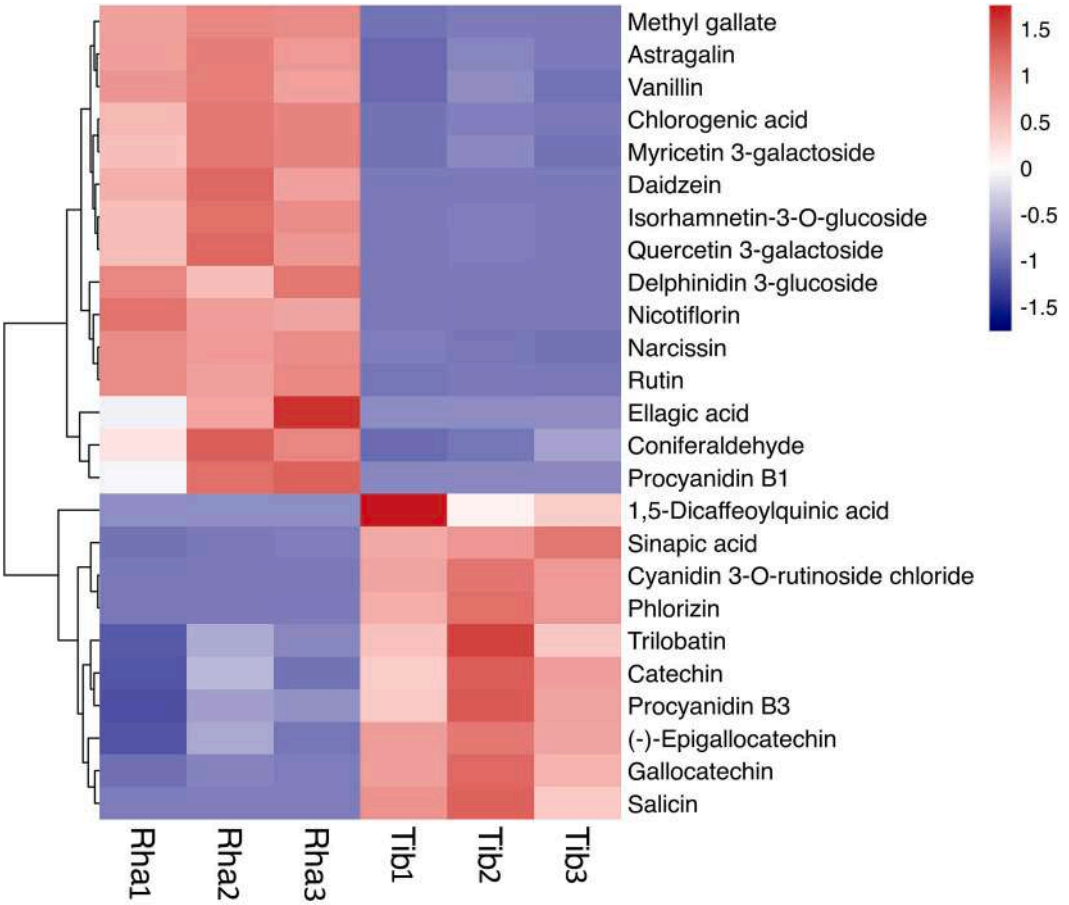

Fig. 3. Hierarchical Clustering of the phenolic compounds that were significantly differentially expressed in Rha and Tib berry. Each line is a phenolic component. The color ranging from blue to red means the component content increasing from low to high. Data showed in the figure are mean ± SD from three replicates. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Composition and contents of flavanols

(−)-Epigallocatechin, catechin, epicatechin, gallocatechin belonging to the flavanols were detected in SBT berry in our results. They constituted 45.2 % of the total phenolic in Tib berry, at the concentration of 39.594 μg/g, which was 2.30-fold than that in Rha berry (Table 1). (−)-Epigallocatechin, which has hormonal regulation activity (Lee et al., 2012), exerted the highest level in this subclass, and it also was the most predominant phenolic substance in Tib berry at the content of 32.798 μg/g, which was consistent with a previous report of epigallocatechin altering from 32.9 to 62.3 μg/g FW in nine SBT cultivar berries originated from Finland, Germany, Slovakia and Russia (Sytarova et al., 2020). But the content of epigallocatechin presented a bit lower level of 15.007 μg/g in Rha berry. Meanwhile, gallocatechin was detected with the amount changing from 0.916 (Tib) to 3.742 μg/g (Rha). Similar to our result, Arimboor and Arumughan (2012) also detected gallocatechin in SBT berries from India, but they did not determine its concentration. Then catechin and epicatechin, which ranging from 0.583 μg/g (Rha) to 1.594 μg/g (Tib) and from 0.738 μg/g (Rha) to 1.460 μg/g (Tib) were also detected in our research. While, concentrations of catechin were significantly lower than 4.8 μg/g of *H. rhamnoides* L. subsp. *Sinensis* berry (Guo et al., 2017). Also, different extraction reagents maybe account for the big variation of contents just as discussed in 3.2.1. For epicatechin, comparably to our results, such a great content variability in SBT berries varying from 0 to 2.4 μg/g was also observed in nine SBT cultivar berries mentioned above (Sytarova et al., 2020).

Notably, all the flavanols detected in our results including (−)-epigallocatechin, catechin, epicatechin and gallocatechin were significantly richer in Tib berry, which was 2.19-, 2.74-, 1.98-, 4.08- fold higher compared with that in Rha berry (Table 1 and Fig. 3). Therefore, we could conclude that Tib berry was better sources of flavanols than Rha berry.

Composition and contents of anthocyanins and proanthocyanidins

The content of total anthocyanins was very low, which from 1.694 (Tib) to 4.65 μg/g (Rha). Only two types of anthocyanins including cyanidin 3-O-rutinoside chloride and delphinidin 3-glucoside were detected in our results. As displayed in Table 1, in Rha berry, delphinidin 3-glucoside was in the amount of 4.652 μg/g, which was higher than 0.5 μg/g in SBT berry from Canada (Hosseinian & Beta, 2007). Cyanidin 3-O-rutinoside chloride was only detected in Tib berry at the content of 1.628 μg/g, which was similar to the content in SBT berry reported by Hosseinian and Beta (2007).

Total amounts of proanthocyanidins were also at very low level, ranging from 0.856 (Rha) to 2.017 μg/g (Tib) (Table 1). This concentration was lower than the report of Yang, Laaksonen, Kallio, and Yang (2016), the extraction agent and purification method maybe the main reason. B-type proanthocyanidins were only identified in all samples, which were the same as the reports of Yang et al. (2016) and Kallio, Yang, Liu, and Yang (2014).

Composition and contents of benzoic acid derivatives and other phenolic classes

There were eight compounds of benzoic acid derivatives, covering ellagic acid, gallic acid, syringic acid, 4-hydroxybenzoic acid, methyl gallate, salicylic acid, vanillic acid, and protocatechuic acid were detected in SBT berries (Table 1). Among them, the most abundant compound was gallic acid, rang from 0.515 μg/g (Tib) to 1.402 μg/g (Rha). This was agreement with a report that changeable gallic acid amounts were recorded reaching from 0.2 to 7.0 μg/g in SBT berries originated from different places of European (Sytarova et al., 2020). Furthermore, protocatechuic acid was also detected in low amounts from 0.215 (Tib) to 0.340 μg/g (Rha), this content was similarly accordance with a range from 0.0 to 21.3 μg/g of six SBT cultivars from Poland and Byelorussia (Zadernowski et al., 2005). Besides, remaining compounds of benzoic acid derivatives were identified in relatively small amounts.

Other subclasses of phenolic compounds, including phenylpropanoids, benzaldehydes, flavanonols, dihydrochalcones, stilbenes, coumarins and isoflavones were all present at very low contents.

In conclusion, flavonols were absolutely the predominant subclass of phenolic compounds in Rha berry, in which narcissin and rutin were the two most abundant substances. Besides, flavonols and flavanols were the two most abundant classes in Tib berry, (−)-epigallocatechin was the richest compound among them, followed by narcissin and rutin. Notably, Tib berry was found to be richer in flavanols and proanthocyanidins than Rha berry. However, Rha berry hold higher levels of the other compounds, especially the class of flavonols. This might mainly due to the genetic diversity, considering they grow in the same environment, as well as the same extract and analytical techniques.

Extracellular and cellular antioxidant activities of SBT berries

There were several methods for testing the extracellular antioxidant activities of phenolic compounds, which based on different mechanisms (Antolovich, Prenzler, Patsalides, McDonald, & Robards, 2002). In our research, DPPH and ABTS radical scavenging capacity, the FRAP and ORAC total antioxidant methods were introduced to examine the antioxidant activity of SBT phenolic extracts counting with various antioxidant mechanism. According to Fig. 4A, the extracellular antioxidant activities of SBT berries varied greatly according to the assay type. The highest value of antioxidant activity were identified by ORAC method, which was 48.83 mg TE./g FW for Rha berry extract and 14.89 mg TE./g FW for Tib berry extract. The ORAC values in our results were lower than 92.36 mg TE./g DW of *H. rhamnoides* L. subsp. *Sinensis* found by Guo et al. (2017). The ABTS radical scavenging activities were 30.37 TE./g FW (Rha) and 28.92 TE./g FW (Tib) (Fig. 4A), which was higher than 8.95 mg TE./g DW of SBT cultivar berries grown in Poland (Tkacz, Wojdylo, Turkiewicz, Bobak, & Nowicka, 2019). Additionally, FRAP value was 25.40 mg TE./g FW for Rha berry and 6.80 mg TE./g FW for Tib berry (Fig. 4A). The FRAP value of SBT cultivar berries grown in Poland reported by (Tkacz et al., 2019) was lower than that in Rha berry of our results. However, the value was higher than that in Tib berry of our results. Similarly, for DPPH radical scavenging activity, the value was 15.37 and 10.74 mg TE./g FW for Rha and Tib berry, separately (Fig. 4A). These values were absolutely higher than the scavenging capacity of nine SBT cultivar berries ranging from 1.08 to 4.67 mg TE/g FW (Sytarova et al., 2020). The varieties of antioxidant activity may result from the genetics, growth conditions, extraction methods or even the expressed type (fresh weight or dry weight).

The antioxidant activities were universally tested using chemical assays. However, these assays could not evaluate the antioxidant performance of tested samples in physiological conditions. So, the CAA method was developed in HepG2 cells, which could reflect the cellular uptake, distribution and metabolism of antioxidants under conditions mimicking biological system (Wolfe & Liu, 2007). But as a kind of liver cells, HepG2 cell line was not ideal for testing the effectiveness of food antioxidants (Kellett et al., 2018). Meanwhile, Caco-2 cells, which have similar morphology and physiology like small intestinal epithelial cells, become a more logical model to assess the antioxidant capacities of dietary compounds (Kellett et al., 2018). Our results indicated that all phenolic extracts of SBT berries showed no cytotoxicity even up to the level at 2000 μg/g FW (Supplementary Fig. 1). And they both exerted high CAA value in two protocols (no PBS or PBS wash) (Fig. 4B). Concerning antioxidant activity of no PBS wash, Rha berry extract showed higher CAA value, which was 1994.68 ± 81.32 μmol QE/100 g FW, followed by Tib with the value of 1075.81 ± 51.03 μmol QE/100 g FW. In terms of antioxidant capacity of PBS wash, Rha berry extracts also possessed the higher antioxidant activity, with CAA value of 1217.16 ± 260.65 μmol QE/100 g FW, which was 1.76- times higher than Tib berry. Our results were significantly higher than the average CAA values of four SBT berry extracts determined by HepG2 cells both in no PBS wash group (354 ± 22 μmol QE /100 g DW) and in PBS group (197 ± 18

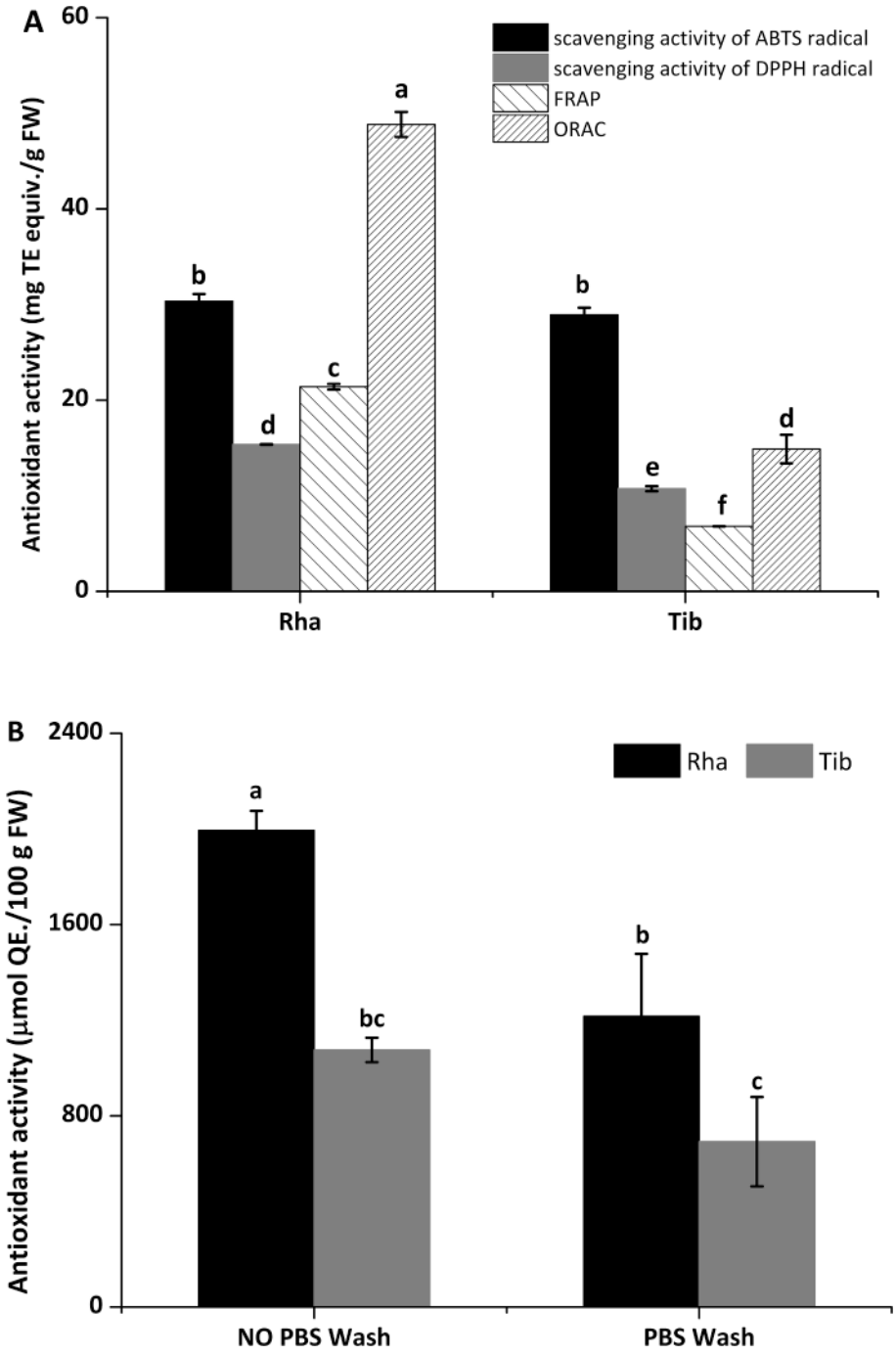

Fig. 4. Extracellular (A) and cellular (B) antioxidant activities of the phenolic extracts from Rha and Tib berries. Bars without letters represent significant differences ($P < 0.05$). Data showed in the figure are mean ± SD from three replicates.

μmol/100 g DW) (Guo et al., 2017). Cell line applied in the experiments maybe the main factors causing these differences, because Caco-2 cells was a more ideal model for testing the antioxidant activities of food antioxidants, especially rich in flavonoids (Kellett et al., 2018). Notably, compared to no PBS wash group, the PBS wash group showed lower antioxidant activities (Fig. 4B). This was because the CAA value with no PBS wash represents the activities of compounds which associated with the cell membrane and entered into the cells. While, PBS wash could reduce the CAA value by removing substances that only loosely bound with the cell membrane (Wolfe & Liu, 2007).

Finally, the chemical assays and cellular antioxidant analysis demonstrated that SBT berry possessed high antioxidant activity. TPC and TFC would be the most essential factor to the antioxidant activities of the SBT berry extracts according to some previous reports (Guo et al., 2017; Rop, Ercisli, Micek, Jurikova, & Hoza, 2014; Tian et al., 2018). Meanwhile, rutin, which is the most abundant phenolic compound in SBT berry, was reported to show correlation with lipid-soluble antioxidant capacity in nine SBT berries from Filand, Germany, Solvakia and Russia (Sytarova et al., 2020). Notably, Rha berry always showed higher antioxidant capacity than Tib berry, probably due to the higher content of TPC, TFC and some pure phenolic compounds in Rha berry. This reminded us that Rha berry may have high nutrition value because of its high antioxidant activity. But the specific components that account for antioxidant capacity of SBT berry, as well as the synergistic and antagonistic effects among individual phenolic compound will need to be identified in the future.

Conclusions

This study revealed that SBT berry contained high concentrations of total phenolics and flavonoids. A total of 49 pure phenolic compounds belonging to 14 subclasses were identified and quantitated. Flavonols definitely was the most predominant subclass in Rha berry, comprising 93.87 % of the phenolic pool. Flavonols and flavanols were the two most abundant subclasses in Tib berry, occupied 48.17 % and 45.20 % of the total phenolic compounds. Among them, rutin and narcissin were present in the most abundant amounts in Rha berry, while, (−)-epigallocatechin was the highest content substance in Tib berry. Additionally, quercetin 3-galactoside, astragalin, kaempferol 3-rutinoside, myricetin 3-galactoside, prunin and morin were firstly identified in SBT berries. Furthermore, phenolic extracts of SBT berry exerted strong antioxidant activities both in chemical assays and in cellular analysis. Furthermore, Rha berry showed higher antioxidant activity than Tib, which might attribute to its highest phenolic contents. This reminded us that Rha berry hold a great potential to serve as the resource of a natural antioxidant dietary supplement used in food industry.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

This work has been funded by Natural Science Foundation of Gansu Province (20JR10RA514); China-Russia International Science and Technology Cooperation Base of Sea Buckthorn Processing Technology in Gansu Province (Science and Technology Department of Gansu Province); Innovation Fund Project of University in Gansu Province (2021B-118).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fochx.2022.100397.

References

Antolovich, M., Prenzler, P. D., Patsalides, E., McDonald, S., & Robards, K. (2002). Methods for testing antioxidant activity. *Analyst, 127*(1), 183–198.

Arimboor, R., & Arumughan, C. (2012). Effect of polymerization on antioxidant and xanthine oxidase inhibitory potential of sea buckthorn (*H. rhamnoides*) proanthocyanidins. *Journal of Food Science, 77*(10), C1036–C1041.

Boudet, A. M. (2007). Evolution and current status of research in phenolic compounds. *Phytochemistry, 68*(22–24), 2722–2735.

Everette, J. D., Bryant, Q. M., Green, A. M., Abbey, Y. A., Wangila, G. W., & Walker, R. B. (2010). Thorough study of reactivity of various compound classes toward the Folin-Ciocalteu reagent. *Journal of Agricultural and Food Chemistry, 58*(14), 8139–8144.

Fatima, T., Kesari, V., Watt, I., Wishart, D., Todd, J. F., Schroeder, W. R., … Krishna, P. (2015). Metabolite profiling and expression analysis of flavonoid, vitamin C and tocopherol biosynthesis genes in the antioxidant-rich sea buckthorn (*Hippophae rhamnoides* L.). *Phytochemistry, 118*, 181–191.

Guo, R., Guo, X., Li, T., Fu, X., & Liu, R. H. (2017). Comparative assessment of phytochemical profiles, antioxidant and antiproliferative activities of Sea buckthorn (*Hippophae rhamnoides* L.) berries. *Food Chemistry, 221*, 997–1003.

Hakkinen, S. H., Karenlampi, S. O., Heinonen, I. M., Mykkanen, H. M., & Torronen, A. R. (1999). Content of the flavonols quercetin, myricetin, and kaempferol in 25 edible berries. *Journal of Agricultural and Food Chemistry, 47*(6), 2274–2279.

Heinaaho, M., Pusenius, J., & Julkunen-Tiitto, R. (2006). Effects of different organic farming methods on the concentration of phenolic compounds in sea buckthorn leaves. *Journal of Agricultural and Food Chemistry, 54*(20), 7678–7685.

Hosseinian, F. S., & Beta, T. (2007). Saskatoon and wild blueberries have higher anthocyanin contents than other Manitoba berries. *Journal of Agricultural and Food Chemistry, 55*(26), 10832–10838.

Ji, M., Gong, X., Li, X., Wang, C., & Li, M. (2020). Advanced research on the antioxidant activity and mechanism of polyphenols from *hippophae* species-a review. *Molecules, 25*(4).

Jia, Q., Zhang, S., Zhang, H., Yang, X., Cui, X., Su, Z., & Hu, P. (2020). A comparative study on polyphenolic composition of berries from the tibetan plateau by UPLC-Q-Orbitrap MS system. *Chemistry & Biodiversity, 17*(4), e2000033.

Jiang, F., Guan, H., Liu, D., Wu, X., Fan, M., & Han, J. (2017). Flavonoids from sea buckthorn inhibit the lipopolysaccharide-induced inflammatory response in RAW264.7 macrophages through the MAPK and NF-kappaB pathways. *Food Function, 8*(3), 1313–1322.

Kallio, H., Yang, W., Liu, P., & Yang, B. (2014). Proanthocyanidins in wild sea buckthorn (*Hippophae rhamnoides*) berries analyzed by reversed-phase, normal-phase, and hydrophilic interaction liquid chromatography with UV and MS detection. *Journal of Agricultural and Food Chemistry, 62*(31), 7721–7729.

Kellett, M. E., Greenspan, P., & Pegg, R. B. (2018). Modification of the cellular antioxidant activity (CAA) assay to study phenolic antioxidants in a Caco-2 cell line. *Food Chemistry, 244*, 359–363.

Kim, J. S., Kwon, Y. S., Sa, Y. J., & Kim, M. J. (2011). Isolation and identification of sea buckthorn (*Hippophae rhamnoides*) phenolics with antioxidant activity and alpha-glucosidase inhibitory effect. *Journal of Agricultural and Food Chemistry, 59*(1), 138–144.

Kumar, M. S., Dutta, R., Prasad, D., & Misra, K. (2011). Subcritical water extraction of antioxidant compounds from Seabuckthorn *(Hippophae rhamnoides*) leaves for the comparative evaluation of antioxidant activity. *Food Chemistry, 127*(3), 1309–1316.

Lee, Y. H., Kwak, J., Choi, H. K., Choi, K. C., Kim, S., Lee, J., … Yoon, H. G. (2012). EGCG suppresses prostate cancer cell growth modulating acetylation of androgen receptor by anti-histone acetyltransferase activity. *International Journal of Molecular Medicine, 30*(1), 69–74.

Li, H., Ruan, C., Ding, J., Li, J., Wang, L., & Tian, X. (2020). Diversity in sea buckthorn (*Hippophae rhamnoides* L.) accessions with different origins based on morphological characteristics, oil traits, and microsatellite markers. *PLoS One, 15*(3), e0230356.

Lian, Y., & Chen, X. (1996). Systematic classification of Seabuckthorn. *Hippophae (Chinese), 9*(1), 15–24.

Liu, Y., Fan, G., Zhang, J., Zhang, Y., Li, J., Xiong, C., … Lai, X. (2017). Metabolic discrimination of sea buckthorn from different *Hippophae* species by (1)H NMR based metabolomics. *Scientific Reports, 7*(1), 1585.

Ma, X., Yang, W., Marsol-Vall, A., Laaksonen, O., & Yang, B. (2020). Analysis of flavour compounds and prediction of sensory properties in sea buckthorn (*Hippophaee rhamnoides* L.) berries. *International Journal of Food Science & Technology, 55*(4), 1705–1715.

Olas, B. (2018). The beneficial health aspects of sea buckthorn (Elaeagnus *rhamnoides* (L.) A.Nelson) oil. *Journal of Ethnopharmacology, 213*, 183–190.

Pop, R. M., Socaciu, C., Pintea, A., Buzoianu, A. D., Sanders, M. G., Gruppen, H., & Vincken, J. P. (2013). UHPLC/PDA-ESI/MS analysis of the main berry and leaf flavonol glycosides from different Carpathian *Hippophae rhamnoides* L. varieties. *Phytochemical Analysis, 24*(5), 484–492.

Rop, O., Ercisli, S., Micek, J., Jurikova, T., & Hoza, I. (2014). Antioxidant and radical scavenging activities in fruits of 6 sea buckthorn (*Hippophae rhamnoides* L.) cultivars. *Turkish Journal of Agriculture and Forestry, 38*(2), 224–232.

Sharma, S., Ali, A., Ali, J., Sahni, J. K., & Baboota, S. (2013). Rutin: Therapeutic potential and recent advances in drug delivery. *Expert Opinion on Investigational Drugs, 22*(8), 1063–1079.

Stobdan, T., Korekar, G., & Srivastava, R. B. (2013). Nutritional attributes and health application of seabuckthorn *(Hippophae rhamnoides* L.) – a review. *Current Nutrition and Food Science, 9*(2), 151–165.

Sytarova, I., Orsavova, J., Snopek, L., Mlcek, J., Byczynski, L., & Misurcova, L. (2020). Impact of phenolic compounds and vitamins C and E on antioxidant activity of sea buckthorn (*Hippophae rhamnoides* L.) berries and leaves of diverse ripening times. *Food Chemistry, 310*, Article 125784.

Tian, Y., Liimatainen, J., Alanne, A. L., Lindstedt, A., Liu, P., Sinkkonen, J., … Yang, B. (2017). Phenolic compounds extracted by acidic aqueous ethanol from berries and leaves of different berry plants. *Food Chemistry, 220*, 266–281.

Tian, Y., Puganen, A., Alakomi, H. L., Uusitupa, A., Saarela, M., & Yang, B. (2018). Antioxidative and antibacterial activities of aqueous ethanol extracts of berries, leaves, and branches of berry plants. *Food Research International, 106*, 291–303.

Tiitinen, K. M., Hakala, M. A., & Kallio, H. P. (2005). Quality components of sea buckthorn (*Hippophae rhamnoides*) varieties. *Journal of Agricultural and Food Chemistry, 53*(5), 1692–1699.

Tkacz, K., Wojdylo, A., Turkiewicz, I. P., Bobak, L., & Nowicka, P. (2019). Anti-oxidant and anti-enzymatic activities of sea buckthorn (*Hippophae rhamnoides* L.) fruits modulated by chemical components. *Antioxidants (Basel), 8*(12), 618.

Treml, J., Večeřová, P., Herczogová, P., & Šmejkal, K. (2021). Direct and indirect antioxidant effects of selected plant phenolics in cell-based assays. *Molecules, 26*(9), 2534.

Wang, K., Xu, Z., & Liao, X. (2021). Bioactive compounds, health benefits and functional food products of sea buckthorn: A review. *Critical Reviews in Food Science and Nutrition, 30*, 1–22.

Wei, J., Su, T., Su, H., Jiang, Y., Li, J., & Bi, Y. (2022). Comparative assessment of phenolics, antioxidant and antiproliferative activities between *Hippophae rhamnoides* ssp. *sinensis* and *H. tibetana* leaf in Qinghai-Tibet Plateau. *Food Bioscience, 46*. https://doi.org/10.1016/j.fbio.2021.101507

Wolfe, K. L., & Liu, R. H. (2007). Cellular antioxidant activity (CAA) assay for assessing antioxidants, foods, and dietary supplements. *Journal of Agricultural and Food Chemistry, 55*(22), 8896–8907.

Xiang, J., Apea-Bah, F. B., Ndolo, V. U., Katundu, M. C., & Beta, T. (2019). Profile of phenolic compounds and antioxidant activity of finger millet varieties. *Food Chemistry, 275*, 361–368.

Yang, W., Laaksonen, O., Kallio, H., & Yang, B. (2016). Proanthocyanidins in Sea Buckthorn (*Hippophae rhamnoides* L.) Berries of Different Origins with Special

Reference to the Influence of Genetic Background and Growth Location. *Journal of Agricultural and Food Chemistry, 64*(6), 1274–1282.
Zadernowski, R., Naczk, M., Czaplicki, S., Rubinskiene, M., & Szałkiewicz, M. (2005). Composition of phenolic acids in sea buckthorn (*Hippophae rhamnoides* L.) berries. *Journal of the American Oil Chemists Society, 82*(3), 175–180.
Zou, Y., Lu, Y., & Wei, D. (2004). Antioxidant activity of a flavonoid-rich extract of *Hypericum perforatum* L. *in vitro*. *Journal of Agricultural and Food Chemistry, 52*(16), 5032–5039.