SUPPLEMENTARY MATERIAL

Chemical composition and antioxidant capacity of extracts from the whole berry, pulp and seed of *Hippophaë rhamnoides ssp. yunnanensis*

Longkai Shi, Li Zheng, Chenwei Zhao, Qingzhe Jin and Xingguo Wang*

Collaborative Innovation Center of Food Safety and Quality Control in Jiangsu Province, National Engineering Research Center for Functional Food, School of Food Science and Technology, Jiangnan University, Wuxi, 214122, China.

Address correspondence to Xing-Guo Wang at Collaborative Innovation Center of Food Safety and Quality Control in Jiangsu Province, National Engineering Research Center for Functional Food, School of Food Science and Technology, Jiangnan University, Wuxi, 214122, China. (E-mail: wangxg1002@gmail.com, telephone: +86-13382226199).

Abstract: In the present study, profiles of fatty acid, sn-2 fatty acid, triglyceride, phytochemical (including tocopherol/tocotrienol, phytosterol, flavonoid, carotenoid and polyphenol) and antioxidant capacity of extracts from the whole berry, pulp and seed of *Hippophaë rhamnoides ssp*. *yunnanensis* were investigated and compared. The distributions of fatty acid, sn-2 fatty acid and triglyceride that was identified using ultra−performance liquid chromatography tandem quadrupole time of flight mass spectrometry (UPLC-Q-TOF/MS) showed obvious differences among the different parts of the *ssp. yunnanensis*. The whole berry and pulp extracts exhibited high flavonoid, carotenoid and polyphenol contents, whereas, the seed portion expressed high tocopherol/tocotrienol and phytosterol concentrations. Results deduced from this study demonstrated that *ssp*. *yunnanensis* is a rich source of unsaturated fatty acid and bioactive minor component, which should be further developed and utilized by breeders and planters.

Keywords: *Hippophaë rhamnoides ssp*. *yunnanensis*, Fatty acid, Sn-2 fatty acid, Triglyceride, Phytochemical, Antioxidant capacity

Experimental

Materials and chemicals

A mixture standard of 37 fatty acid methyl esters, tocopherol and tocotrienol standards, phytosterol standard, quercetin, β-carotene, gallic acid, 5α-cholestane and BSTFA + TMCS (v/v=99:1), as well as fluorescein sodium salt (FL), 2, 2'-azobis (2-methylpropionamidine) dihydrochloride (AAPH), 2, 2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), 2, 4, 6-tris(2-pyridyl)-s-triazine (TPTZ), 2, 2-diphenyl-1-picrylhydrazyl (DPPH) and (±)-6-hydroxy-2, 5, 7, 8-tetramethylchromane-2-carboxylic acid (Trolox) used for in *vitro* antioxidant tests were all purchased from Sigma-Aldrich (Bellefonte, PA, USA) and stored at -20 °C in darkness.

HPLC grade n-hexane, isopropanol, methanol, ethanol, dichloromethane and ethyl acetate were obtained from Sigma (St. Louis, MO, USA). Ultrapure water was prepared by a Milli-Q system purchased from Millipore (Billerica, MA, USA). Other inorganic reagents (analytical grade unless otherwise stated) were all provided by Sinopharm Medicine Holding Co., Ltd. (Shanghai, China). SB berries of *Hippophaë rhamnoides ssp. yunnanensis* (No. Yunnan-03) were collected from Yunnan province of China in September, 2017. The mature berries were immediately frozen after picking and transported to the laboratory for analysising.

SB whole berry, pulp and seed extracts preparation

Parts of the frozen SB berries were thawed at room temperature and then mechanically crushed. The seeds of the fruits were manually separated from the fractured seriflux and rinsed with distilled water. The isolated SB pulp and seed, together with the crushed SB whole berry were individually lyophilized to a constant weight before extraction.

Supercritical CO_2 extraction was operated in according to the procedures described below: 200 g of the freeze-dried, crushed SB whole berry, pulp and seed samples were individually extracted using a Waters SFE 100 CO_2 extraction system equipped with a 2 L extraction vessel (Waters Corp., Milford, MA, UK). The extraction temperature, pressure, time and flow rate of CO_2 were set at 40 °C, 30 MPa, 90 min and 0.5 L/min, respectively. The temperature of the heat exchanger was set at 50 °C, and the temperature and pressure of the separator were set at 40 °C and 9 MPa, respectively. High purity carbon dioxide (99.999%) was used as the supercritical carrier solvent. The obtained three kinds of extracts were individually stored in brown bottles at -20 °C until needed.

Fatty acid, sn-2 fatty acid and triglyceride

Fatty acid, sn-2 fatty acid and triglyceride were determined based on our previously published methods (Jin

et al. 2016, Zhang et al. 2016), respectively.

Tocopherol and tocotrienol

Approximately 500 mg of the extracts were individually weighted in a 10 mL brown volumetric flask and dissolved with n-hexane to the volume. Then the mixed solution was filtered through a 0.22 μm nylon syringe filter and 10 μL of the solution was injected into a Waters 2695 HPLC for analysis. A Waters Spherisorb® NH_2 analytical column (4.6 mm × 250 mm, 5 μm) from Waters Corp. (Milford, MA, UK) was used for LC separation. LC conditions were as follow: mobile phase, n-hexane/isopropanol (98:2 v/v); flow rate: 0.8 mL/min; column temperature: 40 °C; injection volume: 10 μL; excitation and emissions wavelengths: 298 and 330 nm, respectively.

Phytosterol

Approximately 250 mg of the extracts were individually weighted in a 15 mL glass centrifuge tube. 0.5 mL of 0.1 mg/mL 5α-cholestane (dissolved in n-hexane) and 2 mL of 2 mol/L KOH-Ethanol solution were added. The mixture was heated at 85 °C for 1 h and then cooled at ambient temperature, 2 mL of pure water and 5 mL of n-hexane were added. The top layer was carefully transferred into a 50 mL centrifuge tube, and the extraction process was repeated twice with 5 mL of n-hexane each. All the extracts were collected together and dried under a gentle flow of nitrogen. Then silylated with 0.3 mL of BSTFA + TMCS at 75 °C for 30 min, filtered through a 0.22 μm nylon syringe filter and 1 μL of the solution was injected into a Thermo Scientific ISQTM GC-MS for analysis. A DB-5 column (30 m × 0.25 mm × 0.25 μm) from Agilent Technologies (Wilmington, NC, USA) was used for GC separation. Oven temperature was set initially at 200 °C (0.5 min hold), then increased to 300 °C at 10 °C/min and held at 300 °C for 18 min. Helium (99.999% purity) was used as carrier gas in a constant flow of 1.2 mL/min and the injection volume was 1 μL with an autosampler in split mode (split ratio was set at 100:1). The temperatures of injector, transfer line and ion source were set at 280, 280 and 250 °C, respectively.

Total flavonoid

Total flavonoid contents of the extracts were analysed using a aluminum chloride colorimetric method. Briefly, approximately 500 mg of the extracts were individually extracted by 5 mL of methanol, and the top layer was transferred into a 10 mL brown volumetric flask. 1 mL of $AlCl_3$ solution (5%) was added and the volume was adjusted to the mark with methanol. The mix was incubated in darkness for 20 min and the absorbance at 415 nm was measured. The amount of total flavonoid content was calculated by plotting a calibration curve with quercetin as a reference standard.

Total carotenoid

Total carotenoid contents of the extracts were measured using a colorimetric method with modifications. Briefly, approximately 200 mg of the extracts were individually weighted in a 25 mL brown volumetric flask and dissolved with dichloromethane to the volume. Then the mixed solution was properly diluted, and the absorbance at 450 nm was measured. The amount of total carotenoid content was calculated by plotting a calibration curve with β-carotene as a reference standard.

Total polyphenol

The solid phase extraction and Folin-Ciocalteu method were used for total polyphenol determination. Briefly, 1 g of the extracts were individually weighted in a 10 mL glass tube. 6 mL of n-hexane were added and the mixture was loaded on a 500 mg Sepax Generik Diol tube (Sepax Technologies, Inc., Newark, DE, USA), which was precondition with 6 mL of methanol and 6 mL of n-hexane. Next, the SPE column was purified with 2 × 3 mL of n-hexane and 4 mL of n-hexane/ethyl acetate (v/v=9:1). At last, 10 mL of methanol were added and collected, and 3 mL of this solution was mixed with 0.5 mL of Folin-Ciocalteu reagent before adding 1 mL of sodium carbonate solution (10%). Water was added to bring the final volume to 10 mL. The absorbance was read at 765 nm after 2 h reaction in dark place. The amount of total polyphenol content was calculated by plotting a calibration curve with gallic acid as a reference standard.

In vitro antioxidant capacity

In order to evaluate the free radical scavenging capacities of the samples, 4 g of the extracts were individually mixed with 5 mL of methanol, the mixture was vigorously stirred for 20 min in darkness, then centrifuged at 4500 rpm for 5 min. The supernatant was carefully transferred into a 30 mL brown vial, and the extraction process was repeated three times with 5 mL of methanol each. All the methanolic sample extracts were individually collected together and stored at -20 °C until analysis.

Four kinds of commonly authorized and used methods (DPPH, FRAP (ferric reducing antioxidant potential), ABTS and ORAC (oxygen radical absorption capacity)) were selected and assessed in this study. The absorbance recorded were translated into the antioxidant activity using Trolox (a water-soluble analogue of vitamin E) worked as a reference standard, and the final results were expressed as the TEAC (antioxidant equivalent to Trolox) content, namely μmol TE/100 g.

DPPH radical scavenging capacity

2 mL of the diluted methanolic sample extract and 3 mL of DPPH reagent (0.1 mmol/L) were added to a centrifuge tube. The mixture was shaken for 1 min and placed in darkness for 2 h at room temperature after

which the absorbance was determined against a reagent blank at 517 nm using an UV1902 spectrophotometer (Shanghai Aucy Technology Instrument Co., Ltd, Shanghai, China) with a 1 cm quartz cell. The DPPH radical scavenging capacity of the sample solution was expressed as inhibition percentage and calculated using the following formula: inhibition percentage (%) = [1 - (A517 of sample)/(A517 of blank)] × 100%.

Ferric reducing ability

FRAP reagent contained 25 mL of acetate buffer (0.1 mol/L, pH 3.6), 2.5 mL of TPTZ solution (10 mmol/L) dissolved in 40 mmol/L HCl and 2.5 mL of $FeCl_3$ solution (20 mmol/L) was daily prepared and warmed at 37 °C. Next, 0.3 mL of the diluted methanolic extract and 3 mL of the FRAP reagent were added to a 10 mL volumetric flask and made up to volume with distilled water. Then, the blue solution was kept at room temperature for 10 min and centrifuged at 10,000 rpm for 10 min, the supernatant was carefully transferred into a 10 mL brown vial. The absorbance was measured at 593 nm against a reagent blank using an UV1902 spectrophotometer with a 1 cm quartz cell. The difference between sample absorbance and blank absorbance was calculated and used to determine the FRAP radical scavenging capacity.

ABTS radical scavenging capacity

ABTS working solution contained 25 mL of ABTS reagent (7 mmol/L) and 440 μL of potassium persulfate solution (2.45 mmol/L) was daily prepared. The mixture was placed in darkness for 12-16 h, then diluted with methanol to the absorbance of 0.7 ± 0.02 at 734 nm before used. The final reaction solution was consisted of 20 μL of the methanolic extract and 3 mL of the ABTS working solution. The mixture was shaken for 1 min and placed in darkness for 20 min at room temperature after which the absorbance was determined against a reagent blank at 734 nm using an UV1902 spectrophotometer with a 1 cm quartz cell. The ABTS radical scavenging capacity of the sample solution was expressed as inhibition percentage and calculated using the following formula: inhibition percentage (%) = [1 - (A734 of sample)/(A734 of blank)] × 100%.

ORAC assay

150 μL of FL reagent (0.0816 μmol/L) in 75 mmol/L phosphate buffer (pH = 7.4) and 25 μL of the diluted methanolic extracts were added into the transparent 96-wells ELISA plate. The mixture was shaked for 2 min and kept at 37 °C in the darkness for 10 min, and the reaction was initiated by addition of 25 μL of AAPH solution (153 mmol/L). The fluorescence decay was measured at 37 °C every 3 min at 525 nm emission and 485 nm excitation wavelengths, using a Thermo Scientific™ Multiskan™ GO fluorescence spectrophotometer (Thermo Scientific, Austin, TX, USA). The area under the curve (AUC) was calculated for each sample by integrating the relative fluorescence curve. The net AUC of the targets were obtained by subtracting the AUC of

the blank. The calculation equation obtained from the correlation between the net AUC and Trolox standard was calculated and used to determine the ORAC radical scavenging capacity.

Data analysis

All the sample preparation and analysis were performed in triplicate, and results were reported as means ± standard deviations (SD). Statistical analysis was performed using Origin 9.0 and SPSS 20.0. Data were compared by one-way analysis of variance (ANOVA), and significant differences between the variables of the extracts were determined by Duncan's multiple-range tests at $P<0.05$.

Table S1 Fatty acid and sn-2 fatty acid compositions of the SB whole berry, pulp and seed extracts

Item	Fatty acid (%)			Sn-2 fatty acid (%)		
	Whole berry	Pulp	Seed	Whole berry	Pulp	Seed
$C_{14:0}$	0.34 ± 0.01^{a}	0.35 ± 0.01^{a}	0.10 ± 0.01^{b}	N. D.^{a}	N. D.^{a}	N. D.^{a}
$C_{15:0}$	0.09 ± 0.01^{a}	0.09 ± 0.01^{a}	0.14 ± 0.01^{b}	N. D.^{a}	N. D.^{a}	N. D.^{a}
$C_{16:0}$	28.65 ± 1.16^{a}	32.63 ± 1.16^{b}	8.63 ± 0.41^{c}	2.48 ± 0.78^{a}	2.86 ± 0.40^{b}	1.45 ± 0.30^{c}
$C_{16:1}$ (n7)	27.07 ± 1.23^{a}	28.97 ± 1.16^{a}	0.50 ± 0.03^{b}	36.10 ± 3.63^{a}	37.35 ± 2.59^{a}	0.46 ± 0.01^{b}
$C_{18:0}$	1.68 ± 0.01^{a}	1.20 ± 0.03^{b}	2.56 ± 0.01^{c}	1.37 ± 0.15^{ab}	1.65 ± 0.36^{a}	1.17 ± 0.25^{b}
$C_{18:1}$ (n9)	22.51 ± 1.11^{a}	23.53 ± 1.33^{a}	21.65 ± 1.07^{a}	40.26 ± 4.05^{a}	43.78 ± 0.64^{a}	23.03 ± 0.11^{b}
$C_{18:1}$ (n7)	7.57 ± 0.05^{a}	7.24 ± 0.38^{a}	1.88 ± 0.05^{b}	6.30 ± 0.28^{a}	6.31 ± 0.35^{a}	N. D.^{b}
$C_{18:2}$ (n6)	7.25 ± 0.05^{a}	3.91 ± 0.01^{b}	36.63 ± 0.01^{c}	9.49 ± 0.86^{a}	5.18 ± 0.05^{b}	45.10 ± 0.18^{c}
$C_{18:3}$ (n3)	4.60 ± 0.05^{a}	1.86 ± 0.01^{b}	27.52 ± 0.01^{c}	4.00 ± 0.44^{a}	2.87 ± 0.07^{b}	28.79 ± 0.25^{c}
$C_{20:1}$ (n9)	0.15 ± 0.01^{a}	0.15 ± 0.05^{a}	0.22 ± 0.02^{b}	N. D.^{a}	N. D.^{a}	N. D.^{a}
$C_{20:3}$ (n3)	0.09 ± 0.01^{a}	0.07 ± 0.01^{a}	0.17 ± 0.01^{b}	N. D.^{a}	N. D.^{a}	N. D.^{a}
SFA	30.76 ± 2.24^{a}	34.27 ± 2.13^{a}	11.45 ± 1.01^{b}	3.85 ± 0.52^{a}	4.50 ± 0.76^{a}	2.62 ± 0.55^{b}
MUFA	57.30 ± 3.38^{a}	59.89 ± 4.16^{a}	24.24 ± 2.03^{b}	82.66 ± 2.92^{a}	87.44 ± 0.88^{b}	23.49 ± 0.11^{c}
PUFA	11.94 ± 1.09^{a}	4.84 ± 0.52^{b}	64.32 ± 5.03^{c}	13.49 ± 1.31^{a}	8.05 ± 0.12^{b}	73.89 ± 0.44^{c}

$C_{14:0}$, myristic acid; $C_{15:0}$, pentadecanoic acid; $C_{16:0}$, palmitic acid; $C_{16:1}$ (n7), palmitoleic acid; $C_{18:0}$, stearic acid; $C_{18:1}$ (n9), oleic acid; $C_{18:1}$ (n7), vaccenic acid; $C_{18:2}$ (n6), linoleic acid; $C_{18:3}$ (n6), γ-linolenic acid; $C_{20:1}$ (n9), eicosaenoic acid; $C_{20:3}$ (n3), eicosatrienoic acid; SFA, saturated fatty acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid

Values of individual fatty acid and sn-2 fatty acid percentages in the same row with different letters are significant difference at $P<0.05$

Values of fatty acid and sn-2 fatty acid percentages are expressed as mean ± sd (n=3)

N. D., not detected

Table S2 Triglyceride profile of the SB whole berry, pulp and seed extracts measured by UPLC-Q-TOF/MS

Item	Mass ($[M + NH_4]^+$) (m/z)	$[DAG]^+$ fragment ions (m/z)	Triglyceride (%)		
			Whole berry	Pulp	Seed
PoOPo	846.68	575.5, 547.5	13.44 ± 0.98[a]	13.67 ± 1.05[a]	0.58 ± 0.01[b]
OPoO	874.78	575.5, 603.5	10.69 ± 0.85[a]	12.73 ± 0.92[a]	6.99 ± 0.54[b]
OLO	900.79	601.5, 603.5	3.17 ± 0.02[a]	1.29 ± 0.01[b]	12.50 ± 0.96[c]
OPoL	872.68	573.5, 601.5	1.69 ± 0.01[a]	N. D.[b]	10.31 ± 0.82[c]
LLnL	894.69	597.5, 599.5	1.58 ± 0.01[a]	N. D.[b]	10.52 ± 0.61[c]
LLnO	896.69	599.5, 601.5	1.76 ± 0.01[a]	N. D.[b]	12.76 ± 0.47[c]
LPoL	870.68	573.5, 599.5	1.98 ± 0.01[a]	1.11 ± 0.01[b]	7.98 ± 0.69[c]
LOL	898.69	601.5, 599.5	2.67 ± 0.02[a]	N. D.[b]	15.95 ± 2.54[c]
LnPoLn	866.68	571.5, 595.5	0.34 ± 0.01[a]	N. D.[b]	5.79 ± 0.17[c]
LnLLn	892.69	597.5, 595.5	0.48 ± 0.01[a]	N. D.[b]	6.82 ± 0.65[c]
POPo	848.68	575.5, 549.5	17.64 ± 2.19[a]	18.96 ± 1.38[a]	2.50 ± 0.01[b]
PoPPo	820.67	549.5, 547.5	11.47 ± 1.33[a]	12.86 ± 1.11[a]	N. D.[b]
OSO	904.79	605.5, 603.5	2.98 ± 0.01[a]	3.23 ± 0.28[a]	N. D.[b]
OLS	902.79	601.5, 603.5	3.98 ± 0.02[a]	4.23 ± 0.36[a]	N. D.[b]
OPO	876.78	577.5, 603.5	13.58 ± 0.69[a]	16.62 ± 1.44[b]	5.13 ± 0.21[c]
POP	850.78	577.5, 551.5	10.47 ± 0.37[a]	12.46 ± 0.58[b]	1.43 ± 0.01[c]
PSO	878.78	577.5, 605.5	2.08 ± 0.01[a]	2.84 ± 0.02[b]	0.74 ± 0.01[c]
UUU			37.80 ± 3.55[a]	28.80 ± 2.94[b]	90.20 ± 4.55[c]
SUU			49.65 ± 5.74[a]	55.76 ± 3.77[a]	7.63 ± 0.49[b]
SSU			12.55 ± 1.68[a]	15.30 ± 1.19[b]	1.88 ± 0.01[c]

Po, palmitoleic; P, palmitic; S, stearic; O, oleic; L, linoleic; Ln, linolenic; UUU, triunsaturated triacylglycerol; SUU, diunsaturated triacylglycerol; SSU, monounsaturated triacylglycerol

Values of triglyceride percentages in the same row with different letters are significant difference at $P<0.05$

Values of triglyceride percentages are expressed as mean ± sd (n=3)

Regiospecificity of the fatty acid in the triglyceride is not determined

N. D., not detected

Table S3 Phytochemical content of the SB whole berry, pulp and seed extracts

Item	Mean ± SD (n=3)		
	Whole berry	Pulp	Seed
Tocopherol (mg/100g)			
α-tocopherol	143.25 ± 2.61[a]	164.95 ± 1.16[b]	124.60 ± 3.26[c]
α-tocotrienol	25.04 ± 0.19[a]	28.57 ± 0.84[b]	22.98 ± 2.73[a]
γ-tocopherol	38.67 ± 1.13[a]	25.49 ± 0.24[b]	88.63 ± 0.52[c]
Sum	206.96 ± 4.28[a]	219.02 ± 0.57[b]	236.21 ± 6.51[c]
Phytosterol (mg/100g)			
Campesterol	22.17 ± 0.45[a]	18.23 ±0 .53[b]	28.09 ± 1.41[c]
β-Sitosterol	579.43 ± 18.62[a]	398.26 ± 16.29[b]	749.47 ± 18.81[c]
Δ^5-Avenasterol	32.45 ± 0.69[a]	N. D.[b]	229.80 ± 16.52[c]
Cycloartenol	112.62 ± 3.11[a]	147.95 ± 12.78[b]	103.16 ± 9.79[a]
Gramisterol	3.86 ± 0.10[a]	N. D.[b]	31.16 ± 19.57[c]
Others	111.11 ± 6.57[a]	147.80 ± 42.95[b]	108.11 ± 6.43[a]
Sum	861.64 ± 24.74[a]	712.23 ± 27.46[b]	1249.78 ± 28.53[c]
Flavonoid (mg QE/100g)	40.18 ± 0.14[a]	46.30 ± 1.16[b]	3.80 ± 0.10[c]
Carotenoid (mg/100g)	204.58±1.99[a]	215.34±2.44[b]	15.83±0.22[c]
Polyphenol (mg GAE/kg)	329.26 ± 2.79[a]	426.74 ± 3.16[b]	71.22 ± 0.15[c]

Values of phytochemical contents in the same row with different letters are significant difference at $P<0.05$

N. D., not detected

Table S4 In *vitro* antioxidant capacity of the SB whole berry, pulp and seed extracts

In *vitro* antioxidant capacity	Mean ± SD (n=3)		
(μmol TE/100 g)	Whole berry	Pulp	Seed
ORAC	8639.25 ± 39.36[a]	6253.99 ± 62.52[b]	11033.23 ± 146.57[c]
ABTS	869.42 ± 19.64[a]	1249.38 ± 8.71[b]	633.85 ± 5.09[c]
FRAP	308.47 ± 9.62[a]	496.79 ± 15.92[b]	160.51 ± 1.76[c]
DPPH	193.42 ± 6.71[a]	279.75 ± 10.03[b]	158.01 ± 3.99[c]

Values of in *vitro* antioxidant capacities in the same row with different letters are significant difference at $P<0.05$

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