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Comparative assessment of phytochemical profiles, antioxidant and antiproliferative activities of Sea buckthorn (*Hippophaë rhamnoides* L.) berries

Ruixue Guo [a], Xinbo Guo [a,b,*], Tong Li [b], Xiong Fu [a], Rui Hai Liu [b,*]

[a] School of Food Science and Engineering, South China University of Technology, Guangzhou 510640, China
[b] Department of Food Science, Cornell University, Ithaca, NY 14853, United States

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ABSTRACT

Phytochemical profiles, antioxidant and antiproliferative activities of berry extracts were evaluated and compared in four subspecies of Sea buckthorn (*Hippophaë rhamnoides* L.). Among the subspecies, *Hippophaë rhamnoides* L. subsp. *sinensis* exhibited highest total phenolics content (38.7 ± 1.3 mg GA equiv./g DW) and corresponding total antioxidant activity. Whereas maximum cellular antioxidant and antiproliferative activities were determined in *Hippophaë rhamnoides* L. subsp. *yunnanensis*. Total antioxidant activity was significantly associated to total phenolics, isorhamnetin-3-rutinoside and isorhamnetin-3-glucoside. The cellular antioxidant activity and antiproliferative activity of phytochemicals were fairly correlated to phenolic acids and flavonoid aglycones. Lower median effective dose (EC_{50}) of individual compounds against human liver cancer HepG2 cells proliferation studies confirmed the better correlation between antiproliferative activity of Sea buckthorn extracts and flavonoid aglycones, including isorhamnetin, quercetin and kaempferol.

1. Introduction

Previous epidemiological studies have shown that significant associations among additive and synergistic interactions of phytochemicals especially phenolics and flavonoids contributed to consumers health and well-being (Liu, 2003). Advice to consumers is that a diet rich in bioactive compounds from a wide variety of foods may help in the reduction of risks associated with major chronic diseases, such as cardiovascular disease, cancer, diabetes and age-related function decline (Liu, 2013a). Like other food resources, berries have been reported as a rich source of phenolics, flavonoids, phenolic acids, anthocyanins and tannins. These naturally occurring bioactive constituents are powerful antioxidant, anticancer, anti-aging, antimicrobial, anti-inflammatory and anti-neurodegenerative ingredients (Liu, 2013b; Nile & Park, 2014).

Sea buckthorn *(Hippophaë rhamnoides* L.), is a deciduous shrub belong to botanical family Elaeagnaceae. It is wildly distributed in Asia and Europe as a pioneer plant used in water and soil conservation, and for land reclamation because of nitrogen-fixing root nodules (Khan, Akhtar, & Mahmood, 2010). Sea buckthorn berries are widely used as functional food supplement, source of jam and food coloring material (i.e. "Sea buckthorn yellow" pigment obtained from berries after juice extraction) in food industry (Beveridge, Li, Oomah, & Smith, 1999).

Sea buckthorn berries are rich in natural antioxidants including phenolics, flavonoids, ascorbic acid, tocopherols, fatty acids, carotenoids and organic acids (Tiitinen, Hakala, & Kallio, 2005). The berries' extract has been utilized for nutritional and medicinal purposes for centuries in Asia and Russia, such as nutraceuticals, cosmeceuticals and marketed herbal dietary supplements for prevention of cardiovascular and cerebrovascular diseases (Bal, Meda, Naik, & Satya, 2011; Beveridge et al., 1999; Xu, Kaur, Dhillon, Tappia, & Dhalla, 2011). Recently, research was mostly focused on the identification of compounds in Sea buckthorn extracts. The main identified components are ascorbic acid, carotenoids and various phenolics, including proanthocyanidins, gallic acid, ursolic acid, caffeic acid, cumaric acid, ferulic acid, catechin and epicatechin derivatives, quercetin, kaempferol, and isorhamnetin glycoside derivatives (Arimboor, Kumar, & Arumughan, 2008; Bal et al., 2011; Ma et al., 2016; Teleszko, Wojdylo, Rudzinska, Oszmianski, & Golis, 2015). *In vitro* antioxidant activity was reported to be closely related to the high content of ascorbic acid and total phenolics (Gao, Ohlander, Jeppsson, Björk, &

* Corresponding authors at: School of Food Science and Engineering, South China University of Technology, Guangzhou 510640, China (X. Guo), Department of Food Science, Cornell University, Ithaca, NY 14853, United States (R.H. Liu).
E-mail addresses: xbg720@gmail.com (X. Guo), rl23@cornell.edu (R.H. Liu).

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Trajkovski, 2000; Kim, Kwon, Sa, & Kim, 2011; Rosch, Bergmann, Knorr, & Kroh, 2003), whereas the anticancer activity has been explored towards human liver cancer cells, breast cancer cells and colon cells and the effects were dramatically diversified depending on different composition of extracts (Grey, Widen, Adlercreutz, Rumpunen, & Duan, 2010; Olsson, Gustavsson, Andersson, Nilsson, & Duan, 2004).

Like other plant species, genetic variation, growth condition, degree of maturity and harvesting season can affect on phytochemical concentration, *in vitro* and *in vivo* activities in Sea buckthorn (Gao et al., 2000; Zheng, Kallio, & Yang, 2016). Though, phytochemical composition and *in vitro* antioxidant activity have been reported in Sea buckthorn, little is know about the cellular antioxidant and the antiproliferative activity of phytochemical extracts especially the phenolic fraction. The association among bioactivities of total phenolics and identified compounds has not been investigated. Furthermore, there is a scarcity of literature on the comparison of phytochemicals and their bioactivities from different subspecies of Sea buckthorn. Thus, the present work is aimed to make a comprehensive comparison of the phytochemical composition of four different subspecies of Sea buckthorn and to link these findings with extracellular and cellular antioxidant activity and antiproliferative activity against human liver cancer cells HepG2 combined with a correlation analysis.

2. Materials and methods

2.1. Chemicals and reagents

Quercetin (QE), gallic acid (GA), Folin-Ciocalteu reagent, 2,2′-azobis-amidinopropane (ABAP), and dichlorofluorescin diacetate (DCFH-DA) were purchased from Sigma-Aldrich Inc. (St. Louis, USA). Protocatechuic acid (PA), ferulic acid (FA), epicathin (Epi), catechin (CE), QE glycosides, isorhamnetin (IS) glycosides and kaempferol (KA) glycosides were purchased from Weikeqi Biological (Chengdu, China). Human liver cancer cells HepG2 were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). Williams' medium E (WME), fetal bovine serum and other cell culture reagents were purchased from Gibico U.S. Biotechnology Co. All the other chemicals and solvents were of analytical grade.

2.2. Sample preparation

Four subspecies of Sea buckthorn *(Hippophaë rhamnoides* L.), viz: *H. rhamnoides* L. subsp. *sinensis* (Sinensis), *H. rhamnoides* L. subsp.*yunnanensis* (Yunnanensis), *H. rhamnoides* L. subsp. *mongolica* (Mongolica) and *H. rhamnoides* L. subsp. *turkestanica* (Turkestanica) were used in the present study. The berries of all four subspecies of Sea buckthorn were supplied by the Northwest Institute of Plateau Biology, Chinese Academy of Sciences. Berries were washed in running tap water and freeze-dried before extraction.

2.3. Extraction of free and bound phytochemicals

Phytochemicals of Sea buckthorn berries were extracted using method reported previously by Guo, Li, Tang, and Liu (2012) with modification. Berries were skimmed before extraction, and 80% acetone was applied to extract the free phytochemicals. All extracts were reconstituted using 10% methanol. For the bound fraction, 4 M NaOH was added into the dry residua for digestion. The mixture was acidified to pH 2 using concentrated hydrochloric acid, and supernatants were re-extracted by ethyl acetate. The ethyl acetate fraction was evaporated to dryness, followed by the addition of 10% methanol to reconstitute bound phytochemicals. Both extractions were stored at −40 °C for further analysis.

2.4. Determination of total phenolics

A Folin-Ciocalteu colorimetric method was adopted to determine the total phenolics following the method explained previously (Liu & Sun, 2003; Zhang & Liu, 2015). Data is expressed as milligram gallic acid equivalent per gram of dry weight of berries (mg GA equiv./g DW) in triplicate.

2.5. Determination of total flavonoids

The flavonoids in free and bound fractions were determined by the borohydride/chloranil protocol (SBC) as reported before (He, Liu, & Liu, 2008). Final values were reported in milligram catechin equivalent per gram of dry weight (mg catechin equiv./g DW) of berries by measuring the absorbance at 490 nm using a UV Visible Spectrophotometer.

2.6. Determination of phytochemical composition by RP-HPLC

The phytochemical composition of Sea buckthorn was assessed by RP-HPLC technique using a Waters 2998 Photodiode Array Detector (Waters Co., USA) at 370 nm and 280 nm wavelengths with a C18 column (250 × 4.6 mm, 5 μm) maintained at 35 °C (Ma et al., 2016; Teleszko et al., 2015). The flow rate of the binary elution phase (A: 0.1% trifluoroacetic acid in water, B: 50% acetonitrile-49.8% water-0.2% trifluoroacetic acid) was 1.0 mL/min using gradient elution as follows: 0–5 min (95% A), 5–40 min (95–75% A), 40–47 min (75–62% A), 47–49 min (62–55% A), 49–51 min (55% A), 51–70 min (55–20% A), 70–75 min (20–5% A), 75–77 min (5–95% A), 77–90 min (95% A). Measured values were expressed as milligrams per 100 g of dry weight of berries (mg/100 g DW).

2.7. Quantification of in vitro antioxidant activity

The total antioxidant activity were evaluated by the oxygen radical absorbance capacity (ORAC) and the peroxyl radical scavenging capacity (PSC) assays as described previously (Adom & Liu, 2005; Huang, Ou, Hampsch-Woodill, Flanagan, & Prior, 2002). ORAC value was expressed as micromoles of Trolox equivalent per gram of dry weight of berries (μmol Trolox equiv./g DW), whereas to determine PSC, vitamin C was employed as calibration standard. Results were expressed as micromoles of vitamin C (Vit. C) equivalent per gram of dry weight of berries (μmol Vit. C equiv./g DW).

2.8. Cell culture

Human liver cancer cells HepG2 were cultured in WME medium supplemented with 5% fetal bovine serum, 10 mM Hepes, 2 mM L-glutamine, 5 μg/mL insulin, 0.05 μg/mL hydrocortisone, 50 units/mL penicillin, 50 μg/mL streptomycin and 100 μg/mL gentamycin as described previously (Liu et al., 1994). HepG2 cultures were maintained at 37 °C in humidified atmosphere of 5% CO_2.

2.9. Cellular antioxidant activity of phytochemical extracts of Sea buckthorn

The cellular antioxidant activity (CAA) assay was used to quantify the cellular antioxidant capacity of Sea buckthorn berry extracts as explained previously (Wolfe & Liu, 2007). The CAA

value was presented as micromoles of quercetin equivalent per 100 g of dry weight of berries (μmol quercetin equiv./100 g DW).

2.10. Cytotoxicity and antiproliferative activity assays

Methylene blue colorimetric method was adopted to study the antiproliferative effects of phytochemicals towards human cancer cells HepG2 following the method reported previously (Felice, Sun, & Liu, 2009). In antiproliferation test, cells were seeded at a density of 2.5×10^4 per well using 100 μL of diluted extracts in growth medium at various concentrations for 72 h. Subsequently, the viable cell numbers were computed by the methylene blue assay. For the cytotoxicity evaluation, cells were seeded at a density of 4.0×10^4 per well in the berry extracts for 24 h, then stained for viable number counting (Yoon & Liu, 2008). The median effective dose (EC_{50}) of cell proliferation and half maximal cytotoxicity concentration (CC_{50}) were used to express the antiproliferative activity and cytotoxicity of phytochemicals.

2.11. Statistical analysis

Data were presented as mean ± standard deviation (SD) for triplicate analysis and statistical analyzed by IBM SPSS statistical software 21.0 (SPSS Inc., Chicago, IL) with p-value < 0.05 considered significant in comparison between two experimental groups. Correlation coefficients between bioactivities and identified phytochemical constituents were evaluated by Pearson's correlation.

3. Results and discussion

3.1. Phenolic and flavonoid contents in subspecies of Sea buckthorn

Table 1 demonstrated that the average contents of total, free and bound phenolics in four subspecies were 32.6 ± 4.5, 32.3 ± 4.5 and 0.32 ± 0.07 mg GA equiv./g DW, respectively. Subspecies Sinensis depicted the highest total and free phenolics, followed by Yunnanensis, Mongolica and Turkestanica, ranging from 27.6 ± 1.9 to 38.7 ± 1.3, and 27.2 ± 1.9 to 38.4 ± 1.4 mg GA equiv./g DW, respectively. However, Turkestanica was found rich in bound phenolics (0.41 ± 0.03 mg GA equiv./g DW) compared to corresponding subspecies. In addition, there were significant differences among the Sinensis, Yunnanensis and Turkestanica in free and total phenolic contents (p < 0.05), while no significant difference was noted in the measured values of bound phenolics in all subspecies. Low concentration of bound phenolics indicated their less contribution to total phenolics at an average of 1%, whereas free phenolic fraction contributed about 99% to total phenolics.

General trend of the flavonoid contents in Sea buckthorn subspecies was almost similar to phenolics (Table 1). Highest levels of total flavonoid content were found in Sinensis, followed by Yunnanensis and Mongolica, with the lowest in Turkestanica, ranging from 34.9 ± 1.2 to 51.5 ± 0.9 mg catechin equiv./g DW. Comparatively, the average concentration of total flavonoids fraction was 44.6 ± 6.7 mg catechin equiv./g DW, followed by free (44.3 ± 6.6 mg catechin equiv./g DW) and bound fractions (0.35 ± 0.10 mg catechin equiv./g DW). The total, free and bound flavonoid contents in Sinensis were 51.5 ± 0.9, 51.0 ± 0.8 and 0.46 ± 0.06 mg catechin equiv./g DW, respectively, whereas Turkestanica exhibited the lowest concentration of free and total flavonoids except bound fraction. Additionally, total flavonoid contents were significantly different (p < 0.05) in Sinensis, Mongolica and Turkestanica. The average contribution of bound fraction to total flavonoids was around 8.3%. This indicated that bound fractions in all subspecies were much lower compared to the free fractions. The percentage contribution of total flavonoids to phenolics ranged from 74.1 to 85.0% with an average of 80.5%, indicating flavonoids as the main component.

3.2. Quantification of phytochemical composition in four Sea buckthorn subspecies

It was reported previously that isorhamnetin-3-*O*-rutinoside (I3R), isorhamnetin-3-*O*-glucoside (I3G), quercetin-3-*O*-rutinoside (Q3R), quercetin-3-*O*-glucoside (Q3G), isorhamnetin-3-*O*-glucoside-7-*O*-rhamnoside (I-3-G-7-Rh), isorhamnetin-3-*O*-sophoroside-7-*O*-rhamnoside (I-3-S-7-Rh), kaempferol-3-*O*-sophoroside-7-*O*-rhamnoside (K-3-S-7-Rh), IS, QE, KA, GA, FA, PA, Epi and CE were the main compounds in Sea buckthorn (Arimboor et al., 2008; Ma et al., 2016; Rosch et al., 2003; Teleszko et al., 2015). Thus, the contents of them were examined in the phytochemical extracts using standard calibration.

A total of fifteen phenolic compounds (Table 2), classified into four categories, phenolic acids, flavones, flavonoid-monoglycosides and flavonoid-diglycosides were found only in the free fractions of all Sea buckthorn subspecies, using RP-HPLC technique. Among these, total flavonoid-diglycosides were dominant with 233 ± 46 mg/100 g DW, followed by total flavonoid-monoglycosides, phenolic acids and flavones (147 ± 24, 62.9 ± 23.4 and 30.9 ± 5.5 mg/100 g DW, respectively). I-3-G-7-Rh was the highest average component (148 ± 29 mg/100 g DW), followed by I3R, K-3-G-S-7-Rh and I-3-S-7-Rh (58.6 ± 16.5, 45.0 ± 11.6 and 39.7 ± 22.6 mg/100 g DW, respectively).

As presented in Table 2, the concentration of I-3-G-7-Rh was varied from 112 ± 6 (in Mongolica) to 187 ± 10 mg/100 g DW (in Yunnanensis) with significant difference (p < 0.05). These values were comparatively higher than reported in methanol-water extracts of Sea buckthorn (Yang, Halttunen, Raimo, Price, & Kallio, 2009). Compound K-3-S-7-Rh was maximum in Turkestanica (61.6 ± 1.2 mg/100 g DW), while minimum in Sinensis (34.1 ± 2.7 mg/100 g DW) (p < 0.05). This compound was verified in Sea buckthorn berries (Rosch, Krumbein, Mugge, & Kroh, 2004) while was quantified for the first time in the present study. Present concentration of I-3-S-7-Rh was ranged from 15.2 ± 0.7 (in Mongolica) to 74.6 ± 1.5 mg/100 g DW (in Yunnanensis) (p < 0.05).

Table 1
Total phenolic, total flavonoid contents and percentage contribution of free and bound fractions to the total (mean ± SD, n = 3).

	Phenolics (mg GA equiv./g DW)			Flavonoids (mg catechin equiv./g DW)		
Subspecies	Free	Bound	Total	Free	Bound	Total
Sinensis	38.4 ± 1.4 a* (99.2)#	0.31 ± 0.04 e	38.7 ± 1.3 a	51.0 ± 0.8 a (99.1)	0.46 ± 0.06 d	51.5 ± 0.9 a (77.9)†
Yunnanensis	32.9 ± 2.1 b (99.0)	0.32 ± 0.01 e	33.2 ± 2.1 b	47.4 ± 3.6 ab (99.2)	0.37 ± 0.05 d	47.7 ± 3.6 ab (84.8)
Mongolica	30.7 ± 2.4 bc (99.2)	0.24 ± 0.03 e	30.9 ± 2.4 bc	44.2 ± 3.2 b (99.5)	0.21 ± 0.03 d	44.4 ± 3.2 b (85.0)
Turkestanica	27.2 ± 1.9 d (98.5)	0.41 ± 0.03 e	27.6 ± 1.9 cd	34.5 ± 1.3 c (98.9)	0.38 ± 0.04 d	34.9 ± 1.2 c (74.1)
Average	32.3 ± 4.5	0.32 ± 0.07	32.6 ± 4.5	44.3 ± 6.6	0.35 ± 0.10	44.6 ± 6.7 (80.5)

* Values with different letters differ significantly at p < 0.05.
\# Values in parentheses indicate percentage contribution to the total.
† Values in parentheses indicate percentage contribution of flavonoids to phenolics.

Table 2
Contents of phenolics in Sea buckthorn subspecies (mg/100 g DW) (mean ± SD, n = 3).

Phenolic content (mg/100 g DW)	Subspecies				
	Sinensis	Yunnanensis	Mongolica	Turkestanica	Average
Total phenolic acids	37.9 ± 1.2 d	97.9 ± 2.8 a	69.4 ± 2.2 b	46.3 ± 2.2 c	62.9 ± 23.4
GA *(1)*	20.1 ± 1.4b	28.7 ± 2.6 a	15.1 ± 0.3 c	15.3 ± 0.7 c	19.8 ± 5.7
PA *(2)*	12.1 ± 0.1 d	64.6 ± 1.6 a	51.5 ± 1.9 b	29.3 ± 2.0 c	39.3 ± 20.2
FA *(3)*	5.72 ± 0.25 a	4.71 ± 0.23 b	2.85 ± 0.08 c	1.77 ± 0.11 d	3.76 ± 1.55
Total flavones	25.2 ± 0.3 d	39.4 ± 1.3 a	31.8 ± 0.4 b	27.3 ± 0.9 c	30.9 ± 5.5
CE *(4)*	8.18 ± 0.91 b	11.9 ± 1.1 a	8.32 ± 0.18 b	7.60 ± 0.34 b	8.99 ± 1.84
Epi *(5)*	1.70 ± 0.04 b	4.51 ± 0.19 a	0.82 ± 0.06 c	1.51 ± 0.06 b	2.14 ± 1.41
QE *(6)*	4.02 ± 0.24 c	6.38 ± 0.20 a	6.67 ± 0.15 a	4.95 ± 0.17 b	5.51 ± 7.04
KA *(7)*	1.02 ± 0.03 d	1.50 ± 0.04 a	1.30 ± 0.04 b	1.11 ± 0.02 c	1.23 ± 0.19
IS *(8)*	10.3 ± 0.5 c	15.1 ± 0.2 a	14.7 ± 0.5 a	12.2 ± 0.7 b	13.1 ± 2.0
Total flavonoid-monoglycosides	179 ± 4 a	148 ± 2 b	147 ± 5 b	113 ± 2 c	147 ± 24
Q3R *(9)*	28.8 ± 1.4 c	23.0 ± 0.8 d	44.6 ± 1.2 a	35.3 ± 1.6 b	32.9 ± 8.2
Q3G *(10)*	40.2 ± 1.4 b	49.5 ± 3.0 a	37. 8 ± 2.1 b	31.2 ± 2.9 c	39.7 ± 7.0
I3R *(11)*	84.0 ± 2.2 a	58.8 ± 1.1 b	52.9 ± 1.5 c	38.7 ± 2.3 d	58.6 ± 16.5
I3G *(12)*	26.0 ± 1.4 a	17.1 ± 1.7 b	11.4 ± 0.7 c	7.61 ± 0.40 d	15.5 ± 7.0
Total flavonoid-diglycosides	212 ± 4 b	311 ± 12 a	208 ± 1 b	199 ± 7 b	233 ± 46
K-3-S-7-Rh *(13)*	34.1 ± 2.7 c	49.6 ± 1.4 b	34.8 ± 1.3 c	61.6 ± 1.2 a	45.0 ± 11.6
I-3-S-7-Rh *(14)*	43.7 ± 1.5 b	74.6 ± 1.5 a	15.2 ± 0.7 d	25.5 ± 0.8 c	39.7 ± 22.6
I-3-G-7-Rh *(15)*	134 ± 4 c	187 ± 10 a	158 ± 3 b	112 ± 6 d	148 ± 29
Total phenolics	454 ± 9 b	597 ± 14 a	456 ± 5 b	385 ± 9 c	473 ± 78

Values with different letters in the same row indicated a significant difference ($p < 0.05$). Where compounds 1–15 are represented as: GA: gallic acid (*1*), PA: protocatechuic acid (*2*), FA: ferulic acid (*3*), CE: catechin (*4*), Epi: epicathin (*5*), QE: quercetin (*6*), KA: kaempferol (*7*), IS: isorhamnetin *(8)*, Q3R: quercetin-3-*O*-rutinoside (*9*), Q3G: quercetin-3-*O*-glucoside (*10*), I3R: isorhamnetin-3-*O*-rutinoside (*11*), I3G: isorhamnetin-3-*O*-glucoside (*12*), K-3-S-7-Rh: kaempferol-3-*O*-sophoroside-7-*O*-rhamnoside (*13*), I-3-S-7-Rh: isorhamnetin-3-*O*-sophoroside-7-*O*-rhamnoside (*14*), I-3-G-7-Rh: isorhamnetin-3-*O*-glucoside-7-*O*-rhamnoside (*15*).

The same compound was reported between 1.1 and 13.6 mg/100 g in fresh berries of Sea buckthorn (Yang et al., 2009). However, this variation may be due to the genetic diversity, different harvesting time and analytical techniques.

The flavonoid-monoglycosides were the second highest components in the analyzed samples. I3R was dominant in this group with maximum concentration ranged from 38.7 ± 2.3 (in Turkestanica) to 84.0 ± 2.2 mg/100 g DW (in Sinensis). These values were similar as reported in other varieties of Sea buckthorn (Yang, Halttunen, Raimo, Price, & Kallio, 2009). The I3G content was ranged from 7.61 ± 0.40 to 26.0 ± 1.4 mg/100 g DW, with highest value in Sinensis and lowest in Turkestanica. These values were the same as previously reported (Grey et al., 2010). Measured level of Q3R ranged from 23.0 ± 0.8 (in Yunnanensis) to 44.6 ± 1.2 mg/100 g DW (in Mongolica) ($p < 0.05$), which was higher than other reported extraction methods (Grey et al., 2010). Compound Q3G appeared at levels of 31.2 ± 2.9 (in Turkestanica) to 49.5 ± 3.0 mg/100 g DW (in Yunnanensis). The value of this compound was 9.0 mg/L in juice of berries, and 2.4–10.0 mg/100 g in fresh berries according to the literatures (Rosch et al., 2003; Yang et al., 2009).

Five flavones were quantified in all subspecies and ranked in the order of IS, CE, QE, Epi and KA. The contents of Epi and CE ranged from 0.82 ± 0.06 (in Mongolica) to 4.51 ± 0.19 mg/100 g DW (in Yunnanensis) and 7.60 ± 0.34 (in Turkestanica) to 11.9 ± 1.1 mg/100 g DW (in Yunnanensis), respectively, compared to that of 2.8–5.2 mg/L and 19–26 mg/L based on fresh weight of berries (Rosch et al., 2003). Highest amounts of IS and KA were found in Yunnanensis (15.1 ± 0.2 and 1.50 ± 0.04 mg/100 g DW, respectively), whereas Sinensis contained lowest concentration of these compounds. The increasing order of QE content was Mongolica > Yunnanensis > Turkestanica > Sinensis.

Total phenolic acid content was highest in Yunnanensis, followed by Mongolica, Turkestanica and Sinensis. GA and PA were maximum in Yunnanensis (28.7 ± 2.6 and 64.6 ± 1.6 mg/100 g DW, respectively). The concentration of GA was lower than previous report (Gao et al., 2000). However, PA and FA contents were in agreement with reported data (Arimboor et al., 2008; Rosch et al., 2003).

On the whole, total phenolic compounds in free fractions were ranged from 385 ± 9 (in Turkestanica) to 597 ± 14 mg/100 g DW (in Yunnanensis). Flavonoid components exhibited >80% contribution, which was in accordance to the trend that flavonoids contributed to total phenolics in phytochemicals, ranging from 74% to 85% (Table 1).

3.3. Total antioxidant activity

Antioxidant and antiproliferative activities were studied in free fractions, because of the high concentration of phytochemicals compared to corresponding bound fractions. The results showing measured values of ORAC and PSC assays were given in Fig. 1. These are sensitive, rapid and precise chemical antioxidant evaluation assays, which reflect the oxygen radical absorbing and peroxyl radical scavenging capacity using dichloroflurescin diacetate as fluorescein probe to monitor reaction (Adom & Liu, 2005; Huang et al., 2002).

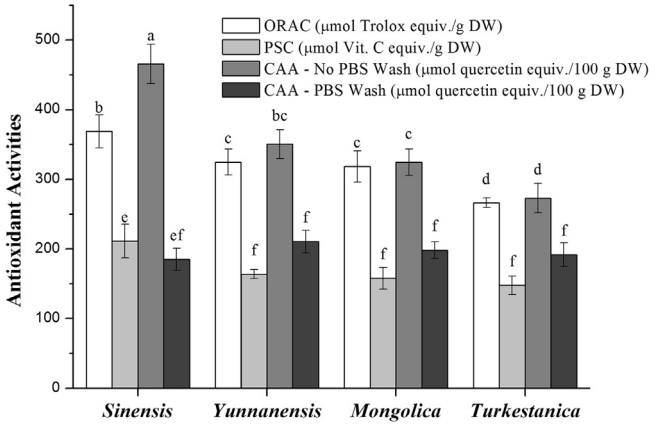

Fig. 1. ORAC, PSC and CAA values of extracts in four Sea buckthorn subspecies (mean ± SD, n = 3). Bars with different letters differ significantly at $p < 0.05$.

For the samples analyzed, the ORAC value was highest in Sinensis, followed by Yunnanensis, Mongolica and lowest in Turkestanica, ranged from 266 ± 7 to 369 ± 24 μmol Trolox equiv./g DW. Correlation analysis (Table 3) revealed that, the ORAC value was significantly associated with the total phenolics ($R^2 = 0.975$, $p < 0.05$), total flavonoids ($R^2 = 0.973$, $p < 0.05$), total flavonoid-monoglycosides ($p < 0.01$) and compound I3R ($R^2 = 0.972$, $p < 0.05$).

The PSC value was varied from 148 ± 13 to 211 ± 24 μmol Vit. C equiv./g DW with an average of 170 ± 29 μmol Vit. C equiv./g DW in all samples. Again the Sinensis exhibited maximum peroxyl radical scavenging capacity at 1.24-fold to average, followed by Yunnanensis, Mongolica and Turkestanica (0.96, 0.93 and 0.87-fold to average, respectively). According to the statistic correlation analysis, the total PSC value was also closely related to the phenolics contents ($R^2 = 0.961$, $p < 0.05$), compound I3R ($R^2 = 0.974$, $p < 0.05$) and I3G ($R^2 = 0.958$, $p < 0.05$) as shown in Table 3. The ORAC value and PSC value showed a relatively correlation with each other ($R^2 = 0.900$). The correlation analysis indicated that, phenolics and flavonoids had significant contribution to the *in vitro* antioxidant activity of phytochemicals.

3.4. Cellular antioxidant activity

The CAA assay is used to quantify the antioxidant activity for food extracts and dietary supplements at the cell level. This method includes a PBS wash protocol and a no PBS wash protocol for HepG2 cells after treated by samples to represent the complexity of biological system, including the cellular absorption, metabolism and distribution of antioxidants (Wolfe & Liu, 2007). This may lead to a good predication of antioxidant activity *in vivo*. The PBS wash protocol evaluates whether phytochemicals could easily pass through cells or not.

Regardless of whether cells were washed with PBS or not between the antioxidant and the ABAP treatment, phytochemical extracts from four subspecies could inhibit the increase emission of fluorescence due to the formation of DCF (Fig. 1). It was clear that, the subspecies Sinensis was leading with the inhibition capacity of 466 ± 28 μmol quercetin equiv./100 g DW, followed by Yunnanensis, Mongolica and Turkestanica (350 ± 21, 325 ± 19 and 273 ± 21 μmol quercetin equiv./100 g DW, respectively). Significant differences existed among Sinensis, Yunnanensis and Turkestanica subspecies without washing the cell with PBS ($p < 0.05$), but not found between Yunnanensis and Mongolica. When PBS wash protocol was followed, the average CAA values of four extracts were decreased to 197 ± 18 μmol quercetin equiv./100 g DW. CAA was highest in Yunnanensis (211 ± 16 μmol quercetin equiv./100 g DW), followed by Mongolica, Turkestanica and Sinensis (198 ± 12, 192 ± 17 and 186 ± 16 μmol quercetin equiv./100 g DW, respectively) without significant differences among subspecies.

According to the statistic analysis (Table 3), the CAA values without PBS wash were significantly correlated to the total phenolics ($R^2 = 0.994$, $p < 0.01$), total flavonoid mono-glycosides ($R^2 = 0.960$, $p < 0.05$), compound I3R ($R^2 = 0.998$, $p < 0.01$) and I3G ($R^2 = 0.985$, $p < 0.05$). The values also had significant relations to ORAC ($R^2 = 0.957$, $p < 0.05$) and PSC value ($R^2 = 0.986$, $p < 0.05$). However, when cells were washed with PBS, the CAA values didn't show any significant associations with total phenolics contents. Moreover, the CAA quality of all the subspecies was reduced but with an link to total phenolic acids ($R^2 = 0.991$, $p < 0.01$), total flavones ($R^2 = 0.993$, $p < 0.01$), identified compound PA ($R^2 = 0.968$, $p < 0.05$), KA ($R^2 = 0.993$, $p < 0.01$) and IS ($R^2 = 0.918$), which could explain the strongest cellular antioxidant activity of the Yunnanensis subspecies composed of highest phenolic acids and flavonoid aglycones than others.

Table 3
Pearson correlation coefficient (probability) among phenolics and antioxidant and antiproliferative activities.

Correlation	ORAC	PSC	CAA-No	CAA-wash	Antiproliferative activity (EC_{50})
ORAC	–	0.900	0.957*	−0.176	0.031
PSC	0.900	–	0.986*	−0.517	0.455
CAA-No	0.957*	0.986*	–	−0.375	0.300
CAA-Wash	−0.176	−0.517	−0.375	–	−0.916
EC_{50}	0.031	0.455	0.300	−0.916	–
TPC	0.975*	0.961*	0.994**	−0.268	0.198
TFC	0.973*	0.797	0.887	0.055	−0.175
TPA	−0.051	−0.422	−0.269	0.991**	−0.944
TFF	−0.059	−0.413	−0.263	0.993**	−0.919
TFM	0.999**	0.908	0.960*	−0.210	0.056
TFD	0.180	−0.069	0.064	0.851	−0.671
GA	0.383	0.215	0.327	0.649	−0.445
PA	−0.235	−0.612	−0.471	0.968*	−0.970*
FA	0.928	0.863	0.925	−0.018	0.022
CE	0.203	−0.076	0.065	0.873	−0.723
Epi	0.123	−0.022	0.080	0.738	−0.473
QE	−0.259	−0.653	−0.523	0.836	−0.954*
KA	−0.096	−0.469	−0.318	0.993**	−0.953*
IS	−0.274	−0.659	−0.524	0.918	−0.970*
Q3R	−0.336	−0.386	−0.424	−0.279	−0.026
Q3G	0.556	0.269	0.414	0.684	−0.625
I3R	0.972*	0.974*	0.998**	−0.329	0.243
I3G	0.942	0.958*	0.985*	−0.255	0.237
K-3-S-7-Rh	−0.836	−0.650	−0.707	0.219	0.112
I-3-S-7-Rh	0.353	0.253	0.344	0.557	−0.308
I-3-G-7-Rh	0.350	−0.052	0.116	0.853	−0.893

Note: The phenolic profiles are TPC (total phenolics contents), TFC (total flavonoids contents), TPA (total phenolic acids), TFF (total free flavonoid aglycones), TFM (total flavonoid-monoglycosides), TFD (total flavonoid-diglycosides) and individual phenolics.

* Correlation is significant at the 0.05 level (2-tailed).

** Correlation is significant at the 0.01 level (2-tailed).

3.5. Inhibition effects against human liver cancer HepG2 cell proliferation

The antiproliferative activity and cytotoxicity were evaluated by the methylene blue assay, and results were represented as EC_{50} and CC_{50}, separately. Fig. 2A demonstrated the proliferation of HepG2 cells was inhibited in a dose-independent manner by all extracts of all the subspecies. Sinensis, Yunnanensis and Mongolica exhibited significant difference while between Sinensis and Turkestanica no significant variation was observed. As presented in Table 4, the EC_{50} was ranged from 0.85 ± 0.07 (in Yunnanensis) to 3.31 ± 0.22 mg/mL (Sinensis) on the dry weight basis of berries, respectively. It is well known that a lower EC_{50} reflects a stronger proliferative inhibitory effect. Thus, the Yunnanensis subspecies appeared to show the strongest antiproliferative activity, followed by Mongolica, Turkestanica and Sinensis. For the cytotoxicity studies, the CC_{50} was ranged from 8.31 ± 0.86 (in Sinensis) to 16.8 ± 0.8 mg/mL (in Yunnanensis). These values were quite higher than the EC_{50}, which indicated that the anticancer activity of Sea buckthorn was attributed to the antiproliferation effects rather than the cytotoxicity of all the four subspecies.

To further explore the relations among phytochemical components and their inhibition effects, fifteen pure compounds identified in Sea buckthorn subspecies were subjected to the antiproliferation test (Table 4). In our findings, weak antiproliferative activity was observed in the flavonoid-diglycosides, K-3-S-7-Rh, I-3-S-7-Rh, and I-3-G-7-Rh (EC_{50} > 800 μM) although they exhibited large amount in extracts, the same situation as found in Epi (EC_{50} > 800 μM). However, other individual compounds showed effective inhibition of HepG2 human cancer cell proliferation (Fig. 2B, Table 4). The EC_{50} ranked in the order of IS (29.0 ± 4.1 μM), KA (57.3 ± 6.1 μM), QE (80.0 ± 3.4 μM), GA (129 ± 13 μM), Q3G (209 ± 4 μM), I3G (244 ± 5 μM), Q3R (249 ± 8 μM), FA (272 ± 9 μM), CE (311 ± 8 μM), I3R (323 ± 13 μM) and PA (349 ± 23 μM). Among them, the flavonoid aglycones, QE, KA and IS showed the highest antiproliferative activity with EC_{50} below 80 μM. Particularly, IS had extremely low EC_{50} (29.0 ± 4.1 μM). Phenolic acid GA was observed to have meaningful EC_{50} around 125 μM, compared to FA and PA. Likewise, quercetin-monoglycosides Q3R and Q3G showed strong inhibition effects when compared with the isorhamnetin-monoglycosides I3R and I3G, while comparable antiproliferative activity was observed in CE and I3R.

Correlation analysis given in Table 3, revealed good association of antiproliferative activity with the total phenolic acids (R^2 = 0.944) and total flavonoid aglycones (R^2 = 0.919) content. Furthermore, similar relations of antiproliferative activity were noted with the amount of identified compounds, like PA (R^2 = 0.970, p < 0.05), QE (R^2 = 0.954, p < 0.05), KA (R^2 = 0.953, p < 0.05) and IS (R^2 = 0.970, p < 0.05). It has been reported that anticancer activity of Sea buckthorn extracts was attributed to the high content of triterpenoids and anthocyanins components (Grey et al., 2010; Olsson et al., 2004). Although phenolic acids and flavonoid aglycones were in lower quantities in phytochemicals in Sea buckthorn extracts, they showed a significantly correlation with the antiproliferative activity. More relevantly, the molar concentrations of main effective compounds IS, KA, QE, PA, GA, FA, Q3R, Q3G, I3R and I3G (calculated in Table 2 and Table 4) in the half maximal antiproliferation concentration of extracts were in the range of 0.4–1.2 μM, 0.04–0.12 μM, 0.2–0.5 μM, 2.6–5.8 μM, 11–39 μM, 0.2–1.0 μM, 0.3–1.8 μM, 0.9–2.9 μM, 0.8–4.5 μM and 0.3–1.8 μM, respectively. And at these concentrations the inhibition effects of pure compound were rather weak (Fig. 2B), suggesting that the antiproliferative activity of extracts were not caused by a single component but by the synergistic effects of phytochemicals.

Table 4
Antiproliferative activities and cytotoxicities of samples' extracts and individual phenolic compound in Sea buckthorn towards human liver cancer cell HepG2 (mean ± SD, n = 3).

	Names	EC_{50}	CC_{50}
Subspecies mg/mL	Sinensis	3.31 ± 0.22	8.31 ± 0.86
	Yunnanensis	0.85 ± 0.07	16.8 ± 0.8
	Mongolica	1.21 ± 0.11	12.7 ± 1.0
	Turkestanica	3.03 ± 0.19	8.73 ± 0.09
Individual compound μM	GA	129 ± 13	331 ± 24
	PA	349 ± 23	386 ± 9
	FA	272 ± 9	319 ± 21
	CE	311 ± 8	520 ± 19
	QE	80.0 ± 3.4	202 ± 10
	KA	57.3 ± 6.1	98.6 ± 3.2
	IS	29.0 ± 4.1	76.2 ± 8.0
	I3R	323 ± 13	654 ± 27
	I3G	244 ± 5	734 ± 33
	Q3R	249 ± 8	602 ± 22
	Q3G	209 ± 4	399 ± 16

*where EC_{50} is the median effective dose of cell proliferation and, while CC_{50} refers to the half maximal cytotoxicity concentration. The concentration of extracts in different subspecies is expressed as mg/mL based on the dry weight of berries, and the effects of identified individual compounds are expressed on the basis of molar concentration (μM) of identified individual compound.

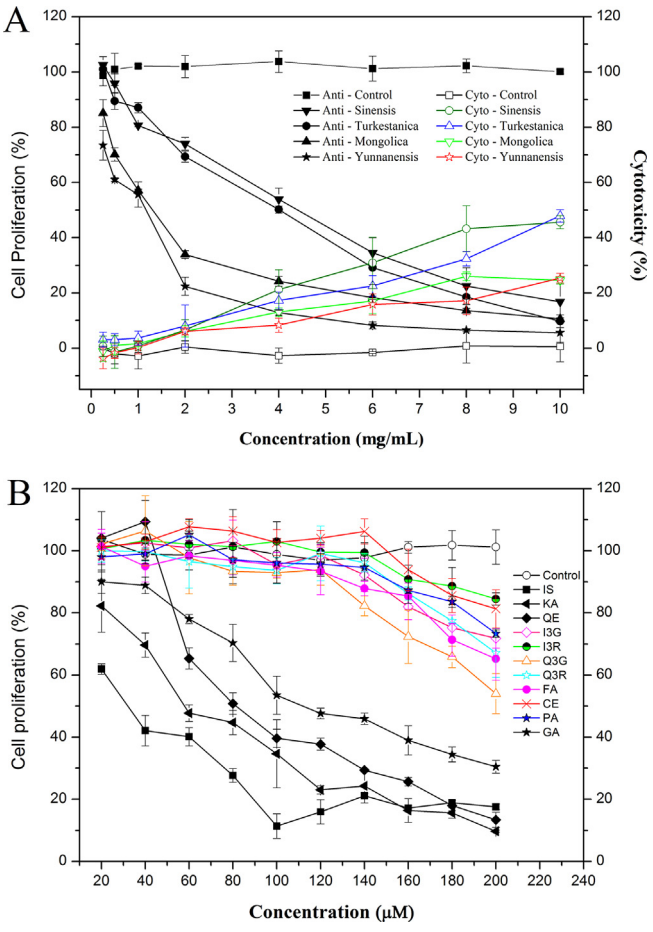

Fig. 2. Antiproliferative activity of extracts and individual phenolic compounds in four Sea buckthorn subspecies towards human liver cancer cell HepG2 (mean ± SD, n = 3).

4. Conclusion

The present study revealed that four subspecies of Sea buckthorns were rich in phenolics and flavonoids along with potential antioxidant and antiproliferative activities. Comparatively, Sinensis subspecies had the highest phytochemical contents with significant oxygen and peroxyl radical scavenging activity, while Yunnanensis was leading in cellular antioxidant activity and antiproliferative activity against human cancer HepG2 cells. The extracellular antioxidant activity was closely associated with total phenolics and flavonoids within the extracts, while cellular antioxidant activity and antiproliferation towards HepG2 were significantly correlated with the total phenolic acids and flavonoid aglycones. Moreover, phenolic acids, quercetin, kaempferol, isorhamnetin and their mono-glycoside derivatives were more effective in HepG2 cell proliferation inhibition although they were in low concentrations. This confirmed that the antiproliferative effects of extracts were attributed to the inherent combination and complex interaction of phytochemical components instead of one or two abundant constituents. These findings provided the foundation for comprehensive application of Sea buckthorn in antioxidant and anticancer dietary supplement synthesis and utilization in food industry.

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