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How *in vitro* gastrointestinal digestion impacts the phenolic profile and bioactivities of North American sea buckthorn fruit and seeds†

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North American sea buckthorn (*Hippophae rhamnoides* L.) shows great commercial potential as a bioactive-rich fruit. The pomace and seeds of sea buckthorn fruit are rich sources of a variety of polyphenols with promising antioxidant and biological potential. However, the modification of these phenolics through the gastrointestinal tract is unknown and can significantly affect their bioactivities. Therefore, the pomace and seeds of Newfoundland cultivated sea buckthorn were subjected to *in vitro* simulated gastrointestinal (GI) digestion and their phenolic composition was monitored across oral, gastric, small and large intestine digestion. Moderate phenolic bioaccessibility was recorded for sea buckthorn pomace and seeds, with phenolic release rates of 35.34 and 44.86% after small intestinal digestion. This fraction contained a variety of proanthocyanidins, while phenolic acids and their derivatives were generally released at the earlier oral and gastric digestion. The phenolic profile of sea buckthorn digestion products was considerably different from undigested samples, reflecting on improved rate of inhibition of metabolic enzymes linked to type 2 diabetes and obesity in some cases, as well as oxidative protection to supercoiled DNA against the action of hydroxyl radicals. However, the capacity of samples to protect LDL-cholesterol from oxidative damage was decreased after digestion. These results represent an estimate of physiological effects, pending validation through *in vivo* human studies. Further research should concentrate on investigating the absorption and transport of polyphenols involved across the human gut by using relevant physiological models.

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Introduction

Sea buckthorn (*Hippophae rhamnoides* L.) is a traditional shrub bearing small oval berries widely used for medicinal purposes. These berries are rich in α- and γ-linolenic acids, vitamins, and antioxidants such as carotenoids and polyphenols. In countries like Russia and Germany, sea buckthorn has an established market for value-added products such as pulp oil, commonly used in nutraceuticals and cosmetics. The Asian and European markets serve as models for exploring this fruit crop's commercial potential.[1]

In North America, sea buckthorn has been cultivated experimentally for over 20 years. Despite efforts to develop it as a commercial crop in the U.S. and parts of Canada, the plant remains relatively unknown, with knowledge gaps in its cultivation, berry composition, and market potential.[2] Recently, sea buckthorn berries grown in Newfoundland, Canada, were characterized for their polyphenolic composition and biological potential.[3] The pomace (pulp and skins) contains phenolic acids and derivatives like *o*-methylgallic, ferulic, and ellagic acids, while the seeds are rich in (+)-catechin.[3] These compounds are potent antioxidants that protect biomolecules such as LDL-cholesterol and DNA. An *in vivo* mouse study[4] reported that diet supplementation with (−)-epicatechin gallate reduced the formation of atherosclerotic plaques. In the same study, a RAW264.7 macrophage model showed that this effect was due to the suppression of LDL-c oxidation, inhibiting inflammatory cytokines and foam cells leading to plaque formation.[4] On the other hand, the complexity and multifactorial nature of cancer poses a significant hurdle to establishing a cause-effect relationship through *in vivo* studies. Oxidative damage to the DNA molecule is one of the hypotheses to explain cancer initiation and progression. Hydroxyl radicals are well-known for causing DNA strand breaks. In this context, the excessive proliferation of ROS is believed to overwhelm DNA repair systems, potentially leading to mutagenesis and cancer development.[5] Polyphenols also serve as natural enzyme inhibitors targeting α-glucosidase and pancreatic lipase, commonly used in antidiabetic and anti-obesity therapies.[3] However, the

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digestibility of sea buckthorn fractions and their ability to retain health benefits during gastrointestinal (GI) digestion remain unclear.

Phenolic compounds are typically released in the stomach, where low pH breaks weak bonds between phenolics and other food components, and in the large intestine, where insoluble-bound phenolics travel with fiber, structural proteins, and polysaccharides.[6] Insoluble-bound phenolics make up 26% and 19% of all phenolics in Newfoundland's sea buckthorn pomace and seeds, respectively.[3] Their resistance to early digestion renders most phenolics non-bioaccessible in the small intestine, preventing intestinal absorption. However, polyphenols may play a role in preventing gut dysbiosis by stimulating the proliferation of beneficial microorganisms, although more research is needed to clarify these mechanisms.[7]

Understanding the modification of phenolic profiles across the GI tract is essential for uncovering potential health effects of sea buckthorn fractions. These insights can inform techniques to optimize the fruit's benefits and translate findings into commercial applications. For instance, individual phenolics deemed as highly bioaccessible could be selected for the development of phenolic mixtures. By estimating phenolic transformation in a simulated GI model, it is possible to explore the co-formulation of phenolic-based nutraceuticals employing encapsulation systems to enhance resistance to degradation and bioefficiency.[8]

The present study aimed to simulate the gastrointestinal digestion process of Newfoundland's sea buckthorn pomace and seeds using an *in vitro* model to evaluate changes in polyphenolic composition at each digestion stage. The antioxidant effects on key biomolecules (*e.g.*, LDL-cholesterol and supercoiled DNA) and the inhibition of enzymes (α-glucosidase and pancreatic lipase) were also monitored throughout the process.

Materials and methods

Material

Authentic phenolic standards, the compounds 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), 2,2′-azo-bis(2-methylpropanimidamide dihydrochloride) (AAPH), 2,4,6-tris(2-pyridyl)-*S*-triazine (TPTZ), *p*-nitrophenyl glucopyranoside, 4-nitrophenyl octanoate, and bile salt were purchased from Thermo Fisher Scientific (Nepean, ON, Canada). Human low-density lipoprotein (LDL), supercoiled plasmid pBR322 DNA, α-glucosidase from *Saccharomyces cerevisiae* (≥10 units per mg of protein) and lipase from porcine pancreas (100–500 units per mg of protein) used in the biological assays were also obtained from Thermo Fisher Scientific (Nepean, ON, Canada). The enzymes α-amylase, pepsin, pancreatin, and Viscozyme-L used in the *in vitro* digestion model, Folin-Ciocalteu's phenol reagent and other solvents and chemicals of analytical and chromatographic grade were acquired from Sigma-Aldrich Ltd (Oakville, ON, Canada).

Sample preparation

Ripe sea buckthorn berries (*Hippophae rhamnoides* L.) were sourced from the St. Phillip's region located in the eastern Avalon Peninsula, Newfoundland (47°37′38″ North, 52°51′02″ West). Seeds were manually separated from pomace (pulp and skins), which was freeze-dried (Freezone 6, Labconco, Kansas City, MO, USA) for 72 h. Dried pomace and seeds were ground using a coffee grinder (CBG5 series, Black and Decker, Brockville, ON, Canada) to particle size 0.500–0.550 mm. Powdered sea buckthorn pomace and seeds were defatted with hexane (1 : 5, w/v) in a Waring blender (33BL73, Waring Products, New Hartford, CT, USA), being subsequently stored at −20 °C until further experimentation.

Simulated *in vitro* gastrointestinal digestion

A sequential *in vitro* digestion model comprising oral, gastric, small intestine, and large intestine phases was used to assess the release pattern and bioaccessibility of phenolics in sea buckthorn pomace and seed powders, according to Ma *et al.*[9] Digestion buffer composition (10 mL each) was as follows:

- *Oral* (pH 6.5): 75 U mL^{-1} α-amylase and 0.75 mM calcium chloride dissolved in 0.01 M phosphate buffered saline (PBS, pH 7.4).
- *Gastric* (pH 2.0): 2000 U mL^{-1} pepsin and 0.075 M calcium chloride in PBS.
- *Small intestine* (pH 7.4): 100 U mL^{-1} pancreatin and 10 mM bile salt containing 0.3 mM calcium chloride in PBS.
- *Large intestine* (pH 4.0): 30 μL of the Viscozyme-L enzyme blend (cellulase, hemicellulase, arabanase, β-glucanase, and xylanase) in PBS.

Digestion was initiated by adding 1 g of powdered sample to the oral digestion buffer, following incubation (MaxQ 4000, Thermo Scientific, Waltham, MA, USA) for 10 min at 37 °C under constant stirring (200 rpm). Adjustments of pH for each phase were performed in a pH meter (FisherBrand, AB315, Waltham, MA, USA) using 5M HCl and 5M NaOH solutions. Then, the orally-digested sample was added to the gastric buffer and incubated for 2 h under the same conditions. Small intestine digestion followed by the addition of the gastric-digested sample to the small intestine buffer and incubation for 3 h. Lastly, the remaining sample was added to the large intestine buffer and incubated for 16 h. The supernatant of each digestion stage (digestion extracts) was collected and immediately placed in ice to cease enzymatic reaction, followed by centrifugation (Thermo Scientific Sorvall LYNX 6000 Superspeed centrifuge, Thermo Fisher Scientific, Pittsburgh, PA, USA) at 4000*g* for 10 min. Centrifuged digestion extracts were made to 15 mL with deionized water and stored at 4 °C for further analysis within 2 days.

Total phenolic content (TPC)

Digestion extracts were assessed for TPC, according to Singleton and Rossi,[10] with modifications. Samples were mixed with Folin–Ciocalteu reagent (phosphomolybdic acid

and sodium tungstate) in a 96-well microplate (25 μL of each), followed by the addition of ultrapure water (200 μL). After reacting for 5 min, 10% sodium carbonate (25 μL) was added, and the plate incubated in the dark for 1 h at room temperature. Absorbance was read at 725 nm in a microplate reader (BioTek Synergy Mx, BioTek Instruments, Winooski, VT, USA). Results were calculated with a gallic acid standard curve (0–136 mg L^{-1}) and expressed as mg of gallic acid equivalents per gram of sample (mg GAE g^{-1}). Phenolic bioaccessibility was calculated according to eqn (1).

$$\text{Bioaccessibility}\,(\%) = \left(\frac{\text{TPC of digestion extract}}{\text{TPC of undigested sample}}\right) \times 100 \quad (1)$$

Total flavonoid content (TFC)

TFC of digestion extracts was measured according to Chandrasekara and Shahidi.[11] Samples (1 mL) were mixed with 5% sodium nitrite (0.3 mL) in a Falcon tube and reacted for 5 min. Then, 10% aluminum chloride (0.3 mL) was added and after 1 min, 1M sodium hydroxide was included (2 mL). The mixture reacted in the dark for 15 min at room temperature, with the resulting absorbance read at 510 nm in a UV-visible spectrophotometer (HP8452 A, Agilent Technologies, Palo Alto, CA, USA). Results were calculated with a catechin standard curve (0–90 mg L^{-1}) and expressed as mg of catechin equivalents per gram of sample (mg CE g^{-1}). Flavonoid bioaccessibility (%) was calculated according to eqn (1), replacing TPC for TFC values.

Identification and quantification of phenolic compounds by high performance liquid chromatography with ultraviolet detection coupled with time-of-flight mass spectrometry (HPLC-UV-MS-TOF)

Digestion extracts were assessed for their individual phenolic composition, according to Danielski and Shahidi.[12] HPLC Agilent 1260 system components included a quaternary pump (G1311A), a degasser (G1379A), an ALS automatic sampler (G1329A), an ALS Therm (G1130B), a Colcom column compartment (G1316), a diode array detector (DAD, G4212B), and a system controller attached to a ChemStation Data handling system (Agilent Technologies, Palo Alto, CA, USA). An Agilent 6230 TOF system (TOF LC/MS) with electrospray ionization (ESI) was coupled to the HPLC and used in negative mode. Mass spectrometry (MS) scan range was set to m/z 100–2000, drying nitrogen gas at 350 °C, flow at 12 L min^{-1}, and nebulizer gas pressure at 70 psi. Data was analyzed in the Agilent LC-MSD software. Before injection, extracts (10 μL) were filtered using a 0.45 μm PTFE membrane syringe filter (Thermo Scientific, Rockwood, TN, USA).

Separation of individual phenolics occurred in a Synergi Fusion LC-18 column (50 × 2 mm, 4 μm, Phenomenex), where the binary mobile phase was composed of 0.1% formic acid in water (A) and methanol (B) at a flow rate of 0.150 mL min^{-1}. The elution gradient followed the order: 0 min – 90% A; 5 min – 10% A; 7 min – 10% A; 10–17 min – 90% A; then, mobile phase A was increased to 100% at 17 min, with column equilibration from 17 to 22 min. Detection of flavonoids and phenolic acids occurred at 280 nm. Compounds were identified by comparison of their retention times and ion fragmentation patterns with those of authentic standards of phenolic acids (protocatechuic, *p*-coumaric, *p*-hydroxybenzoic, *trans*-cinnamic, syringic, sinapic, gallic, caffeic, ferulic, and ellagic acids), flavonoids (quercetin, (+)-catechin, (−)-epicatechin), and the stilbene resveratrol. The standards were also used to construct calibration curves for quantification. Tandem mass spectrometry (MS^n) and literature data were used for tentatively identifying other compounds and their quantification was based on their corresponding aglycones. Results were reported as μg g^{-1} of sample.

Retention of biological activity

All digestion extracts were tested for antioxidant and biological activities and compared with their undigested counterparts. These results were used for calculating the levels of activity retention (%) after each digestion stage, according to eqn (1) with the appropriate adjustments.

Antioxidant capacity

2,2-Diphenyl-1-picrylhydrazyl (DPPH) scavenging activity. Digestion extracts were analyzed for DPPH antiradical activity, according to Shahidi *et al.*,[13] with modifications.[14] In a 96-well microplate, samples (260 μL) were allowed to react with a 0.10 mM DPPH methanolic solution (absorbance of 0.5–0.6 at 515 nm) for 30 min at room temperature in the dark. Absorbance was recorded at 515 nm. A 100 mg L^{-1} Trolox solution was used for building a calibration curve, and results were expressed as micromolar Trolox equivalents per gram of sample (μmol TE g^{-1}).

2,2′-Azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) scavenging activity. ABTS radical scavenging was measured according to Naczk *et al.*,[15] with modifications outlined by Rufino *et al.*[16] In a microplate, samples (10 μL) were mixed with 290 μL of freshly-prepared ABTS radical solution (88 μL of 140 mM potassium persulfate with 5 mL of 7 mM ABTS stock solution, with absorbance of 0.7 at 734 nm), reacting for 6 min in the dark. The absorbance was read at 734 nm in a microplate reader. Results were calculated by using an ascorbic acid standard curve (30–280 mg L^{-1}) and expressed as micromolar ascorbic acid equivalents per gram of sample (μmol AAE g^{-1}).

Ferric reducing antioxidant power (FRAP). The FRAP assay followed the protocol reported by Kumari *et al.*,[17] with some modifications.[14] Digestion extracts (20 μL) were mixed with freshly-prepared FRAP reagent (280 μL) in a microplate and incubated (MaxQ 4000, Thermo Scientific, Waltham, MA, USA) at 37 °C for 30 min. FRAP reagent consisted of 10 mM 2,4,6-Tris(2-pyridyl)-*S*-triazine (TPTZ) diluted in 40 mM HCl (2.5 mL), 20 mmol L^{-1} iron chloride(III) hexahydrate (2.5 mL), and sodium acetate buffer (25 mL). The absorbance increase was read at 593 nm in a microplate reader. Results were calculated by using an ascorbic acid calibration curve (15–150 mg

L^{-1}) and expressed as micromolar ascorbic acid equivalents per gram of sample (μmol AAE g^{-1}).

Biological activity

α-Glucosidase inhibitory activity. This determination was performed according to Danielski and Shahidi.[12] In Eppendorf tubes, the enzyme α-glucosidase (10 U mL^{-1}, 5 μL) was incubated with samples (10 μL) and 0.1 M PBS (pH 6.8) at 37 °C for 20 min. Then, 10 μL of the substrate (*p*-nitrophenyl glucopyranoside) were included with further incubation for 30 min. The reaction was terminated by adding 650 μL of 1 M sodium carbonate to the tubes. The absorbance of the product formed (*p*-nitrophenol, yellow) was measured at 410 nm in a UV-visible spectrophotometer. Sample blanks were prepared (no enzyme), as well as a control (no sample). The α-glucosidase inhibitory activity (%) was calculated according to eqn (2).

$$\%\ \alpha\text{-Glucosidase inhibition} = \left[1\left(\frac{ABS_{410\ sample} - ABS_{410\ sample\ blank}}{ABS_{410\ control} - ABS_{410\ control\ blank}}\right)\right] \times 100 \quad (2)$$

Pancreatic lipase inhibitory activity. This determination was performed according to Danielski and Shahidi.[12] In test tubes, pancreatic lipase (5 mg mL^{-1}, 100 μL) was incubated with samples (100 μL) and 1 M Tris-HCl acetate buffer (pH 8.5, 4 mL) at 37 °C for 25 min. Subsequently, 100 μL of the substrate (5 mM 4-nitrophenyl octanoate dissolved in dimethyl sulfoxide) was added with additional incubation for 25 min. The reaction was terminated by placing tubes into an ice bath. The absorbance of the product formed (*p*-nitrophenol, yellow) was measured at 412 nm in a UV-visible spectrophotometer. Sample blanks were prepared (no enzyme), as well as a control (no sample). The pancreatic lipase inhibitory activity (%) was calculated according to eqn (3).

$$\%\ \text{Pancreatic lipase inhibition} = \left[1 - \left(\frac{ABS_{412\ sample} - ABS_{412\ sample\ blank}}{ABS_{412\ control} - ABS_{412\ control\ blank}}\right)\right] \times 100 \quad (3)$$

Inhibition of cupric ion-induced human low-density lipoprotein (LDL) peroxidation. The capacity of bioaccessible fractions (small intestine extracts) of sea buckthorn pomace and seeds to protect LDL-cholesterol from cupric ion-induced peroxidation was carried out according to Ambigaipalan and Shahidi.[18] Human LDL was prepared by dialysis in a tube with a molecular weight cut-off of 12–14 kDa at 4 °C under nitrogen atmosphere for 12 h in the dark. Then, dialyzed LDL was diluted to 0.05 mg mL^{-1} with 10 mM PBS (pH 7.4). Diluted LDL (800 μL) was placed in Eppendorf tubes with the samples (100 μL) and incubated at 37 °C for 15 min. The initial incubation was followed by the addition of 100 μM cupric sulfate (100 μL) to induce oxidation. Tubes were returned to incubation for a maximum of 22 h. Absorbance was read in 2 h intervals at 234 nm in a UV-visible spectrophotometer to monitor the progression of conjugated dienes (CD) formation. Sample blanks contained only the sample and ultrapure water, while the LDL blank and the negative control contained no sample. Inhibition of CD formation (%) was calculated by eqn (4).

$$\%\ \text{CD inhibition} = \left[\left(\frac{ABS_{234\ control} - (ABS_{234\ sample} - ABS_{234\ sample\ blank})}{ABS_{234\ control} - ABS_{234\ LDL\ blank}}\right)\right] \times 100 \quad (4)$$

Inhibition of peroxyl and hydroxyl radical-induced supercoiled DNA strand scission. The capacity of bioaccessible fractions (small intestine extracts) of sea buckthorn pomace and seeds to mitigate radical-induced supercoiled DNA strand scission was carried out according to Ambigaipalan and Shahidi.[18] For the assay, radical solutions consisted of 5 μL of 0.5 mM $FeSO_4$ and 5 μL of 1 mM H_2O_2 (hydroxyl radical) and 17.5 mM 2,2′-azobis(2-methylpropanimidamide dihydrochloride (AAPH)) diluted in PBS (peroxyl radical). Samples were used undiluted for the hydroxyl assay and diluted 1 : 10 (v/v) with PBS for the peroxyl assay in aliquots of 5 μL. In Eppendorf tubes, samples were mixed with 5 μL of 0.5 mM PBS (pH 7.4), 5 μL of supercoiled pBR322 plasmid DNA (50 μg mL^{-1}), and 10 μL of either hydroxyl or peroxyl radical solution, being further incubated at 37 °C for 1 h. Subsequently, 5 μL of loading dye composed of 0.25% bromophenol blue, 0.25% xylene cyanol, and 50% glycerol was added to the tubes and the mixture (10 μL) was loaded onto an agarose gel (0.7%) stained with SYBR safe (10 μL). The electrophoresis system (model B1A, Owl Separation Systems Inc., Portsmonth, NH, USA) was filled with Tris-acetate-EDTA (TAE, pH 8.5) buffer and set at 80 V for 120 min. The chamber was connected to a power supply (model 300 V, VWR International Inc., West Chester, PA, USA). The blank consisted of DNA and PBS, while the control contained DNA, PBS, and radical solution. DNA bands were detected in the Bio-Rad ChemiDoc image system (Bio-Rad Laboratories, Hercules, CA, USA), and the intensity of bands (% area) was determined. Supercoiled DNA retention (%) was calculated using eqn (5).

$$\%\ \text{Supercoiled DNA retention} = \left[\left(\frac{\text{area of supercoiled DNA with free radical and sample}}{\text{area of supercoiled DNA in blank}}\right)\right] \times 100 \quad (5)$$

Statistical analysis

Statistical analysis was performed with IBM SPSS 27.0 for MacOS (SPSS Inc., Chicago, IL, USA). For detection of significant differences in the mean values of dependent variables across samples, one-way analysis of variance (ANOVA) was used. Mean comparison for significantly different results was carried out by the Tukey's HSD test ($p < 0.05$). All results were reported as mean ± standard deviation (SD) of triplicate values.

Results

Phenolic and flavonoid bioaccessibility

TPC of digested sea buckthorn varied between 2.112–3.931 and 6.322–13.51 mg GAE g^{-1} for the pomace and seed frac-

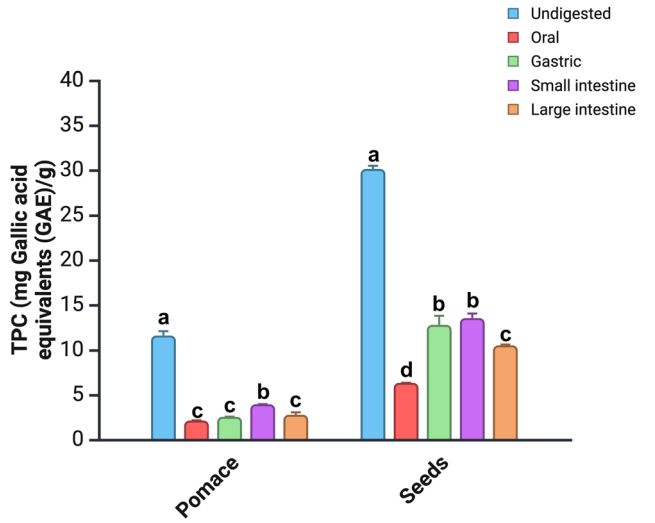

Fig. 1 Total phenolic content (TPC) of sea buckthorn pomace and seeds throughout the simulated *in vitro* gastrointestinal digestion process. Data reported as mean values for each sample ± standard deviation (n = 3). Different lowercase letters (within the same fruit fraction) indicate significant differences (p < 0.05) by the Tukey's HSD test. Created with BioRender.

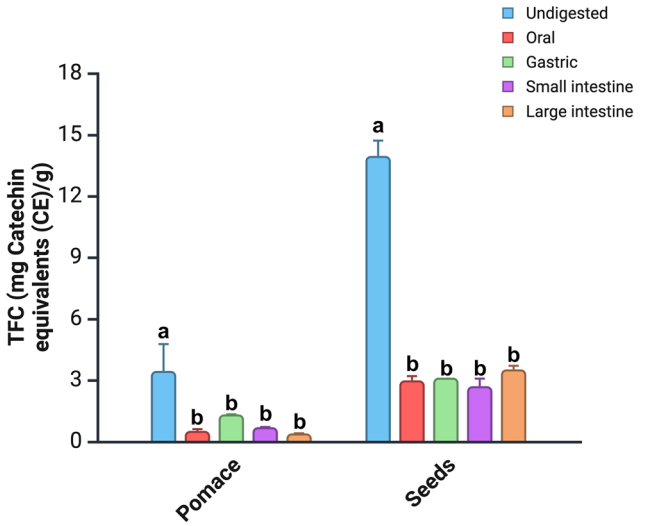

Fig. 2 Total flavonoid content (TFC) of sea buckthorn pomace and seeds throughout the simulated *in vitro* gastrointestinal digestion process. Data reported as mean values for each sample ± standard deviation (n = 3). Different lowercase letters (within the same fruit fraction) indicate significant differences (p < 0.05) by the Tukey's HSD test. Created with BioRender.

tions, respectively, where the lowest phenolic release occurred upon oral digestion, while the highest phenolic release was registered at the small intestine stage in both samples (Fig. 1).

With a gradual phenolic release observed throughout the digestion process, both samples reached their bioaccessibility peak after the small intestine phase (Table 1), with 35.34% (pomace) and 44.86% (seeds). This indicates that of phenolic compounds in sea buckthorn can be released at key steps of the digestive process, making them bioaccessible and increasing their chances of intestinal absorption. A similar outcome was reported by Guo *et al.*[19] when evaluating the bioaccessibility of phenolic compounds from a Chinese sea buckthorn cultivar (Subsp. *Sinensis*). The seedless berries were subjected to a similar *in vitro* GI digestion protocol, and their TPC results revealed that the highest phenolic release occurred upon small intestinal digestion, followed by the large intestine and gastric stage, respectively.

TFC of digested sea buckthorn pomace ranged from 0.3788 to 1.309 mg CE g^{-1}, where the lowest and highest values were found after large intestine and gastric digestion, respectively (Fig. 2). Digested seeds showed TFC values of 2.692–3.513 mg CE g^{-1} with the lowest and highest amounts recorded upon small and large intestine digestion, respectively. These results translated into flavonoid bioaccessibility rates of 20.99–44.86% and 19.28–25.17% for sea buckthorn pomace and seeds, respectively (Table 1).

Flavonoids, especially flavan-3-ols, have been documented as being unstable to alkaline conditions (around pH 7.4), which may explain the low TFC registered by digested seeds after small intestine digestion.[6] However, TFC variation across digestive stages of sea buckthorn samples were minor and not statistically significant (p < 0.05).

Table 1 Phenolic and flavonoid bioacessibility (%) of sea buckthorn pomace and seeds after each digestion phase of *in vitro* simulated gastrointestinal digestion

Sea buckthorn fraction	Digestion phase	Phenolic bioaccessibility	Flavonoid bioaccessibility
Pomace	Oral	18.98 ± 0.3^{d}	$14.67 \pm 3.3^{c,d}$
	Gastric	22.75 ± 0.2^{c}	38.04 ± 0.2^{a}
	Small intestine	35.34 ± 0.1^{a}	19.86 ± 0.3^{b}
	Large intestine	24.78 ± 3.5^{b}	11.01 ± 0.1^{d}
Seeds	Oral	20.99 ± 0.008^{c}	21.33 ± 2.0^{c}
	Gastric	42.35 ± 4.7^{a}	22.27 ± 0.01^{b}
	Small intestine	44.86 ± 2.3^{a}	19.28 ± 3.7^{d}
	Large intestine	34.88 ± 0.4^{b}	25.17 ± 1.7^{a}

Data reported as mean ± standard deviation (n = 3). Different lowercase letters in the column (within the same fruit fraction) indicate significant differences (p < 0.05) by the Tukey's HSD test.

Phenolic profile

Following *in vitro* GI digestion, a total of 15 phenolic compounds were identified in sea buckthorn pomace and seeds, including seven phenolic acids, one flavonoid, and seven tannins (four hydrolysable tannins and three proanthocyanidins), as shown in Table 2. In digested pomace, the highest number of phenolics was released at the oral step (5), followed by gastric and small intestine (four in each), and large intestine (3) steps. In digested seeds, the small intestine phase warranted the highest number of phenolics (5), followed by gastric (4), oral (3), and large intestine (2) phases.

Flavonoids were the most affected group by GI digestion. Apigenin, (+)-catechin, gallocatechin, quercetin sulfate, querce-

Table 2 Identification and quantification of phenolic compounds of digested samples of sea buckthorn pomace and seeds

Phenolic Compound	$[M-H]^-$ (m/z)	RT (min)	MS^2 ion fragments	Sea buckthorn pomace (μg g^{-1})				Sea buckthorn seeds (μg g^{-1})			
				O	G	S	L	O	G	S	L
Phenolic acids											
trans-Cinnamic acid[a]	147	1.5	126, 137			679 ± 7[a]	459 ± 2[b]	170 ± 5[a]	650 ± 2[b]		465 ± 1[a]
Vanillic acid[a]	167	2.1	103,121, 137	73.0 ± 0.4[d]	3630 ± 30[a]						
O-Methylgallic acid	183	1.1	121, 139, 154, 168				99.2 ± 1[c]		86.9 ± 0.5[d]		96.9 ± 0.5[b]
Ferulic acid[a]	193	10.3	133, 173, 178	91.9 ± 1[c]	26.0 ± 1[d]					55.7 ± 6.2[d]	
Syringic acid[a]	195	1.3	129, 155							205 ± 10[c]	
Dihydroxycaffeic acid lactone	357	13.4	279, 291, 311				1310 ± 14[a]				
Hydroxyferulic acid hexoside	371	13.2	293, 325						957 ± 5[a]		
Flavonoid											
(+)-Catechin[a]	289	8.8	133, 137, 183, 193, 230, 245, 275		147 ± 3[b]						
Tannins											
3,4,8,9,10-Pentahydroxydibenzo [*b*, *d*] pyran-6-one	275	1.4	173, 219, 229	[b]							
Ellagic acid derivative	389	5.3	137, 265, 300, 311, 339, 357	215 ± 0.04[b]							
Ellagic derivative	389	12.5	137, 265, 300, 311, 339, 357		110 ± 2[c]	144 ± 0.1[d]		66.2 ± 0.1[c]	367 ± 12[c]		
HHDP hexoside	480	13.9	137, 249, 279, 297, 318, 325, 339, 371, 448	459.3 ± 2.0[a]							
Digalloyl hexoside	484	12.7	137, 297, 311, 325, 339, 357, 371, 448							229 ± 3[b]	
B-type proanthocyanidin dimer	593	13.6	137, 249, 297, 311, 325, 357, 371					163 ± 1[a,b]			
A-type procyanidin trimer	861	14.1	137, 219, 297, 430, 536, 728, 783			319 ± 1[c]				293 ± 1[a]	
B-type proanthocyanidin trimer	897	13.5	137, 249, 339, 448.3, 554, 746, 840			351 ± 8[b]				225 ± 5[b]	
Number of identified phenolics				5	4	4	3	3	4	5	2
Total (μg g^{-1})				839	3915	1494	1868	399	2061	802	562
Total (O + G + S + L)				8115				3824			

RT = retention time, O = oral phase, G = gastric phase, S = small intestine phase, and L = large intestine phase. Sample chromatograms from each digestion stage can be found in Fig. S1 (Appendix A).† [a] Identified with authentic standards. [b] Compound identified in the sample, but below the limit of quantification.

tin-3-*O*-(6″-benzoyl)-β-galactoside, catechin-*O*-dihexoside, and mearnsetin-diglucoside were detected in undigested sea buckthorn samples,[3] but only (+)-catechin was still present after the digestion process. According to Cheung *et al.*,[21] monomeric catechins display higher stability under gastric than intestinal conditions, which may explain the detection of (+)-catechin exclusively in the gastric phase of digested sea buckthorn pomace. Additionally, the phenolic acids *p*-hydroxybenzoic, protocatechuic, caffeic, *p*-coumaric, and hydroxygallic gallic acids, as well as derivatives of *p*-coumaric (*p*-coumaroyl malonyldihexoside) and caffeic acids (hydroxycaffeic acid, caffeoylquinate shikimate derivative, and tri-*O*-caffeoylshikimic acid) were only detected prior to *in vitro* GI digestion. The same was observed for ellagic derivatives II and IV, ellagic acid pentoside, and A-type procyanidin.[3]

Besides (+)-catechin, phenolics detected in both undigested and digested sea buckthorn samples included *trans*-cinnamic, *o*-methylgallic, and ferulic acids, as well as 3,4,8,9,10-pentahydroxydibenzo[*b*,*d*]pyran-6-one. In undigested sea buckthorn, *trans*-cinnamic acid was only detected in the seeds, while after digestion this phenolic acid was found in both pomace (small and large intestine phases) and seeds (oral, gastric, and large intestine phases). *o*-Methylgallic and ferulic acids, previously found in the original extracts, were retained in sea buckthorn pomace and seeds in the post-digestion stage. (+)-Catechin and 3,4,8,9,10-pentahydroxydibenzo[*b*,*d*]pyran-6-one were not retained following GI digestion of the seeds. However, both compounds were identified in digested sea buckthorn pomace, not present prior to the digestive process.

Ten compounds were exclusively found in digested sea buckthorn pomace and seeds. From those, only two simple phenolic acids were detected (vanillic acid in orally- and gastric-digested pomace and syringic acid in small intestine-digested seeds). *trans*-Cinnamic, ferulic, and *o*-methylgallic acids (also detected in the pre-digested samples) complete the group of simpler phenolic acids of digested samples. The digestive fraction corresponding to pomace's large intestine phase contained dihydroxycaffeic acid lactone, while hydroxyferulic acid hexoside was observed in the gastric phase of the seeds.

Ellagitannins included ellagic derivative, found in the pomace (oral, gastric, and small intestine phases) and seeds (oral and gastric phases), and HHDP hexoside, released in the oral phase of digested pomace. The latter was tentatively identified by its parent ion at *m*/*z* 480 and fragmented at *m*/*z* 318, which can indicate the loss of hexose moiety (162 Da), as suggested in the literature.[22] Small intestine-digested seeds showed a compound eluting at RT 12.7 min with $[M - H]^-$ at *m*/*z* 484 and MS^2 ion fractions at *m*/*z* 325 and *m*/*z* 339, indicating the detachment of hexose and digalloyl moieties, respectively. Those parameters are consistent with the identification of digalloyl hexoside.[22] A-Type procyanidin trimer and B-type proanthocyanidin trimer were released upon small intestine digestion of the pomace and seeds, even though they were not detected in the original samples. In addition, a B-type proanthocyanidin dimer ($[M - H]^-$ at *m*/*z* 593) was detected in orally-digested seeds. According to Lin *et al.*,[22] this molecule is formed by the polymerization of epigallocatechin units, accounting for 30 carbon, 24 hydrogen, and 13 oxygen atoms in total.

Digestion promoted a considerable modification in the phenolic profile of sea buckthorn fractions. Before, 11 different phenolics were identified in the pomace and 18 in the seeds.[3] After digestion, the pomace remained with 11 compounds, although not necessarily the same ones, while the seeds declined to 10 compounds and mostly retained more complex phenolics (Table 2). A diagram summarizing the changes in the phenolic profiles of digested sea buckthorn fractions can be found in the Appendix (Fig. S1†).

Phenolic quantification following *in vitro* GI digestion of sea buckthorn samples is found in Table 2. Gastric-digested pomace showed the highest concentration of total polyphenols (sum of individual phenolics), with 3914.8 μg g^{-1} due to a major contribution of vanillic acid (3631.4 μg g^{-1}), which represented 93% of the total. The large intestine (1867.8 μg g^{-1}), small intestine (1493.5 μg g^{-1}), and oral (839.0 μg g^{-1}) phases followed in decreasing order of total polyphenol content. The ellagitannin HHDP hexoside, an ellagic acid glycoside, was the predominant polyphenol released after oral digestion (459.3 μg g^{-1}), while *trans*-cinnamic acid (679.1 μg g^{-1}) and dihydroxycaffeic acid lactone (1310.0 μg g^{-1}) were the main compounds in the small intestine and large intestine phases, respectively.

The gastric phase was also responsible for the largest phenolic content of digested sea buckthorn seeds, with 2061.2 μg g^{-1}. Hydroxyferulic acid hexoside (957.0 μg g^{-1}) was the main contributor in this stage. The sum of individual phenolics in the small intestine phase accounted for 802.4 μg g^{-1}, followed by the large intestine (562.1 μg g^{-1}) and oral (398.7 μg g^{-1}) phases. *trans*-Cinnamic acid was the main polyphenol in both oral (169.6 μg g^{-1}) and large intestine (465.1 μg g^{-1}) stages, while A-type procyanidin trimer (293.4 μg g^{-1}) was the most representative compound in the small intestine stage. This procyanidin along with B-type proanthocyanidin trimer were exclusively detected in the extracts corresponding to the small intestine and large intestine phases.

Reducing activity

The DPPH and ABTS antiradical activities of digested sea buckthorn samples were favored in comparison to their pre-digestion levels (Table 3). The reducing capacity of sea buckthorn pomace reached its peak at different digestion stages depending on the antioxidant activity assay employed. Upon gastric and small intestine digestion, the pomace powder showed enhanced scavenging of DPPH (41.45 μmol AAE g^{-1}) and ABTS (206.6 μmol AAE g^{-1}) radicals, respectively, while ferric reducing power (26.74 μmol AAE g^{-1}) peaked after large intestine digestion. In contrast, digested seeds showed the highest activity retention in the large intestine (402.1%) for DPPH scavenging, while ABTS scavenging and ferric reducing activity were better retained in the small (85.54 and 29.17%, respectively) and large intestine (84.99 and 29.33%, respectively) phases.

Table 3 Reducing activity of undigested and digested sea buckthorn pomace and seeds

Sample	Digestion Phase	DPPH (μmol TE g^{-1})	Average activity retention (%)	ABTS (μmol AAE g^{-1})	Average activity retention (%)	FRAP (μmol AAE g^{-1})	Average activity retention (%)
Sea buckthorn pomace	Undigested	8.173 ± 0.1^{c}	—	105.7 ± 3.4^{c}	—	109.7 ± 0.4^{a}	—
	Oral	34.26 ± 1.5^{b}	419.2	37.41 ± 0.2^{e}	35.37	10.32 ± 0.01^{d}	9.401
	Gastric	41.45 ± 1.2^{a}	507.1	60.66 ± 1.0^{d}	57.35	16.24 ± 0.07^{c}	14.79
	Small intestine	7.024 ± 0.6^{c}	85.94	206.6 ± 5.9^{a}	195.3	15.61 ± 0.5^{c}	14.22
	Large intestine	28.73 ± 2.3^{b}	351.55	155.2 ± 1.9^{b}	146.7	26.74 ± 0.1^{b}	24.36
Sea buckthorn seeds	Undigested	12.67 ± 0.4^{c}	—	289.0 ± 0.2^{a}	—	187.4 ± 1.1^{a}	—
	Oral	45.92 ± 0.7^{a}	362.2	49.07 ± 0.01^{d}	16.97	10.47 ± 0.03^{d}	5.587
	Gastric	36.07 ± 0.2^{b}	284.6	70.83 ± 0.6^{c}	24.50	18.31 ± 0.05^{c}	9.769
	Small intestine	34.42 ± 4.3^{b}	271.6	247.2 ± 0.1^{b}	85.54	54.67 ± 1.3^{b}	29.17
	Large intestine	50.97 ± 4.3^{a}	402.1	245.7 ± 0.7^{b}	84.99	54.99 ± 1.7^{b}	29.33

Data reported as mean ± standard deviation (n = 3). Different lowercase letters in the column (within the same fruit portion) indicate significant differences (p < 0.05) by the Tukey's HSD test. Abbreviations: DPPH – 2,2-diphenyl-1-picrylhydrazyl, ABTS – (2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)), FRAP – ferric reducing antioxidant power; TE – Trolox equivalents; and AAE – ascorbic acid equivalents.

Retention of bioactivities

Inhibition of α-glucosidase and pancreatic lipase. The inhibitory activity of digested sea buckthorn pomace and seeds toward α-glucosidase ranged from 32.93 to 68.94% and 22.50 to 58.70%, respectively (Fig. 3a). Both samples reached their lowest inhibitory activity upon the oral phase, while the highest rates were achieved upon the small intestine and large intestine phases.

Meanwhile, digested sea buckthorn samples were less effective at inhibiting the activity of pancreatic lipase, in which results ranged from 11.99 to 18.98% and 8.635 to 18.91% for the pomace and seeds, respectively (Fig. 3b). Similar to what was observed in terms of α-glucosidase inhibition, the samples corresponding to the small intestine phase presented the highest inhibitory efficacy. Pancreatic lipase is a metabolic enzyme that catalyzes the breakdown of triacylglycerols into monoacylglycerols and free fatty acids, which can be absorbed and incorporated to the adipose tissue. This enzyme is a target for drugs intended for weight management.

Inhibition of LDL-c and DNA oxidation. The bioaccessible fractions (small intestine phase) of digested sea buckthorn pomace and seeds were evaluated for their ability to inhibit cupric ion-induced LDL-c peroxidation (Fig. 4). Both samples displayed capacity to hamper oxidative damage to LDL-c by 42.36 (pomace) and 38.57% (seeds). The inhibition capacity was reduced by the digestive process, since phenolic extracts obtained from undigested sea buckthorn pomace and seeds demonstrated higher rates of inhibition (up to 88.90%).

The bioaccessible fractions of digested sea buckthorn samples were also assessed for their ability to mitigate DNA oxidation induced by hydroxyl and peroxyl radicals. Digestion positively affected the ability of sea buckthorn samples to retain supercoiled DNA when incubated with hydroxyl radicals (57.15–69.64%), with similar results to those obtained for their undigested counterparts (Fig. 5). On the other hand, the undigested phenolic extracts showed higher efficacy at retaining supercoiled DNA in the presence of peroxyl radicals (up to 79.84%) than digested sea buckthorn pomace (25.46%) and seeds (33.77%).

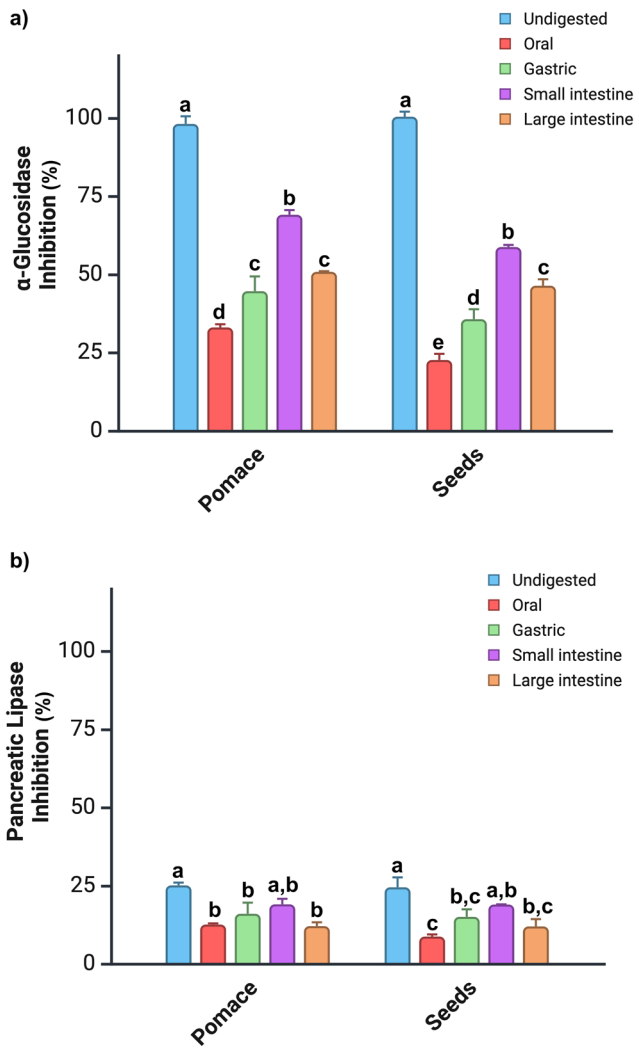

Fig. 3 Inhibitory activity of digested sea buckthorn pomace and seeds toward (a) α-glucosidase and (b) pancreatic lipase. Data reported as mean values for each sample ± standard deviation (n = 3). Different lowercase letters (within the same fruit fraction) indicate significant differences (p < 0.05) by the Tukey's HSD test. Created with BioRender.

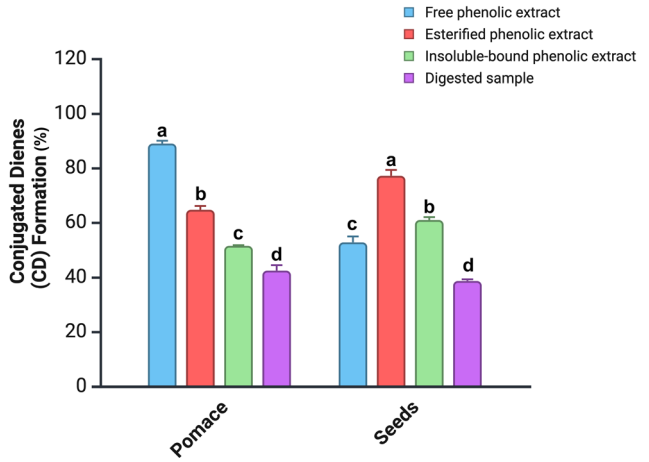

Fig. 4 Inhibition (%) of conjugated dienes formation from LDL-cholesterol by digested (a) sea buckthorn pomace and (b) seeds and their respective undigested phenolic extracts. Data reported as mean values for each sample ± standard deviation (n = 3). Different lowercase letters (within the same fruit fraction) indicate significant differences ($p < 0.05$) by the Tukey's HSD test. Results from free, esterified, and insoluble-bound phenolic extracts of undigested sea buckthorn powders were previously published elsewhere[3] and were reproduced in the graphs for comparison purposes. Created with BioRender.

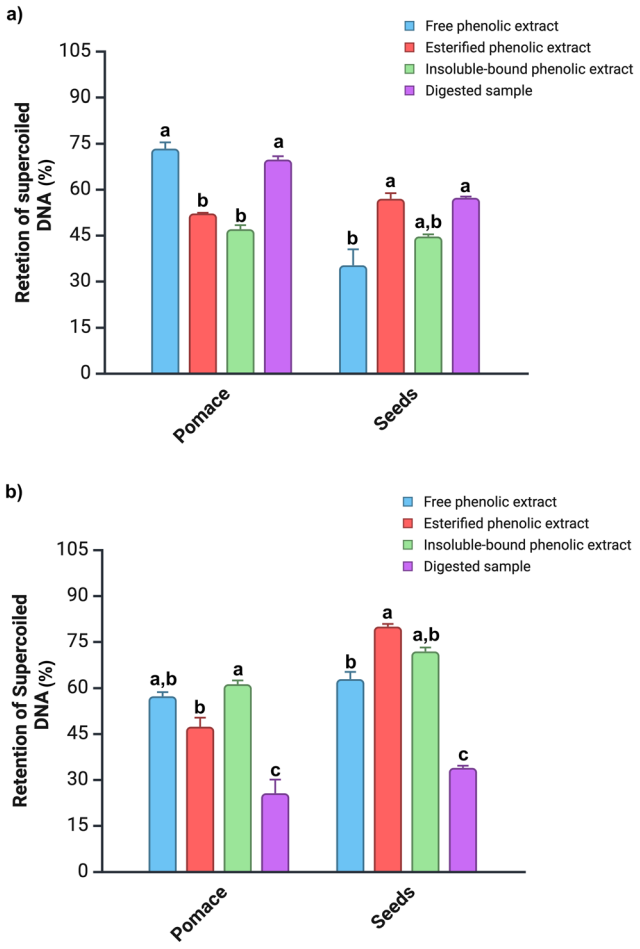

Fig. 5 Retention of supercoiled DNA (%) promoted by undigested extracts and digested sea buckthorn pomace and seeds by scavenging hydroxyl (a) and peroxyl (b) radicals. Data reported as mean values for each sample ± standard deviation (n = 3). Different lowercase letters (within the same fruit fraction) indicate significant differences ($p < 0.05$) by the Tukey's HSD test. Results from free, esterified, and insoluble-bound phenolic extracts of undigested sea buckthorn powders were previously published elsewhere[3] and were reproduced in the graphs for comparison purposes. A representative image of the DNA gel used for the analysis can be found in Fig. S2 (Appendix A).† Created with BioRender.

Discussion

Sea buckthorn seeds showed higher total phenolics than the pomace in both pre-digested and digested states. Fruit seeds are typically richer in polyphenols and other antioxidants due to their protective roles. These molecules protect seeds from oxidative damage, pathogens, and environmental stressors.[3,13] Other reducing substances (*e.g.*, ascorbic acid, carotenoids, tocols) may also contribute to total reducing activity, potentially overestimating phenolic concentration.[3,13] Therefore, phenolics from digested sea buckthorn were also analyzed chromatographically.

Significant changes in the phenolic composition of sea buckthorn fractions occurred between pre- and post-digestion. Pre-digested pomace and seeds mainly contained simpler phenolic acids and flavonoids, as reported by Danielski and Shahidi.[3] Post-digestion, more complex polyphenols were observed. Phenolic acids such gallic and caffeic acids and flavan-3-ols are highly susceptible to digestive conditions, particularly in the small intestine's alkaline environment.[20] This could explain the absence of flavan-3-ols and the reduction of simple phenolic acids after digestion.

The release of *trans*-cinnamic, ferulic, and *o*-methylgallic acids during the gastric phase is noteworthy, as simple phenolic acids are more likely to absorbed in the stomach.[20] Dimeric and trimeric proanthocyanidins can form during digestion through monomeric catechin complexation.[20] Sea buckthorn seeds were rich in (+)-catechin before digestion.[3] Post-digestion, catechin was undetected, while proanthocyanidin dimers and trimers were present, possibly due to catechin polymerization or the inability of the extraction process to release these compounds from the undigested samples.

Phenolic acids and simple flavonoids were mainly released under gastric conditions, whereas proanthocyanidins were released more under intestinal conditions. The stomach's acidic environment favors release of small phenolics,[23] while proanthocyanidins are relatively stable during gastric and intestinal phases, undergoing extensive metabolism in the colon *via* gut microbiota.[24] This behavior can vary with the food matrix. For example, proanthocyanidins in wild cereal grains are minimally released in the small intestine and become bioaccessible only in the colon.[25]

DPPH scavenging activity peaked during gastric digestion of pomace but during oral and large intestine digestion for seeds. In these stages, ellagic acid derivatives and *trans*-cinnamic acid were abundant. *trans*-Cinnamic acid, along with

A-type procyanidin trimer and *o*-methylgallic acid, was prominent in digestion extracts with effective ABTS scavenging and ferric reducing activity. These compounds likely contributed to the increased antiradical activity and reducing capacity post-digestion. Many of these phenolics, though present pre-digestion, increased in concentration after digestion, enhancing the antioxidant capacity of sea buckthorn pomace and seeds.

Pomace and seed extracts from the small intestine phase were rich in A-type procyanidin trimer and B-type proanthocyanidin trimer, while *trans*-cinnamic and *o*-methylgallic acids were prominent in the large intestine phase. These compounds likely contributed to inhibiting α-glucosidase, an enzyme involved in carbohydrate digestion and a target for type 2 diabetes treatments. Proanthocyanidins are effective α-glucosidase inhibitors, benefitted by their 5,6,7-trihydroxyflavone structure of the A-ring and hydroxylation on the B-ring. Such features allow them to interact with the enzyme, typically promoting mixed inhibition.[26] The presence of sea buckthorn proanthocyanidins in the small intestine may provide gastrointestinal benefits.

LDL cholesterol oxidation triggers atherosclerosis. Oxidized LDL (Ox-LDL) is cytotoxic, promoting foam cell formation and plaque development. Ox-LDL also damages endothelial cells, compromising their integrity. LDL oxidation occurs in the arterial sub-endothelial region, where antioxidant defenses are depleted. Lipid peroxidation initiates with conjugated diene formation, producing unstable radicals that break into low-molecular-weight aldehydes, coupling with apolipoprotein B-100 to form Ox-LDL. Dietary antioxidants, such as phenolic compounds, enhance defenses against oxidized species.[27,28] Compared to phenolic extracts from undigested sea buckthorn fractions, digesta extracts showed reduced ability to mitigate LDL-c oxidation. Whereas free and esterified phenolic extracts from sea buckthorn pomace and seeds could inhibit conjugated diene formation by 90 and 77%, respectively, digested counterparts could only supress 43 and 38%, respectively.

Supercoiled DNA can be damaged by free radicals, causing strand breaks, base modification, and cross-linking. Excessive free radical propagation can overwhelm DNA's repair mechanisms, leading to mutagenesis. Hydroxyl radicals, being highly reactive, cause chain-breaking reactions in biomolecules, while peroxyl radicals primarily target polyunsaturated fatty acids, breaking into secondary reactive products.[20] Digested sea buckthorn samples were particularly effective against highly reactive hydroxyl radicals.

Conclusion

Simulated gastrointestinal digestion of sea buckthorn pomace and seeds showed the small intestinal stage releases the most phenolics, predominantly proanthocyanidins, while gastric and large intestine digestions produce mainly phenolic acids. This alters the phenolic profile compared to undigested samples. Digested samples inhibited α-glucosidase more effectively than pancreatic lipase, consistent with undigested sea buckthorn. The bioaccessible fraction showed lower inhibition of LDL-cholesterol oxidative damage than phenolic extracts from undigested samples. Digested fractions showed enhanced DNA protection from hydroxyl radicals. The *in vitro* digestion model used was limited by the absence of a colonic fermentation step. Future studies should incorporate this step to provide insights into the influence of gut microbiota on phenolic bioaccessibility. Moreover, *in vitro* models can only estimate physiological effects, which must be validated by *in vivo* results, preferably in human subjects. Our future research would focus on investigating the absorption, distribution, metabolism, and excretion (ADME) of bioaccessible sea buckthorn phenolics using physiologically relevant models for a comprehensive understanding of their bioavailability.

Author contributions

Renan Danielski: conceptualization, methodology, investigation, data analysis, writing.

Fereidoon Shahidi: conceptualization, supervision, funding acquisition, writing – review.

Conflicts of interest

The authors declare no conflict of interest.

Data availability

All data used for this study appear in the illustrated figures, and the raw data can be made available upon request.

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