

## In Vitro Gastrointestinal Digestion Model to Monitor the Antioxidant Properties and Bioavailability of Phenolic Antioxidants from Elderberry

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**ABSTRACT** | The sustainability of the elderberry antioxidants during gastrointestinal (GI) digestion was monitored using an in vitro GI digestion model involving pepsin digestion (to simulate gastric digestion) and pancreatin digestion (to simulate small intestine conditions). The potential bioavailability of elderberry antioxidants after GI digestion was further determined by employing a membrane model using dialysis tubing. Our results showed that simulated GI digestion significantly decreased 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity of the elderberry antioxidant extract, but had no effect on its total phenolic content (TPC) and oxygen radical absorbance capacity (ORAC) value. After GI digestion, more than 75% of phenolic content in the elderberry extract was absorbable according to our simulated dialysis experiment. The impact of GI digestion on individual antioxidants in the elderberry extract was determined by reverse-phase high performance liquid chromatography (HPLC). Ten individual antioxidants were identified and quantified by HPLC with chlorogenic acid, rutin, catechin, *p*-coumaric acid, and quercetin as the major phenolic compounds in the elderberry extract. GI digestion increased the concentrations of chlorogenic acid and *p*-coumaric acid but decreased epicatechin and quercetin 3-glucoside content in the elderberry extract.

**KEYWORDS** | Antioxidant; Bioavailability; Elderberry; Gastrointestinal digestion model

**ABBREVIATIONS** | DPPH, 2,2-diphenyl-1-picrylhydrazyl; GI, gastrointestinal; HPLC, high performance liquid chromatography; ORAC, oxygen radical absorbance capacity; TPC, total phenolic content

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## 1. INTRODUCTION

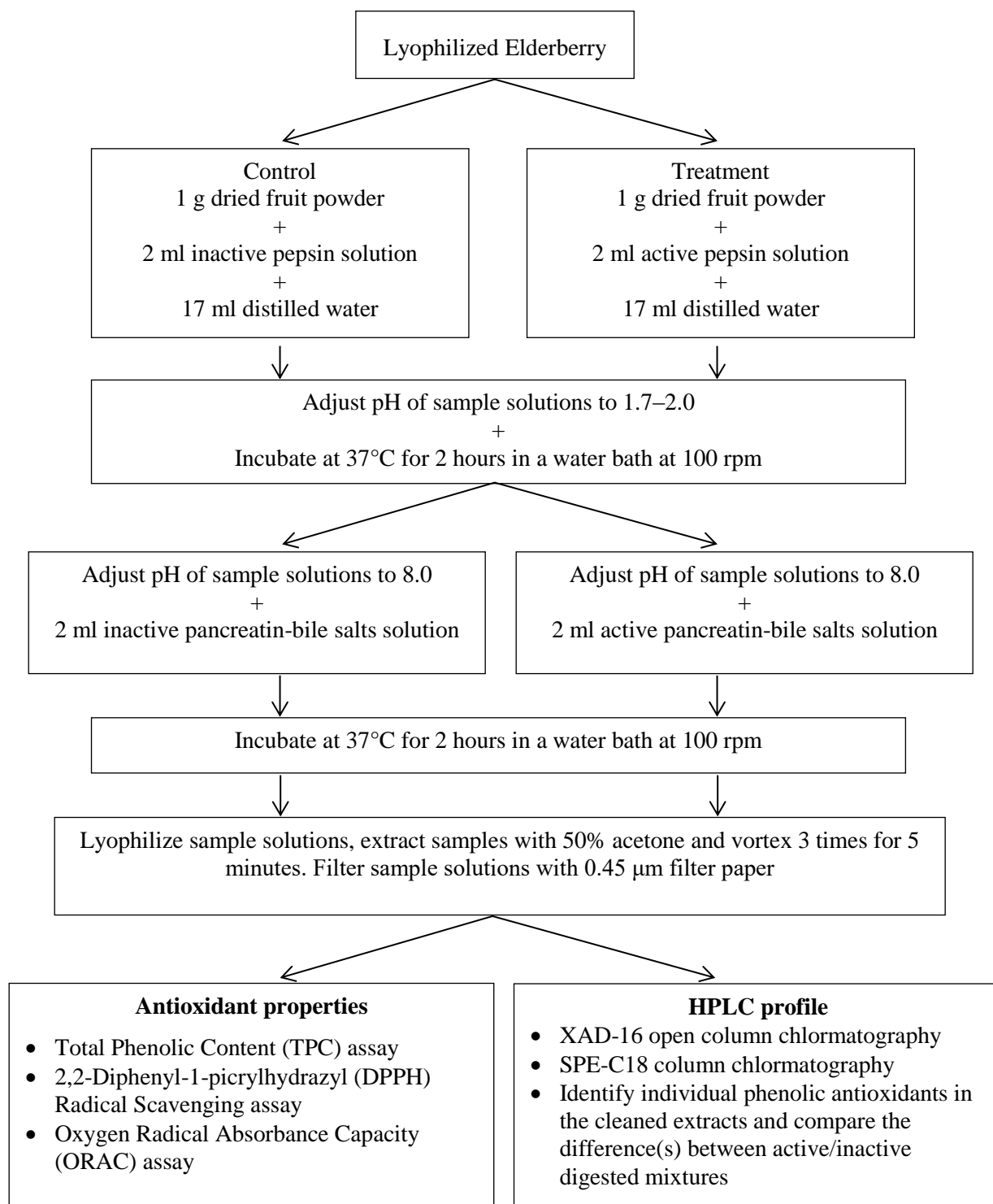
The elderberry fruit (*Sambucus nigra L.*) is a rich source of phenolic compounds. Evidence is accumulating which supports the health promoting quality of phenolic antioxidants participating in redox reactions ubiquitously occurring in biological milieus, including the plasma, tissues, organs, and gastrointestinal environments. Dietary phenolic compounds may facilitate homeostasis of oxidative stress once consumed. However, a limiting factor of the health promoting phenolic compounds is their stability, possible degradation during digestion, and poor bioavailability. The bioavailability of phenolic compound-derived metabolites from elderberry in humans has been detected, albeit in low concentrations [1–3]. With the heightened interest in health promoting role that bioactive compounds play, screening the potential candidate compounds efficiently and cost-effectively with an in vitro digestive model which mimics biological digestive system is useful for determining the fate of the compounds in the early stage of research [4, 5]. To this end, the goal of this study was to investigate how gastrointestinal (GI) digestion affects elderberry antioxidants in regards to their antioxidant activities, chemical structure, stability, and potential bioavailability using an in vitro GI digestion and membrane model.

Understanding the instability and degradation of dietary phenolic compounds within the GI tract is important for clarifying factors affecting their activity and bioavailability [6, 7]. For example, it has been hypothesized that the cyanin ionic form existing at neutral pH in the small intestine is less stable and degrades rapidly into unknown metabolites [8]. On the contrary, anthocyanins are stable in their acidic form typically present in the gastric milieu [9, 10]. Due to this dynamic environment the stability of cyanins may be the reason for their limited absorption

across gastric or intestinal walls and subsequent metabolic fate. Information on bioavailability of specific phenolic compounds can be obtained by conducting human and animal absorption studies [11]. However, there remains a need to take advantage of in vitro gastrointestinal models which can be executed more rapidly and are typically an inexpensive approach to assess bioavailability.

In the present study, an in vitro digestive model, similar to other methods previously used on phenolic compounds [10, 12, 13], was designed which provides a rapid, inexpensive preliminary strategy to investigate the elderberry and its associated bioactive compounds. In vitro GI models are useful because they can be customized to mimic or simulate biological digestive processes, useful for elucidating and monitoring the biofunctional status and bioavailability of specific components [10, 14]. During digestion anthocyanins are exposed to a wide range of pH conditions in the gastric and intestinal tract, both of which may affect their bioavailability [10]. The stomach is one of the metabolic sites of digestion of phenolic compounds as well as a site where cyanins are absorbed through the gastric membrane for transport in the plasma. Anthocyanins are exposed to pepsin in the acidic gastric milieu. The pancreatic digestion occurs in the small intestine which is the site of the majority of cyanin absorption.

For the purposes of this study, both the effect of live and inactivated pepsin in the stomach on the functional properties of elderberry crude extract were compared, such as their antioxidant capacity in regards to oxygen radical absorbance capacity (ORAC) and ability to scavenge reactive free radicals such as, 2,2-diphenyl-1-picrylhydrazyl (DPPH). Similarly, the small intestinal pancreatic enzyme in both the inactivated and live forms was assessed for its influence on the antioxidant properties of the elderberry crude extract. This is important because most of pre-



**FIGURE 1. Flow chart for the in vitro digestion and antioxidant analysis of elderberry.** See text (Section 2.3) for further description.

vious studies used crude extracts for antioxidant evaluation where the important effects of GI digestion on dietary antioxidant bioaccessibility were missed. In addition, the digested elderberry mixture was further evaluated for their potential absorption in GI tract using a dialysis tubing model for GI simulation. The possible modification of individual elderberry phenolic compounds during simulated GI digestion was also monitored by high performance liquid chromatography (HPLC) profiling.

## 2. MATERIAL AND METHODS

### 2.1. Materials

Pancreatin, pepsin, gallic acid, Folin–Ciocalteu's phenol reagent, caffeic acid, delphinidin chloride, and malvidin chloride were purchased from Sigma–Aldrich (St. Louis, MO, USA). Formic acid was obtained from Fisher (Fair Lawn, NJ, USA). Bile salts, fluorescein, malvidin 3-*O*-galactoside, cyanidin 3-glucoside, quercetin 3-glucoside, chlorogenic acid, ferulic acid, and *p*-coumaric acid were acquired from Fluka (Buchs, Switzerland). Apigenin, curcumin, catechin, epicatechin, epicatechin gallate, epigallocatechin, epigallocatechin gallate, hesperitin, indole-3-carbinol, kaempferol, luteolin, myricetin, naringenin, and resveratrol were obtained from LKT laboratories (St. Paul, MN, USA). DPPH and pelargonidin chloride were purchased from Aldrich (Milwaukee, WI, USA). Trolox (6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid), quercetin hydrate, and rutin were purchased from ACROS (Geel, Belgium). 2,2'-Azobis (2-amidinopropane) dihydrochloride (AAPH) was purchased from Wako (Richmond, VA, USA).

### 2.2. Sample and Standard Preparation

Samples of organic elderberries (*Sambucus Nigra L.*) were obtained from Starwest Botanicals, Inc. (Sacramento, CA, USA). The elderberry samples were frozen and chopped into small pieces, stored overnight at  $-80^{\circ}\text{C}$ , and lyophilized with a freeze-dryer (Labconco Freeze Dry System Lyph Lock 6, Kansas City, MO, USA). The samples were milled into fine powder and then used for the in vitro digestion and dialysis study, after which antioxidant activity assays and HPLC were performed. Twenty six antioxidant

standards were included in HPLC analysis to identify antioxidants in the elderberry. All antioxidant standards were dissolved in 100% methanol.

### 2.3. In Vitro Gastrointestinal Digestion Simulation

An overview of the in vitro digestion process utilized is presented in **Figure 1** according to our previous publication. Specifically, the procedure was comprised of a pepsin-HCl digestion for 2 h at  $37^{\circ}\text{C}$  (to simulate gastric digestion) and a pancreatin digestion with pancreatin and bile salts for 2 h at  $37^{\circ}\text{C}$  (to simulate small intestinal digestion) [15]. One gram of pepsin was dissolved in 100 ml distilled water to make the pepsin solution, whereas 0.4 g of pancreatin and 2.5 g of bile salts were dissolved in 100 ml distilled water to make the pancreatin-bile salt solution. The control (treated with inactive enzymes) and treatment (treated with active enzymes) sample groups were treated with these solutions as indicated above. For the control group, the solutions were boiled then cooled down to room temperature before treatment, thus deactivating the digestive enzymes. The procedure was as follows: 1 g of dried fruit powder was mixed with 2 ml pepsin solution (inactive for the control group; active for the treatment group) and 17 ml distilled water. The mixture was adjusted to pH 1.7–2.0 with HCl and incubated at  $37^{\circ}\text{C}$  for 2 h in a shaking water bath.

After the above incubation, 2 ml from each digested mixture was transferred into a 15 ml centrifuge tube and stored in a  $-20^{\circ}\text{C}$  freezer (the mixture fractions were then extracted by aqueous acetone for antioxidant evaluation). The remaining mixture would go to next pancreatin/bile salts digestion where the mixture was adjusted to pH 8.0 with 1 N NaOH, and 2 ml pancreatin-bile salts solution was added. The mixtures were digested in the same shaking water bath for 2 h. Following the digestion process, the digested mixtures were stored at  $-80^{\circ}\text{C}$  overnight, and then freeze-dried. The lyophilized mixtures were then extracted with aqueous acetone (1:1, vol/vol) and vortexed 3 times for 5 min each. The extracts were then filtered using Whatman filter paper (0.45  $\mu\text{m}$ ) and diluted with aqueous acetone to a concentration of 50 mg/ml. These samples were then used for antioxidant evaluation including TPC assay, DPPH radical scavenging assay, and ORAC assay (see Sections 2.5, 2.6, and 2.7).

## 2.4. In Vitro Bioavailability Simulation

The experiment was conducted according to an established method with slight modification [16]. A portion (0.5 g) of the lyophilized mixture after above-described simulating digestion (with active enzymes) was mixed with 10 ml of 1 M NaHCO<sub>3</sub> and transferred to a cellulose dialysis tubing (Sigma-Aldrich). The dialysis mixture was then placed into a flask containing NaHCO<sub>3</sub> (pH 7.5) and incubated in a water bath at room temperature for 1 h with stirring. After dialysis, both solutions inside (fraction IN) and outside (fraction OUT, the colon bioavailable fraction) fractions of the dialysis tubing were frozen and lyophilized for further antioxidant assays.

## 2.5. Total Phenolic Content (TPC) Assay

TPC was evaluated using a Beckman DU 640 spectrophotometer (Beckman Coulter, Fullerton, CA, USA) with Folin–Ciocalteu's phenolic reagent [17]. Samples collected from in vitro digestion were diluted to 25 mg/ml with aqueous acetone. Gallic acid was used as a standard for preparing the standard curve (0.1, 0.2, 0.3, 0.4, and 0.5 mg/ml in aqueous acetone). All the samples and standards were run in triplicate. Each test tube contained 25 µl of a sample or standard and 250 µl distilled water. 750 µl of the Folin–Ciocalteu's phenol reagent was then added to each tube and mixed thoroughly using a vortex mixer. Then, 500 µl of 200 mg/ml sodium carbonate was added to each tube and vortexed. Samples and standards were incubated for 2 h at room temperature in the dark. Absorbance was detected at 765 nm using a spectrophotometer and the TPC of a sample was expressed as milligrams of gallic acid equivalents (GAE)/g fruit weight.

## 2.6. ORAC Assay

ORAC was used to measure the antioxidant activity of a sample to scavenge peroxy radical and therefore protect the fluorescent molecule from radical attack. The assay was conducted as described by Zhou et al. [17]. The digested samples were diluted with aqueous acetone to a concentration of 1.3 mg/ml. All samples and standards were run in triplicate. The mixture of fluorescein and samples or standards was placed in a 96-well microplate. The plate was incubated for 15 min at 37°C and 35 µl AAPH was then

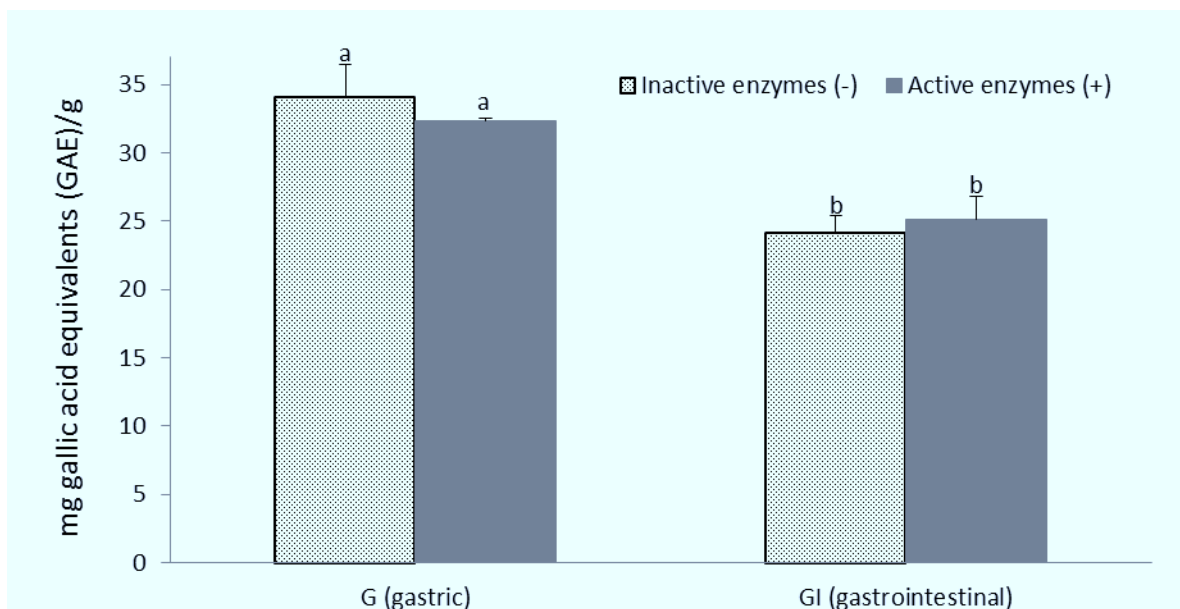
added to each sample and fluorescence was measured every 5 min for 100 min at an excitation wavelength of 485 nm and an emission wavelength of 520 nm. Results were expressed as µmol trolox equivalent (TE)/gram fruit weight.

## 2.7. DPPH Radical Scavenging Assay

The modified protocol was based on Brand-Williams et al. [18]. This assay measured the ability of the samples to quench DPPH radicals using trolox as the standard. The digested samples were diluted to 10 mg/ml with pure ethanol and then centrifuged at 6900 g for 20 min to eliminate residues. 100 µl of each sample or standard solutions was mixed with 150 µl of DPPH radical solution in a 96-well microplate and absorbance was measured at room temperature every 5 min for 2 h at 500 nm, using an HTS 7000 Bio Assay Reader (Perkin Elmer, Norwalk, CT, USA). All samples were run in triplicate. Results were expressed as µmol trolox equivalents (TE)/gram fruit weight.

## 2.8. Antioxidant Purification and HPLC Analysis

To profile antioxidant compounds in the extracts obtained from in vitro digested mixture (by active and inactive enzymes), XAD-16 (20–60 mesh, Sigma-Aldrich, St. Louis, MO, USA) open column chromatography was used to remove sugars followed by solid phase extraction (Thermo Scientific HyperSep SPE-C18, Fair Lawn, NJ, USA) column to further remove other contaminants [19]. HPLC (C18 column, 250 × 4.6 mm; 4 µm) with diode array detection (Hitachi High Technologies America, Schaumburg, Illinois, USA) was then used to analyze individual antioxidants in the cleaned extracts using antioxidant standards. The samples were eluted with methanol (mobile phase A) and formic acid aqueous solution (1:99, vol/vol, mobile phase B) under room temperature at a flow rate of 1 ml/min. Gradient program was set as follows: 10–30% A, 0–6 min; 30–50% A, 7–15 min; 50–70% A, 16–25 min; 70–100% A, 26–35 min; 100% A, 36–40 min. Samples and standards were profiled at a wide range of wavelengths (200–800 nm), with the optimal wavelength selected for comparison. Profiles of standards and samples were compared and matched on the basis of their retention time and ultraviolet (UV) light absorbance.



**FIGURE 2. Total phenolic content of elderberry digested by heat-inactivated and active digestive enzymes (mg GAE/g dry weight sample).** TPC of the samples was determined in triplicate and expressed in mg GAE/g dry weight sample. Texture bar represents the control group treated with the heat-inactivated enzymes, and grey bar represents the treatment group treated with active enzymes. Bars with different superscripts indicate significant difference ( $p < 0.05$ ).

## 2.9. Statistical Analysis

Antioxidant data obtained from the above performed assays was analyzed via SPSS using independent samples t-test. Data for each parameter is reported as mean  $\pm$  SD. Outcomes were compared using  $p \leq 0.05$  as a cutoff point for statistical significance.

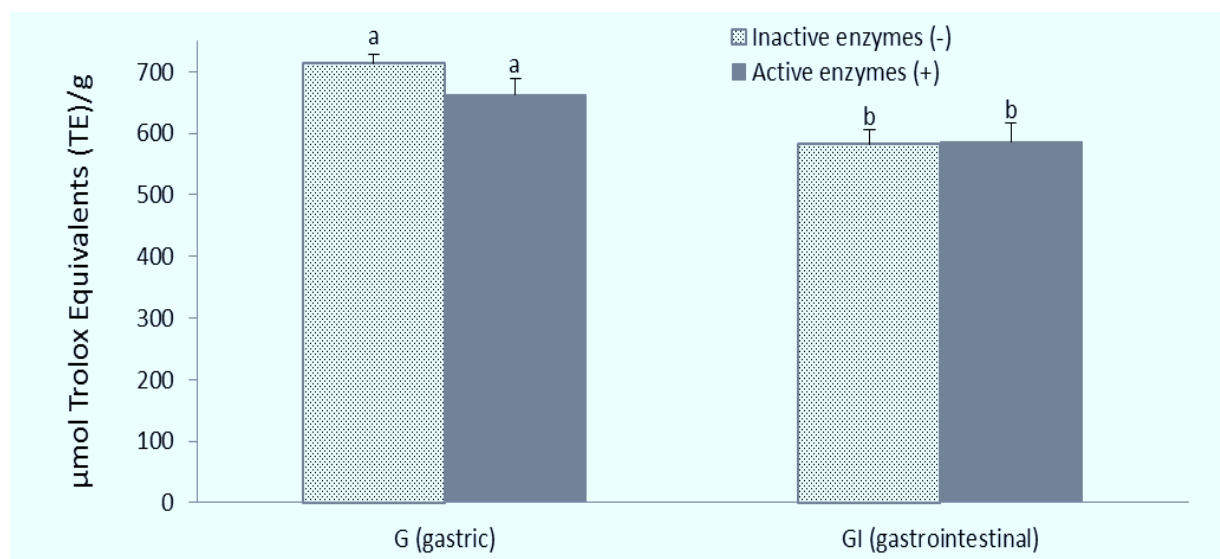
## 3. RESULTS AND DISCUSSION

### 3.1. TPC of the Elderberry Preparations

TPC is commonly used to determine the amount of accessible phenolic antioxidants in a sample [20, 21]. As shown in **Figure 2**, the TPC values of the elderberry extract after first step stomach digestion simulation with inactive (G-) and active pepsin (G+) were 34.1 mg and 32.3 mg gallic acid equivalents (GAE) per gram (dry weight) of the elderberry mixture, respectively. After sequential intestinal digestion simulation, the TPC values of the digested

mixture were 24.0 mg (treated with inactive pancreatic enzymes, GI-) and 25.2 mg GAE (treated with active pancreatic enzymes, GI+) per gram, respectively. The TPC contained in our elderberry sample (on dry weight) are comparable to the previous studies where 24 varieties of American elderberry samples contained 2.7–5.8 mg GAE per gram fresh weight [22, 23]. During the digestion simulation sequentially with pepsin and pancreatic juice, no significant difference was detected between the TPC of the elderberry mixtures treated with inactive and active digestive enzymes. Our results suggest that the GI digestion process did not show significant impact on the extractability (accessibility) of the elderberry. This is in contrast to a previous study showing that GI digestion improves the accessibility of wolfberry phenolics [19]. Indeed, studies of different fruits have shown varying results during the digestion [24–26]. Nevertheless, the result indicates that the process of digestion does not seem to destroy or reduce the amount of accessible phenolic antioxidants in elderberry.





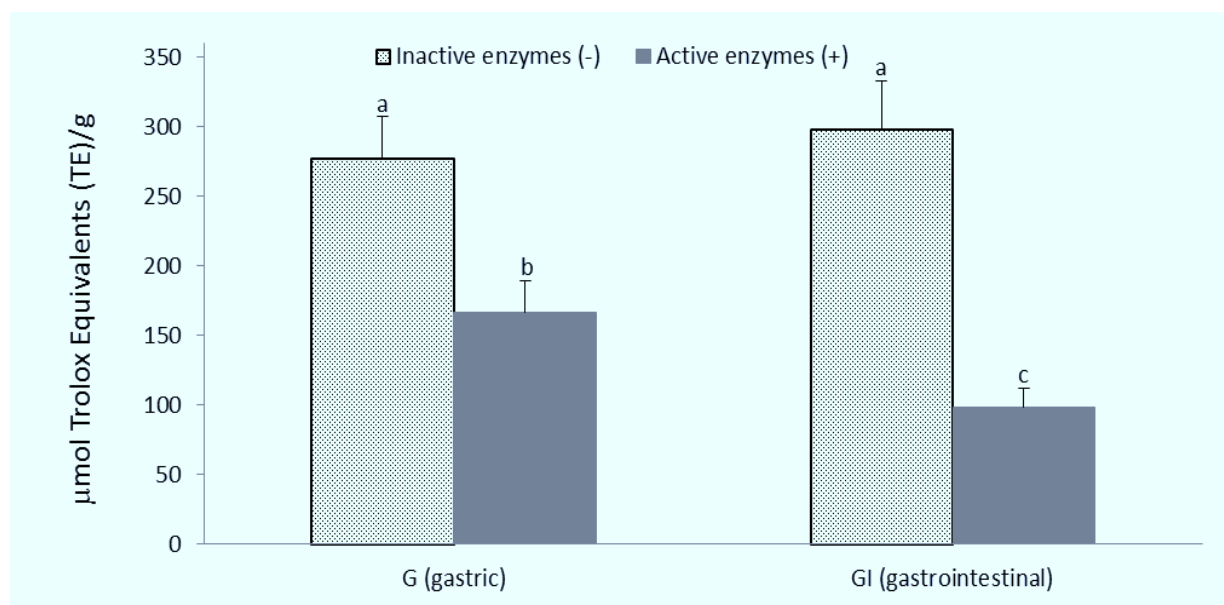
**FIGURE 3. Oxygen radical absorbance capacity of elderberry digested by heat-inactivated and active digestive enzymes.** ORAC of the samples were determined in triplicate and expressed as trolox equivalents ( $\mu\text{mol/g}$  dry weight sample). Texture bar represents the control group treated with the heat-inactivated enzymes, and grey bar represents the treatment group treated with active enzymes. Bars with different superscripts indicate significant difference ( $p < 0.05$ ).

### 3.2. ORAC of the Elderberry Preparations

ORAC assay was used to determine how GI digestion would affect the peroxy radical scavenging capacity of elderberry. Trolox was used as the standard with results expressed as micromoles of trolox equivalents (TE) per gram dry weight of the digested mixture. As presented in **Figure 3**, the ORAC values of the elderberry powder after sequential gastric and intestinal digestion simulation were 712.8 (G-) versus 661.8 (G+) and 581.2 (GI-) versus 587.1 (GI+)  $\mu\text{mol TE/gram}$  of the digested mixture, respectively. Similar to the effect of GI digestion on TPC of elderberry, we found no significant difference between the ORAC of the elderberry mixtures treated with inactive and active digestive enzymes. Although many studies have shown positive correlation between TPC and ORAC on various fruits or vegetables, higher total phenolic content does not always correspond to higher ORAC [27, 28]. In our case of elderberry, it appears that the TPC and ORAC of elderberry are positively correlated, and both are not significantly affected by GI digestions.

### 3.3. DPPH Radical Scavenging Activity of the Elderberry Preparations

Antioxidant capacity of the digested elderberry was further determined by DPPH radical scavenging assay which uses trolox as the antioxidant standard and expressed as  $\mu\text{mol TE/gram}$  (dry weight) of the digested mixture. As shown in **Figure 4**, the DPPH radical scavenging activity of the elderberry powder after sequential gastric and intestinal digestion simulation were 276.1 (G-) versus 166.5 (G+) and 297.8 (GI-) versus 98.5 (GI+)  $\mu\text{mol TE per gram}$  of the digested mixture, respectively. The DPPH radical scavenging activity of the elderberry was reduced by 39.7% (G- versus G+,  $P < 0.05$ ) after gastric digestion and by 66.9% (GI- versus GI+,  $P < 0.05$ ) after GI digestion. Our data showed that GI digestion significantly reduced DPPH radical scavenging activity of the elderberry mixture. This is in contrast to the results for TPC and ORAC where GI digestion had no significant effect. Our result is in contrast to the result a previous study found that digestion process increased DPPH scavenging activity on 25 commer-



**FIGURE 4. DPPH radical scavenging capacity of elderberry digested by heat-inactivated and active digestive enzymes.** The activity of the samples were determined in triplicate and expressed as trolox equivalents ( $\mu\text{mol/g}$  dry weight sample). Texture bar represents the control group treated with the heat-inactivated enzymes, and grey bar represents the treatment group treated with active enzymes. Bars with different superscripts indicate significant difference ( $p < 0.05$ ).

cially available fruit juices [29]. DPPH radical scavenging assay has been commonly used for antioxidant activity evaluation [30]. However, the assay which differs from ORAC assay by the type of radical produced, scavenging method and measurement. It is not unusual that DPPH radical scavenging activity is not positively correlated with TPC or ORAC for an antioxidant sample, especially when the sample contains a variety of antioxidants that have different mechanisms of action [31]. Previous studies have shown varying effects of digestion process on DPPH radical scavenging capacities of various fruits [26, 32]. The result indicated that the digestion process might have changed the composition of individual phenolic compounds but not the overall phenolic content (TPC) in elderberry mixture.

### 3.4. In Vitro Bioavailability Simulation of Phenolic Compounds in the Elderberry Preparations

Dialysis tube was used for evaluating and simulating the absorption of food bioactive compounds. The

simulation experiments showed that 75.6% of total phenolic compounds can be dialyzed (Table 1), indicating that the majority of phenolic compounds in elderberry after active GI digestion could be absorbed. The antioxidant activities of the outside portion (absorbed) were also higher than the inside portion (not absorbed). Evidence is accumulating in consensus that phenolic compounds such as anthocyanins are rapidly absorbed and excreted and that they are absorbed with poor efficiency [33, 34]. Indeed, the bioavailability of anthocyanins is very low with values reported to be less than 0.1% of the consumed amount excreted in the urine [33, 35]. However, in this dialysis study, most of the digested elderberry phenolic compounds were absorbed in the simulating conditions, which the same model has been commonly used for absorption evaluation. Further animal experiments are needed to confirm whether our finding in dialysis tubing can be translated into in vivo physiological environments. It is speculated that a significant portion of phenolic compounds being consumed could remain in the gastrointestinal tract.



**TABLE 1. Antioxidant properties of elderberry phenolic compounds after GI digestion and dialysis for absorption simulation**

Assay	Inside the Tube (relative percentage)	Outside the Tube (relative percentage)	Significance (p value)
TPC	24.40 ± 1.32	75.60 ± 1.46	< 0.01
DPPH	41.79 ± 1.29	58.21 ± 0.38	< 0.05
ORAC	38.97 ± 2.15	61.03 ± 0.77	< 0.01

**TABLE 2. Concentrations of detected antioxidants in elderberry samples after GI digestion by active or inactive enzymes**

Detected Antioxidants	Samples (mg/g dry weight)	
	Inactive Enzyme	Active Enzyme
Caffeic acid	0.23	0.15
Chlorogenic acid*	5.62	12.04
<i>p</i> -Coumaric acid*	1.24	1.97
Ferulic acid	0.41	0.59
Quercetin*	2.24	1.32
Catechin	3.42	4.02
Epicatechin	0.58	0.07
Rutin	9.01	11.32
Cyanidin 3-glucoside	0.53	0.29
Quercetin 3-glucoside	0.36	ND

*Note: the HPLC analyses were conducted in triplicate; ND denotes "not detected"; \* indicates significant difference ( $p < 0.05$ ) between the samples with or without active digestion.*

Therefore, there is a possibility that these compounds are modified into molecular structures that are not identified with available analytical methods, rather than being absorbed [33]. Anthocyanin derived compounds may be found in the gastrointestinal tract as a consequence of the intestinal microflora which have been shown in vitro to modify phenolic compounds [33, 36–38].

HPLC profile of elderberry antioxidants during simulated GI digestion. Antioxidants in the samples were individually compared with the selected antioxidant standards based on HPLC retention time and spectra. Matching peaks were then quantified by peak integrate calculation. Ten individual antioxidants were identified and quantified in milligram of antioxidant per gram dry weight sample as shown in **Table 2**. Antioxidants chlorogenic acid, rutin, catechin, *p*-coumaric acid, and quercetin were the major phenolic compounds in both elderberry samples. Other phenolics including caffeic acid, ferulic acid, epicatechin, and two anthocyanins cyanidin 3-glucoside and quercetin 3-glucoside were also identi-

fied with smaller amounts. The amounts of phenolic acids chlorogenic acid and *p*-coumaric acid in elderberry after digestion with active enzymes were significantly higher than those in the sample digested with inactive enzymes, while the epicatechin concentration was opposite, and quercetin 3-glucoside was not detected in the sample with active enzyme digestion. Collectively, our results suggest that GI digestion could impact release of phenolic compounds differently.

In summary, in vitro models provided a useful tool for rapidly determining the chemical forms of dietary phenolic compounds and their bioactive properties in the dynamic GI environment. Overall, the in vitro findings obtained in this study have demonstrated that GI digestion significantly decreased DPPH radical scavenging activity of elderberry but had no effect on its TPC and ORAC values. After GI digestion, more than 75% of elderberry antioxidants were potentially absorbable in our simulated dialysis tubing experiment. However it is well appreciated that true physiological impact and efficacy of dietary

phenolic compounds may only be speculated pending in vivo studies; the results of this study indicated that such studies are warranted.

## REFERENCES

1. Cao G, Prior RL. Anthocyanins are detected in human plasma after oral administration of an elderberry extract. *Clin Chem* 1999; 45(4):574–6.
2. Murkovic M, Abuja PM, Bergmann AR, Zirngast A, Adam U, Winklhofer-Roob BM, et al. Effects of elderberry juice on fasting and postprandial serum lipids and low-density lipoprotein oxidation in healthy volunteers: a randomized, double-blind, placebo-controlled study. *Eur J Clin Nutr* 2004; 58(2):244–9. doi: 10.1038/sj.ejcn.1601773
3. Milbury PE, Cao G, Prior RL, Blumberg J. Bioavailability of elderberry anthocyanins. *Mech Ageing Dev* 2002; 123(8):997–1006. doi: S0047637401003839
4. Yi W, Akoh CC, Fischer J, Krewer G. Absorption of anthocyanins from blueberry extracts by caco-2 human intestinal cell monolayers. *J Agric Food Chem* 2006; 54(15):5651–8. doi: 10.1021/jf0531959.
5. Fazzari M, Fukumoto L, Mazza G, Livrea MA, Tesoriere L, Marco LD. In vitro bioavailability of phenolic compounds from five cultivars of frozen sweet cherries (*Prunus avium* L.). *J Agric Food Chem* 2008; 56(10):3561–8. doi: 10.1021/jf073506a.
6. Steinert RE, Ditscheid B, Netzel M, Jahreis G. Absorption of black currant anthocyanins by monolayers of human intestinal epithelial Caco-2 cells mounted in ussing type chambers. *J Agric Food Chem* 2008; 56(13):4995–5001. doi: 10.1021/jf0703670h.
7. Wu X, Pittman HE, 3rd, Prior RL. Fate of anthocyanins and antioxidant capacity in contents of the gastrointestinal tract of weanling pigs following black raspberry consumption. *J Agric Food Chem* 2006; 54(2):583–9. doi: 10.1021/jf052108+.
8. Seeram NP, Bourquin LD, Nair MG. Degradation products of cyanidin glycosides from tart cherries and their bioactivities. *J Agric Food Chem* 2001; 49(10):4924–9. doi: jf0107508.
9. McDougall GJ, Fyffe S, Dobson P, Stewart D. Anthocyanins from red wine: their stability under simulated gastrointestinal digestion. *Phytochemistry* 2005; 66(21):2540–8. doi: 10.1016/j.phytochem.2005.09.003.
10. McDougall GJ, Dobson P, Smith P, Blake A, Stewart D. Assessing potential bioavailability of raspberry anthocyanins using an in vitro digestion system. *J Agric Food Chem* 2005; 53(15):5896–904. doi: 10.1021/jf050131p.
11. McGhie TK, Ainge GD, Barnett LE, Cooney JM, Jensen DJ. Anthocyanin glycosides from berry fruit are absorbed and excreted unmetabolized by both humans and rats. *J Agric Food Chem* 2003; 51(16):4539–48. doi: 10.1021/jf026206w.
12. Ohrvik V, Witthoft C. Orange juice is a good folate source in respect to folate content and stability during storage and simulated digestion. *Eur J Nutr* 2008; 47(2):92–8. doi: 10.1007/s00394-008-0701-3.
13. Perez-Vicente A, Gil-Izquierdo A, Garcia-Viguera C. In vitro gastrointestinal digestion study of pomegranate juice phenolic compounds, anthocyanins, and vitamin C. *J Agric Food Chem* 2002; 50(8):2308–12. doi: jf0113833.
14. Kapolna E, Fodor P. Bioavailability of selenium from selenium-enriched green onions (*Allium fistulosum*) and chives (*Allium schoenoprasum*) after 'in vitro' gastrointestinal digestion. *Int J Food Sci Nutr* 2007; 58(4):282–96. doi: 10.1080/09637480601154335.
15. Gil-Izquierdo A, Gil MI, Ferreres F, Tomás-Barberán FA. In vitro availability of flavonoids and other phenolics in orange juice. *J Agric Food Chem* 2001; 49(2):1035–41. doi: 10.1021/jf0000528.
16. Dias MI, Morales P, Barreira JCM, Oliveira MBPP, Sánchez-Mata MC, Ferreira ICFR. Minerals and vitamin B9 in dried plants vs. infusions: assessing absorption dynamics of minerals by membrane dialysis tandem in vitro digestion. *Food Biosci* 2016; 13:9–14. doi: http://dx.doi.org/10.1016/j.fbio.2015.11.004.
17. Zhou K, Su L, Yu L. Phytochemicals and Antioxidant Properties in Wheat Bran. *J Agric Food Chem* 2004; 52(20):6108–14. doi: 10.1021/jf049214g.
18. Brand-Williams W, Cuvelier ME, Berset C. Use of a free radical method to evaluate antioxidant activity. *LWT - Food Sci Tech* 1995; 28(1):25–30. doi: 10.1016/s0023-6438(95)80008-5.
19. Chiang C-J, Kadouh H, Zhou K. Phenolic compounds and antioxidant properties of gooseberry as affected by in vitro digestion. *LWT - Food Sci Tech* 2013; 51(2):417–22. doi: http://dx.doi.org/10.1016/j.lwt.2012.11.014.
20. Zheng W, Wang SY. Antioxidant Activity and

- Phenolic Compounds in Selected Herbs. *J Agric Food Chem* 2001; 49(11):5165–70. doi: 10.1021/jf010697n.
21. Javanmardi J, Stushnoff C, Locke E, Vivanco JM. Antioxidant activity and total phenolic content of Iranian *Ocimum* accessions. *Food Chem* 2003; 83(4):547–50. doi: 10.1016/s0308-8146(03)00151-1.
22. Özgen M, Scheerens JC, Reese RN, Miller RA. Total phenolic, anthocyanin contents and antioxidant capacity of selected elderberry (*Sambucus canadensis* L.) accessions. *Pharmacogn Mag* 2010; 6(23):198–203. doi: 10.4103/0973-1296.66936.
23. Lee J, Finn CE. Anthocyanins and other polyphenolics in American elderberry (*Sambucus canadensis*) and European elderberry (*S. nigra*) cultivars. *J Sci Food Agric* 2007; 87(14):2665–75. doi: 10.1002/jsfa.3029.
24. Bermúdez-Soto MJ, Tomás-Barberán FA, García-Conesa MT. Stability of polyphenols in chokeberry (*Aronia melanocarpa*) subjected to in vitro gastric and pancreatic digestion. *Food Chem* 2007; 102(3):865–74. doi: 10.1016/j.foodchem.2006.06.025.
25. Tavares L, Figueira I, Macedo D, McDougall GJ, Leitão MC, Vieira HLA, et al. Neuroprotective effect of blackberry (*Rubus* sp.) polyphenols is potentiated after simulated gastrointestinal digestion. *Food Chem* 2012; 131(4):1443–52. doi: 10.1016/j.foodchem.2011.10.025.
26. Wootton-Beard PC, Moran A, Ryan L. Stability of the total antioxidant capacity and total polyphenol content of 23 commercially available vegetable juices before and after in vitro digestion measured by FRAP, DPPH, ABTS and Folin–Ciocalteu methods. *Food Res Intl* 2011; 44(1):217–24. doi: 10.1016/j.foodres.2010.10.033.
27. Frontela-Saseta C, López-Nicolás R, González-Bermúdez CA, Peso-Echarri P, Ros-Berruazo G, Martínez-Graciá C, et al. Evaluation of antioxidant activity and antiproliferative effect of fruit juices enriched with Pycnogenol® in Colon carcinoma cells: the effect of in vitro gastrointestinal digestion. *Phytother Res* 2011; 25(12):1870–5. doi: 10.1002/ptr.3625.
28. Scalzo J, Politi A, Pellegrini N, Mezzetti B, Battino M. Plant genotype affects total antioxidant capacity and phenolic contents in fruit. *Nutrition* 2005; 21(2):207–13. doi: 10.1016/j.nut.2004.03.025.
29. Ryan L, Prescott SL. Stability of the antioxidant capacity of twenty-five commercially available fruit juices subjected to an in vitro digestion. *Int J Food Sci Tech* 2010; 45(6):1191–7. doi: 10.1111/j.1365-2621.2010.02254.x.
30. Villano D, Fernandez-Pachon MS, Moya ML, Troncoso AM, Garcia-Parrilla MC. Radical scavenging ability of polyphenolic compounds towards DPPH free radical. *Talanta* 2007; 71(1):230–5. doi: 10.1016/j.talanta.2006.03.050.
31. Velioglu YS, Mazza G, Gao L, Oomah BD. Antioxidant activity and total phenolics in selected fruits, vegetables, and grain products. *J Agric Food Chem* 1998; 46(10):4113–7. doi: 10.1021/jf9801973.
32. Hua Li XYW, P.H. Li, Y. Li and H. Wang. Comparative study of antioxidant activity of grape (*Vitis vinifera*) seed powder assessed by different methods. *J Food Drug Anal* 2008; 16(6):1–7.
33. McGhie TK, Walton MC. The bioavailability and absorption of anthocyanins: towards a better understanding. *Mol Nutr Food Res* 2007; 51(6):702–13. doi: 10.1002/mnfr.200700092.
34. Manach C, Williamson G, Morand C, Scalbert A, Remesy C. Bioavailability and bioefficacy of polyphenols in humans. I. Review of 97 bioavailability studies. *Am J Clin Nutr* 2005; 81(1 Suppl):230S–42S. doi: 10.1093/ajcn/81.1.230S.
35. Prior RL, Wu X. Anthocyanins: structural characteristics that result in unique metabolic patterns and biological activities. *Free Radic Res* 2006; 40(10):1014–28. doi: 10.1080/10715760600758522.
36. Aura AM, Martin-Lopez P, O'Leary KA, Williamson G, Oksman-Caldentey KM, Poutanen K, et al. In vitro metabolism of anthocyanins by human gut microflora. *Eur J Nutr* 2005; 44(3):133–42. doi: 10.1007/s00394-004-0502-2.
37. Fleschhut J, Kratzer F, Rechkemmer G, Kulling SE. Stability and biotransformation of various dietary anthocyanins in vitro. *Eur J Nutr* 2006; 45(1):7–18. doi: 10.1007/s00394-005-0557-8.
38. Keppler K, Humpf HU. Metabolism of anthocyanins and their phenolic degradation products by the intestinal microflora. *Bioorg Med Chem* 2005; 13(17):5195–205. doi: 10.1016/j.bmc.2005.05.003.