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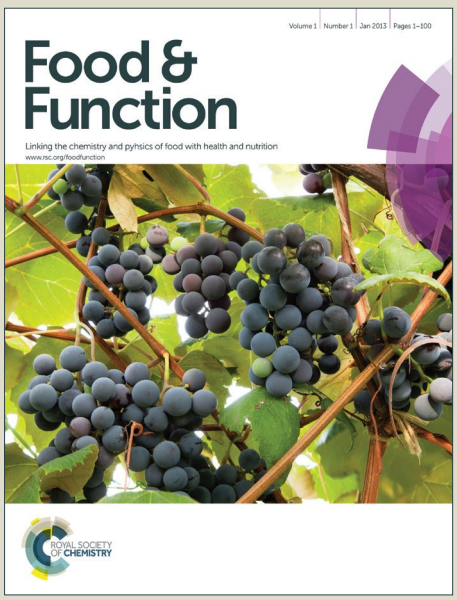

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Effects of boiling and *in vitro* gastrointestinal digestion on the antioxidant activity of *Sonchus oleraceus* leaves

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Abstract

Leaves of *Sonchus oleraceus* L. are especially rich in phenolic compounds and have potent extractable antioxidants. However, it is not known how their antioxidant activity changes after cooking and gastrointestinal digestion. We recorded the profile of phenolics and their associated antioxidant activity in both raw and boiled *S. oleraceus* leaf extracts after *in vitro* gastric and intestinal digestion, and quantified their antioxidant potentials using Caco-2 and HepG2 cells. Boiling significantly diminished the oxygen radical absorbance capacity (ORAC) and concentrations of ascorbate and chicoric acid in the soluble fractions. In contrast, 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging and concentrations of caftaric and chlorogenic acids were unaffected. Phenolics in the soluble fraction were absorbed into cultured human cells and exerted antioxidant activity. Only chlorogenic acid content remained stable during gastrointestinal digestion. *S. oleraceus* appears to be an excellent dietary source of phenolic antioxidants.

Keywords: *Sonchus oleraceus*, boiling, gastrointestinal digestion, phenolics, Caco-2 cells, HepG2 cells

Introduction

Leaves of the smooth sow thistle, *Sonchus oleraceus* L., are an important component of the traditional Māori diet.[1] Ingestion of the thistle, known locally as pūhā, has been postulated to have contributed to the lower incidence of colorectal adenomas in Māori than in New Zealanders of European origin.[1] The lower cancer rates occur despite a greater prevalence of oncogenic risk factors among Māori, including higher intakes of red meat, saturated fat and alcohol, and a higher frequency of obesity.[2,3]

The leaves of *S. oleraceus* are rich sources of assorted phenolic compounds and ascorbic acid. [4–6] The phenolic compounds include chlorogenic, chicoric and caftaric acids, all potent antioxidants.[4–6] These concentrated phenolics explain why exceptionally high antioxidant activities (5.8 ascorbic acid molar equivalents), up to three-fold higher on a dry weight basis than those of frozen blueberry fruit, have been recorded for methanolic extracts of the leaves.[5] Indeed, a recent study has explored the potential to use *S. oleraceus* cell cultures to exploit these high concentrations of phenolics for the commercial extraction of antioxidants.[7]

Vegetative *S. oleraceus* shoots are typically consumed by Māori after boiling for 5 – 30 minutes.[8] However, the effects of cooking on antioxidant concentration in the leaves are unknown. Moreover, previous studies have measured antioxidant potential of the leaves using chemical assays of the leaf extracts, which does not necessarily translate into an effective antioxidant following ingestion; the compounds need to retain their antioxidant activities through the processes of cooking, gastrointestinal digestion, absorption, and transport to the target tissues. Nothing has been documented about the fate of antioxidants in *S. oleraceus* leaves during the digestion process. It is not known whether they are released from the leaf matrix under physiological conditions of the digestive tract, nor whether the antioxidant activity is retained.

Here, we quantify levels of ascorbate, hydroxycinnamic acids, total phenolics and antioxidant activities of extracts of raw and boiled *S. oleraceus* leaves and their ‘soluble fractions’ obtained from *in vitro* gastric and intestinal digestion, and then employ the cellular antioxidant assay (CAA) to identify cellular uptake and antioxidant activity. We hypothesized that the chief

antioxidants in *S. oleraceus* leaves would survive cooking and *in vitro* gastrointestinal digestion, and are absorbed into human cells wherein they exert an antioxidant effect.

Materials and methods

Chemicals

2,2'-Azobis(2-amidino-propane) dihydrochloride (AAPH) and chlorogenic acid were purchased from Sapphire Bioscience (Hamilton, New Zealand). 2,2'-Azobis(2-methylpropionamididine) dihydrochloride (ABAP), 2',7'-dichlorofluorescin diacetate (DCFH-DA), 2,2-diphenyl-1-picrylhydrazyl **(**DPPH), caftaric acid, chicoric acid, Dulbecco's Modified Eagle's Medium (DMEM), fluorescein disodium, Folin–Ciocalteu reagent, Hank's balanced salt solution (HBSS), pepsin from porcine gastric mucosa, pancreatin from porcine pancreas, phosphate buffered saline solution (PBS) and quercetindihydrate were purchased from Sigma-Aldrich (St Louis, MO). 6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) and Na_2CO_3 were purchased from Thermo Fisher Scientific Australia (Scoresby, Australia). Tannic acid was purchased from Carl Roth Gmbh (Karlsruhe, Germany). Ascorbate assay kit (#700420) from Cayman Chemical Company (Ann Arbor, MI, USA). Quercetin was kindly provided by Plant and Food Research, Chemistry Department, University of Otago, Dunedin, New Zealand. Human hepatocellular carcinoma (HepG2) cells were gratefully obtained from the Pathology Department at the University of Otago, Dunedin, New Zealand. Human epithelial colorectal adenocarcinoma cells (Caco-2) cells were kindly provided by Victoria University of Wellington, Wellington, New Zealand. All solvents were of HPLC grade.

Plant materials

Plants were grown from seeds obtained from selfed F1 generation plants, which themselves had been raised from seeds collected from wild populations at Acacia Bay (S 38º 42', E 176º 02') in New Zealand. The plants were grown in an unheated glasshouse under natural light at Victoria University of Wellington, Wellington, New Zealand for 84 d during January to April 2011 (summer).

125

126 **Phytochemical extraction and boiling**

127

128 The leaves from nodes 5 and 6 were removed from 48 plants. Leaves were bisected
129 longitudinally, one half (untreated control) was used for phytochemical analysis and the other for
130 boiling and gastrointestinal digestion. To study the effects of cooking leaf portions were
131 subdivided equally, and one half was boiled in water at 100°C for 5 min, and the other half used
132 raw. The 5-min duration was chosen because this is considered to be the minimum time for
133 cooking *S. oleraceus* leaves and would, therefore, yield information on the maximum antioxidant
134 potential from this prepared food source.

135

136 Material intended for phytochemical extraction was snap frozen in liquid nitrogen, ground to a
137 fine powder, and dissolved in methanol:ddH_2O:acetic acid (70:23:7, v/v/v) to obtain a 10% (w/v)
138 slurry. Aliquots were centrifuged at 24000 g for 5 min, and supernatants stored under nitrogen at
139 −20°C. Phytochemical analyses were performed within 7 d of extraction. All results are
140 presented on a dry weight basis.

141

142 **Gastric and intestinal digestion**

143

144 Artificial gastric juice and intestinal fluid were prepared as described in the British
145 Pharmacopoeia (1988).[9] Simulated gastric juice contained 34.2 mM NaCl, 92.4 µM pepsin and
146 80 mM HCl at pH 1.2. Simulated intestinal fluid was made using 50 mM KH_2PO_4, 15.4 mM
147 NaOH, 1.1 g L^{-1} of pancrease powder, and adjusted to pH 7.5. Digestion was performed in a six-
148 station Erweka DT 600 Dissolution Tester (Erweka International AG, Basel, Switzerland) at
149 37°C with a paddle speed of 50 rpm according to the protocol for release of drug from
150 conventional solid dosage forms.[9] Boiled and raw leaf portions were cut into approximately 1
151 cm^2 pieces (9 g) and incubated in 900 mL artificial gastric juice for 1 h. The remaining leaf
152 fraction was then recovered and resuspended in 900 mL intestinal fluids for 1 h. For the soluble
153 fraction, aliquots of the fluids were withdrawn 60 min into each digestion, centrifuged at 24000 g
154 for 5 min, and supernatant stored at –20°C under nitrogen. The 'soluble fraction' as defined here
155 was the supernatant obtained by centrifugation of the artificial gastrointestinal solution following

in vitro gastrointestinal digestion.[10] A blank was prepared using the same chemicals treated identically but without the leaf material.

Total phenolics assay

Concentrations of total phenolics were measured using the Folin–Ciocalteu method, modified after Waterhouse (2002).[11] Duplicate 1:2 serial dilutions of samples or a 0 – 60 µM tannic acid standard series were introduced into the wells of 96-well plates. Each well held 25 µL sample, standard or blank (ddH_2O), plus 125 µL of 0.1 M Folin-Ciocalteu reagent. Plates were incubated in the dark on an orbital shaker (10 rpm) at room temperature for 3 min, and then 125 µL 0.6 M Na_2CO_3 added to each well and incubated for a further 30 min. Absorbance at 760 nm was read using an EnSpire 2300 multimode reader (PerkinElmer, San Jose, CA). Total phenolic concentrations were expressed as mg tannic acid equivalents g^{-1}.

DPPH scavenging assay

DPPH scavenging capacity was measured for duplicate serial dilutions of the samples as described by Philpott *et al*. (2003)[12] using 1mM Trolox as the standard. A 200 µL aliquot of 100 µM DPPH in methanol was added to 50 µL sample or standard, incubated in darkness on an orbital shaker for 30 min, and absorbance measured at 515 nm. Antioxidant activity was estimated as µmol Trolox equivalents g^{-1} by comparing the sample EC_{50}, with that of the standard. EC_{50} being the concentration of sample or standard which resulted in a 50% reduction in A_{515}.

ORAC assay

The ORAC-fluorescein assay, adapted for manual handling, was performed on serial dilutions of samples in black 96-well plates (Dávalos*et al*., 2004).[13] Into each well were dispensed 120 µL of 117 nM fluorescein disodium in 75 mM phosphate buffer at pH 7.4, and 20 µL of sample or Trolox in the same phosphate buffer. Plates were incubated at 37°C for 5 min, and then 60 µL of 40 mM AAPH added to generate peroxyl radicals. Wells were excited at 485 nm, and

fluorescence emission at 538 nm was read at minute intervals over 1 h using an EnSpire 2300 multimode reader, with 5 s shaking between readings. The area under the fluorescence decay curve (AUC) of samples was calculated, from which the AUC of the antioxidant-free blank (75 mM phosphate buffer) was subtracted. ORAC values for samples were expressed as µmol Trolox equivalents g^{-1} using regression equations between net AUC and Trolox concentration.

Ascorbate assay

Ascorbate concentration was measured using a commercial kit (#700420 from Cayman Chemical Company, Ann Arbor, MI, USA). The ground samples were resuspended in methanol:ddH_2O:DTPA (75:22:2.5, v/v/v) to obtain a 0.05% (w/v) dilution. Ascorbate concentration was measured using serial dilutions of samples in black 96-well plates using a 0 – 150 µM ascorbate standard series. Into each well were dispensed 50 µL DTPA and 50 µL sample or ascorbate standard. Then 100 µL of reconstituted "ascorbate substrate" was added to all wells apart from the sample background wells. Ascorbate 'assay buffer' (100 µL) was added to sample background wells. The plates were incubated in darkness at 25°C for 10 min, and then 50 µL of 'ascorbate developer' was added to all the wells. The plates were incubated in darkness at 25°C for 5 min, and well contents were excited at 345 nm, and fluorescence emission at 425 nm was read using an EnSpire 2300 multimode reader. Ascorbate concentrations of samples were expressed as ascorbate mg g^{-1} using the standard curve for ascorbate 0 – 150 µM.

Online reverse phase HPLC-DPPH scavenging

The HPLC method described by Ou *et al*. (2013)[6] was used to separate and quantify phenolic compounds in samples. We used an Agilent Technologies 1200 series HPLC system (Agilent Technologies, Palo Alto, CA) equipped with a quaternary pump and a diode array detector. Briefly, samples were injected at 4°C into a reverse phase Alltima C18 column (3 µm 150 x 2.1 mm). Elution (0.2 mL min^{-1}) was performed using a solvent system comprising (A) 1% formic acid and (B) 100% acetonitrile using a gradient starting with 95% A, reducing to 85 % at 15 min; 76% at 27 min, 70% at 40 min, 20% from 41 to 45 min and 95% from 46 to 55 min. The absorption spectra at 320 nm were recorded. The HPLC-separated analytes were reacted

postcolumn with 250 μM DPPH in 100% acetonitrile:0.1 M sodium citrate buffer (50:50, v/v) at pH 7.6, and pumped at 0.2 mL min^{-1}. The induced bleaching was detected photometrically as a negative peak at 518 nm. Identification of the main peaks was confirmed by co-eluting with authentic compounds. Concentrations of the main hydroxycinnamic acids in the eluted fractions were computed using regression equations between concentrations of authentic compounds and areas of their peaks in the chromatograms.

The CAA assay

The CAA assay was performed according to Wolfe and Liu (2007).[14] Human HepG2 and Caco-2 cells were seeded at 6 x 10^4 per well on a 96 well flat-bottom plate in 100 μL of DMEM, and incubated at 37°C for 24 h. DMEM was removed, and the cells were washed with PBS. Samples were evaporated and diluted with DMEM, and 50 μL of 25 mM DCFH-DA was added to 50 μL of the diluted extracts, to yield final concentrations of 1, 3, 10 and 30 g L^{-1} of samples. Cells were treated for 1 h with plant samples or quercetindihydrate standard (at 5, 10, 15 and 20 μM final concentration) in DCFH-DA. A 100 μL aliquot of 600 μM ABAP in HBSS was applied to the cells after PBS wash. The emission fluorescence at 538 nm (following excitation at 485 nm), was measured at 37°C every 5 min for 1 h using a Fluoroskan Ascent microplate fluorometer (Thermo Electron, Franklin, MA). CAA values were calculated as the integral of fluorescence emission using the following equation:

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

where ∫SA and ∫CA are the integrated areas under the curves of fluorescence versus time for the sample and controls, respectively.[14]

Statistical analysis

Differences in antioxidant activity and antioxidant concentrations attributable to boiling and digestion were evaluated using ANOVA with Bonferroni post hoc tests ($P<0.05$). Probit analysis

was performed for CAA dose-response data. All analysis was performed using SPSS 18.0 statistical software.

RESULTS

Effect of boiling on leaf contents

Boiling *S. oleraceus* leaves for 5 min at 100°C did not significantly alter concentrations of total phenolics (ANOVA; P=0.99; Fig. 1a) or antioxidant activity as measured by DPPH scavenging (P=0.99; Fig. 1b) or ORAC (P=0.99; Fig. 1c) in methanolic extracts. None of the main antioxidants–ascorbic, caftaric, chlorogenic and chicoric acids –diminished in concentration after boiling (P>0.1 in each instance; Figs.2a-d).

Effects of *in vitro* gastrointestinal digestion

Digestion of raw leaves released 90% more phenolic compounds into the soluble fraction than had been extractable by methanol from the raw leaves (P<0.001; Fig. 1a). The soluble fraction also showed 110% higher ORAC capacities (P<0.001; Fig. 1c), though DPPH radical scavenging (P=0.64; Fig. 1b), and concentrations of ascorbic (P=0.40; Fig. 2a), caftaric (P=0.03; Fig. 2b) and chlorogenic acids (P=0.29; Fig. 2c) were unaffected. In contrast, the concentration of chicoric acid was 30% lower in the soluble fraction compared to that in the methanolic extract of raw leaves (P<0.001; Fig. 2d).

For boiled leaves, digestion yielded a soluble fraction that contained 60% more total phenolic compounds (P<0.001; Fig. 1a) and which was 110% stronger in DPPH radical scavenging capacities (P=0.002; Fig. 1b) compared to the methanolic extracts. ORAC values (Fig. 1c), and concentrations of ascorbic (Fig. 2a) and chlorogenic acids (Fig. 2b) in the soluble fraction were similar to those in methanolic extracts (P>0.1 in each instance). However, the soluble fraction of boiled leaves was lower in concentrations of caftaric (P=0.02; Fig. 2b) and chicoric acids (P<0.001; Fig. 2d) than the methanolic extracts.

Methanolic extracts and soluble factions of both raw and boiled leaves exhibited antioxidant activity in HepG2 and Caco-2 cells (Fig. 3a,b). The antioxidant activity inside the human cells caused by the soluble fraction from the raw leaves were greater than for boiled leaves; five times greater in HepG2 cells (P=0.004) and seven-fold higher in Caco-2 cells (P<0.001). The soluble fraction of boiled leaves displayed lower antioxidant activity inside Caco-2 cells than did the methanolic extracts of boiled leaves. Inside HepG2 cells, in contrast, the antioxidant activities of both the soluble fraction and the methanolic extract of boiled leaves were similar.

Effect of boiling on the soluble fraction of leaves

The soluble fraction from the *in vitro* digestion of boiled leaves had 40% lower ORAC values, 45% lower concentrations of ascorbate, and 40% lower chicoric acid content than that from raw leaves (P=0.001; Figs.1c, 2a,2d). In contrast, DPPH activities of the soluble fraction were 80% higher for boiled than for raw leaves (P=0.01; Fig. 1b). Concentrations of total phenolics (Fig. 1a), caftaric acid (Fig. 2b) and chlorogenic acid (Fig. 2c) in the soluble fraction were similar between boiled and raw leaves (P>0.1 in each instance).

The boiled leaves yielded lower μmole proportions of chicoric acid (P<0.001; Table 1), but higher proportions of chlorogenic acid (P=0.02) into the soluble fraction than that of raw leaves. The μmole proportion of caftaric acid in the soluble fractions were unaffected by boiling (P=0.6; Table 1).

DISCUSSION

Our data indicate that cooking the leaves by boiling them for 5 min did not appreciably diminish the antioxidant activities prior to *in vitro* gastrointestinal digestion. Significantly, three major phenolic compounds (caftaric, chlorogenic, and chicoric acids) were present in the extracts both before and after digestion. These compounds were absorbed by human cells, whereupon they exhibited apparent antioxidant activity. Collectively, these data argue a compelling case for exploring *S. oleraceus* further as a useful dietary antioxidant supplement to promote human health.

Boiling leaves for 5 min is sufficient to cook them[1,8], but this did not significantly diminish their total concentration of phenolic compounds (Fig. 1a). Of the main hydroxycinnamic acids, only chicoric acid significantly decreased in concentration upon boiling (Fig. 2d); chicoric acid is the most heat labile of the three, and would likely have hydrolysed to yield caftaric and caffeic acids.[15,16] In other crops, boiling has been reported to cause the decline[17] or an increase[18,19] in levels of the available phenolic compounds. Decreased levels of phenolics are often attributed to polymerisation or decomposition of aromatic rings[20], or else to their removal in the water used for boiling.[17] In contrast, boiling may increase the release of phenolic compounds by enhancing the release of cell wall-bound compounds[21], and/or by halting polyphenoloxidase-driven enzymatic oxidation.[18,19]

Boiling did not significantly diminish concentrations of ascorbate as measured in the methanolic extracts of leaves (Fig. 2a) even though ascorbate is water soluble and heat labile. The apparent stability may be because the oxidation of ascorbate into dehydroascorbate (DHA) in water at 100°C is reversible.[22] Furthermore, submerging the leaves in boiling water may restrict contact between leaf ascorbate and oxygen, thus limiting its oxidation. Hot air drying of vegetables, for example, causes more ascorbate loss than does boiling them at the same temperature.[23] It is also possible that boiling for 5 min was insufficient to cause ascorbate loss via leaching into the water; the duration of boiling has been shown to affect the degree of ascorbate leaching in a variety of vegetables.[24,25]

The soluble fraction of the boiled leaves had lower antioxidant activity than did that of raw leaves as detected by ORAC measurements (Fig. 1c). However, this difference was not evident in the measurements of DPPH radical scavenging (Fig. 1b). The soluble fraction from boiled leaves had similar proportions of caftaric acid, higher proportions of chlorogenic acid and lower proportions of chicoric acid than those from raw leaves (Table 1). The DPPH assay would likely overestimate the antioxidant capacity of boiled leaves because caftaric and chlorogenic acids are small molecules with coplanar structures that have better access to the radical of the DPPH molecule than chicoric acid.[26] For example, the molar volumes of caftaric (184 cm^3 mol^{-1}) and chlorogenic acids (214 cm^3 mol^{-1}) are 60% and 30% smaller, respectively, than that of chicoric

acid (www.chemspider.com; accessed April 2015). Thus, measurements of antioxidant activity can vary across different assays, dependent largely on the type and proportion of the compounds that remain in the soluble fraction, as reported during the *in vitro* digestion of *Solanum esculentum*.[27] Thus, more than one assay needs to be used to evaluate the antioxidant activity of a plant food as every method has limitations.[28] Nonetheless, the DPPH assay is a simple, rapid technique, which does not require special equipment and provides useful information on the capacity of phytochemical sources in scavenging free radicals, aiding the screening of potential antioxidant sources. The technique is routinely used in assays of the antioxidant potential of foodstuffs.[26]

Acid hydrolysis of bound phenolics in leaves[29] would likely explain why levels of total phenolics in the soluble fraction were approximately two-fold greater than those in methanolic extracts from untreated leaves (Fig. 1a). The release of phenolics during *in vitro* gastric digestion has been attributed to acid action in a wide variety of fruits and vegetables[30,31]. The glycosylated and esterified phenolic compounds are hydrolysed by the acidic conditions, which exist during gastric simulations.[29]

Gastrointestinal digestion facilitated the release of caftaric, chlorogenic and chicoric acids from the leaves. Consistent with these results, chlorogenic acid has been shown to retain its structure in the stomach of rats[32] and in ileostomized humans on a liquid chlorogenic acid supplement.[33] Furthermore, chlorogenic acid is rapidly absorbed without structural transformations into the plasma in the stomachs of rats[32], in healthy humans[34] and in ileostomized humans.[33] Chlorogenic acid is not hydrolysed by intestinal enzymes during *in vitro* digestion[35], although it is transformed into neochlorogenic acid after 2 h of *in vitro* digestion, attributable to the high pH (7.5) rather than to activities of pepsin or pancreatin.[30] It has been shown previously that the concentrations of chicoric and caftaric acids decline after simulated gastrointestinal digestion, reaching the lowest values in the *in vitro* jejunum phase.[36]

The CAA of undigested and digested raw leaves were measured using Caco-2 and HepG2 cells (Fig. 3) since they are suitable models to study uptake and metabolism of antioxidants by cells, accordingly representing the intestinal epithelium and liver.[37] The *S. oleraceus* digested leaves

had several-fold higher CAA values (2.7 µmol quercetin equiv.g^{-1}, Fig. 3) than those reported for fresh fruits (0.4 ± 0.1 µmol quercetin equiv.g^{-1}) and vegetables (0.02 ± 0.002 µmol quercetin equiv.g^{-1}), including fresh blueberries (0.3 ± 0.06 µmol quercetin equiv.g^{-1}).[14,38,39] Thus, the potential of *S. oleraceus* leaves to protect human cells from oxidative stress is much higher than many plant foods. Confocal laser scanning microscopy of HepG2 cells treated with *S. oleraceus* leaf extract and stained with Naturstoff reagent has confirmed that antioxidants entered the cells, rather than being bound to the cell membranes.[40] Previous reports also verify that chlorogenic acid is readily taken up by human HepG2 cells[41], human Caco-2 cells[42], and protected human neuroblastoma SH-SY5Y cells from oxidative stress.[42] In addition, mouse erythrocytes[43] and granulocytes[44] treated with chlorogenic acid were protected from H_2O_2 induced haemolysis and lipid peroxidation. Chicoric acid, too, was absorbed by neuron-like PC-12 cells extracted from rat pheochromocytoma, which were then protected from oxidative stress and maintained their viability.[45]

Chicoric, chlorogenic and caftaric acids are important dietary low molecular weight antioxidants [46,47] with potent radical scavenging activities *in vitro*.[48,49] Chicoric acid is comparable in activity to certain flavonoids and rosmarinic acid, which are efficient antioxidants.[48] Chicoric acid is several times more effective in scavenging peroxyl radicals than ascorbic acid in *in vitro* human intestinal conditions.[49] Chlorogenic acid was, respectively, three- and seven-fold more potent than ascorbic acid and Trolox in simulated human intestinal conditions.[49] Despite their strong antioxidant activities, studies on the bioavailability and stability of chicoric acid[50] and caftaric acid[51] are far less documented compared to chlorogenic acid. The data presented here confirm that these hydroxycinnamic acids in *S. oleraceus* leaves were stable following gastrointestinal digestion as quantified by *in vitro* and cellular measures of antioxidant activity.

This work represents an important initial step to elucidate the nutraceutical potential of *S. oleraceus*. However, further research is required to examine the effect of enzymes and microbiota in the gut on the structural transformation and antioxidant activities of phenolic compounds. For example, in the small intestine and liver, hydroxycinnamic acids undergo methylation, sulfation and glucuronidation.[52] Those hydroxycinnamic acids that escape absorption in the stomach and small intestine can be metabolised by the colonic microbiota.[53–55]

Esterase activities of human gut bacteria *Lactobacillus johnsonii*, for example, have been shown to hydrolyse chlorogenic acid into quinic and caffeic acids, chicoric acid into caftaric and caffeic acids, and caftaric acid into caffeic and tartaric acids, as evidenced using an *in vitro* gastrointestinal model.[54]

The leaves of *S. oleraceus* contains 32.5 g kg^{-1} fibre on dry weight basis.[56] Levels of inulin-type fructans have not been reported for the leaves of *S. oleraceus*, although low amounts (7 g kg^{-1} fresh weight) are known to be present in its roots.[57] Inulin-type fructans may act as prebiotics, stimulating the growth of bifidobacteria in the human colon. However, the fate of inulin-type fructans from leaves of *S. oleraceus* in the human colon is unknown.

In conclusion, gastrointestinal digestion of *S. oleraceus* leaves resulted in extraction of hydroxycinnamic acids and ascorbate, with corresponding antioxidant activities, and that raw leaves were slightly superior to boiled leaves. The antioxidant activity of hydroxycinnamic acids were stable during *in vitro* gastrointestinal digestion, and displayed antioxidant activity inside HepG2 and Caco-2 cells. Therefore, these *in vitro* studies demonstrate that *S. oleraceus* raw leaves are potentially an excellent dietary antioxidant source.

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Table 1 Proportionate contributions of three main hydroxycinnamic acids to the total phenolic acid pool in the soluble fractions of raw and boiled *Sonchus oleraceus* leaves

	Contribution (%)[1]		
	Caftaric acid	**Chlorogenic acid**	**Chicoric acid**
Raw	31±2 a	21±1 b	48±1 a
Boiled	33±1 a	24±0 a	43±0 b

[1] Values are means ± SE (n=6). Different letters in the same column indicate statistically significant differences ($P<0.05$)

552 **Figure captions**

553

554 **Fig. 1.** Changes in (a) concentration of total phenolic compounds, (b) DPPH radical scavenging
555 activity and (c) ORAC activity in the methanolic extracts of raw and boiled leaves of *Sonchus*
556 *oleraceus*, and in the soluble fraction following *in vitro* gastrointestinal digestion. Means ± SE
557 (n=6). Bars with different letters are significantly different (P<0.05).

558

559 **Fig. 2.** Concentration of (a) ascorbic, (b) caftaric, (c) chlorogenic and (d) chicoric acids in the
560 methanolic extracts of raw and boiled leaves of *Sonchus oleraceus*, and in the soluble fraction
561 following *in vitro* gastrointestinal digestion. Means ± SE (n=6). Bars with different letters are
562 significantly different (P<0.05).

563

564 **Fig. 3.** Cellular antioxidant activity measured in (a) HepG2 and (b) Caco-2 cells incubated with
565 the methanolic extracts of raw and boiled leaves of *Sonchus oleraceus*, and the soluble fraction
566 following *in vitro* gastrointestinal digestion. Means ± SE (n=6). Bars with different letters are
567 significantly different (P<0.05).

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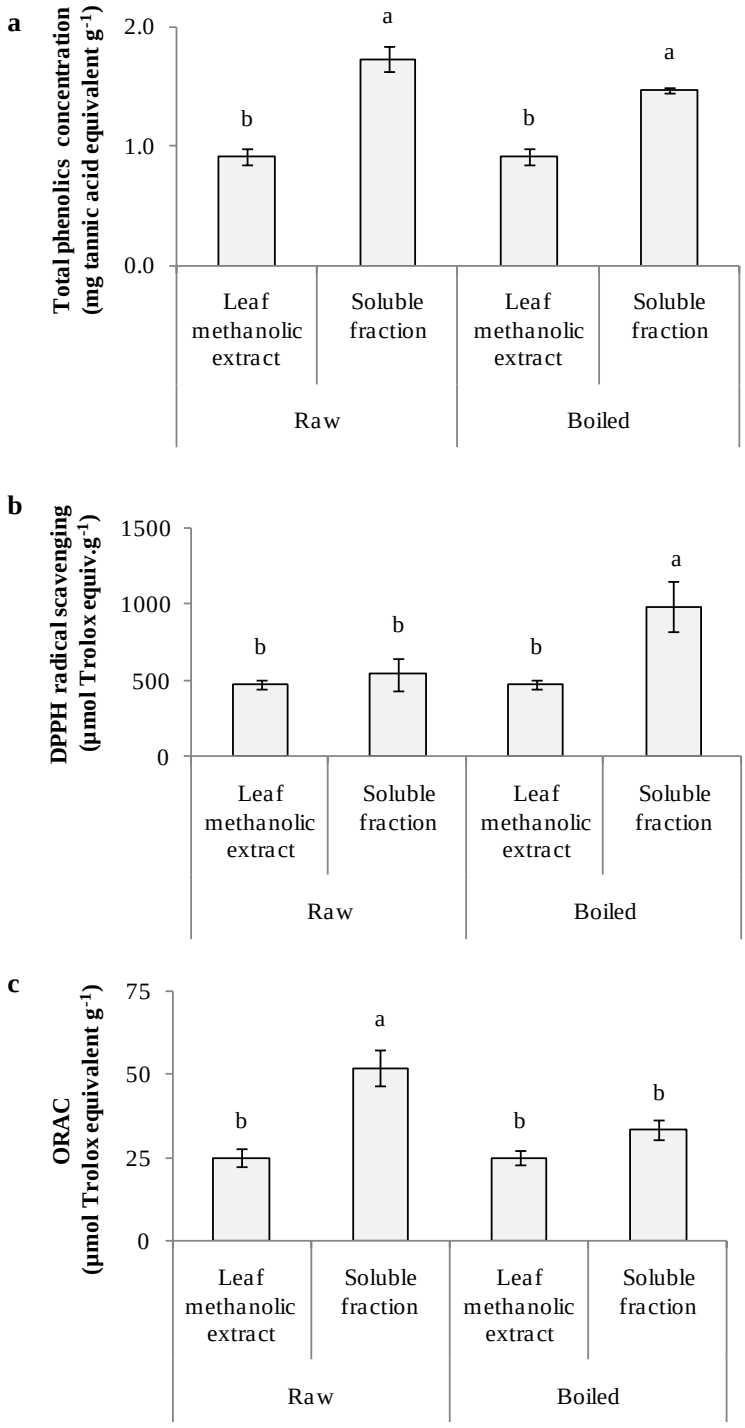

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584 **Fig. 1.**

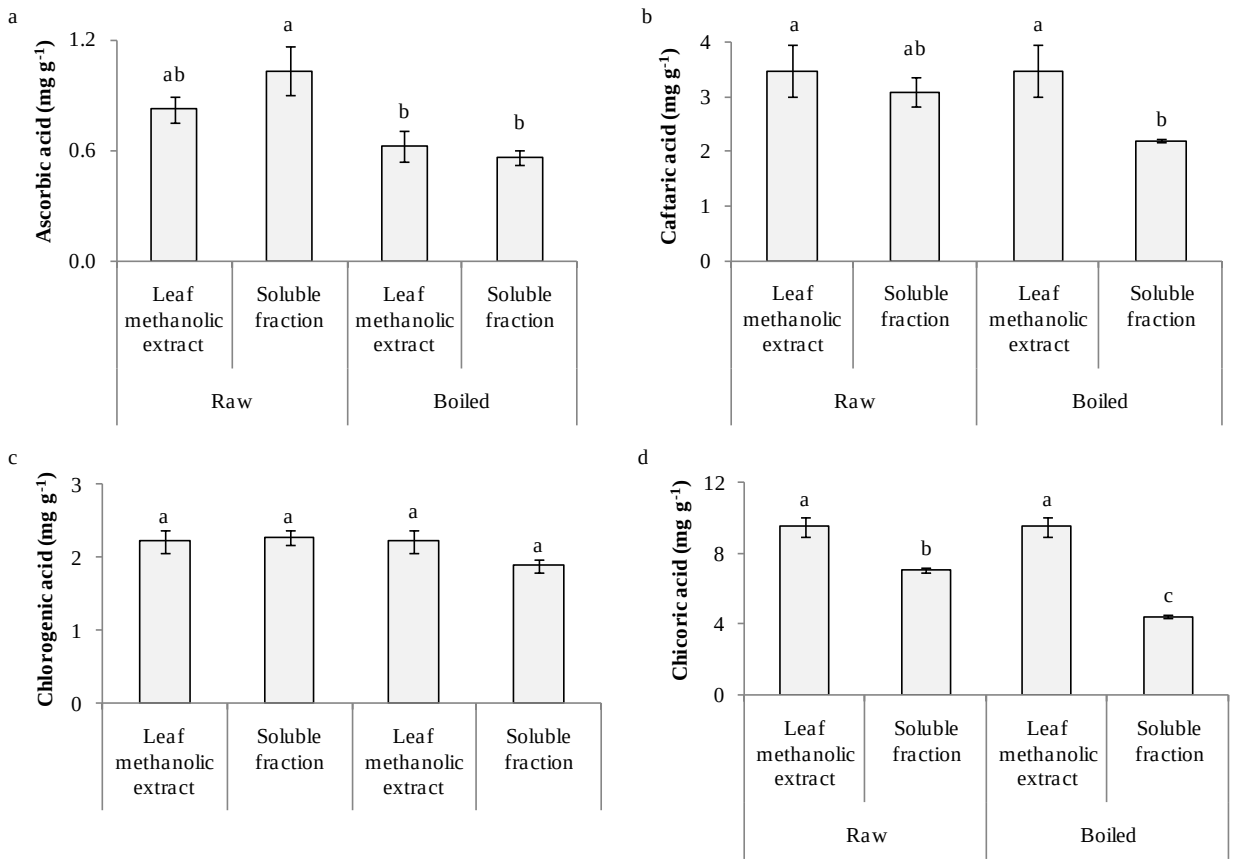

Fig. 2.

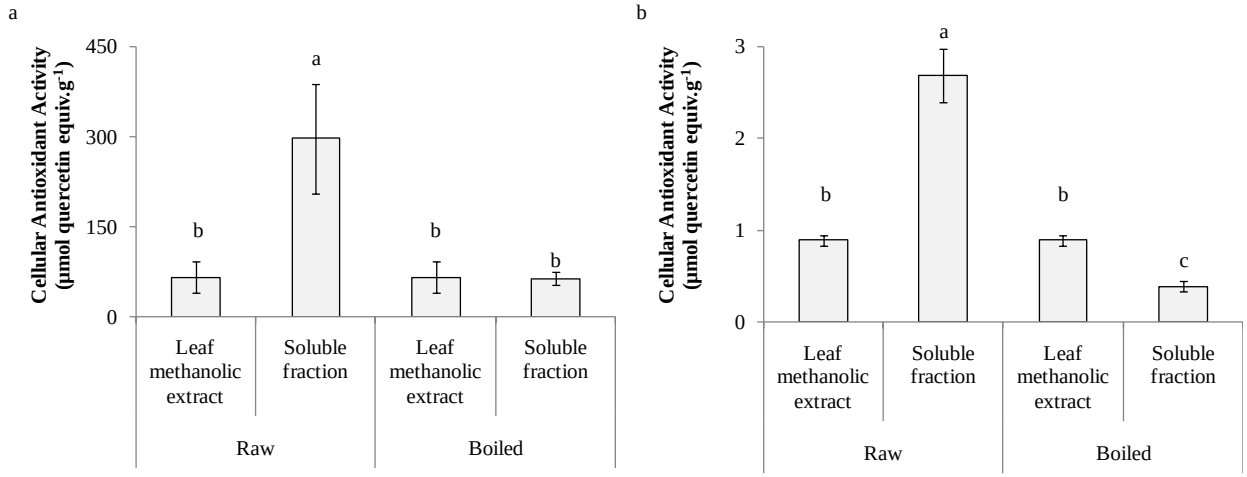

Fig. 3.

Table of contents entry

Soluble fractions released by *in vitro* gastrointestinal digestion of *S. oleraceus* leaves exhibited antioxidant activity in cultured human cells.