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Effect of simulated gastrointestinal digestion and fermentation on polyphenolic content and bioactivity of brown seaweed phlorotannin-rich extracts.

Giulia Corona^{1,2,*}, Maria Magdalena Coman^{2,3}, Yuxuan Guo², Sarah Hotchkiss⁴, Chris Gill⁵, Parveen Yaqoob², Jeremy P.E. Spencer² and Ian Rowland²

¹*Health Sciences Research Centre, University of Roehampton, London SW15 4JD, UK*

²*Department of Food and Nutritional Sciences, University of Reading, Reading, RG6 6AP, UK*

³*School of Biosciences and Veterinary Medicine, University of Camerino, Camerino (MC), Italy*

⁴*CyberColloids Ltd. Carrigaline Industrial Estate, Carrigaline, County Cork, Ireland*

⁵*Northern Ireland Centre for Food & Health, University of Ulster, Coleraine, BT52 1AA*

***Corresponding author:** Dr. Giulia Corona, Health Science Research Centre, Life Sciences Department, Whitelands College, University of Roehampton, Holybourne Avenue, London, SW15 4JD . e-mail: giulia.corona@roehampton.ac.uk
Tel: +44 (0)20 8392 3622, fax +44 -(0)208392 3610,

List of abbreviations: CF, colonic fermentation; GID, gastrointestinal digestion; HMW, high molecular weight; LMW, low molecular weight; ND, non-digested; SPE, seaweed polyphenol extract; TP, total polyphenol;

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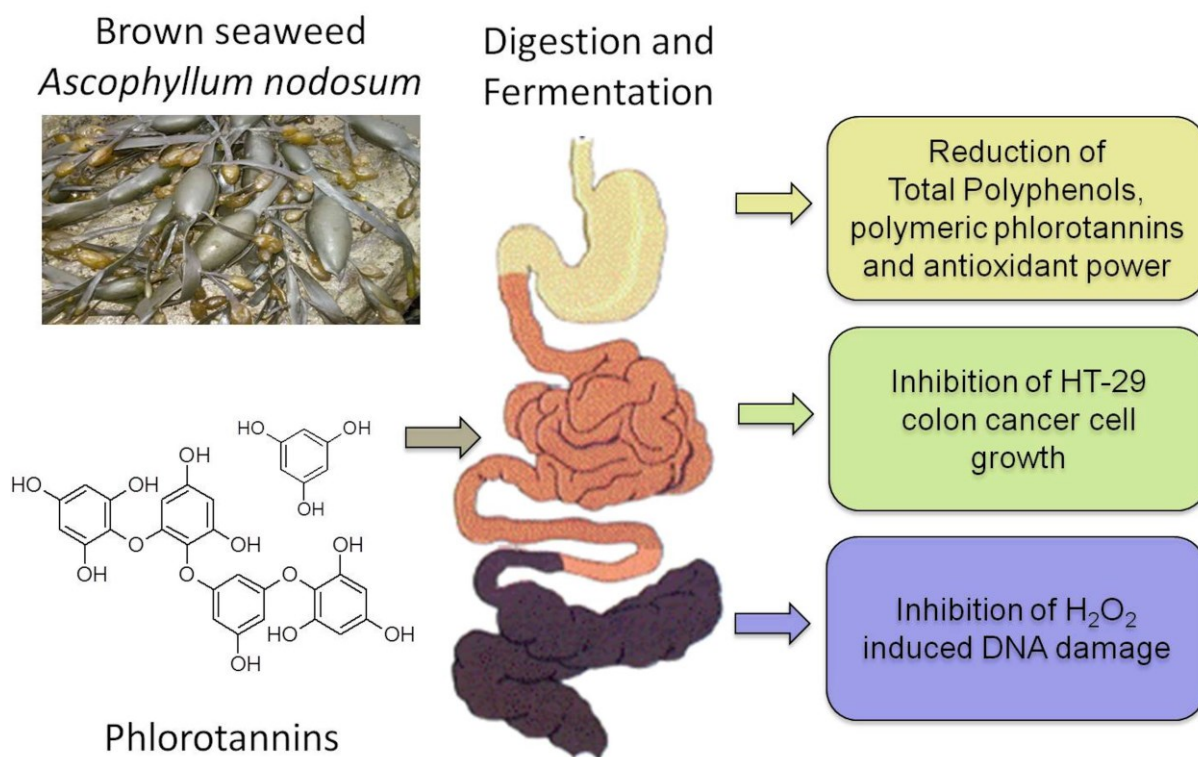
Abstract

Scope: Unlike other classes of polyphenols, there is a lack of knowledge regarding brown seaweed phlorotannins and their bioactivity. We investigated the impact of *in vitro* gastrointestinal digestion and colonic fermentation on the bioactivity of a seaweed phlorotannin extract from *Ascophyllum nodosum* and its high molecular weight (HMW) and low molecular weight (LMW) fractions.

Methods and Results: The highest phlorotannin and total polyphenol (TP) concentration was observed in the HMW fraction. Antioxidant capacity broadly followed phlorotannin and TP levels, with HMW having the highest activity. Both gastrointestinal digestion (GID) and colonic fermentation (CF) significantly affected phlorotannin and TP levels, and antioxidant capacity of the extract and fractions. Despite this, in HT-29 cells, all GID extracts significantly inhibit cell growth, whereas CF extracts effectively counteracted H₂O₂ induced DNA damage.

Conclusion: Although phlorotannins, TP levels and antioxidant power of the extracts were strongly reduced after *in vitro* digestion and fermentation, their anti-genotoxic activity and cell growth inhibitory effect in colon HT-29 cells was maintained and enhanced. HMW was the most effective fraction, indicating that the high molecular weight phlorotannins potentially exert a stronger beneficial effect in the colon.

This study examines the impact of in vitro gastrointestinal digestion and colonic fermentation on the stability and bioactivity of a seaweed phlorotannins. An ethanolic extract from *Ascophyllum nodosum* rich in phlorotannins, and its high molecular weight (HMW) and low molecular weight (LMW) fractions were utilized. Both gastrointestinal digestion (GID) and colonic fermentation (CF) significantly affected polymeric phlorotannins, total polyphenol levels, and antioxidant activity of the extract and fractions. Despite this, the anti-genotoxic activity and cell growth inhibitory effect of the extracts in colon HT-29 cells was maintained and enhanced. HMW was the most effective fraction, indicating that the high molecular weight phlorotannins potentially exert a stronger beneficial effect in the colon.



Introduction

Seaweeds have been traditionally consumed for centuries in coastal regions of Asian countries [1], and more recently European countries including Ireland have shown a renewed interest in seaweeds that were once a traditional part of the diet [2-5]. The gastronomic use of seaweeds has expanded into the European cuisine, and various species of edible seaweeds are now increasingly used worldwide as a source of components with potential industrial applications [6-8].

Seaweeds are a rich source of a range of bioactive compounds [9-11], including polyphenolic compounds [12]. Polyphenols found in algae [13, 14] show some similarities to those present in land plants [13-15]. Brown seaweeds contain a characteristic class of compounds called phlorotannins [16-19], which are only found in brown seaweeds [20]. and can account for 5 to 15 % of the plant dried weight [15]. They are oligomers and polymers of phloroglucinol units, and their molecular weight can vary greatly, from 126 Da to 650KDa [12, 19]. Phlorotannins are being increasingly investigated for their vast array of bioactivities [14, 21, 22] such as antioxidant [23-27], anti-inflammatory [24, 28, 29], antibacterial [30], anticancer [31-35], and antidiabetic [31, 36, 37], showing promising potential to develop seaweed-derived products rich in bioactive components with commercial potential for food and pharma applications [38, 39]. However the gastrointestinal tract may largely impact the structure of phlorotannins during digestion and this can have an impact on their bioactivity [40, 41]. The ability of phlorotannins to act as effective bioactive molecules *in vivo* will be dependent on the degree of their biotransformation and conjugation during absorption from the gastrointestinal (GI) tract, in the liver and finally in cells [40, 42]. In view of this, the way phlorotannins are modified and metabolised during gastrointestinal digestion and colonic fermentation, needs to be considered, because

it will affect their bioactivity [43]. We have recently shown that after consumption of a phlorotannin-rich capsule a range of oligomeric metabolites appear in plasma and are excreted in urine, and the data suggest that extensive gastrointestinal modifications occur prior to absorption [42]. In the present study, the *in vitro* digestion/fermentation and their impact on antioxidant and anti-genotoxic activity of extracts from *Ascophyllum nodosum* (a common brown alga in British Isles) was investigated. An ethanol-based seaweed polyphenol extract (SPE) and its High Molecular Weight (HMW) fraction and Low Molecular Weight (LMW) fraction were produced from the seaweed and were digested (GID) *in vitro* to mimic the condition of the human gastrointestinal tract [44, 45] and fermented (CF) by using a pH-controlled, stirred, batch-culture system reflective of the distal region of the human large intestine [46]. We have assessed the impact of digestion and fermentation on the *in vitro* antioxidant capacity, and anti-genotoxic potential of the extracts in HT-29 cells.

Materials and methods

Seaweed material. Fresh *Ascophyllum nodosum* was supplied by The Hebridean Seaweed Company, Isle of Lewis, Scotland in March 2011. The seaweed biomass was harvested by hand to ensure quality, cleaned of any contaminating sand and fouling organisms and then shipped refrigerated to a processing facility in France where it was immediately chopped and frozen.

Preparation of seaweed extracts. A novel seaweed polyphenol extract (SPE) from *Ascophyllum nodosum* was produced by CEVA (France) using a proprietary solvent based extraction system that was specifically adapted for use with fresh or frozen

Ascophyllum nodosum. The extraction step was carried out at room temperature overnight, using an ethanol/water (60/40) solvent mix and 400kg of defrosted seaweed. Post extraction, insoluble material (>100µm) was removed through a series of filtration steps followed by ethanol removal and concentration using a vacuum evaporator. Approximately half of the produced extract was then fractionated using tangential flow ultra-filtration to produce two further extracts of high molecular weight (HMW, >10KDa) and low molecular weight (LMW, 1-10KDa) range, and with varying polyphenol content. The phlorotannins in the extract and fractions were characterized by NP-HPLC analysis. Total polyphenols were quantified using the Folin-Ciocalteu method as equivalents of phloroglucinol units.

Simulated Gastro-Intestinal Digestion: The procedure was adapted from Mills et al. and McDougall et al. [44, 45] and conducted as previously described [42]. This method consists of two sequential stages: gastric digestion and small intestinal digestion with a dialysis step. Seaweed extracts and fractions (SPE, HMW, LMW) were dissolved (10g in 30 ml acidified water pH=2) and pepsin (320 U/ml) was added. Samples were incubated at 37 °C for 2 h on a shaker covered with foil to exclude light. The pH was adjusted to 7.5 by adding few drops of 6M NaOH, and pancreatin (4 mg/ml) and bile extracts (25 mg/ml) were added. The samples were incubated at 37 °C for 2 h on a shaker, transferred into the dialysis tubing (100-500 Da, cut-off, 1.8 ml/cm, Spectra/Por, Biotech) and dialysed overnight at 4 °C against water (4 L) to remove low molecular weight digested materials. The dialysis fluid was changed and dialysis continued for additional 2 h. Digested samples (GID) were freeze-dried and stored at -20 °C. Aliquots of digested and dialyzed extracts were used in the subsequent *in vitro* assays and cell culture treatments.

Colonic Fermentation (Batch Culture): An aliquot of the digested extracts (GID) was subjected to *in vitro* colonic fermentation procedure, using the previously described method [42]. Batch-culture fermentation vessels (300 ml; one vessel per treatment) were autoclaved and filled with 135 ml sterilized basal medium. Medium was stirred and gassed overnight with O₂-free N₂. Before addition of the digested extracts (equivalent to 1.5 g of undigested extracts), the temperature inside the vessels was set to 37 °C by a circulating water bath and the pH was controlled at 6.8 by an electrolab pH controller, in order to mimic conditions in the distal region of the human large intestine (anaerobic; 37 °C; pH 6.8). Vessels were inoculated with 15 ml faecal slurry (1:10, w/v) and batch cultures were run for 24 h. Fermented samples were centrifuged at 13200 rcf for 10 minutes and supernatants were transferred into the dialysis tubing (100-500 Da, cut-off, 1.8 ml/cm, Spectra/Por, Biotech) and dialysed overnight at 4 °C against water (4 L) to remove low molecular weight fermented materials. The dialysis fluid was changed and dialysis continued for additional 2 h. Samples were stored at -20 °C until subsequent analysis (NP-HPLC analysis, *in vitro* assays and cell culture treatments).

NP-HPLC analysis: The phlorotannin levels of the non-digested (ND) digested (GID) and fermented (CF) extracts were analyzed by normal phase HPLC analysis as previously described [42], using an HPLC 1100 series (Agilent) equipped with LiChrospher Si60-5 column. The compounds were detected at a wavelength of 268 nm. All data were analyzed by ChemStation software. The phloroglucinol standard was injected at 0.1-100 µg/ml and phlorotannins in the extracts were analysed as phloroglucinol equivalents.

LC-MS analysis: Further characterization of the non-digested (ND) digested (GID) and fermented (CF) extracts was carried out in the negative ion mode using LC-

MS/MS utilizing electrospray ionisation (ESI) as previously described as previously described [42].

Total polyphenol content (Folin-Ciocalteu). Total polyphenol (TP) content of the non-digested (ND) digested (GID) and fermented (CF) extracts was determined by the Folin-Ciocalteu's method adapted from Yuan et al. [47]. 100 µl of sample were mixed with 50 µl of 2M Folin-Ciocalteu's reagent and left at room temperature for 3 min. Then 200 µl of 20% Na₂CO₃ were added and the mixture incubated at room temperature in the dark for 45 min before transferring 150 µl to a 96 microwell plate in duplicate. Absorbance was measured at 730 nm on a GENios TECAN microplate reader using phloroglucinol as the standard. TP content of the extracts was expressed as µg/mg phloroglucinol equivalents.

Antioxidant capacity (TEAC assay). The antioxidant capacity of the ND, GID and CF extracts was determined by the Trolox Equivalent Antioxidant Capacity (TEAC assay) assay method [48] as follows: A stable stock solution of ABTS^{•+} was produced by reacting a 7 mmol/L aqueous solution of ABTS with 2.45 mmol/L potassium persulfate (final concentration) and allowing the mixture to stand in the dark at room temperature for 12–16 h before use. An ABTS^{•+} working solution was obtained by diluting the stock solution in ethanol to an absorbance of 0.70 (730nm). 15 µl of each sample were mixed with 450 µl of ABTS^{•+} working solution and left at room temperature for 5 min. 150 µl of sample solutions were transferred to microwell plate in duplicate and absorbance was measured at 730 nm on the GENios TECAN microplate reader. Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) was used as the standard for a calibration curve. Results from TEAC assay were quantified as µmol/mg Trolox equivalents.

HT-29 cell culture: HT-29 human colorectal adenocarcinoma cells were cultured in high glucose (4.5g/L) Dulbecco's modified Eagle's medium (DMEM) containing Na pyruvate, supplemented with 10 % heat-inactivated bovine serum, 2 mM L-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin. Cells were kept in 75cm² culture flasks at 37°C, 5% CO₂ and passaged once a week. Spent media was replaced 1 day after seeding and every 2-3 days thereafter.

Treatment of HT-29 cells for DNA damage assay (COMET): HT-29 cells were prepared as follows: 1 x 10⁶ cells/well were seeded on 6 well plates (0.5ml/well) and incubated overnight at 37 °C, 5% CO₂ before treatment. Cells were treated with 100µg/ml of extracts and incubated at 37 °C, 5% CO₂ for 24h. After the incubation time the media was removed, and cells were trypsinized, washed with PBS, centrifuged (1800rpm, 5 min), re-suspended in serum-free media and counted with trypan blue staining. The cell concentration was adjusted to 1.5 x 10⁶ cells/ml and subjected to the Comet assay.

COMET assay: An alkaline COMET assay was performed as described by Gill et al. [49] on HT-29 cells suspended in 1 ml phosphate buffer saline (PBS). Cells aliquots were incubated for 5 min on ice in presence or absence of hydrogen peroxide (75µM). Each sample was analyzed in triplicate, along with positive and negative controls. Cells were washed with PBS and centrifuged (380 g for 8 minutes) to remove residual H₂O₂, re-suspended in 0.85% low melting agarose and added into fully frosted slides coated with normal melting agarose. Slides were dried (4 °C, 10 min) and cells were lysed by incubating with lysis buffer containing 1% Triton X-100 (4 °C, 1 h). Slides were transferred into the electrophoresis tank, covered with alkaline buffer and allowed to unwind for 20 minutes prior to run (20 minutes, 26 volts, 300 mA at 4 °C). After 3 washes in neutralizing buffer the slides were stained

with ethidium bromide (20µg/ml), dried and analysed using an Epi-fluorescent Nikon microscope connected with a digital camera. Imaging was performed using a computerized image analysis system (Komet 5.5, Andor Bio-Imaging). Fifty cells per slide were analyzed randomly and the data were presented as the percentage of tail DNA.

Treatment of HT-29 cells for cell growth assay (SRB): HT-29 cells were seeded at low confluence in 24-well plates (2.5×10^4 per well) and were treated with the extracts (100, 250, 500 µg/ml) or vehicle 8 h after seeding. Following 48 h incubation (37 °C, 5% CO₂), cells were fixed and cell biomass was determined using the SRB assay [50].

SRB assay: sulforodhamine B (SRB) assay was performed as previously described [50]. Briefly, cells were fixed by the addition of 125 µl ice-cold TCA (10 % final concentration; 4 °C; 1 h). After fixing, media was removed, cells were washed and total biomass was determined using SRB (250 µl of 0.4 % SRB; 0.5 h).

Unincorporated dye was discarded by washing with 1 % acetic acid, whilst cell incorporated dye was solubilised using Tris Base (10 mM, pH 10.5). Dye incorporation, reflecting cell biomass, was measured at 492 nm, using a GENios microplate reader (TECAN, Reading, UK).

Statistical analysis: The statistical evaluation of the results was performed using GraphPad Prism version 7 (GraphPad Software, San Diego, CA, USA). Grouped data format was used, and the statistical analysis performed was a two-way analysis of variance (ANOVA) followed by a Bonferroni post-hoc t-test. Statistical significance was set at $P < 0.05$.

Results

Seaweed polyphenol extract characterization: The chromatogram (Figure 1) illustrates the traces obtained by NP-HPLC with diode array detection after injecting a water solution of the SPE and the HMW (>10KDa) and LMW (1-10KDa) fractions. The 3 chromatograms show a number of peaks eluting, predominantly at 20-70 min, the elution time being directly related to the molecular weight of the phlorotannins, where low molecular weight components will elute early and high molecular weight components will appear at later retention times. A calibration curve of phloroglucinol was used to quantify all the phlorotannins in the samples as phloroglucinol equivalents. The SPE, HMW and LMW extracts comprised a wide range of molecular weight of phloroglucinol derivatives with a total phlorotannin concentration of 358.9 µg/mg (SPE), 938.2 µg/mg (HMW), 374.5 µg/mg (LMW), as phloroglucinol equivalents.

***In vitro* digestion and characterisation:** SPE, HMW and LMW were subjected to *in vitro* digestive and fermentative processes and simulation of absorption into the circulation (Figure 2). , Samples from the procedure were analysed by NP-HPLC with diode array detection. Figure 3 shows the traces obtained for the 3 extracts (SPE, HMW and LMW) when undigested (black trace, ND), digested (grey trace, GID) and fermented (black dashed trace, CF) aliquots were injected. For all 3 extracts, we observed a reduction of the components eluting at 20-70 min after GID (grey line), which is limited for HMW (5.4%), and much more important for SPE (59.0%) and LMW (52.8%). After the CF procedure (black dashed line) we observed a further reduction of the peaks eluting at 20-70 minutes, with reduction levels of 96.8 % (SPE), 89.9 (HMW) and 68.0% (LMW). In parallel, we observe the appearance and/or increase of some large peaks eluting at earlier timepoints, 0-

20min. In our Normal Phase chromatographic conditions, the elution times are correlated to the size/molecular weight of the phlorotannin units, and therefore our results indicated the progressive reduction of the larger molecular weight polymeric phlorotannins into low molecular weight forms (oligomeric units, degradation products) during simulated gastrointestinal digestion and *in vitro* colonic fermentation. It is interesting to note that the very high molecular weight component present in the HMW fraction (≥ 1 KDa) were reduced to a very limited extent under gastric and small intestinal conditions (5.4%), whereas they were more significantly reduced during colonic fermentation conditions (89.9%).

Additionally, the samples were analysed by LC-MS/MS utilizing electrospray ionisation (ESI) in negative ion mode (Supplementary tables 1 and 2) as previously described [42]. In both GID and CF extracts, we found a range of newly formed metabolites, and we were able to identify molecular ions and fragments corresponding to hydroxytrifupalol A (405), the C-O-C dimer of phloroglucinol (247), the dimer diphlorethol/ difucol (249) and 7-hydroxyeckol (387), in accordance with what observed in our previous study [42].

Total Polyphenol content and antioxidant capacity.

The three extracts (SPE, HMW and LMW) undigested (ND), digested (GID) and fermented (CF) were assessed for TP content (Figure 4A) and for *in vitro* antioxidant capacity (Figure 4B) using the TEAC assay method. As shown in Figure 4A, before digestion (ND) the high molecular weight fraction HMW had the highest TP content (441.6 $\mu\text{g}/\text{mg}$), followed by SPE (211.9 $\mu\text{g}/\text{mg}$) and LMW (155.3 $\mu\text{g}/\text{mg}$). The TP content of all extracts was significantly reduced by GID ($P < 0.001$ vs. ND), with reduction levels of 81.7% (SPE), 40.4% (HMW) and 64.7% (LMW). After CF, the TP levels were further reduced ($P < 0.001$ vs. ND) to 99.5% (SPE), 86.5% (HMW) and

92.5% (LMW) of the initial levels measured in the ND extracts. In accordance with TP results, the antioxidant test (Figure 4B) showed that before digestion the high molecular weight fraction HMW had the highest TEAC value (3.7 $\mu\text{mol}/\text{mg}$), followed by SPE (2.6 $\mu\text{mol}/\text{mg}$) and LMW (2.1 $\mu\text{mol}/\text{mg}$). Digestion significantly reduced the TEAC value of all extracts ($P < 0.001$ vs. ND), with reduction levels of 79.5% (SPE), 27.4% (HMW) and 68.7% (LMW). After CF, the TEAC values were further reduced ($P < 0.001$ vs. ND) to 94.8% (SPE), 82.4% (HMW) and 84.0% (LMW) of the initial levels measured in the ND extracts. It is interesting to note that TP levels and TEAC values of the HMW fraction were affected less than the HMW fraction by the GID procedure.

DNA damage in human colon cells HT29 (COMET assay)

HT-29 cells were exposed to the GID and CF extracts (100 $\mu\text{g}/\text{ml}$, 24 h) and we tested the ability of each extract (SPE, HMW, LMW) to reduce H_2O_2 induced cellular DNA damage (COMET assay). As shown in Figure 5, a low level of DNA damage (% of DNA in tail) was measured in the controls whereas in cells challenged with H_2O_2 , the DNA damage was significantly increased ($P < 0.001$). Among the extracts subjected to the GID procedure, only HMW was able to significantly reduce DNA damage compared to H_2O_2 alone ($P < 0.01$), whereas SPE and LMW were not effective ($P > 0.05$). When analysing the effects of the extracts subjected to CF procedure, however, all extracts were significantly effective ($P < 0.001$) in reducing the DNA damage induced by H_2O_2 , despite the loss of antioxidant capacity of the CF extracts. The statistical analysis also highlighted that within each group (GID and CF) the effect of HMW was significantly higher than SPE and LMW ($P < 0.05$).

Cell growth inhibition in human colon cells HT29 (SRB assay):

HT-29 cells were exposed to the GID (Figure 6A) and CF (Figure 6B) extracts (100, 250 and 500 µg/ml, 72 h) and we tested the ability of each extract (SPE, HMW, LMW) to induce cell growth inhibition by measuring changes in the cell biomass (SRB assay).

Fig.6A shows that the lower concentration tested (100 µg/ml) was ineffective ($P>0.05$), whereas at higher doses all GID extracts significantly inhibited ($P<0.01$) HT-29 cell growth, with HMW being the most effective. In contrast, when the cells were treated with the CF extracts (Figure 6B), only HMW was able to significantly inhibit ($P<0.05$) the growth of the cells at the concentrations 250 and 500 µg/ml. It is interesting to note that the HMW seems to exert the strongest effects in both GID and CF groups, and again it is worth highlighting the fact that the CF extract is able to exert a significant effect despite the loss of antioxidant capacity.

Discussion

Over the past few years, significant interest has developed in bioactives from brown seaweed [11, 51] and phlorotannins have gained attention for their potential beneficial effects for human health [11]. However, due to the many analytical challenges faced when conducting phlorotannin analysis, knowledge on phlorotannin bioavailability, gastrointestinal modifications and their impact on bioactivity is scarce or absent. We recently reported [42] for the first time that seaweed phlorotannins are metabolized to small oligomeric units, and their time of appearance in urine and plasma is indicative of a predominant large intestinal metabolic transformation. The analysis of phlorotannins is challenging due to high range of molecular weight

present, and their characterisation is complicated further by the lack of commercially available standards, therefore in our recent work we made use of both reverse phase (RP) and normal phase (NP) HPLC chromatography coupled to diode array (DAD) and mass spectrometry detection to try and understand how phlorotannins are digested and absorbed into the circulation, and what kind of modifications can occur in the small and large intestine prior to absorption [42]. NP-HPLC coupled to DAD is indeed a simple and useful tool for the analysis of oligomeric and polymeric phlorotannins [12], and we used it in the present study to characterize phlorotannins in the 3 extracts, and to understand the modifications they are subjected to during gastrointestinal digestion and colonic fermentation at different degrees of polymerization. Our results highlight a large reduction in the amount of the polymeric forms eluting a late retention times in our NP method. The forms with molecular weight > 10KDa were only marginally affected by the GID condition, whereas they were largely reduced following CF in the presence of the large intestinal microbiota. In addition, further characterization via LC-MS/MS analysis of the GID and CF extracts has indicated the presence of some oligomeric phlorotannins (hydroxytrifupalol A, diphloretol/difucol, 7-hydroxyeckol, C-O-Cdimer of phloroglucinol), in addition to a range of newly formed, unknown metabolites. In parallel, using traditional *in vitro* tests for TP content and antioxidant scavenging capacity, we observed a reduction of both after *in vitro* GID and CF. These results imply that the putative beneficial health effects of polyphenolic compounds cannot be meaningfully assessed by measuring their total polyphenolic content and scavenging potential without taking into consideration the impact of digestive and fermentative processes in the gastro-intestinal tract. In the upper gastrointestinal tract, where GID occurs, dietary polyphenols are exposed to specific pH conditions, and act as

substrates for a number of enzymes found both in the stomach and small intestine [40]. Further transformations can occur in the colon, where the enzymes of the gut microbiota act to breakdown complex polyphenolic structures to smaller units.

Bacterial enzymes may catalyse many reactions including hydrolysis, dehydroxylation, demethylation, ring cleavage and decarboxylation as well as rapid de-conjugation [52].

In addition, our study made use of a simple cell culture system (colonic HT-29 cells), to assess changes in bioactivity of phlorotannins after digestion and fermentation.

We stress the attention on the fact that despite the significant reduction of phlorotannins, TP and antioxidant potential in GID conditions, the HMW extract exerted a significant protective effect in counteracting H₂O₂-induced DNA damage. In addition, after CF all extracts showed a further reduction of TP and antioxidant capacity, thus they were able to significantly reduce H₂O₂-induced DNA damage, indicating how the potential beneficial effects of the extracts are not linked to their *in vitro* antioxidant potential. Our results are consistent with observations of (poly)phenolic compounds from other terrestrial dietary sources [53-56]. Our results also highlight the strong impact of GID and CF on the ability of the extracts to induce cell growth inhibition on HT-29 cells. In this case, all GID extracts exerted a significant effect, whereas after CF only HMW was able to significantly inhibit the growth of the cells. Therefore we highlight the differential effects of GID and CF, with CF more strongly influencing the anti-genotoxic potential of the extract, whereas GID has more strongly impacted the potential of the extracts to induce cell growth inhibition. Our data indicates that the gut microbiota metabolism of the seaweed extracts increased their ability to counteract the H₂O₂ induced DNA damage, whereas it reduced the ability of the extracts to inhibit cell growth.

Therefore, the present work gives some important insights on the role of gastrointestinal digestion and colonic biotransformation on phlorotannin bioactivity in the colon, providing a basis for further investigating the seaweed-derived bioactive components *in vivo*, and help to elucidate their mechanism of action.

Accepted Article

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Figure Legends:

Figure 1. Chromatographic separation of phlorotannins contained in the seaweed polyphenol extract (SPE), High Molecular Weight (HMW) and Low Molecular Weight (LMW) fractions by Normal-Phase HPLC with diode array detection (268nm).

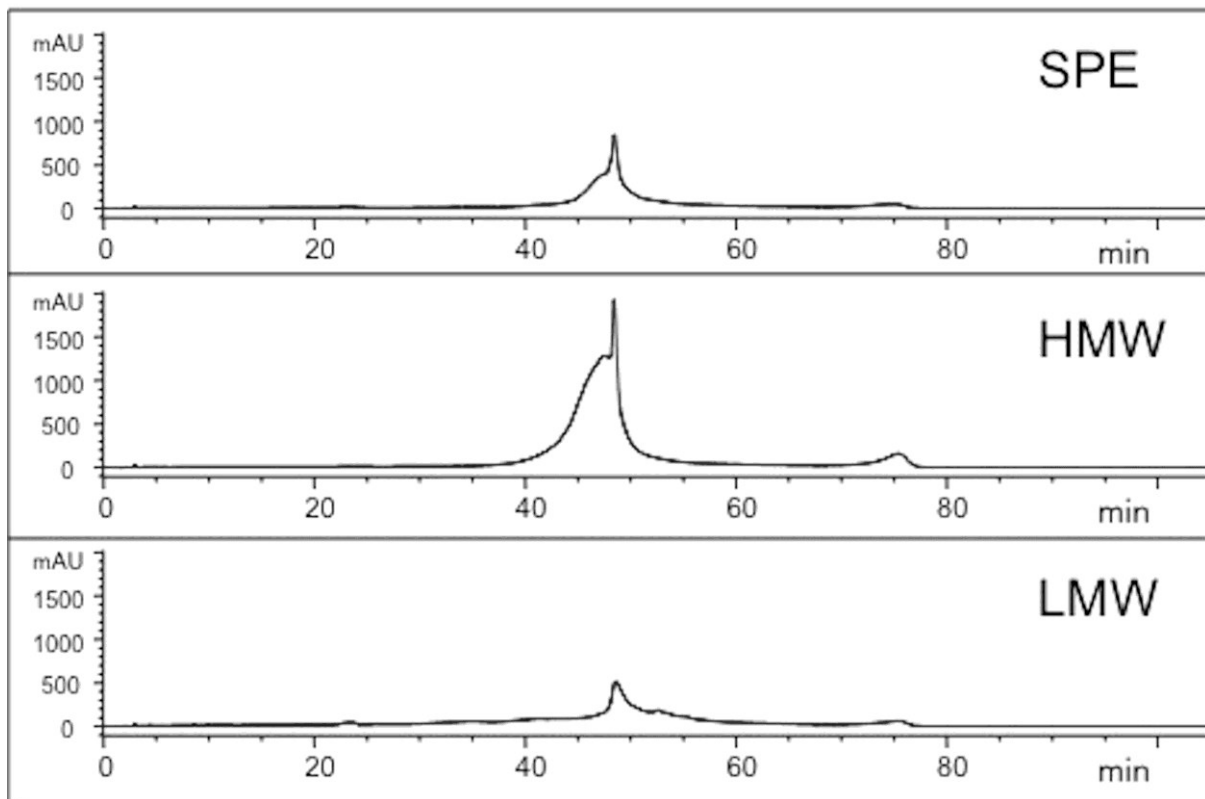


Figure 2. Digestion scheme. Schematic illustration of the simulated Gastro-Intestinal Digestion (GID) and Colonic Fermentation (CF) procedures.

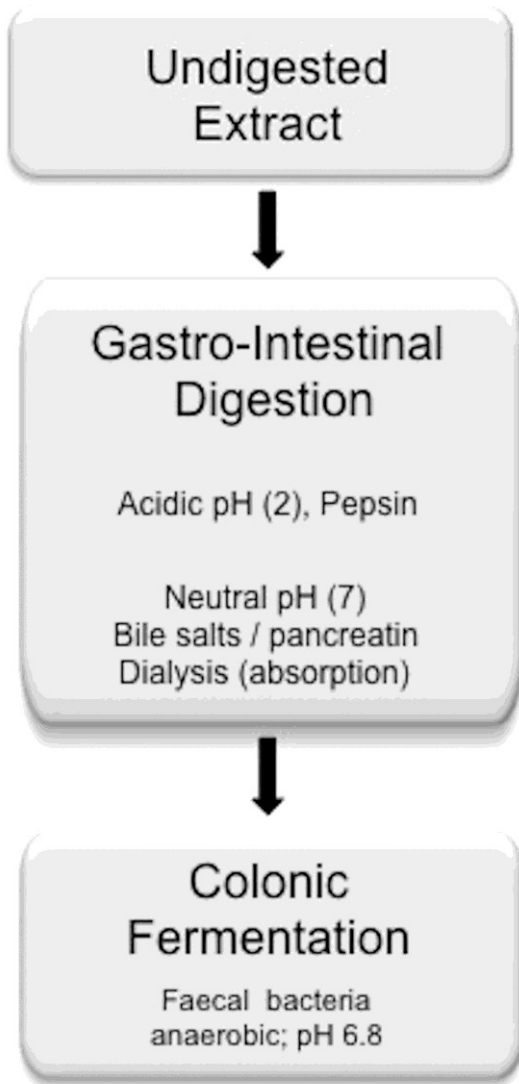


Figure 3. HPLC analysis/digestion. Normal-Phase HPLC analysis (268nm) of the extracts showing the impact of digestion and fermentation on the phlorotannin content. Black line = Non-Digested (ND); grey line = Gastro-Intestinal Digestion (GID); black dashed line = Colonic Fermentation (CF).

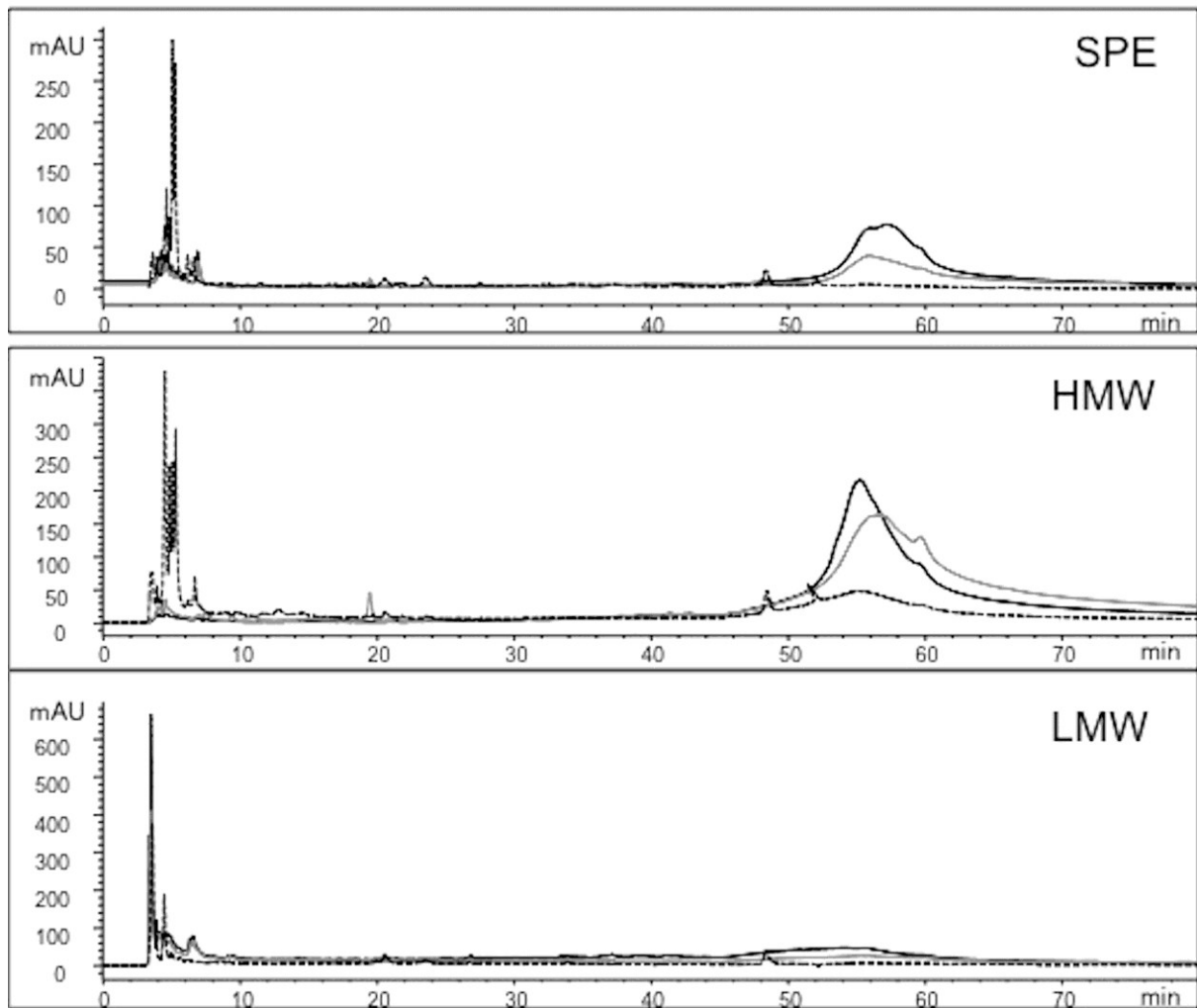


Figure 4. *In vitro* activity of seaweed extracts. Total Polyphenol content (A) and Trolox Equivalent Antioxidant Capacity (B). Data are means of 3 separate experiments, each performed in 3 replicates ($n = 9$), and presented as mean \pm SEM. Statistical analysis of grouped data was conducted by Two-way ANOVA, using a Bonferroni post-hoc test to analyse simple effects of the digestion procedure in each extracts. In all groups, $P < 0.001$ vs. ND.

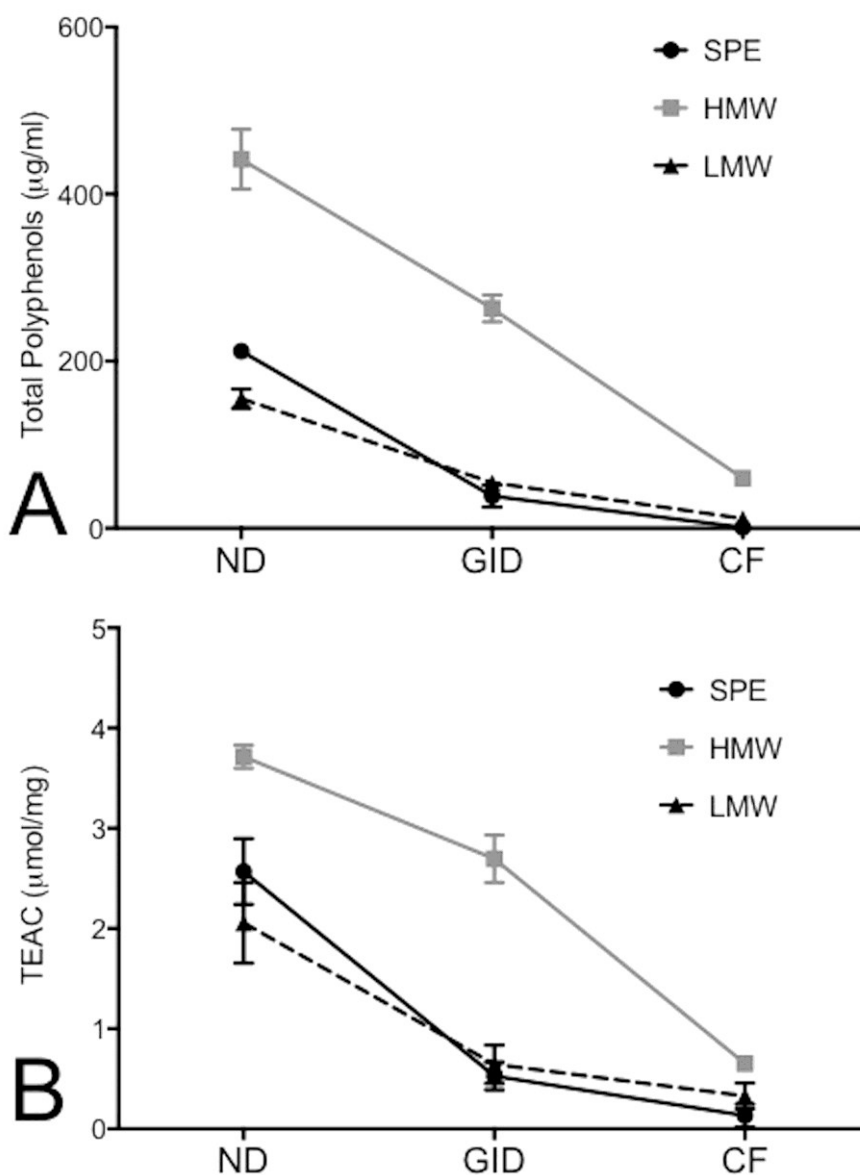


Figure 5. DNA damage (% cells in tail) measured by COMET assay in HT-29 cells treated with digested (GID) and fermented (CF) seaweed extracts. Data are means of 3 separate experiments, each performed in 3 replicates ($n = 9$), and presented as mean \pm SEM. Statistical analysis of grouped data was conducted by Two-way ANOVA, using a Bonferroni post-hoc test to analyse simple effects of the treatments for each digestion procedure. A = $P < 0.05$ vs. control; a = $P < 0.05$ vs. H_2O_2

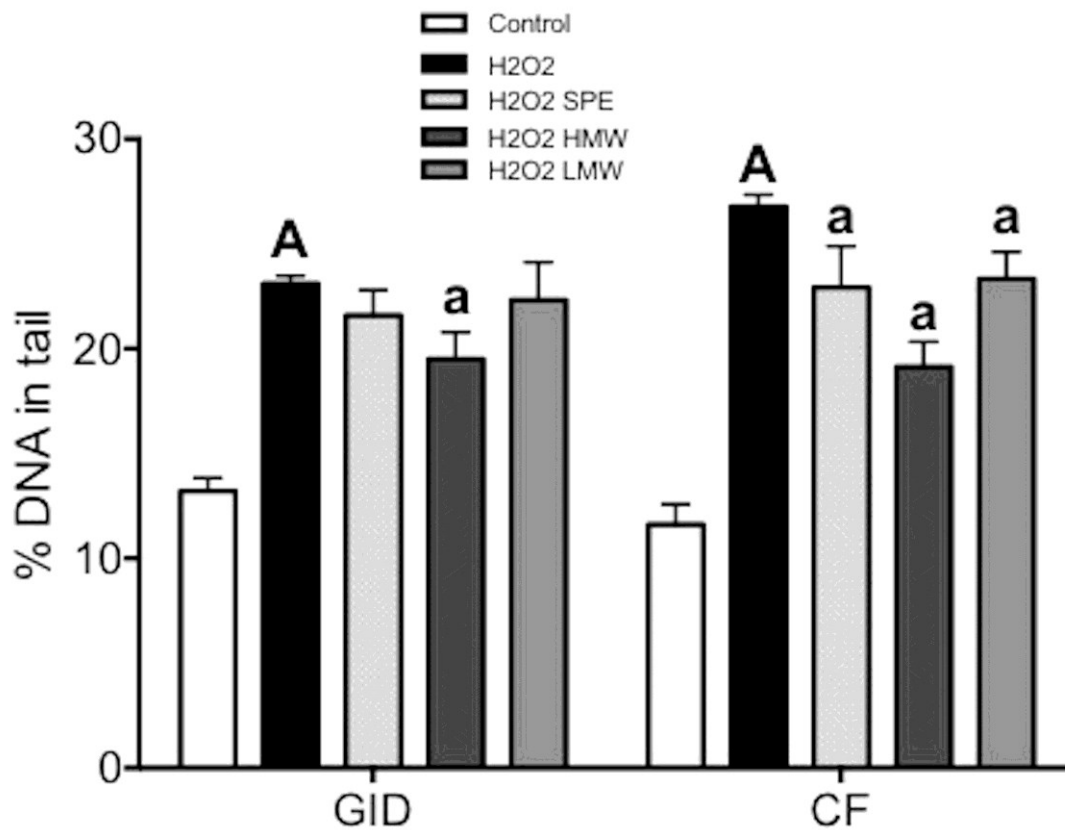
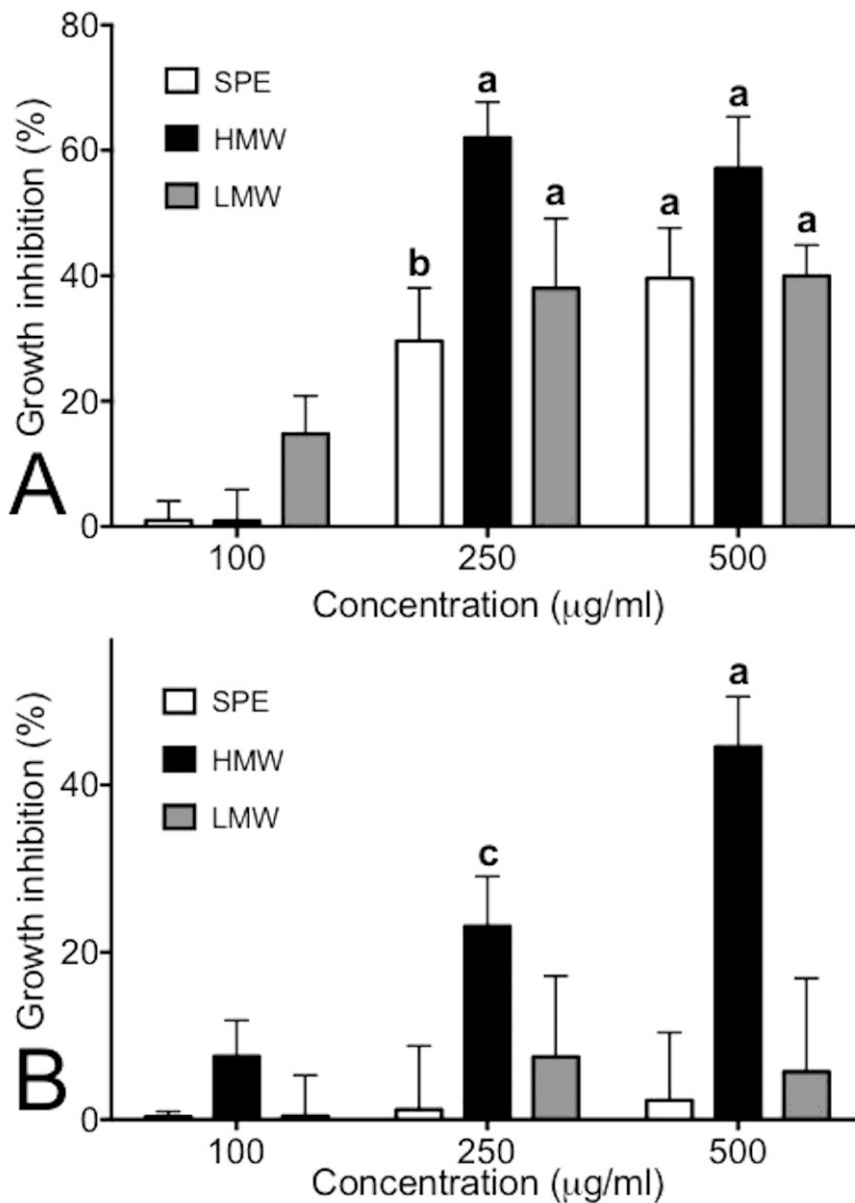


Figure 6. SRB assay. Cell growth inhibition induced by in Ht-29 cells pretreated with SPE, HMW and LMW extracts (100, 250 and 500 $\mu\text{g/ml}$) for 24h. GID extracts (A) and CF extract (B). Data are means of 3 separate experiments, each performed in 3 replicates ($n = 9$), and presented as mean \pm SEM. Statistical analysis was conducted by Two-way ANOVA with Bonferroni post-hoc test. $a = P < 0.05$ vs. control; $A = P < 0.05$ vs. control H_2O_2 .



Author contributions:

G.C., J.P.E.S, P.Y. and I.R. designed the research. G.C. organized and coordinated all parts of the work. S.H. overviewed the preparation of seaweed materials and extracts. M. M.C. and Y.G. aided with the experimental work. G.C. analysed and summarised all the data. G.C. drafted the manuscript, C.G. and I.R revised the manuscript. I.R. had primary responsibility for final content.

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Conflict of interest statement:

All authors declare no conflicts of interest.