

Project No 262519 Seaweed derived anti-inflammatory agents and antioxidants

SWAFAX

Aims and Objectives

The overall aim of the proposal is to provide the SME partners with the information, expertise and capacity to produce, from seaweed sources, polyphenol extracts with valuable biological activities and to exploit the commercial possibilities of these extracts.

The following scientific objectives were designed to address these aims:

1. To prepare a food grade polyphenol extract of *Ascophyllum nodosum* for use in human trials plus novel *Ascophyllum* polyphenol extracts for exploratory *in vitro* studies and to characterize the extracts in particular their polyphenol profiles. Originally it was planned to prepare extracts of other seaweed species but in consultation with the SME partners, it was decided to focus on *Ascophyllum*
2. To evaluate bioavailability of the components of the food grade polyphenol extract in a short-term human trial,
- 3 To conduct a chronic human dietary trial to assess the biological activity of the food grade seaweed polyphenol extract in terms of plasma antioxidant capacity, reduction of oxidative damage to DNA, modulation of inflammatory responses and reduction on chronic, low level inflammation *in vivo*.
4. To screen the novel seaweed extracts for antioxidant and anti inflammatory activity *in vitro*.

1. Production and analysis of food grade and other novel seaweed polyphenol extracts (WP2)

A range of novel seaweed extracts were produced as part of the SWAFAX project:

- 3 food grade polyphenol rich extracts from *Ascophyllum nodosum* were produced at pilot scale for use in the *in vivo* studies.
- 7 bioactive (polyphenol and others) rich extracts from *Ascophyllum nodosum* were produced at laboratory scale for use in the *in vitro* studies.
- 1 food grade polyphenol rich extract from *Ascophyllum nodosum* was produced at pilot scale in order to validate the commercial viability of the process.

1.1. Production of food grade polyphenol rich extracts for in vivo studies.

A polyphenol rich extract from *Ascophyllum nodosum* was produced and characterised. The extract was produced at the pilot scale, food grade, facility at CEVA in France (www.ceva.fr). The extract was produced using a solvent based (EtOH:water) extraction system that was specifically developed for use with *Ascophyllum nodosum*. The extract was then fractionated using tangential flow ultra filtration to produce two further extracts of varying molecular weight range and with varying polyphenol content. Each extract was given a unique identifier code that was used by all partners throughout the study:

CC number	Detail
CC2575	Basic extract produced using solvent extraction system
CC2576	>10 kDa fraction of CC2575
CC2577	1-10 kDa fraction of CC2575

Each extract was characterised in terms of its major structural and bioactive components. Also, heavy metal (inorganic arsenic, lead, mercury, cadmium and tin) and microbiological analysis was carried out in order to ensure that each extract was safe for consumption.

1.2. Production of delivery capsule for *in vivo* studies.

For the *in vivo* studies (acute bioavailability study and in a long term intervention study (See section 3) a blended polyphenol rich extract was produced (CC2574). The blend was formulated so as to maximise the polyphenol content (>100mg per day) but also to minimise the level of iodine to within accepted regulatory guidelines (< 500µg per day). NB: some European seaweeds, including *Ascophyllum nodosum*, are naturally high in iodine. The formulation was approved by the ethics committees overseeing the research at both UU and UREAD. The daily dose for the *in vivo* studies was set at one 400mg capsule per day.

Blending was carried out at the food grade CEVA facilities in France. 400mg doses of the *Ascophyllum* blend (CC2574) and 400mg doses of the placebo material (Avebe Maltodextrin – MD14PS) were packed into white, opaque, vegetarian capsules by Irish Seaweeds, Belfast, UK (www.irishseaweeds.com) before delivery to UU and UREAD.

A microbiological safety testing strategy was put in place to ensure that the capsules were fit for human consumption and that they remained stable for the duration of the long term *in vivo* study. Capsules (Seaweed extract CC2574 and Placebo) were tested for microbial safety prior to the start of the *in vivo* studies and were also monitored for microbial stability at 2 weekly intervals during the long term human study.

1.2.1 Analysis of phlorotannin content of SPE capsule (WP2)

The SPE was characterized by normal phase HPLC and LC-MS analysis

The phlorotannins in the seaweed polyphenol extract (SPE) CC2574 were analyzed by normal phase HPLC analysis (Koivikko R, 2008) using an HPLC 1100 series equipped with LiChrospher Si60-5 column (250 mm × 4.0 mm ID, 5 µm particle size from HICHROM (LISP60-5-250AF), fitted with a guard column LiChrospher Si60-5 from HICHROM (LISP60-5-10C5). The mobile phase contained A: 82% dichloromethane + 2% methanol + 2% acetic acid in water and B: 96% methanol + 2% acetic acid in water and was pumped through the column at 1 ml/min. 10 µl of samples were injected and analyzed by the gradient program which were (min/%B): 0/0, 30/17.6, 45/30.7, 50/87.8, 60/87.8, 80/0, 105/0 for detection of all compounds. 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 capsules were analysed as phloroglucinol equivalents.

The chromatogram (Figure 1) illustrates the trace obtained by injecting a water solution of the SPE. The chromatogram shows a number of peaks (20-70 min) representing different high molecular weight phlorotannins, the characteristic phenolics in brown seaweeds.

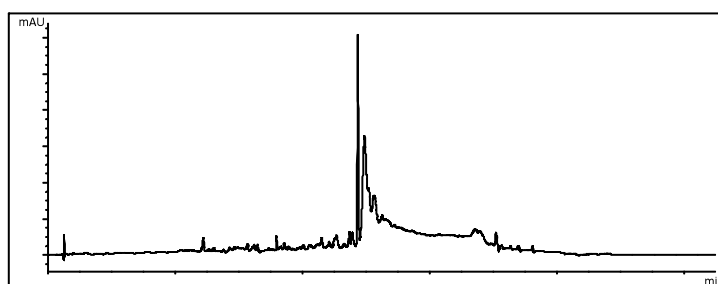


Figure 1. NP-HPLC chromatogram of phlorotannins in a food grade seaweed polyphenol extract capsule ($\lambda = 268$ nm).

Longer phlorotannin polymers, which consisted of more hydroxy groups, resulted in tighter attachment to the column material. Consequently, shorter compounds were released earlier than longer compounds. Owing to a lack of phlorotannin standards, the calibration curve of phloroglucinol was used to quantify the phlorotannins contained in the SPE as phloroglucinol equivalents, as shown in Table 1

Table 1. Quantification of phlorotannins contained in the SPE (phloroglucinol equivalents).

Retention time	Area	Conc.(µg/ml) PHL EQ
24.446	132.7	78.24
26.165	21.7	12.79
31.46	60.3	35.55
32.512	60.3	35.55
33.045	38.7	22.82
35.928	68.8	40.57
37.128	49.5	29.19
37.807	26.5	15.63
40.152	43.2	25.47
41.345	46.7	27.54
43.058	183.4	108.14
44.258	67.3	39.68
45.391	249.9	147.35
46.602	35.5	20.93
47.476	144.5	85.20
47.889	148.3	87.44
48.732	1344.5	792.75
49.819	3498.3	2062.68
51.351	1419.7	837.09
52.628	2939.4	1733.14
TOTAL (µg/ml)		6237.74
TOTAL (µg/mg capsule)		311.89

This table indicates that the seaweed polyphenol extract comprised a wide range of molecular weight of phloroglucinol derivatives with a total phlorotannins concentration of 311.89 µg/mg quantified as phloroglucinol equivalents.

Further characterization of the SPE CC2574 was achieved with LC-MS/MS utilizing electrospray ionisation (ESI). This consisted of an Agilent 1200 HPLC system equipped with a binary pump, degasser, auto-sampler, thermostat, column heater, photodiode array detector and an Agilent 1100 Series LC/MSD Mass Trap Spectrometer. Separation of samples was achieved using a Zorbax SB C18 column (2.1 x 100 mm; 1.8 µm) (Agilent) Mass spectrometry data were collected in a non-targeted fashion, by acquiring full spectrum data in negative ion mode from m/z 100 to 1000. The data were then analysed by searching for the theoretical mono-isotopic masses corresponding to possible phlorotannins oligomers and the presence of ions (1-6) which could correspond to phlorotannins. The ion 1 with [M-H]⁻ at m/z 405 correspond to the trimer hydroxytrifuhalol A, where the MS² fragment 387 corresponded to the loss of one molecule of water (18), a characteristic pattern of phlorotannins fragmentation. Compound 2 ([M-H]⁻ at m/z 497) was considered to be a phlorotannin tetramer composed of 4 phloroglucinol units, such as tetrafucol or fucodiphlorethol and also in this case the fragment 479 corresponds to the loss of a molecule of water. The ion 3 has a [M-H]⁻ at m/z 247 corresponding to a C-O-C dimer of phloroglucinol as previously indicated by Nwosu et al (2012). The ion 4 (387) corresponds to the trimer 7-hydroxyeckol, and we observe the presence of a fragment at 369 deriving from the loss of one molecule of water. Isomers 5 and 6 with [M-H]⁻ at m/z 249 were also observed, which can correspond to the dimers diphlorethol and difucol.

1.3. Production of novel bioactive rich extracts for in vitro studies.

A range of bioactive rich extracts from *Ascophyllum nodosum* were produced at laboratory scale and characterised. The key objectives of this phase of the work were to (i) evaluate the effects of different extraction techniques on the yield and composition of polyphenols and other key bioactives; (ii) optimise key reaction parameters; (iii) evaluate any differences in yield and composition of Hebridean and Irish *Ascophyllum nodosum* (as relevant to the SME partners); (iv) conduct a preliminary

evaluation of seasonal effects. In all cases, the extraction techniques were developed with potential scale up and commercial application in mind *i.e.* practical larger scale alternatives for processing steps and equipment used were considered when the methodology was developed.

The following extraction techniques were developed and optimised at laboratory scale: (i) aqueous extraction; (ii) solvent extraction using food grade solvents and (iii) enzyme assisted extraction using commercially available food grade enzymes. In all cases, key reaction parameters were investigated and optimised.

1.3.1 Aqueous extractions: The effects of variations in several reaction parameters were studied - (i) temperature, (ii) time, (iii) solvent:seaweed ratio, (iv) stirring, (v) absence/presence of light, and (vi) use of acidic vs alkaline conditions and (vii) particle size. It was found that the solvent/seaweed ratio had the most noticeable effect on extraction yield. The use of acidic or alkaline conditions also improved extraction yield, however, it is well documented that such conditions can lead to the polymerisation of the phlorotannins and these processes would require the use of an anti-oxidant additive in order to proceed further with them. Ascorbic acid was used as an anti oxidant in subsequent alkaline studies.

1.3.2. Enzyme assisted extractions: A range of commercially available proteases (Flavourzyme, ProteAX (Umamizyme), Neutrase and Alcalase) and carbohydrases (Viscozyme and Celluclast) were used as a pre-treatment to phlorotannin extraction in order to break down the structure of the seaweed and to release bound phlorotannins. Key reaction parameters were optimised as with the aqueous extractions. All reaction conditions were set according to the optimal conditions outlined for each enzyme in the company literature. All the enzymes successfully solubilised the seaweed matrix with the soluble matter content in all enzyme extracts being greater than that in the water only control. However, the effect on phlorotannin yield was not positive in all cases. It is possible that the protein content of the enzymes interfered with the extraction by binding with the phlorotannins and thus no further investigation of the effects of enzymes was conducted for this project.

1.3.3. Solvent extractions: most work was carried out using ethanol and ethanol:water solvent mixes although the use of another food grade solvent IPA and non-food grade solvent methanol were investigated for comparative reasons. Key reaction parameters were optimised as with the aqueous extractions but with the addition of methanol:water ratio. Solvent extraction protocols in general resulted in high total extract yields and high polyphenol content in the extracts. However, analysis of the salt content (NaCl, KCl, and Cl) of various extracts showed that up to half of the mass of the dried extracts could be attributed to salts. Further work was carried out to address the removal of these salts, so as to improve the overall yield of phlorotannins in the extracts.

The efficiency of extraction technique was evaluated on the basis of total extract yield, polyphenol content (quantified using the Folin-Ciocalteu method using phloroglucinol as a standard), radical scavenging ability (using DPPH assay) and presence of non-bioactive components *e.g.* mineral salts which could interfere with potential bioactivity of the extract. NB: high salts content was not desired due to potential negative effects on cells in the *in vitro* culture assays. Where necessary, centrifugation and/or dialysis steps were introduced to concentrate and/or remove unwanted components *e.g.* mineral salts.

The following seven optimised extracts were produced for evaluation in the *in vitro* studies:

CC Number	Detail
CC2681	Water extraction at laboratory scale
CC2698	NaOH extraction with dialysis step at a laboratory scale
CC2700	Ethanol water extraction containing all soluble fractions including fucoxanthin at laboratory scale
CC2699	Ethanol water extraction containing water soluble portion only at laboratory scale
CC2719	Ethanol water extraction containing water soluble portion only at laboratory scale
CC2720	Ethanol water extraction containing all soluble fractions including fucoxanthin at laboratory scale

Each extract was characterised in terms of its major structural components (carbohydrates, sugars, moisture, sodium & potassium chloride), total polyphenol content, polyphenol molecular weight range and radical scavenging ability (DPPH assay). This analysis was carried out at CyberColloids. Heavy metal analysis and iodine and iodide analysis was also carried out by MacCaulay Scientific Consulting Ltd., Scotland.

1.4. Pilot scale production of food grade polyphenol rich extract.

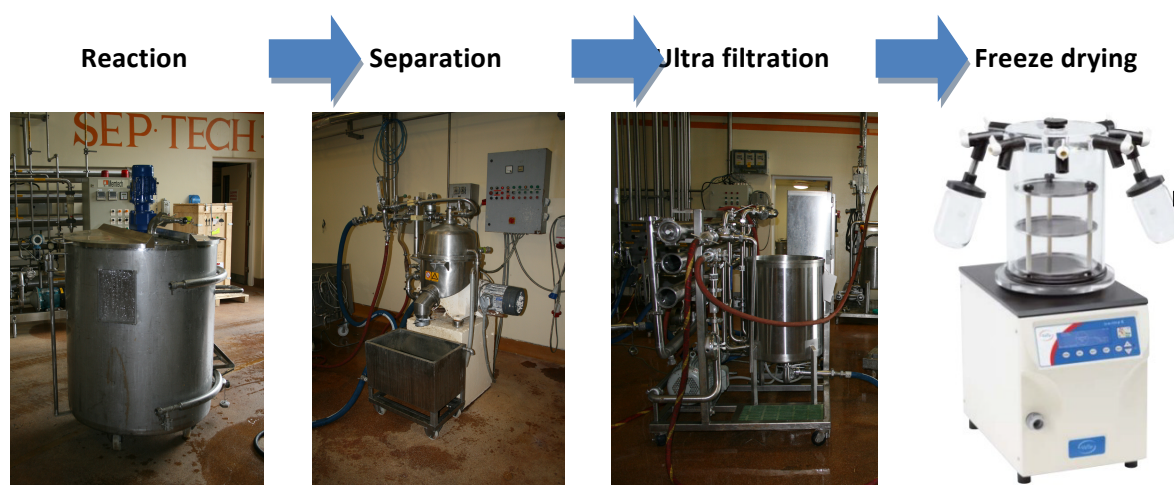
The SME participants made the decision to validate the scaled up production of the sodium hydroxide based extraction process that was developed at laboratory scale. There were a number of reasons why this process was chosen including: the polyphenol yield, relative ease of processing, safety and cost compared to solvent and other non alcohol based processes. Also, the process could be relatively simply and cost effectively implemented at the production facilities of the relevant SME partners.

Prior to scale up, the following parameters were optimised taking into account the availability and process specifications of equipment at the intended scale up facility (MTL, Fermoy, Ireland) and also the production requirements and potential capability at the relevant SME partners: pH, extraction time, seaweed:solvent ratio, ascorbic acid concentration, and number of extractions.

A schematic of the process is given below but in brief, this involved: a reaction phase in a stirred container during which key reaction parameters were monitored and adjusted accordingly; a separation phase to remove the spent seaweed and large particles; ultrafiltration to remove salts and other unwanted low molecular weight substances and freeze drying.

As anticipated, a number of observations and considerations were raised during the transfer of the process from lab scale to pilot scale. Large differences in processing volumes and equipment performance can often result in process parameters not scaling as predicted, however, CyberColloids maintained the necessary experience to overcome such difficulties and to modify the process accordingly.

Schematic of the pilot scale process



Approximately 80 litres of liquid extract was produced at the MTL facilities (CC2812). Some of the extract was frozen for freeze-thaw and stability trials and some of the extract was freeze dried at CyberColloids. Freeze dried samples were analysed major structural components (carbohydrates, sugars, moisture, sodium & potassium chloride), total polyphenol content and polyphenol molecular weight range. This analysis was carried out at CyberColloids. Heavy metal analysis and iodine and iodide analysis was also carried out by MacCaulay Scientific Consulting Ltd., Scotland. The microbiological safety of the product was assessed by Enva Ireland. Freeze dried samples were also monitored for product stability *i.e.* the antioxidant potential in terms of radical scavenging ability (DPPH assay) was monitored at regular intervals until the end of the SWAFAX project. CC2812 was not assessed in any of the *in vitro* or *in vivo* studies at UU and UREAD as the extract was produced during the latter stages of the project.

CC Number	Detail
CC2812	NaOH extraction using membrane separation at pilot scale

1.5. Technology transfer for the production and analysis of novel seaweed polyphenol extracts WP6

Transfer of the SWAFAX project technology to the SME participants involved a number of key elements: (i) transfer of all laboratory derived procedures for extraction and analysis of seaweed extracts; (ii) transfer of the pilot scale process; (iii) recommendations for commercial production - based on identified scale up requirements of the SMEs in terms of annual production requirements and expected process economics. Technology transfer was implemented via a series of discussions, interviews, site visits, training sessions and technical transfer documents & reports.

All information concerning the laboratory methods for extraction and analysis was detailed in a series of reports that outlined: the developmental stages and rationale; finalised step by step protocols; information on optimisation steps; list of equipment, reagents and analytical equipment. Information concerning (ii) and (iii) was contained in a standalone detailed technical transfer document that outlined: process development; process optimisation; scale up requirements and specifications; mass balance; critical process parameters; product definitions and specifications including suitable quality control parameters and test procedures required for production; analytical methods and list of equipment suppliers.

2. Bioavailability study (WP3)

2.1. Study design

Ethical approval for the bioavailability study was obtained before initiation of study from the University of Reading ethics committee. Exclusion criteria for subjects were smokers, BMI <18 or >30, abnormal liver function and haematology, alcohol intake of >21 units/wk, gastrointestinal disease or chronic gastrointestinal disorders, consumption of antibiotics in previous 3 months before study, females who are pregnant or intending to become pregnant. In total 24 volunteers were recruited - 1224 females (6 aged 18-30 years and aged 30-65 years) and 12 males (6 aged 18-30 years and 6 aged 30-65 years). Subjects were asked to follow a low phenolic diet for 1 day prior to the study day. On the study day, the subjects were cannulated and a baseline blood sample was taken. Volunteers were asked to consume a capsule (400mg) containing 100mg of polyphenols. Blood samples were collected at 0,1,2,3,4,6,8 and 24 hours after ingestion of capsule containing the food grade SPE and urine samples were collected at baseline, 0-8 and 8-24 h after the ingestion. During the day, volunteers were provided with a lunch and dinner of low phenolic content.

2.2. Extraction of polyphenols from urine samples (WP4.1)

The total volume of urine in each period was measured and divided into three aliquots of 15 ml. One aliquot was kept without any added preservatives, one aliquot contained 600 µl of 5% thymol and 600 µl of 2M sodium acetate, and another aliquot contained 1% formic acid. The urine samples were centrifuged at 3000 rpm for 10 min at 4 °C and subdivided into 1 ml aliquots and stored at -80 °C. Urine samples were prepared as follows: 10 µl of internal standard solution were added to 250 µl urine. Samples were analyzed with and without enzymatic treatment (glucuronidase/sulfatase, 37°C, 40min). 1ml of 100% methanol acidified with 0.5% acetic acid was added, samples were mixed and centrifuged for 15min at 16,100xg at 4°C, supernatants were transferred to a new tube and dried on a speedvac. Dried samples were resuspended in 125µl of mobile phase, completely dissolved, centrifuged and transferred to vials for HPLC-DAD and LC-MS analysis.

2.3. Extraction of polyphenols from blood samples.

Blood was collected in EDTA tubes and centrifuged at 3000 rpm for 15 min at 4°C. The plasma was subsequently divided into two aliquots, one was kept without any preservative, and one contained 1 mg/ml ascorbic acid. Aliquots were stored at -80 °C until analysis. Plasma samples were prepared as follows: 10 µl of internal standard solution (resorcinol) were added to 450ul of plasma, then 50ul of 1.2M acetic acid were added and samples were mixed. Samples were analyzed with and without enzymatic treatment (glucuronidase/sulfatase, 37°C, 40min). 1ml of 100% methanol acidified with 0.5% acetic acid was added and samples were centrifuged for 15min at 16,100xg at 4°C and supernatants were collected. This step was repeated 3 times (last time with 50% methanol acidified with 0.5% acetic acid) and the supernatants were dried using a speedvac. The pellets were dissolved with 125µl of mobile phase and transferred to vials for HPLC analysis.

2.4. HPLC-DAD analysis.

Samples analysis was carried out with a Hewlett-Packard 1100 series liquid chromatography system (Hewlett-Packard, Palo Alto, CA) equipped with a diode array detector (HP Chem Station Software system). Samples were analyzed by reverse-phase HPLC using a Nova-Pak C18 column (4.6 x 250mm) with 4 µm particle size. The temperature of the column was maintained at 30 °C. The mobile phases consisted of a mixture of aqueous methanol 5 % in 0.1 % hydrochloric acid 5M (A) and a mixture of aqueous acetonitrile 50 in 0.1 % hydrochloric acid 5M (B) and were pumped through the column at 0.7 ml/min. The following gradient system was used (min / % B): 0/5, 5/5, 40/50, 55/100, 59,9/100, 60/5, with 10 min post-run for both compound and metabolite detections. The eluent was monitored by photodiode array detection at 280nm and spectra of products obtained over the 200–600 nm range. Peaks were characterized by their retention time and spectra characteristics. A calibration curve of phloroglucinol was constructed using an authentic standard (0.1–100 µg/ml) and was found

to be linear with correlation coefficient of 0.995. Metabolites were quantified as phloroglucinol equivalents.

2.5. Results.

HPLC-DAD analysis of the urine (Table 2) and plasma (Table 3) with and without glucuronidase/sulfatase treatment showed the presence of a variety of metabolites (Figure 2) absent in the baselines (before SPE ingestion) in urine from 15 volunteers out of 24 and in plasma from 14 out of 24. Some metabolite peaks were present in samples with and without enzymatic treatment, and therefore could be assigned to un-conjugated metabolites. Some other metabolite peaks were present only in samples without enzymatic treatment or were only appearing in samples enzymatically treated, and were attributed to conjugated forms (glucuronides and/or sulfates). In urines some metabolites were found in samples collected at 0-8h after capsule ingestion, but the majority of the metabolites was found in samples collected at 8-24h.

Table 2. Analysis of seaweed metabolites in urine samples from the bioavailability study

NO ENZYME TREATMENT		UM1	UM2	UM3	UM4	UM5	UM6	UM7	UM8	UM9	UM10	UM11	UM12	UM13	UM14	UM15	UM16	UM17	UM18
VOLUNTEER	time (h)																		
30	0 -- 8				15.3														
44	8 -- 24						24.3												
49	8 -- 24		122.5														8.3		
50	8 -- 24		118.5			12.9				9.8									
23	8 -- 24												5.9	2.0					
17	8 -- 24								6.2										
11	8 -- 24	180.6		203.0															
30	8 -- 24		122.4		33.5														
36	8 -- 24		211.4											4.6					
22	8 -- 24													7.3					
40	8 -- 24											11.8							
ENZYME TREATMENT (glucuronidase/sulfatase)		UM1	UM2	UM3	UM4	UM5	UM6	UM7	UM8	UM9	UM10	UM11	UM12	UM13	UM14	UM15	UM16	UM17	UM18
VOLUNTEER	time (h)																		
44	0 -- 8														2.3				
26	0 -- 8														14.2				
46	0 -- 8														3.6				
24	0 -- 8														7.9				
30	0 -- 8														17.3				
28	0 -- 8														23.5				
44	8 -- 24						25.3	43.8							8.7				
43	8 -- 24														25.0				
49	8 -- 24		121.7																
50	8 -- 24		131.9													4.9		4.8	13.4
23	8 -- 24		70.6		28.3								4.2	2.2					
11	8 -- 24	205.5		218.9															
30	8 -- 24										9.7								
36	8 -- 24													1.8					
22	8 -- 24													5.9					
40	8 -- 24											10.8							

metabolites were quantified as phloroglucinol equivalents (µg/ml)

Table 3. Analysis of seaweed metabolites in plasma samples from the bioavailability study

NO ENZYME TREATMENT																			
VOLUNTEER	time (h)	PM1	PM2	PM3	PM4	PM5	PM6	PM7	PM8	PM9	PM10	PM11	PM12	PM13	PM14	PM15	PM16	PM17	PM18
11	2																		
48	2				0.4														
49	2															0.1	0.1		
17	3																0.3		
33	3					0.2													
48	3				0.9														
33	4					0.3													
22	4														0.4			0.4	
24	6																		0.2
30	6					0.5													
44	8																		
17	8																0.2		
33	8					0.6													
11	8									0.7									
30	8					0.4													
26	8			0.7															
44	24										0.2								
46	24		9.5				15.1							0.2					0.2
24	24					0.2													
34	24					1.0													
34	24													1.0					
50	24												4.1						
36	24									0.5		2.2							
ENZYME TREATMENT (glucuronidase/sulfatase)																			
VOLUNTEER	time (h)	PM1	PM2	PM3	PM4	PM5	PM6	PM7	PM8	PM9	PM10	PM11	PM12	PM13	PM14	PM15	PM16	PM17	PM18
17	3																		
33	3							0.3											
34	4	7.4																	
24	6																		0.1
17	24																		0.8
33	24							0.3											
44	24									0.1									
46	24	35.8					41.2							0.3					0.2

metabolites were quantified as phloroglucinol equivalents ($\mu\text{g/ml}$)

Some metabolites (M3 and M4) show similar UV spectra characteristics and might therefore be structurally related (Figure 2). In plasma (**Table 3**) some metabolites were found in samples collected at 2, 3 and 4h after capsule ingestion, but the majority of the metabolites was found in samples collected at later time-points (6-24h). This could be due to a high colonic metabolism, following fermentation of high molecular weight phlorotannins in the large intestine. The analysis of urine and plasma samples from the chronic intervention also showed the presence of a variety of metabolites in samples from some volunteer but not all. Bioavailability is a critical factor influencing in vivo biological activity and this study provides a basis for further investigating the seaweed-derived bioactive components in the body after ingestion and their mechanism of action in vivo.

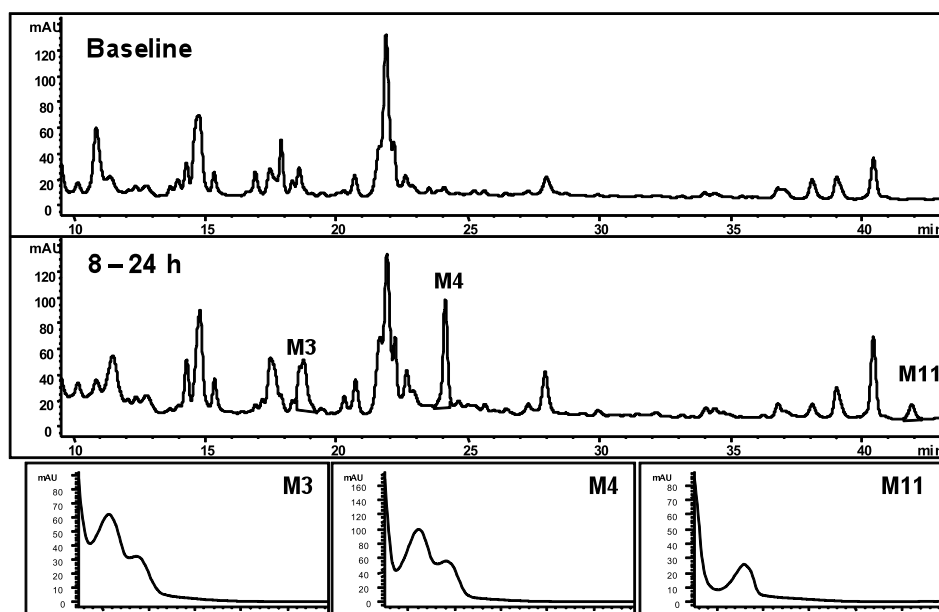


Figure 2. HPLC chromatogram (280nm) showing an example of urine analysis and the characterization of some metabolites (peaks M3, M4 and M11).

3. Long term intervention study with SPE CC2574

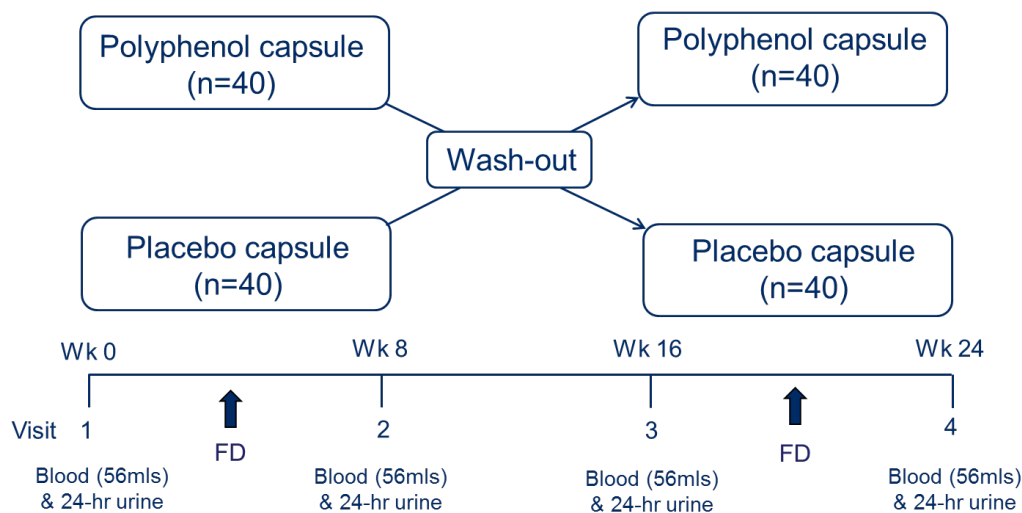
3.1 Study design

Ethical approval (REC/11/0077) was obtained from the local ethical committee prior to commencement of the study. Sufficient subjects were screened to ensure the study began with 80 healthy volunteers (39male/41female) aged 30-65 yrs apparently healthy, non-smoking, BMI>25, omnivores, who did not habitually use vitamin or mineral supplementation. Pregnant and lactating women, vegetarians and vegans and lactose intolerant individuals were excluded from the study, as were those with chronic medical complications such as diabetes, cardiovascular diseases, autoimmune/ inflammatory disorders or those who had chronic medication use including anti-inflammatory agents.

A double blind randomised cross-over design study was employed to test the efficacy of the seaweed SPE) CC2574 (Figure 3). Eighty subjects were randomised to 2 groups of 40 subjects each starting on either a SPE capsule or a maltodextrin placebo control capsule (Avebe MD14P) daily for an 8-week period. Both groups received each eight-week course of supplements interspersed by an 8-week washout period. Fasting blood and urine samples were collected from each subject at 4 time points during the study, equivalent to commencement/conclusion of each of the two treatment phases.

Figure 3

Study design



3.2 Blood samples and urine processing

Fasting blood samples were collected before and after each phase (week 1, week 8, week 16, week 24) by venepuncture into EDTA- or lithium heparin- containing tubes as required. Whole blood samples were aliquoted and lymphocytes isolated using Histopaques-1077, according to the manufacturer's instructions (Sigma Diagnostics, St Louis, MO, USA) and plasma samples prepared by centrifugation at 1000 x g for 10 min, 4 °C. Serum samples were prepared by centrifugation at 2000 x g for 10 min, 4 °C. Lymphocytes were stored frozen in liquid nitrogen, while whole blood, plasma, serum samples were immediately stored at -80 °C. Samples were stored independently as primary and backup samples at -80 °C. The subjects were asked collect urine (24 h) preceding each visit, urine samples were aliquoted and stored (-20 °C) until analysis.

3.3 Dietary assessment

The habitual diet of the 80 subjects was monitored during treatment period (placebo/active) by administration of a 4-day “food diary” assessment at the mid-point. These diaries are routinely used in our Centre and have been validated. The information on dietary composition of the subjects’ intake was generated using the nutritional software package NETWISP.

3.4. Polyphenol analysis in blood and urine:

(See section 2.3 above)

All samples were assessed against baseline (ie before SPE consumption). Baseline samples showed no detectable SPE metabolites

3.5 Plasma total oxidant capacity (TOC)

Total oxidative capacity (TOC) measures total peroxide levels in plasma, by the reaction of endogenous peroxides with peroxidases, using tetramethylbenzidine (TMB) as the chromogenic substrate (Josephy, 1982). TMB blue colour turns to yellow after addition of the stop solution and can be measured photometrically at 450 nm. For the assay protocol, 10µl of standard (hydrogen peroxide) and samples were incubated with 200µl of the reaction mixture (containing buffer, TMB substrate and peroxidase) in uncoated microtiter plates and incubated at room temperature for 15 min. 50µl of stop solution (H₂SO₄) were added to all wells and the absorbance at 450 nm was measured using a microplate reader (GENIOS Tecan). Hydrogen peroxide standard solutions for calibration curve were freshly prepared before use.

3.6 Lymphocyte DNA damage

A number of recent dietary intervention trials have reported the ability of single dietary components containing various mixtures of phytochemicals (e.g. simple phenols, flavonols, catechins, tannins) to decrease DNA damage in lymphocytes or to increase their ability to resist damage (Gill *et al.*, 2004; Riso *et al.*, 2005). The impact of seaweed phenolic consumption on DNA damage in lymphocytes was determined in 78 subjects using the comet assay as described in Gill *et al.*, (2007) for basal levels of DNA strand breaks and also the susceptibility of the cells to oxidative stress by *in vitro* challenge with hydrogen peroxide (150µm) was assessed.

3.7 Oxidative stress markers and lipid profile

The isoprostanes are a unique series of prostaglandin-like compounds formed *in vivo* via a non-enzymatic mechanism involving the free radical-initiated peroxidation of arachidonic acid (Milne *et al.*, 2005). Isoprostanes have been established as chemically stable, highly specific and reliable biomarkers of *in vivo* oxidative stress, and were measured in frozen serum samples using a commercial 8-Isoprostane EIA Kit Item no. 516351, Cayman Chemicals. This assay is based on the competition between 8-isoprostane and an 8-isoprostane acetylcholinesterase (Ache) conjugate (8-Isoprostane Tracer) for a limited number of 8-isoprostane-specific rabbit antiserum binding sites consequently 8-isoprostane concentrations are measured as a function of turbidity (absorbance). Plasma total cholesterol, HDL cholesterol and triglycerides were measured Instrument Laboratory ILAB 600 (Warrington, UK) autoanalyzer using commercial kits (Roche diagnostics, Lewis, UK) according to kit manufacturer’s protocols with plasma LDL cholesterol was calculated using the other three lipid profile parameters and the Friedewald formula.

3.8 Measurement of Inflammatory markers, Cytokines and CRP

Intracellular cytokine levels in lymphocyte and monocyte populations and TF expression were assessed using a whole blood labelling method that utilises flow cytometry namely the Fast immune system (B&D) in accordance with manufacturer’s instructions for 78 subjects at all time points. The method was used to measure intracellular IL-1β, IL-2, IL-6, IL-10, IL-12, IFN-γ and TNFα expression in mononuclear cells. These cytokines were chosen for analysis because of evidence suggesting that they are associated with inflammation and CVD risk. Briefly, whole blood was

incubated with either lipopolysaccharide or phorbol 12-myristate 13-acetate to activate monocytes and lymphocytes respectively. Cells were labelled with the appropriate cell surface antibody and cytokine-specific antibody and analysed on a Gallios flow cytometer (Beckman Coulter). The number and percentage of each cell type expressing the cytokine as well the mean channel fluorescence was recorded.

C-reactive protein an acute phase protein synthesized by the liver in response to inflammatory stimuli, especially the cytokine IL-6.). Serum C-reactive protein concentrations were determined on an Instrument Laboratory ILAB 600 (Warrington, UK) autoanalyzer using a commercial kit in accordance with manufacturers instructions.

3.9 Statistical analysis

All biological measurements were carried out at the end of the intervention, in batches containing equal numbers of active and control phase samples in each batch, and the researchers were blinded to these samples during analyses. All values are expressed as mean $\pm$ SD, unless otherwise specified. The mean values are reported for all subjects (n=78) during their treatment phase (SPE capsule) and during their placebo control (maltodextrin) phase. All biochemical analyses were conducted in duplicate and the mean values taken as the final result. For all markers the results are presented as treatment effects. This was undertaken by calculating individual differences between pre- and post-values for both control and treatment phases for each subject. Paired T tests were then carried out on the difference scores (post-treatment value minus pre-treatment values) between treatment (SPE capsule) and placebo control phase (maltodextrin). Significance level was set at $p < 0.05$. All statistical analyses were performed using the SPSS

3.9 Results

Eighty subjects (males n=39, females n=41) began the randomised, double-blind, crossover trial to assess the anti-inflammatory efficacy of the SPE. The study population had a mean group age of 42.7 ± 7.1 years and a BMI 30.2 ± 3.9 kg/m². The study had an overall compliance of 97% with 78 subjects completing the 24 week study, two subjects withdrew from the study at the midpoint for personal reasons and compliance was not significantly different by treatment group or time period.

Table 4 Nutrient intake of subjects

Macronutrient	Placebo	SPE	Significance
Energy (kcal/d)	1949 $\pm$ 590	2057 $\pm$ 684	NS
Protein (g/d)	79.4 $\pm$ 28	79.9 $\pm$ 25	NS
Fat (g/d)	78.6 $\pm$ 27	79.9 $\pm$ 25	NS
Carbohydrate (g/d)	220 $\pm$ 72	231 $\pm$ 84	NS

Table 4 reports the dietary analysis of habitual intake (midpoint) during both the control and treatment periods of the RDBCT. Total average energy and carbohydrate levels varied by less than 5% across the treatments periods, while the levels of consumption of protein and fat remained virtually unchanged. Consequently we were able to determine that intervention with a seaweed phenolic extract did not negatively affect the consumption patterns in an overweight UK population.

Table 5 Effects of seaweed polyphenol extract on blood lipids and CRP

Blood marker (n=78)	Average baseline value	Placebo treatment effect (post-pre)	SPE treatment effect (post-pre)	Significance
CRP (mg/ml)	2.67 ± 3.9	0.01 ± 3.3	-0.83 ± 4.9 (-31%)	NS
Cholesterol (mmol/l)	5.20 ± 0.77	-0.06 ± 0.57	-0.10 ± 0.57	NS
TAG(mmol/l)	1.51 ± 0.94	0.01 ± 0.82	0.04 ± 0.96	NS
HDL-C (mmol/l)	1.37 ± 0.32	-0.01 ± 0.15	0.03 ± 0.15	NS
LDL-C (mmol/l)	3.16 ± 0.1	-0.08 ± 0.5	-0.06 ± 0.50	NS

The baseline levels for blood lipid parameters were calculated as a mean group average for both pre supplementation sampling points (time points 1 & 3). The group mean average (n=78) for key blood parameters was as follows: blood cholesterol 5.2 ± 0.77 mmol/l; HDL 1.37 ± 0.32 mmol/l; LDL 3.16 ± 0.1 mmol/l and triglycerides 1.51 ± 0.94 mmol/l. The study population displayed a characteristic profile associated with a middle aged population and with an average BMI exceeding 30 kg/m². An 8-week supplementation with seaweed extract did not significant effect any cardiovascular risk marker as is reported Table 5. The acute inflammatory marker CRP was reduced by approximately 31% in response to seaweed treatment however this change did not reach significance.

Table 6 Effect of seaweed polyphenol extract on lymphocyte DNA damage and F2 isoprostanes

Blood marker (n=78)	Average baseline value	Placebo treatment effect (post-pre)	SPE treatment effect (post-pre)	Significance
DNA damage (basal)	6.72 ± 2.48	0.74 ± 2.86	-0.41 ± 3.13	NS
DNA damage +H ₂ O ₂	34.20 ± 7.00	-1.56 ± 6.60	-2.03 ± 6.40	NS
F2 Isoprostanes pg/ml (n=40)	392 ± 219	-10 ± 182	-6 ± 138	NS

8-F2-Isoprostane levels at baseline were 392 ± 219 pg/ml for the population. A high degree of inter-individual variation was observed, and the 8 week SPE intervention did not exhibit a significant effect on this marker of oxidative stress. Basal levels of DNA damage levels as measured by the Comet assay were consistent with previous studies with a mean group average of 6.72 ± 2.48 % Tail DNA. In response to oxidative challenge (150 µM H₂O₂) DNA damage was increased to a mean value of 34.2 ± 7 % tail DNA as reported in Table 6. The 8 week intervention with a seaweed phenolic extract did not result in any significant change in the risk biomarker in the total population. However, in a subset analysis of subjects at greater risk (BMI>30), a significant 23% reduction in basal DNA damage was observed (Figure 4).

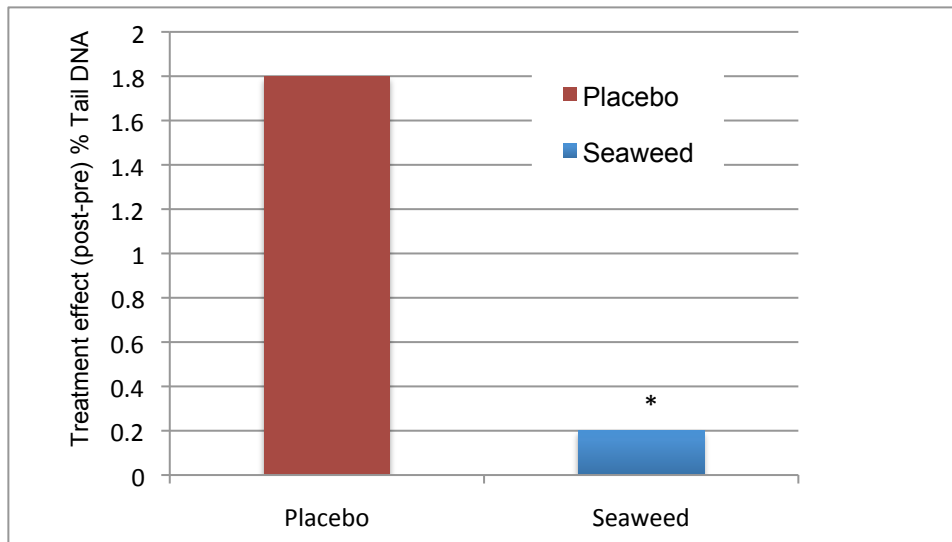


Fig 4 SPE decreased basal DNA damage in lymphocytes in an obese UK population (n=36). Average baseline value = 6.9 ± 1.9 . 23% difference in effect seaweed vs placebo ($p=0.04$, one tailed test)

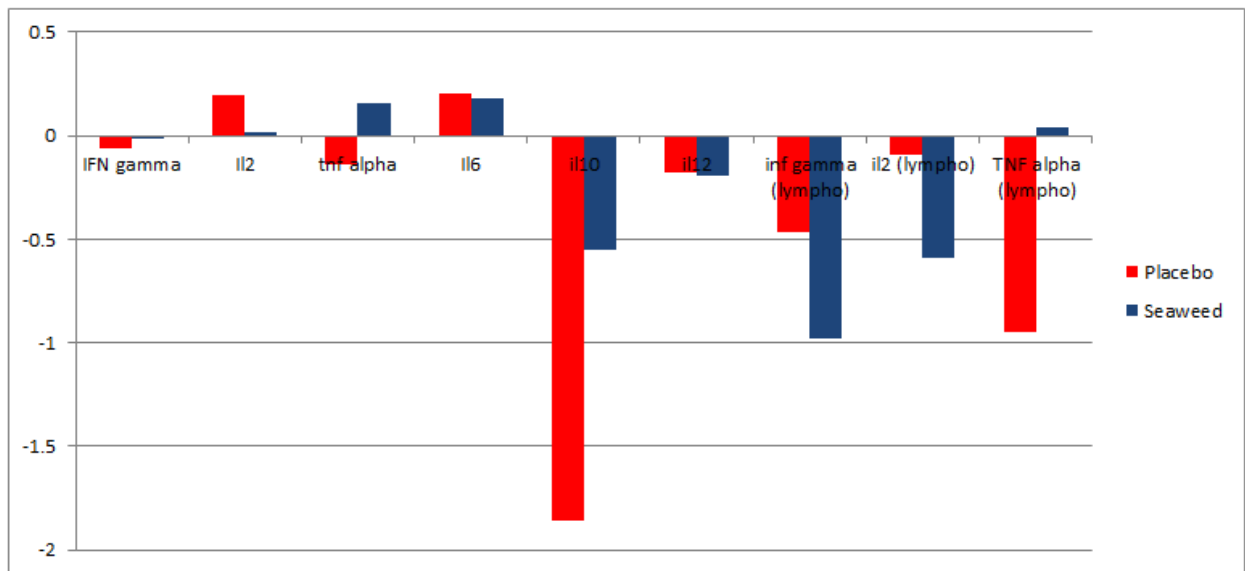


Figure 5 Effect of SPE intervention on cytokines levels in an overweight UK population (N=78)

No significant treatment changes were observed in the monocyte cytokine IFN gamma, IL2, TNF alpha, IL6, IL10, IL12, nor in the lymphocytes IFN gamma, IL2 and TNF alpha in response to seaweed intervention (Figure 5).

Fig 6a

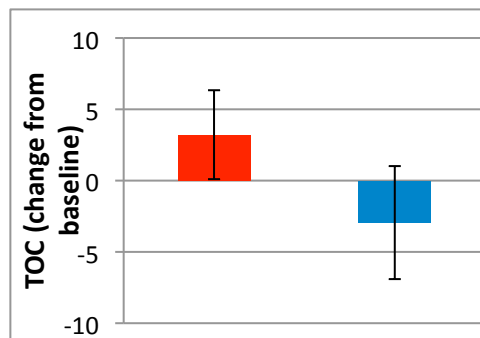
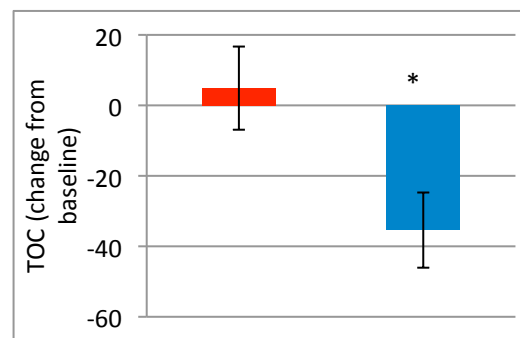


Fig 6b



Placebo

Seaweed

Placebo

Seaweed

Figure 6 Effect of SPE on total oxidative capacity (TOC) in plasma. Fig 6a shows data for all subjects ($n=78$), Fig 6b shows data for subjects with high baseline plasma peroxides ($>100\text{mM}$). TOC was determined by a rapid enzymic assay (POX-ACT). This assay measures endogenous levels of peroxides using tetramethylbenzidine as the chromogenic substrate. * $P<0.05$ compared to baseline

Fig 6a shows the Total Oxidative Capacity (peroxide levels) in plasma samples from all volunteers measured after the placebo and seaweed capsule intervention. When considering the full set of data, there were no significant changes from baseline after both the placebo and seaweed capsule interventions ($p > 0.05$). Interestingly however, when considering a subset of data (Fig 6b) from volunteers having high peroxide levels at baseline (higher than $100\text{ }\mu\text{M}$ before the intervention) a significant reduction (37%) of peroxide levels was observed after intervention with the seaweed capsule ($p < 0.05$). An overproduction of peroxides leads to a dangerous imbalance in the organism and may induce oxidative stress and start pathological mechanisms that can develop several diseases. Our data shows that the chronic SPE administration in volunteers with high peroxide levels could counteract the plasma oxidative imbalance.

Total polyphenols (as phloroglucinol equivalents) excreted in urine (mg/day) and detected in plasma ($\mu\text{g/ml}$) are shown in Table 7. There was a large inter-individual variation in polyphenols excreted - in 43 subjects none were detected in urine and in the remainder the amounts excreted ranged from 5 to 645 mg/day. Similarly, there was a large variation in plasma concentration. These results are consistent with the bioavailability study and reflect differences between subjects in absorption and metabolism of the seaweed phenolics. Such large interindividual differences have been seen with polyphenols from other plant sources (Rowland et al 2000)

Table 7 Urinary and plasma polyphenols

Volunteer	Urine total mg	Total plasma ug/ml
S1	13.56	6
S3	41.48	
S4		9.7
S5		8.5
S7	27.25	
S8		20.4
S10	23.32	
S13	15	
S16	194.01	
S17	24.24	
S18	32.49	22.4
S19	98.28	17
S20		326.8
S24	43.22	
S27	10.34	
S29	5.1	
S30	6.94	
S31	6.21	
S32	157.93	
S33	192.14	
S38	50.89	
S39		147
S40	6.23	30.1
S43	223.53	4439.4
S44	42.83	
S45		846.9
S46	18.26	
S49	81	
S53	19.17	
S56	645.65	9
S57	45.02	
S58	9.43	
S62	102.23	
S63	108.75	
S65	12.67	22.2
S66	182.76	
S67	283.65	
S70	4.66	
S76	124.1	
S77	43.27	
S78		15.9
S81	32.79	
S82	8.57	

Phenolics (POL) in plasma and urine were quantified as phloroglucinol equivalents using HPLC and expressed as ug/ml plasma and mg/d in urine. No value indicates polyphenols were undetectable

4. In vitro activity of seaweed polyphenol extracts (WP 5).

Ten seaweed polyphenol extracts (SPEs, see sections 1.1 & 1.3) were investigated for total polyphenol content and *in vitro* antioxidant and anti-inflammatory activities.

4.1. Total Polyphenol content (Folin-Ciocalteu)

Total polyphenol (TP) content of extracts was determined by the Folin-Ciocalteu's method adapted from Yuan et al. (2005). 100µl of sample were mixed with 50µl of 2M Folin-Ciocalteu's reagent and left at room temperature for 3 min. Then 200µl of 20% Na₂CO₃ were added and the mixture incubated at room temperature in the dark for 45 min before transferring 150µl to a 96 microwell plate in duplicate. Absorbance was measured at 730 nm on a GENios TECAN microplate reader with phloroglucinol as the standard. TP content of the extracts was expressed as µg/mg phloroglucinol equivalents.

As shown in Figure 7 the extract CC2576 (high molecular weight fraction) had the highest TP content (442 µg/mg), followed by CC2575 (basic extract), CC2699, CC2700 and CC2577 and CC2698. Lower TP content was measured for CC2574, CC 2719 and CC2720. Extract CC2681 had the lowest TP content (50.55 µg/mg).

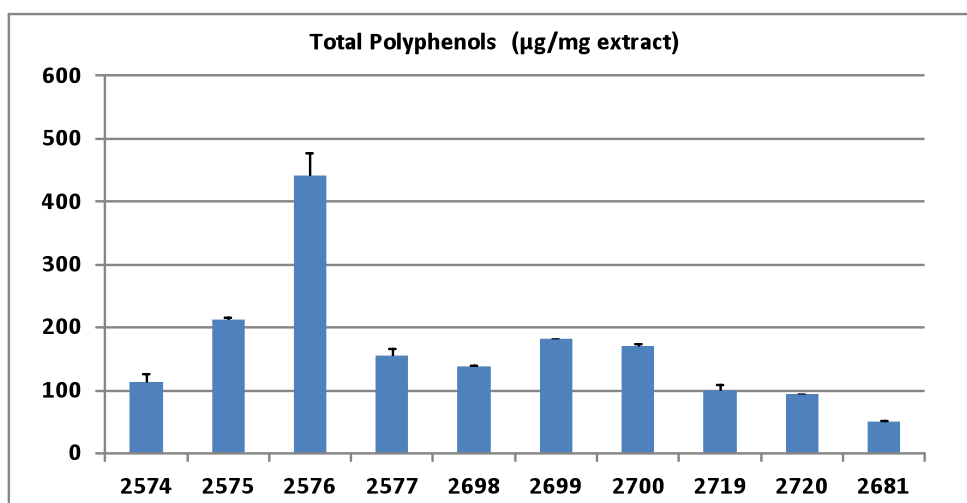


Figure 7. Total polyphenol content of seaweed extracts

4.2. Antioxidant activity (FRAP assay)

The Ferric-Reducing Antioxidant Power (FRAP) assay is a simple test that measures the antioxidant power as the ability to reduce iron, a transition metal that can induce oxidative damage. The higher the FRAP value measured, the higher is the antioxidant potential. (Pellegrini et al., 2003). The FRAP reagent working solution was freshly prepared before use by mixing 300 mM acetate buffer pH 3.6, 10 mM TPTZ (2,4,6-tripyridyl-s-triazine), and 20 mM ferric chloride in a 10 : 1 : 1 ratio. 10 µl of diluted samples were mixed with 300 µl of FRAP reagent. 100 µl of sample solutions were transferred to microwell plates in duplicate and absorbance was measured at 595 nm by GENios TECAN microplate reader. Ascorbic acid was used as the standard for a calibration curve. Results from the FRAP assay were expressed as nmol/mg ascorbic equivalent

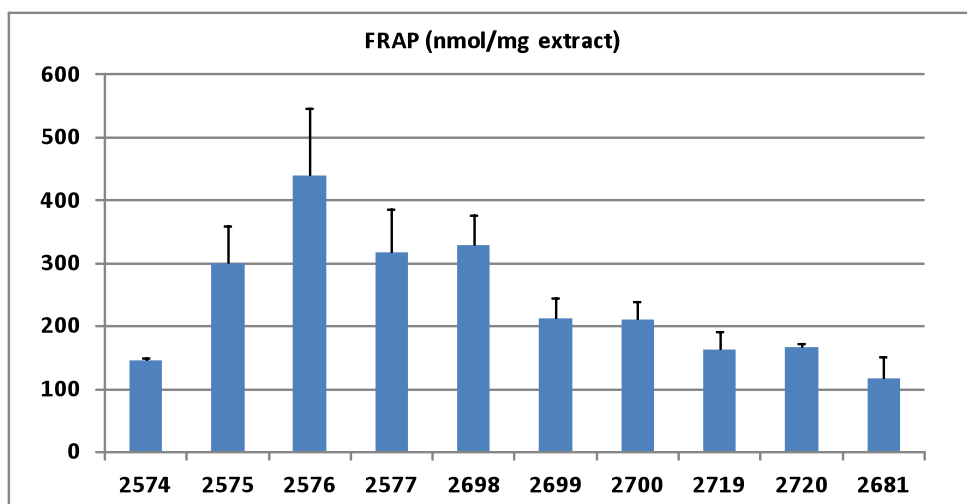


Figure 8. In vitro Ferric-Reducing Antioxidant Power (FRAP) of seaweed extracts

Extract 2576 had the highest FRAP value, followed by CC2698, CC2577, CC2575, CC2699 and CC2700 (Figure 8). A slightly lower FRAP value was measured for CC2720, CC2719 and CC2574. CC2681 had the lowest FRAP value. It is noteworthy that although CC2576 had a TP value double that of CC2575, its FRAP value was only 25% higher. It was also interesting to note that the extracts CC2577 and CC2698 had a lower TP content than CC2699, but higher FRAP value.

4.3. Antioxidant activity (TEAC assay)

The Trolox Equivalent Antioxidant Capacity (TEAC assay) measures the antioxidant power as the ability to quench the 2,2-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt radical cation ($ABTS^{\cdot+}$) and therefore gives an indication of free radical scavenging power. The higher the TEAC value measured, the higher is the antioxidant potential (Pellegrini et al., 2003). A stable stock solution of $ABTS^{\cdot+}$ 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 $ABTS^{\cdot+}$ working solution was obtained by diluting the stock solution in ethanol to an absorbance of 0.70 (730nm). 15 μ l of each sample were mixed with 450 μ l of $ABTS^{\cdot+}$ working solution and left at room temperature for 5 min. 150 μ l 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 μ mol/mg Trolox equivalents.

As shown in Figure 9, the extract CC2576 had the highest TEAC value, followed by CC2575, CC2700, CC2699, CC2577 and CC2698. A slightly lower TEAC value was measured for CC2719, CC2720, and CC2574. CC2681 had the lowest FRAP value. In agreement with the FRAP results, although CC2576 had a TP value double than CC2575, its TEAC value was not doubled. It was also interesting to note that the extracts CC2577 and CC2698 have less scavenging activity (TEAC) but higher iron-reducing power (FRAP) than CC2699 and CC2700.

Summarizing the evidence derived from the TP and anti-oxidant assays, it is clear that the extraction and fractionation procedures had a marked effect on TP and anti-oxidant activity. The ethanol/water extraction method developed at CEVA for extraction of polyphenols from *Ascophyllum nodosum* (sample CC2575) appeared to be more efficient in terms of TP levels than the methods used for the novel extracts. Water extraction (CC2681) gave the lowest TP concentration. When CC2575 was fractionated into high and low molecular weight fractions, the highest TP concentration was seen in the high MW fraction (CC2576), which had 3 x the level in the low MW fraction. Antioxidant activity broadly followed TP levels, with CC 2576 having the highest activity and CC 2681 the lowest although the difference between the high and low MW fractions of CC2575 were not as great as those seen in TP levels

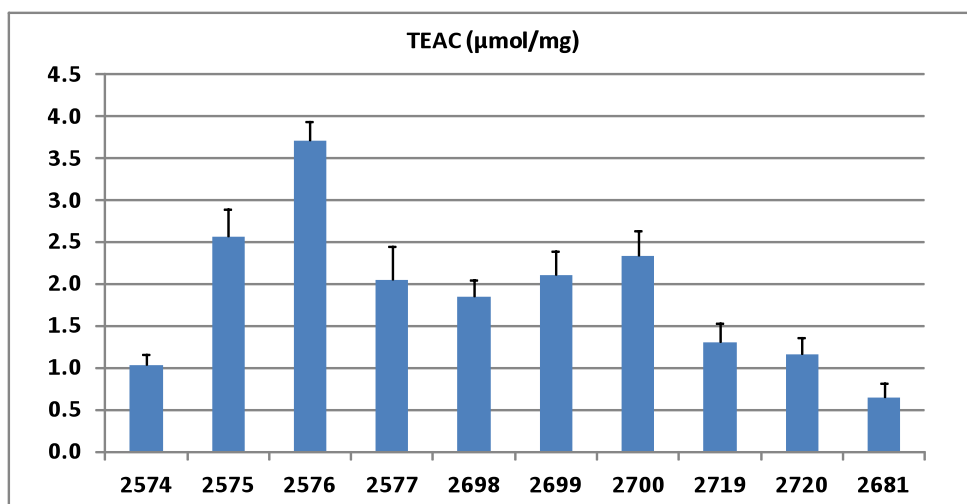


Figure 9. Trolox Equivalent Antioxidant Capacity (TEAC) of seaweed extracts

4.4. Simulated Digestion and Fermentation As is evident from the analytical results for urine and plasma from the human intervention studies, the polyphenols in the seaweed extracts undergo extensive metabolism in the stomach, small intestine and colon. Consequently in addition to the original work plan, the SPEs were subjected to *in vitro* gastric and ileal digestion and colon fermentation procedures, followed by dialysis to simulate absorption into the circulation.

4.4.1. Simulated Gastro-Intestinal Digestion: The procedure was adapted from Mills et al. (2008) and McDougall et al. (2005). This method consists of two sequential stages: gastric digestion and small intestinal digestion with dialysis. 10 g seaweed extracts were dissolved 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 protected from light. 5 ml aliquots (G) were removed. 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 shaker. 5 ml aliquots (SI) were removed. Samples 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 digested material. 5 ml aliquots of dialysis solution (D1) were removed. The dialysis fluid was changed and dialysis continued for additional 2 h. 5 ml aliquots of second dialysis solution (D2) were removed. Samples (SID) were freeze-dried and stored at -20 °C. All the aliquots collected during the digestion procedure were stored at -80 °C before further analysis.

4.4.2. Colonic Fermentation (Batch Culture): The method was adapted from Tzounis et al. (2008). 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 SI+D 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. 7 ml samples were collected at five time points (0, 2, 4, 8 and 24 h), centrifuged at 13,000 rpm at 4 °C for 10 mins and the supernatant was removed and stored at -80 °C for further analysis.

Aliquots of digested/fermented and dialyzed SPEs were used in the subsequent *in vitro* studies to assess potential anti-inflammatory effects in whole blood cultures and to investigate the ability of the seaweed extracts to reduce cellular DNA damage (COMET assays) on lymphocytes (PBMC cells) and human colon cells (HT-29 cell line).

4.5. Anti-inflammatory effects in vitro (cytokine levels in whole blood cultures)

To undertake this work, cultured whole blood containing peripheral blood mononuclear cells (PBMC) was used. We tested the anti-inflammatory effects of the digested materials that represent what has crossed the intestinal barrier and reached the circulation (D1 and D2 from the small intestine and FD from the large intestine). D1 and D2 were mixed following 1:1 dilution prior to use (SI+D). The TP content of the digested materials was measured (Folin-Ciocalteu assay) as described above (see section 5.1).

4.5.1. Methods: Blood samples were collected from fasted healthy donors into heparin tubes, and cultured as follows: a 250 µl blood aliquot was mixed with 250 µl of RPMI media containing antibiotics on a 24 well plate and the dialysates (SID and FD) were added. The extracts tested were 2574 (the extract tested in the human studies) at 5 concentrations, 2575 and 2576, at two concentrations (0.25 and 0.05 µg/ml TP). After incubation at 37 °C for 4 h, LPS (1 µg/ml) or vehicle (negative control) was added to each well and the plate was incubated for 20h at 37°C. At the end of the culture period, samples were centrifuged at 2000 x g for 5min and the supernatants were collected and stored at -20°C. Cytokines (IL-1β, IL-6, IL-8, IL-10 and TNF-α) in the supernatants were measured with Luminex xMAP Technology using commercially available Fluorokine MAP kits (R&D systems) and data analyzed on the xPONENT software.

The statistical evaluation of the results was performed by one-way analysis of variance (ANOVA) followed by a Bonferroni post-hoc t-test using GraphPad InStat version 5 (GraphPad Software, San Diego, CA, USA). Significant changes are indicated as $P < 0.05$.

4.5.2. Results.

Table 8. Cytokine production by whole blood cultures treated with SPEs

Cytokine levels and ratios		IL-8 (pmol/ml)		IL-1β (pmol/ml)		IL-6 (pmol/ml)		IL-10 (pmol/ml)		TNF-α (pmol/ml)		(IL-8 + IL-1β + IL-6 + TNF-α) / IL-10		IL-8 / IL-10	
	Conc (µg/ml TP)	mean	stdev	mean	stdev	mean	stdev	mean	stdev	mean	stdev	mean	stdev	mean	stdev
Small Intestinal Dialysis (SID)															
Negative Control	0	1549.7	748.9	77.0	24.4	758.6	350.5	6.3	4.1	17.3	14.6	404.4	241.9	273.9	196.3
Control	0	51391.6	25419.7	5567.0	3208.8	13295.2	4896.1	1791.4	708.2	1452.0	848.5	44.4	19.5	32.4	15.7
	0.01	36654.0	31413.1	5223.1	3778.1	10422.9	4586.0	1756.4	1080.0	1879.1	1667.2	36.8	21.5	26.4	19.4
	0.025	24468.7	15176.1	4122.9	2969.8	8455.0	3671.3	1727.5	1115.2	1418.9	1404.4	29.4	14.4	20.4	13.0
	0.05	22542.9	8567.6	5763.2	3086.5	11612.9	3889.6	2048.9	1064.1	1687.6	1543.5	26.6	13.5	16.5	11.1
	0.1	20689.9	16431.1	6666.4	5249.2	10466.3	5340.5	1784.3	768.9	2300.0	2422.6	23.4	10.2	13.1	9.7
	0.25	13020.8^a	5565.7	6303.2	3029.1	10780.9	4082.1	2063.4	959.6	2346.8	1824.2	16.1	3.1	6.5^a	3.1
2575	0.05	40277.1	14065.3	5638.0	4576.0	12289.3	7578.7	1985.2	1406.0	1950.2	2372.7	35.9	18.8	25.2	13.7
	0.25	45526.8	36530.8	5693.2	3170.3	11003.1	4653.7	1854.4	701.9	2153.3	1623.1	37.8	27.1	19.3	14.7
	0.05	30040.5	10375.8	5827.0	3267.8	11586.3	5250.8	2031.8	1283.2	1683.7	1685.5	30.2	15.4	19.4	10.6
2576	0.25	29457.6	24237.3	5386.3	2446.6	11025.5	5069.6	1888.6	757.6	1501.4	1100.7	27.1	14.7	17.2	12.3
Fermented Dialysis (FD)															
Negative Control	0	1549.7	748.9	77.0	24.4	758.6	350.5	6.3	4.1	17.3	14.6	404.4	241.9	273.9	196.3
Control	0	54798.8	38520.7	4266.0	1896.3	11014.8	4310.8	1653.1	647.0	1103.7	627.3	49.2	30.5	38.5	26.4
	0.01	41415.6	21852.3	4411.2	3141.4	9549.0	4172.6	1721.6	726.6	1753.0	1221.4	35.2	14.6	26.0	11.5
	0.025	29643.3	12046.4	4957.3	3171.4	10510.9	4732.8	1982.0	1031.3	2288.8	1630.5	27.1	11.0	19.5	8.6
	0.05	39640.7	16438.4	5965.1	3934.8	12987.3	6267.7	2310.1	1193.3	2213.9	1663.1	30.8	14.9	21.2	11.6
	0.1	33260.7	10768.5	4173.5	2528.6	10057.2	4085.4	2425.5	1346.0	1411.7	1327.0	26.4	20.0	19.2	17.0
	0.25	39873.7	19480.8	5118.6	2652.2	11028.4	4445.0	2256.3	1278.8	1594.2	1265.8	31.3	17.2	22.6	14.2
2575	0.05	27926.0	9728.4	5078.8	2909.8	11536.1	6829.2	2197.8	1445.8	1857.2	2101.0	24.5	13.8	15.3	8.1
	0.25	31890.0	19703.7	3594.8	1472.8	7965.1	3156.6	2230.7	1018.8	991.7	1030.4	23.8	16.6	18.0	15.0
	0.05	21055.7	4199.2	4170.7	2451.8	8522.2	4834.0	2186.2	1153.1	1618.0	2037.9	17.4	4.2	11.1	5.0
2576	0.25	36886.9	21312.2	5584.4	3805.5	9181.4	4168.6	1993.8	740.1	1409.0	1128.6	28.0	13.6	20.1	11.4

Cytokine production was measured in the supernatants of whole blood cultures incubated with dialyzed digested (SID) and fermented (FD) seaweed extracts 2574, 2575 and 2576, in the presence of the inflammatory stimulus lipopolysaccharide (1 µg/ml). Statistical analysis was conducted by One-way ANOVA with Bonferroni post-hoc test. a = $P < 0.05$ vs. control

The production of cytokines (IL-1β, IL-6, IL-8, IL-10 and TNF-α) in non-treated non-stimulated blood (negative control) was very low (Table 8). When stimulated by LPS alone (control), the production of all cytokines was induced to different extents. The pretreatment with the dialysates from the digested (SID) and fermented (FD) extracts did not induce a significant alteration on IL-1β, IL-6, IL10 and TNF-α production compared with the LPS stimulated control alone. However, a concentration dependent reduction in the production of the anti-inflammatory cytokine IL-8 was observed. The reduction was significant for the dialyzed digested extract CC2474 at the highest concentration tested (0.25 µg/ml). Pro / anti-inflammatory ratios were also calculated (IL-8 + IL-1β + IL-6 + TNF-α) / IL-10 and IL-8 / IL-10) and found to be consistent with the results obtained for individual cytokines.

Amounts of the dialysates available for other SPEs (CC2681, CC2699, CC2719) were much less than the main extracts, allowing only limited testing for anti-inflammatory activity. No significant effects were seen by comparison with untreated controls.

4.5. PBMC DNA damage (Comet assay):

For the trials on PBMC cells, we used the digested materials that represent what has crossed the intestinal barrier and reached the circulation (D1 and D2 from the small intestine and FD from the large intestine). D1 and D2 were mixed following 1:1 dilution prior to use (SID).

4.5.1. Preparation of PBMC cells from blood: Fasted blood samples were taken from healthy adult donors aged 28–44 years in sodium heparin vacutainer tubes (Greiner Bio-One Ltd, Gloucestershire, UK). Blood was layered over an equal volume of Histopaque -1077 (Sigma, Dorset, UK) and centrifuged at 400 g for 30 min at room temperature. The plasma was removed into a sterile tube and kept for later use. Cells were harvested from the interface, washed with PBS and centrifuged (380g for 8 minutes) The pellet was resuspended in RPMI- 1640 and the cell number was adjusted to 1.5×10^6 cells/ml after trypan blue cell counting.

4.5.2. PBMC cell culture and treatment: PBMC were cultured into 6-well plates (3×10^6 /well) with RPMI media and in the presence of 2.5% autologous plasma and the seaweed extracts SID and FD (CC2574, CC 2575, CC2576) were added (0.25µg/ml TP). The cells were kept in a humidified 5% CO₂ incubator at 37°C for 24h, centrifuged and resuspended in RPMI media. After cell counting (trypan blue) the cell concentration was adjusted to 1.5×10^6 cells/ml and the cell suspension was kept on ice ready for the COMET assay.

4.5.3. COMET assay: An alkaline COMET assay was performed on PBMC cells (pre-treated for 24h as above) 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. The assessment of single strand breaks in DNA was performed as described by Gill et al (2004)

4.5.4. Statistical analysis: The statistical evaluation of the results was performed by one-way analysis of variance (ANOVA) followed by a Bonferroni post-hoc t-test using GraphPad InStat version 5 (GraphPad Software, San Diego, CA, USA). Statistical significance was set at $P < 0.05$.

4.5.5. Results:

As can be seen in Figure 10 (SID) and Figure 11 (FD), untreated control cells exhibited some DNA damage which was slightly decreased in PBMC pre-treated with the dialyzed digested (SID) extracts, significantly in the case of CC2576. In cells challenged with H₂O₂, the DNA damage was elevated by about 2 fold, but this increased level of damage was significantly ameliorated by pre-treatment with the SID extract and fermented (FD) extract CC2576. Non-significant decreases in induced DNA damage were seen with SID and FD CC2575 pre-treated cells.

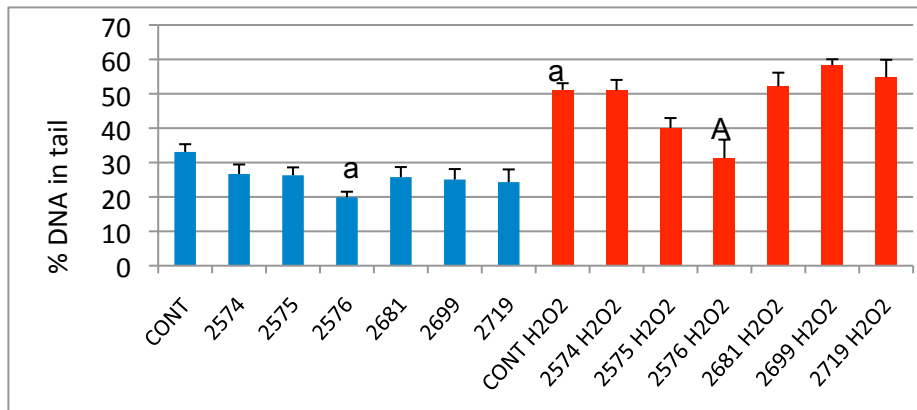


Figure 10. DNA damage measured by COMET assay in PBMC cells treated with dialyzed digested (SID) seaweed extracts. Statistical analysis was conducted by One-way ANOVA with Bonferroni post-hoc test. *a* = $P < 0.05$ vs. control; *A* = $P < 0.05$ vs. control H_2O_2 .

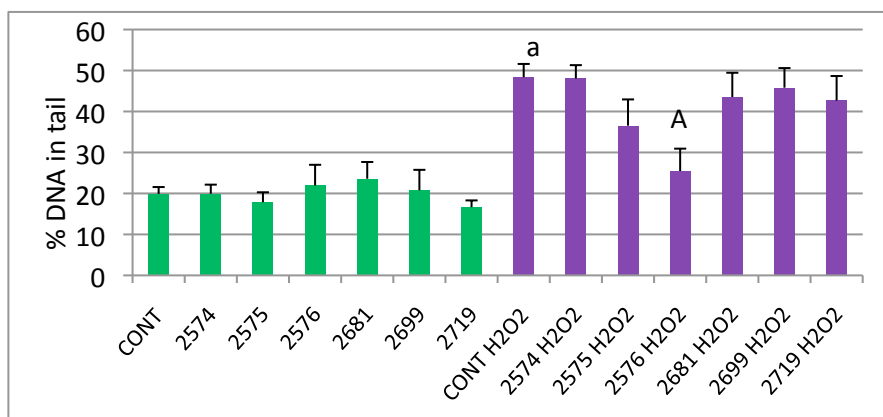


Figure11. DNA damage measured by COMET assay in PBMC cells treated with dialyzed fermented (FD) seaweed extracts. Statistical analysis was conducted by One-way ANOVA with Bonferroni post-hoc test. *a* = $P < 0.05$ vs. control; *A* = $P < 0.05$ vs. control H_2O_2 .

4.6. DNA damage in human colon cells (HT29)

For the Comet assays on colon cancer cells, we tested the digested extracts (SI), that represent what enters the colon after upper GI tract digestion, and the fermented extracts (LI), that represent what is metabolized and biotransformed in the colon by gut bacteria.

4.6.1. HT-29 cell culture and treatment: HT-29 human colon cancer cells were grown on DMEM media and prepared as follows: 1×10^6 cells/well were seeded on 6 well plates (0.5ml/well) and incubated overnight at 37°C , 5% CO_2 before treatment. Cells were treated with $100\mu\text{g/ml}$ of the digested and fermented extracts and incubated at 37°C , 5% CO_2 for 24h. After incubation time remove media, cells were trypsinized, washed with PBS, centrifuged (1800rpm, 5 min), resuspended in serum-free media and counted with trypan blue staining. The cell concentration was adjusted to 1.5×10^6 cells/ml and subjected to the Comet assay as above (Gill et al 2004).

4.6.2. Results:

As shown in Figure 12 (SI) and Figure 13 (LI), a low level of DNA damage (reported as % of DNA in tail) was measured in the controls. In cells challenged with H_2O_2 , the DNA damaged was significantly increased.

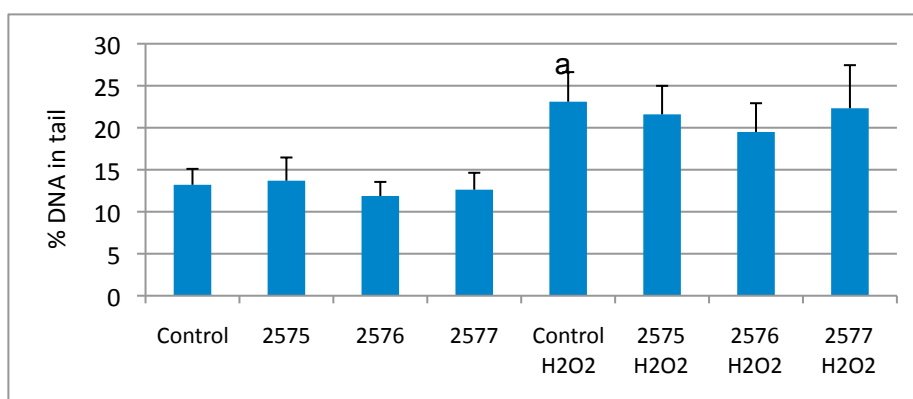


Figure 12. DNA damage measured by COMET assay in HT-29 cells treated with digested (SI) seaweed extracts. Statistical analysis was conducted by One-way ANOVA with Bonferroni post-hoc test. *a* = *P* < 0.05 vs. control.

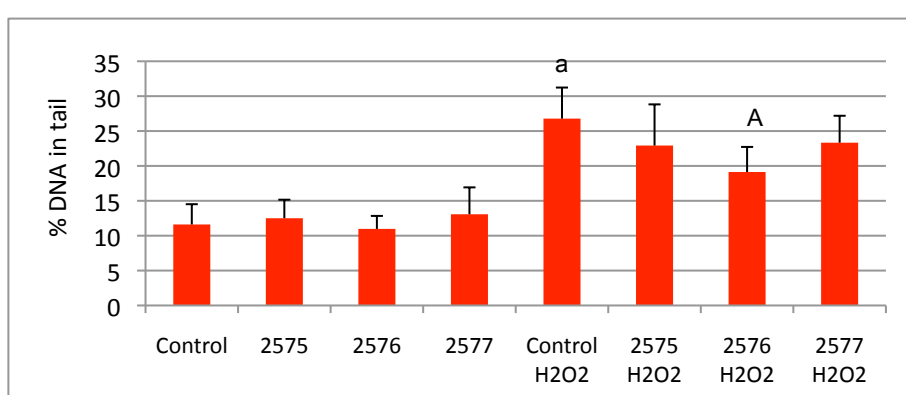


Figure 13. DNA damage measured by COMET assay in HT-29 cells treated with fermented (LI) seaweed extracts. Statistical analysis was conducted by One-way ANOVA with Bonferroni post-hoc test. *a* = *P* < 0.05 vs. control; *A* = *P* < 0.05 vs. control H₂O₂.

The pre-treatment with the digested (SI) extracts (Figure 11) did not induce a significant reduction of the DNA damage in cells challenged and non-challenged with H₂O₂. However, in HT-29 cells treated with the fermented (LI) extracts (Figure 12) the extract CC2576 significantly reduced the DNA damage in cells challenged with H₂O₂ whereas all other extracts tested did not induce any significant protective effect.

5. Conclusions

The project has explored a range of processing methods to extract and fractionate polyphenols from *Ascophyllum nodosum*. It is clear that the extraction and fractionation procedures can have a marked effect on TP and on anti-oxidant activity. The sample extracted with ethanol/water and fractionated to provide a product with predominantly high molecular weight polyphenols had the highest level of polyphenols and the greatest antioxidant activity. Water extraction alone was the least effective method of extracting polyphenols.

When used in the *in vitro* Comet assay for assessing single strand DNA breaks, the high MW extract (after simulated digestion and gut fermentation followed by dialysis to simulate absorbed metabolites) significantly protected human lymphocytes from DNA damage induced by hydrogen peroxide. Similarly, the digested and fermented high MW fraction, representing polyphenol metabolites generated and remaining in the gut, was able to protect human intestinal cells from induced DNA damage.

In vitro assays to assess effects of the digested and fermented extracts on markers of inflammation in blood revealed dose-related decreases in the pro-inflammatory cytokine IL8 and increases (non-significant) in the anti-inflammatory cytokine IL10 with CC2574. The ratio of IL8/IL10 in lymphocytes also decreased significantly in the presence of the digested CC2574 extract, indicating overall potential anti-inflammatory activity of the *Ascophyllum* extract.

In the human dietary intervention study with the blended (high MW+ low MW) SPE extract CC2574, the effects seen were less dramatic than *in vitro*, and significant effects were only apparent in analyses of subsets of the complete cohort. After consumption of SPE extract there was a non-significant decrease in total oxidant capacity in plasma, this became significant when a subset of subjects with a high initial level of peroxides were investigated. Similarly there was a decrease (non-significant) in the level of DNA damage in lymphocytes in subjects given SPE capsules. However in a subset of obese subjects, a significant 23% reduction in lymphocyte DNA damage was seen.

It seems likely that the lack of statistical significance of several of the beneficial trends seen in the main dietary intervention trial was a consequence of the high level of inter-individual variation in absorption and metabolism of the polyphenols that was apparent from the bioavailability study. Such interindividual variation has been seen with many other polyphenol rich plant sources.

6. References

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