Impact of a (poly)phenol-rich extract from the brown algae Ascophyllum nodosum on DNA damage and antioxidant activity in an overweight or obese population: a randomized controlled trial

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1 **Impact of a (poly)phenol-rich extract from the brown algae *Ascophyllum nodosum* on**

2 **DNA damage and antioxidant activity in an overweight/obese population: a randomised**

3 **controlled trial**

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Running head: Ascophyllum nodosum (poly)phenols impact DNA damage in the obese.

Abbreviations used: SPE, seaweed (poly)phenol extract; CRP, C-reactive protein; CVD, cardiovascular disease; ROS, reactive oxygen species; TNF-α, tumour necrocis factor alpha; COX, cyclooxygenase; UUREC, University of Ulster Research Ethics Committee; WISP, Weighed Intake Software Program; TF, tissue factor; TOC, Total oxidative capacity; TMB, tetramethylbenzidine; IL, interleukin; IFN, interferon; TNF, tumour necrosis factor; UHPLC-HRMS: ultra-high performance liquid chromatography-high resolution-mass spectrometry; VIP: variable of importance in projection; OPLS-DA: Orthogonal Partial Least Square Discriminant Analysis.

This trial was registered at clinicaltrials.gov as NCT02295878.

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ABSTRACT

Background: Epidemiological evidence suggests a diet rich in (poly)phenols has beneficial effects on many chronic diseases. Brown seaweed is a rich source of (poly)phenols

Objective: The aim of this study was to investigate the bioavailability and effect of a brown seaweed (*Ascophyllum nodosum*) (poly)phenol extract from on DNA damage, oxidative stress, and inflammation *in vivo*.

Design: A randomised double-blind placebo-controlled crossover trial was conducted in 80 participants aged 30-65 years with a BMI $\geq 25 kg/m^2$. The participants consumed either a 400 mg capsule containing 100 mg of seaweed (poly)phenol and 300 mg maltodextrin or a 400 mg maltodextrin placebo control capsule daily for an 8-week period. Bioactivity was assessed with a panel of blood-based markers including lymphocyte DNA damage, plasma oxidant capacity, C-reactive protein and inflammatory cytokines. To explore the bioavailability of seaweed phenolics, an untargeted metabolomics analysis of urine and plasma samples following seaweed consumption was determined by UHPLC-HR-MS.

Results: Consumption of the seaweed (poly)phenols resulted in a modest decrease DNA damage but only in a subset of the total population who were obese. There were no significant changes in CRP, antioxidant status, inflammatory cytokines. We identified phlorotannin metabolites that are considered potential biomarkers of seaweed consumption including pyrogallol/phloroglucinol-sulfate, hydroxytrifurahol A-glucuronide, dioxinodehydroeckol-glucuronide, diphlorethol sulfates, C-O-C dimers of phloroglucinol sulfate, C-O-C dimers of phloroglucinol and diphlorethol sulfate.

Conclusion: To the best of our knowledge, this work represents the first comprehensive study investigating the bioactivity and bioavailability of seaweed (poly)phenolics in human participants. We identified several potential biomarkers of seaweed consumption. Intriguingly, the modest improvements in DNA damage were observed only in the obese subset of the total

74   population, the subgroup analysis should be considered exploratory as it was not preplanned;

75   therefore, are not powered adequately. Elucidation of the biology underpinning this observation

76   will require participant stratification according to weight in future studies.

77

78   *Key words:* Seaweed, Phenolic compounds, Inflammation, DNA damage, Oxidative stress,

79   bioavailability

80

81 **INTRODUCTION**

82   Diets rich in plant-derived foods may reduce risk of chronic degenerative diseases, including

83 cardiovascular disease (CVD), with the beneficial effects being attributable in part to highly

84 bioactive (poly)phenolic compounds contained therein (1-3). Fruits and vegetables are well-

85 known sources of (poly)phenols, but a less well-known source is Ascophyllum nodosum, a

86 brown algae common to the British Isles that is rich in (poly)phenolic compounds, including,

87 uniquely, phlorotannin (4, 5). Phlorotannins are oligomers of phloroglucinol whose

88 concentration in seaweed is affected by numerous factors including plant size and age, water

89 salinity, nutrient and heavy metal content, in addition to light intensity changes (6-8).

90 *Ascophyllum nodosum* is of interest as it is one of only a few commercially sustainable seaweed

91 species.

92   Phlorotannins – and brown seaweed extracts in general – exhibit beneficial effects on a

93 range of biological processes including modulation of inflammation, reduction of oxidative

94 stress and improvements in cardiovascular function (9-11). However, the evidence base relies

95 heavily on cell line and small animal models, with few studies to date involving humans (12-

96 13). Phlorotannin-rich extracts from brown seaweeds have been shown to be effective in

97 controlling inflammation *via* a number of pathways including inhibition of pro-inflammatory

98 cytokines including tumour necrosis factor (TNF)-α and interleukins (IL)-1β and IL-6 *in vitro*

99 (14). The efficacy of *Ascophyllum nodosum* and other Fucoid species extracts in mitigating the

100 effects of oxidative stress by inhibiting the generation of reactive oxygen species (ROS),

101 preventing DNA damage and in stimulating the production of glutathione in affected cells has

102 been demonstrated in our work and that of other researchers (15-20). Our initial *in vitro* (15)

103 and acute *in vivo* (20) observations of the antioxidant and anti-inflammatory activities of

104 *Ascophyllum nodosum* extract(s) gave rise to the hypothesis that longer term consumption of

105 *Ascophyllum nodosum*-derived (poly)phenols would be of benefit *in vivo*.

106   A few investigations have evaluated – either *in vitro* or *in vivo* – the bioactivity of

107   phlorotannin rich extracts from Fucoid species. For example, safe consumption levels for a

108   (poly)phenol-enriched extract of the brown seaweeds *Ascophyllum nodosum* and *Fucus*

109   *vesiculosus* and its effects on glycaemic response have been determined in clinical trials (21).

110   To the best of our knowledge, we now report the first clinical study aimed at specifically

111   addressing the effects of a phlorotannin-rich extract from *Ascophyllum nodosum* on oxidative

112   damage to DNA, plasma antioxidant capacity, inflammatory responses and chronic, low level

113   inflammation *in vivo*.

114

115   **PARTICIPANTS AND METHODS**

116   **Seaweed material**

117   Fresh *Ascophyllum nodosum* was supplied by The Hebridean Seaweed Company, Isle of Lewis,

118   Scotland, UK, in March 2011. The seaweed biomass was harvested by hand to ensure quality,

119   cleaned of contaminating sand and fouling organisms and then shipped refrigerated to the

120   processing facility (CEVA) in France where it was immediately chopped and frozen.

121

122   **Preparation of food-grade seaweed extracts and capsule**

123   A (poly)phenol-rich seaweed extract from *Ascophyllum nodosum* was produced by CEVA

124   (France) using a food–grade solvent (ethanol:water, 60:40 vol/vol) extraction system that was

125   specifically developed for use with fresh or frozen *Ascophyllum nodosum*. Approximately half

126   of the produced extract was then fractionated using tangential flow ultra-filtration to produce

127   further extracts of varying molecular weight ranges and with varying (poly)phenol content. A

128   standardised blended (poly)phenol-rich *Ascophyllum nodosum* extract was formulated by

129   CEVA (**Table 1**) using 175 mg of extract and 50 mg of high molecular weight fraction (>10

130   kDa cut off) for use in the current study, in order to maximise the seaweed (poly)phenol content

131   (>100 mg per day) available from the extraction of fresh or frozen *Ascophyllum nodosum*

132   against the need to minimise the level of iodine to within accepted regulatory guidelines (<500

133   μg per day) (**Table 1**), heavy metal contamination was also assessed. Maltodextrin (175 mg)

134   was added to the capsule formulation as an excipient. Blending was carried out at the food–

135   grade CEVA facilities in France. Samples of 400 mg of the *Ascophyllum* (poly)phenol–rich

136   blend (SPE) or a placebo containing 400 mg maltodextrin only were packed into identical

137   white, opaque, vegetarian capsules by Irish Seaweeds, Belfast, UK and identically sized and

138   matched capsules were used for the clinical study. The food–grade seaweed capsule was

139   characterized by NP-HPLC and LC-MS analysis and is reported elsewhere (20) (see

140   **Supplemental figures 1 & 2**). Phlorotannins were quantified using the Folin-Ciocalteau

141   Method (22) using phloroglucinol as the standard. In brief, 1 mL of suitability diluted sample

142   was reacted with 1 mL of 40 % Folin Ciocalteu reagent for 5 min, and then made alkaline with

143   the addition of 1 mL of 100 g/L Na_2CO_3. Absorbance was read at 730 nm after the solution

144   had developed for 1 h at room temperature. Phloroglucinol dihydrate (range 0-30 mg/L) was

145   used as a standard and was treated in the same way as samples.

146

147   **Ethics and participants**

148   Ethical approval was received from the Ulster University Research Ethics Committee

149   (UUREC). All participants gave written informed consent. Participants were recruited between

150   May 2011 and August 2011 from Ulster University and the surrounding area. The intervention

151   study ran between August 2011 and February 2012. The study was registered at

152   clinicaltrials.gov as NCT02295878.

153   The study was conducted in 80 participants (age range 30-65 years). All participants were

154   apparently healthy, non-smoking, BMI≥25 kg/m^2, omnivores, who did not habitually use

155   vitamin or mineral supplements, as determined using a pre-screening health and lifestyle

156   questionnaire. Pregnant and lactating women, vegetarians and vegans and lactose–intolerant

157   individuals were excluded from the study, as were those with chronic medical complications

158   such as diabetes, cardiovascular disease, autoimmune/inflammatory disorders, or who had

159   chronic medication use including anti-inflammatory agents.

160

161   **Study design**

162   The study was a 24-week randomised, double-blind, placebo-controlled crossover trial.

163   After obtaining consent, participants were randomly assigned, in blocks of four using a

164   random-number generator (www.randomization.com), to either the intervention or the control.

165   In total, eighty participants were randomised to 2 groups of 40, each starting on either a 400

166   mg seaweed (poly)phenol extract (SPE) capsule containing 100mg of (poly)phenols or a 400

167   mg maltodextrin placebo control capsule (Avebe MD14P) daily for an 8-week period. The

168   participants were supplied with all capsules in weekly labelled capsule boxes at the beginning

169   of each phase, which was interspersed by an 8-week washout phase. During the washout phase,

170   the participants were asked to maintain their habitual diet. Participants were asked to bring any

171   unconsumed capsules to their study appointment at the end of each treatment phase and were

172   also contacted weekly by the study researcher to encourage compliance and to discuss any

173   difficulties they were experiencing.

174

175   **Blood and urine sample collection**

176   Fasting blood samples were collected before and after each phase (week 0, week 8, week

177   16, and week 24) by venepuncture into EDTA, serum or sodium heparin-containing tubes, as

178   required. All blood samples were processed on ice. Lymphocytes were isolated by using

179   Histopaque-1077, according to the manufacturer's instructions (Sigma Diagnostics, St Louis,

180   MO), and plasma samples were prepared by centrifugation at $1000 \times g$ for 10 min at 4 °C.

181 Serum samples were allowed to clot for 30 min at room temperature and then were centrifuged

182 at $2000 \times$ g for 10 min at 4 °C. Whole blood from sodium-heparin treated tubes was prepared

183 according to the manufacturer's instructions (BD Bioscience Fast Immune Cytokine System).

184 Whole blood, plasma, serum and urine samples were immediately stored at -80 °C, whereas

185 lymphocytes were stored frozen in liquid nitrogen. All biological measurements were carried

186 out at the end of the intervention in batches containing equal numbers of active and control

187 phase samples in each batch, and the researchers were blinded to these samples during analyses.

188 A 24 h urine collection occurred at each time point; volume and pH were measured and the

189 sample mixed following which 2×14ml aliquots were removed and centrifuged at 1000 x g

190 for 10 min at 4 °C. Supernatants were stored at -80 °C until required.

191

192 **Questionnaire assessments**

193 All participants completed a health and lifestyle questionnaire assessing their alcohol intake

194 and physical activity levels, as well as a validated 4-day food diary at the mid-point during

195 each treatment phase (active/placebo) of the study. Data on type of food and corresponding

196 weight was entered into a food analysis database (WISP, Weighed Intake Software Program;

197 Tinuviel Software, Warrington, U.K.) by two independent researchers and the dietary

198 composition calculated.

199

200 **DNA damage in peripheral blood mononuclear cells**

201 Peripheral blood lymphocytes, previously isolated and stored in liquid nitrogen, were

202 thawed and screened for basal single strand breaks (SBs) in DNA using the single cell gel

203 electrophoresis (Comet) assay (23) as adapted by Gill *et al.* (24). Spontaneous DNA SBs are

204 associated with an altered cell function and are considered appropriate for the substantiation of

205 health claims in the context of protection against generic DNA damage (25). In addition,

206 resistance to induced DNA damage (SB) was measured in lymphocytes subjected to increased

207 oxidative insult *ex vivo* by pre-treating lymphocytes with 150 µmol H_2O_2/L for 5 min at 4°C,

208 before the measurement of SBs. The mean (percentage DNA in tail) was calculated from 50

209 cells per gel (each sample in triplicate) and the mean of each dataset was used in the statistical

210 analysis.

211

212 **Plasma total oxidative capacity**

213 Total oxidative capacity (TOC) measures total peroxide levels in plasma, by the reaction of

214 endogenous peroxides with peroxidases, using tetramethylbenzidine (TMB) as the

215 chromogenic substrate (26) with spectrophotometric measurement at 450 nm. To 10 µL of

216 standard (freshly-prepared hydrogen peroxide, 0.1 mmol/L) or samples in an uncoated

217 microtiter plate was added 200 µL of reaction mixture (0.05M phosphate-citrate buffer, pH 5.0;

218 TMB solution (1 mg/mL), and peroxidase (>2500U/mL) in a proportion of 100:10:1. Plates

219 were incubated at room temperature for 15 min, following which 50 µL of stop solution (2M

220 H_2SO_4) was added to all wells and absorbance at 450 nm was measured using a microplate

221 reader (GENIOS Tecan). Hydrogen peroxide standard solutions (range 0 - 1 mmol/L) were

222 freshly prepared before use.

223

224 **Lipid profile and serum C-reactive protein**

225 Plasma total cholesterol, HDL cholesterol and triglycerides were measured on an Instrument

226 Laboratory (ILAB) 600 (Warrington, UK) autoanalyzer using commercial kits (Roche

227 diagnostics, Lewis, UK) according to kit manufacturer's protocols. Plasma LDL cholesterol

228 was calculated using the Friedewald formula (27).

229   C-reactive protein was determined on an ILAB 600 autoanalyser using a *Quantex* CRP

230   Ultra-Sensitive commercial kit (0.4-18.3 µg/dL) in accordance with the manufacturer's

231   instructions.

232

233   **Measurement of inflammatory markers**

234   Intracellular cytokine levels in lymphocyte and monocyte populations and tissue factor (TF)

235   expression were assessed using a whole blood labelling method that utilises flow-cytometry

236   (Fast Immune Cytokine System, BD Biosciences) in accordance with manufacturer's

237   instructions for all participants at all time-points. The method was used to measure intracellular

238   IL-1β, IL-2, IL-6, IL-10, IL-12, interferon (IFN)-γ and tumour necrosis factor (TNF)-α

239   expression in mononuclear cells. Briefly, whole blood was incubated with either

240   lipopolysaccharide or phorbol 12-myristate 13-acetate to activate monocytes and lymphocytes,

241   respectively. Cells were labelled with the appropriate cell surface antibody and cytokine-

242   specific antibody and analysed on a Gallios flow cytometer (Beckman Coulter). The number

243   and percentage of each cell type expressing the cytokine, as well as the mean channel

244   fluorescence was recorded. The cytokine profiles were examined by ratio of TNF-α to IL-10,

245   IL-1β to IL-10, IL-6 to IL-10 and CRP to IL-10, according to Laird *et al*. (29).

246

247   **Extraction of (poly)phenols from urine and blood samples.**

248   Urine samples were defrosted, vortexed, centrifuged at 16,110 $\times$ g for 10 min at 5 °C, and

249   passed through 0.45 µm filter discs prior to the analysis of 50 µL aliquots by UHPLC-HR-MS.

250   The extraction of metabolites from the plasma samples was carried out as described previously

251   (28). Briefly, plasma samples were defrosted, vortexed and 400 µL aliquots mixed with 10 µL

252   of ascorbic acid (10%, v/v), and 980 µL of 1% formic acid in acetonitrile. One µg of rutin was

253   added to the samples as internal standard for plasma extraction efficiency. The samples were

254   then vortexed for 1 min and ultrasonicated for 10 min. After centrifugation at 16,110 g for 15

255   min, supernatants were reduced to dryness in vacuo using a concentrator plus (Eppendorf,

256   Hamburg, Germany) and resuspended in 150 µL of distilled water containing 1% formic acid

257   and 50 µL of methanol, which was then centrifuged at 16,110 g for 10 min. Analysis of 10 µL

258   aliquots of the supernatant was by UHPLC-HR-MS. Recovery of the internal standard was 79

259   ± 16% (n=78).

260

261   **Non-targeted analysis of urine and plasma by UHPLC-HR-MS.**

262   Plasma and urine samples were analysed using a Dionex Ultimate 3000 RP UHPLC system

263   comprising of a UHPLC pump, a PDA detector scanning from 200 to 600 nm, and an

264   autosampler operating at 4 °C (Thermo Scientific). The HPLC conditions were previously

265   described by Corona *et al.,* (20) with some modifications. Briefly, reverse phase separations

266   were carried out using a 100 x 2.1 mm i.d. 1.8 µm Zorbax SB C18 (Agilent) maintained at 25

267   °C and eluted at a flow rate of 0.2 mL/min with a 50 min gradient of 3-70% of 0.1% acidic

268   methanol in 0.1% aqueous formic acid. After passing through the flow cell of the PDA detector

269   the column eluate was directed to an ExactiveTM Orbitrap mass spectrometer fitted with a

270   heated electrospray ionization probe (Thermo Scientific) operating in negative ionization

271   mode. Analyses were based on scanning from 100 to 1000 m/z, with in-source collision-

272   induced dissociation at 25.0 eV. The capillary temperature was 350 °C, the heater temperature

273   was 150 °C, the sheath gas and the auxillary gas flow rate were both 25 and 5 units,

274   respectively, and the sweep gas was 4 and the spray voltage was 3.00 kv. Data acquisition and

275   processing were carried out using Xcalibur 3.0 software.

276   Untargeted analysis of the selected urine and plasma samples was performed using mass

277   spectral data from the orbitrap analysis applied to the Compound Discoverer software (version

278   2, Thermo Fisher Scientific Inc.). The Compound Discover application processes the raw data

279 into workflows that can be defined on the basis of the nature of the experimental setup. In our

280 case, the workflow selected was 'untargeted metabolomics workflow' that includes retention

281 time alignment, component detection, grouping, elemental composition prediction, gap filling,

282 hide chemical background (using blanks), ID using mzCloud and ChemSpider and differential

283 analysis. The parameters were adjusted to our experimental conditions. Samples were grouped

284 and labelled according to our experimental design, either before or after supplementation of

285 seaweed capsule, the output, as peak areas for the detected peaks was used to develop a

286 multivariate data analysis by Orthogonal Partial Least Square Discriminant Analysis (OPLS-

287 DA). Analysis of seaweed phenolics in blood and urine was also undertaken by HPLC-DAD

288 analysis (**Supplemental Tables 1 & 2**) in a manner consistent with previous studies (15, 20).

289

290 **Power calculations and statistical analyses**

291   Power calculations were performed for the primary endpoint of the change in DNA damage

292 in peripheral blood mononuclear cells. Based on data from a previous study (24), 72.6

293 participants were needed to detect a 25% change in DNA damage in lymphocytes (α 0.05).

294   All values are expressed as mean $\pm$ SD, unless otherwise specified. The mean values are

295 reported for all participants (n=78) during both treatment phases (SPE, Maltodextrin).

296 Significant associations between several outcomes and confounders including BMI and gender

297 were identified at baseline using bivariate correlations or independent t-tests, as appropriate

298 (TOC & Gender P=0.03, DNA damage & BMI P=0.048, DNA damage & Gender P=0.009).

299 Consequently, data was also analysed by stratification of increasing risk, including overweight

300 participants (n=42) and obese participants (n=36). The subgroup analysis was not preplanned;

301 therefore, are not powered adequately and should be considered exploratory. All biochemical

302 analysis was conducted in duplicates, unless otherwise stated, and the mean values taken as the

303 final result. For all markers, the results are presented as treatment effects. This was undertaken

304 by calculating individual differences between pre– and post– values for both control and

305 treatment phases for each subject. Paired T-tests were then carried out on the difference scores

306 (post-treatment value *minus* pre-treatment values) for both treatment phases (SPE and

307 Maltodextrin). Significance level was set at P<0.05. All statistical analyses were performed

308 using the Statistical Package for the Social Sciences (SPSS) for Windows version 22.0 (SPSS

309 Inc., Chicago, IL, USA).

310

311 **Multivariate Data Analysis**

312 Data were obtained as peak areas from the Compound Discover automatic integration software

313 and consisted of 2194 and 3289 potential metabolites (or features) in urine and plasma samples,

314 respectively. Relative peak areas of the metabolites (normalized by the total urine excretion of

315 each subject) obtained by UPLC-HRMS were imported into MATLAB R2015b (Mathworks,

316 USA). PLS toolbox v.8.5 (Eigenvector, USA) and homemade scripts were used. Principal

317 component analysis (PCA) and hierarchical cluster analysis (HCA) are the most widely used

318 tools to explore similarities and patterns among samples where data grouping are unclear.

319 Moreover, the orthogonal partial least squares discriminate analysis (OPLS-DA) method was

320 performed as a typical supervised multivariate methodology used in metabolomics studies (30,

321 31). Several data pre-processing transformations were performed and evaluated, such as

322 Probabilistic Quote Normalization (PQN), Log transformation, mean centering, pareto scaling

323 and auto-scaling. In our case, PQN and autoscaling were selected as pre-processing techniques

324 to reach the lowest root mean square error (RMSE) in an iterative process. A re-sampling

325 method, cross-validation k-fold cross validation (k=5) was also used to evaluate the number of

326 latent variables (according to the lowest RMSE) and the prediction ability of our models. To

327 reduce the impact of the random split of CV-participants, the mean values of the estimated

328 results were obtained after 20 random 5-fold CV. Urine models provided successful

329   classification results at the cross validation step and achieved good prediction parameters that

330   can be explained by the area under the curve values (1.00 and 0.895). Based on the plasma data

331   set and classes selected a discriminant model could not be developed. Moreover, the Variable

332   Importance in Projection (VIP) is the widely known metric that is used to identify potential

333   markers in metabolomics studies (32). VIP is a weighted sum of squares of the PLS weight

334   which indicates the importance of the variable to the whole model. The cut off VIP value

335   selected in our study was 2. The potential markers were extracted from the two models to

336   compare the results obtained.

337

338   **RESULTS**

339   **Baseline characteristics**

340   Eighty participants (males n=39, females n=41) were enrolled on this 24-week randomised,

341   double-blind, placebo-controlled crossover trial. The intervention was conducted as per the

342   protocol and there were no adverse events associated with the intervention. The study

343   population had a mean group age of 42.7±7.1 years and a mean BMI of 30.2±3.9 kg/m^2. The

344   study had an overall compliance of 97% with 78 participants completing the 24-week study;

345   two participants withdrew from the study at the midpoint for personal reasons and compliance

346   was not significantly different by treatment group or time period (P>0.05) (**Figure 1**). There

347   were no significant differences between the participants in age and physical characteristics at

348   the beginning of either treatment phase (**Table 2**).

349

350   **Habitual dietary intake**

351   Dietary analysis of habitual intake (midpoint) during both treatment phases (SPE,

352   Maltodextrin) of the crossover trial is described in **Table 3**. There were no significant

353   differences between treatment phases for any of the macronutrients or micronutrients analysed

354   indicating that the seaweed phenolic extract did not affect the habitual food consumption

355   patterns in the study population.

356

357   **Effects of seaweed (poly)phenol extract on DNA damage**

358   Basal levels of DNA damage observed in the study were consistent with previous studies

359   with a mean group average of $6.72 \pm 2.48\%$ tail DNA (data not shown). In response to an

360   oxidative challenge with 150 µM H_2O_2, DNA damage was increased to an average of

361   approximately $34 \pm 7\%$ tail DNA in both the placebo and SPE phase in all participants (**Table**

362   **4**). The 8-week intervention with a seaweed phenolic extract resulted in a significant reduction

363   in basal DNA damage, as measured by the Comet assay, in obese participants only (BMI >30

364   kg/m^2), with a significant reduction (P=0.044) in basal DNA damage observed (**Table 4**). A

365   significant reduction was not observed in the total population (n=78) nor in participants

366   classified as overweight (n=42), either in terms of a challenge response or in basal levels. In

367   addition, we noted that consumption of seaweed phenolic extract also significantly reduced

368   (P=0.009) basal DNA damage in males only with a mean group change value of -0.8 ± 2.5 %

369   tail DNA (SPE) compared to $0.9 \pm 2.8\%$ tail DNA in the control. No significant effects were

370   observed for females.

371

372   **Total oxidative capacity of seaweed phenolics**

373   Total oxidative capacity (peroxide levels) in plasma samples from all participants (n=78),

374   in overweight participants (n=42) and in obese participants (n=36) were measured after the

375   placebo and seaweed capsule intervention (**Figure 2**). There were no significant changes from

376   baseline after either treatment phase (SPE, Maltodextrin) in all participants (n=78) nor in

377   overweight (n=42) or obese (n=36) sub groups. However consumption of seaweed phenolic

378   extract also significantly reduced (P=0.018) TOC in females only with a mean group change

379   value of -7.44 ± 29.37 µM peroxides (SPE) compared to 4.33 ± 22.36 µM peroxides in the

380   control. No significant effects were observed for males.

381

Effects of seaweed (poly)phenol extract on blood lipids and CRP

383   Analysis of Pre- and post- values, and the percentage change, for both the placebo phase

384   and the SPE phase for each blood lipid biomarker and CRP indicated that the 8-week

385   supplementation with seaweed (poly)phenol extract did not significantly affect any

386   cardiovascular risk marker **(Table 4).**

387

Effects of seaweed (poly)phenol extract on inflammatory markers

389   No significant differences were measured in any of the inflammatory cytokines in either

390   treatment phase (SPE, Maltodextrin) for all study participants (n=78) (**Figure 4**) and in addition

391   no significant effects on the cytokine profiles were observed.

392

Bioavailability of seaweed phenolics, untargeted analysis

394   Initially an unsupervised PCA model was carried out (**Figure 4A**) on the urine data from all

395   78 participants. The first component of the PC1 vs PC2 scores plot obtained from the data set

396   explained 23.14% of the total variance showing a clear trend among some of the participants

397   compared to the rest. Moreover, a hierarchical cluster analysis was performed to verify this

398   natural aggrupation pattern among the 78 participants suggesting, two distinct clusters among

399   all the participants (**Figure 4B**), accounting for the inter-individual differences associated with

400   variation in the urinary metabolite profiles. Recent studies suggest that individuals can be

401   clustered into distinct groups based on their gut microbiome composition, functional

402   metabolism (33) or individual responses to obtain markers of a specific treatment, minimizing

403   the inter-individual data that are not the target of the study. In order to elucidate the observed

404   variability between these two groups, two supervised models were performed, one with the

405   urinary profiles from all the participants before the seaweed consumption and another one with

406   the urinary profiles from all the participants after seaweed consumption. Both supervised

407   models verified differences between the two groups of individuals (Group 1 and Group 2) and

408   provided successful classification results at the cross validation step and achieved in the pre-

409   treatment and post-treatment samples 95% and 98% sensitivity (participants of the class of

410   interest correctly assigned to their class) and 100% and 100% specificity (participants not

411   belonging to the class of interest were correctly not assigned to that class), respectively

412   (**Supplemental Figure 3**). It is noteworthy that even stratifying for BMI category, the

413   multivariate analysis of the urine profiles did not show clear differentiation of the metabolome

414   between obese or overweight individuals.

415   By using the preliminary information provided by PCA and HCA related to the stratification

416   of the individuals into groups which share a common excretion profile before and after

417   supplementation with seaweed capsules, two data sets were analysed. The individuals were

418   stratified into two groups; Group 1 including 70 participants; and Group 2 including 8

419   participants (S58, S60, S61, S71, S76, S78, S79, S83, all being overweight individuals). Two

420   supervised OPLS-DA models were built to discriminate according to the seaweed treatment in

421   both models (**Figure 5**). In our study we used two latent variables (LV) and the sensitivity and

422   specificity values were set to 100% at the calibration step recognition ability of the two models.

423   These analyses showed that the human urine metabolome in both groups of participants was

424   modified after the 8-week supplementation with seaweed (poly)phenol extract compared to

425   baseline urine metabolic profiles. Using the loadings plot and the variable importance in

426   projection values (VIP) we ascertained important contributors between the modelled classes

427   and therefore identified the compounds responsible for the difference in the urine metabolic

428   profile before and after 8-weeks seaweed (poly)phenol extract consumption in both groups.

429   VIP is the widely known metric that is used to identify potential markers (metabolites) in

430   metabolomics studies. Further, the urine scores plot allowed the identification of those

431   metabolites which appeared after the seaweed (poly)phenol extract intake (**Figure 5**) in both

432   groups of participants.

433

434   The main contributors to metabolome differentiation in the urine before and after seaweed

435   consumption in both groups of participants are described in **Supplemental Tables 3 & 4**.

436   Positive loading values (x axis) and higher VIP values (VIP>2.0) were detected in urine after

437   seaweed consumption and were the responsible of the observed differences between the

438   participants classes (post ingestion vs non ingestion of seaweed (poly)phenol extract). Urine

439   metabolites tentatively identified as pyrogallol/phloroglucinol sulfate, hydroxytrifurahol A-

440   glucuronide and dioxinodehydroeckol glucuronide are considered clear biomarkers of seaweed

441   consumption in Group 1 while C-O-C dimer of phloroglucinol-sulfate, C-O-C-dimer of

442   phloroglucinol, fucophloroethol-glucuronide, diphlorethol sulfates and dioxinodehydroeckol

443   glucuronide are those biomarkers of seaweed consumption in Group 2. All of them have shown

444   high values in the variable importance in the projection (values > 2) (**Table 5).**

445   Based upon the identified seaweed (poly)phenol metabolites, the total amount of seaweed

446   metabolites excreted in urine varied noticeably between participant ranging from 0.001 to

447   4.140 m moles, with a group average of 1.29 +/- 0.88 m moles. Some 25% of the population

448   (19/78) appeared to be low excretors, with urinary seaweed metabolites excretion less than 0.5

449   m moles, while 55% of the population (43/78) were medium excretors (0.5 and 2 m moles) and

450   the remaining 20% of the population (16/78) were high excretors (>2 m moles) (**Supplemental**

451   **Table 5**). Despite exploration using PCA or HCA, the plasma metabolome did not allow a clear

452   differentiation between participants in either treatment phase (data not shown). As a result, it

453 was not possible to define exposure biomarkers in plasma samples across all the participants

454 (n=78).

455

456 **DISCUSSION**

457 The association of seaweed consumption with reduced CVD risk factor has been tested

458 largely within *in vitro* or animal models, with only limited human data allowing substantiation

459 of the proposed beneficial properties of seaweeds (12, 13, 34). The work reported in this paper

460 represents the first comprehensive study of the *in vivo* bioactivity and bioavailability of

461 seaweed (poly)phenolics on biomarkers of inflammation and oxidative stress in overweight

462 and obese individuals. Participant retention rates were high (98%) and self-reported SPE

463 capsule intake (97%) indicated that the intervention was implemented successfully in this

464 group of participants. There was, however, no significant difference between the intervention

465 (SPE capsule) and control (placebo capsule) phases for markers of oxidative stress, antioxidant

466 status or inflammation in the population as a whole (n=78). Stratifying for BMI however

467 revealed a significant albeit modest decrease in DNA damage in the obese population (n=36).

468 This is consistent with Park *et al*. (35) who tested the natural microalgal carotenoid astaxanthin,

469 in 14 healthy females (2 mg extract/d for 8 weeks) and showed a significant reduction in the

470 oxidative damage plasma marker 8-hydroxy-2'-deoxyguanosine. Obese individuals are

471 associated with increased basal DNA damage, higher oxidant status and increased oxidative

472 damage to macromolecules as a group and are thus at higher risk of chronic disease (36-39). In

473 the current work, an overweight/obese population (age 30-65 years, BMI >25 kg/m^2)

474 supplementation with seaweed (poly)phenolics significantly reduced basal levels of

475 lymphocyte DNA damage (by 23%) but only in the obese sub-population (n=36). These two

476 seaweed-extract interventions are consistent with data from *in vitro* studies which demonstrated

477 anti-genotoxic activity for seaweed extracts on a range of cell lines (40,41), including the SPE

478 extract used in our study (15). The biological mechanism underpinning the observed reduction

479 in DNA damage in the obese group could not be determined from our data, however the

480 activation of the cytoprotective Nrf2/ARE pathway may be involved, as seaweed (poly)phenols

481 such as eckol are anti-genotoxic (42) and can activate Nrf2-mediated HO-1 induction (43,44)

482 consistent with effects observed for (poly)phenols from terrestrial sources (45,46). We

483 observed a 28% decrease in CRP levels, as a marker of inflammation, in response to seaweed

484 extract consumption however this change was not significant, in contrast to the observations

485 of Park *et al*. (35). However, a study investigating the effect of consuming *Palmaria palmata*

486 (5 g/day) incorporated into bread found that it significantly increased CRP by 16%, suggesting

487 that *P. palmata* stimulates inflammation rather than reducing it (47). Park *et al*. (35) reported

488 that there was no difference in TNF and IL-2 concentrations, but plasma IFN-γand IL-6

489 increased on week 8 in participants given 8mg astaxanthin. Within our study, we similarly

490 observed no significant changes in IL-2 or TNF, nor did we see an alteration in IFN-γor IL-6.

491 The seaweed extract tested did not affect immune function (cell mediated and humoral immune

492 responses as tested by the cytokine markers) following supplementation.rather than reducing it

493 (47). Park *et al*. (35) reported that there was no difference in TNF and IL-2 concentrations, but

494 plasma IFN-γand IL-6 increased on week 8 in participants given 8mg astaxanthin. Within this

495 study, we similarly observed no significant changes in IL-2 or TNF, nor did we see an alteration

496 in IFN-γor IL-6. The seaweed extract tested did not affect immune function (cell mediated and

497 humoral immune responses as tested by the cytokine markers) following supplementation.

498

499 A comparison of metabolic profiles between urine and plasma samples of 78 individuals who

500 consumed seaweed capsules has allowed evaluation of seaweed molecules potentially

501 responsible for the modest beneficial effects observed *in vivo* as well as identifying biological

502 markers linked to seaweed consumption. We determined differences in the urine metabolite

503   profiles between participants, and thus were able to stratify the individuals into groups that

504   shared common excretion metabolite profiles both pre- and post- seaweed supplementation.

505   Despite substantial inter-individual variation in the concentration of seaweed metabolites

506   excreted in urine (0.001 to 4.140 m moles, the urinary profiles of these two groups were

507   statistically different and we have been able to select the metabolites responsible for this

508   discrimination as potential biomarkers of seaweed consumption (**Table 5**). Our comprehensive

509   multivariate analysis of the metabolite profiles showed the expected person-to-person variation

510   in the 0-24h urinary excretion of seaweed (poly)phenols, which could be attributed to

511   differences in gut microbiota or living conditions amongst study participants. Nonetheless, we

512   could group the 78 participants by ability to metabolize seaweed (poly)phenols. Group 1

513   individuals were characterized by greater excretion of seaweed–derived metabolites such as

514   pyrogallol/phloroglucinol sulfate, hydroxytrifurahol A-sulfate and dioxinodehydroeckol, while

515   Group 2 individuals were characterized by excretion of C-O-C dimer of phloroglucinol-sulfate,

516   C-O-C dimer of phloroglucinol, fucophloroethol-glucuronide, diphlorethol sulfates and

517   dioxinodehydroeckol glucuronide. This inter-individual variation in absorption of seaweed

518   polyphenols is consistent with profiles previously observed in the bioavailability of dietary

519   (poly)phenols such as orange, cranberry and pomegranate juices (48-50).

520

521   The seaweed phenolic metabolites and can be divided into (a) phase II sulphated and

522   glucuronidated metabolites related to the targeted components described earlier by Corona *et*

523   *al.* (20) (**Supplemental Tables 1 & 2**); arguably formed in the liver, and (b) an extended list

524   of unknown compounds which could be potential breakdown products or metabolites of the

525   original seaweed (poly)phenols catabolised by colonic bacteria. However, further investigation

526   would be required to confirm the identity and origin of these unknown compounds.   We

527   previously reported the poor absorption of the high molecular weight phlorotannins in the upper

528   gastrointestinal tract (20) likely results in them reaching the colon and becoming subject to

529   microbial fermentation to lower molecular weight derivatives, as we have recently shown using

530   an in vitro gut microbiota model (15). The urinary metabolite profiles of seaweed phenolics

531   from the low and high excretors clearly indicated a high inter-individual variation in

532   metabolism. It is possible that inter-individual variation in gut microbiota underlie these

533   metabolic changes and were responsible for the observed differences (51-53). The study of

534   seaweed polyphenol(s) bioavailability remains challenging due to the high range of molecular

535   weight compounds present, and their characterisation is complicated further by the lack of

536   commercially available standards. Other limitations of the present study include a relatively

537   short-term intervention, and thus results cannot be extrapolated to long-term chronic

538   consumption. In addition, study participants were mostly recruited from the University of

539   Ulster staff and local residents and thus may not be representative of the general overweight

540   population of Northern Ireland. While lymphocyte DNA damage (spontaneous DNA SBs) is

541   considered appropriate for the substantiation of EFSA health claims in the context of protection

542   against generic DNA damage, a lesion specific enzyme such as (ENDO III) would have been

543   required for a claim related to oxidative DNA damage (54). With respect to the TOC assay it

544   is based on the reaction of endogenous peroxides with hydrogen peroxide, using TMB as the

545   chromogenic substrate, and provides a measure of total peroxide levels in plasma. While this

546   assay lacks specificity in comparison to other measures of oxidative stress, the direct

547   correlation between oxygen radicals and peroxides allows measurement and characterization

548   of the oxidative status/oxidative stress in biological fluids. Moreover the test is characterized

549   by linearity, good precision, and endpoint determination (55). Finally, the study population

550   number may have been too small to yield significant results in intercellular cytokines. These

551   factors should be considered for the design of future studies in this area.

552   In conclusion, this work represents the first comprehensive human study investigating

553   bioavailability and metabolism of seaweed (poly)phenolics. Consumption of SPE decreased

554   DNA damage–albeit to a modest extent–in obese individuals only, with no clear effects on

555   clinical markers of inflammation. Untargeted analysis identified novel urinary biomarkers of

556   seaweed consumption and highlighted a high degree of inter-person variation in the metabolism

557   of seaweed phenolics. Future studies that address the ingestion of seaweed phenolics will need

558   to consider and adjust for these parameters, and this work has highlighted the importance of

559   establishing an individual's capacity for metabolising (poly)phenols.

560

575

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Table 1. Key components of phlorotannin rich basic seaweed extract, High Molecular Weight (HMW) seaweed extract fraction, and blend used for intervention seaweed polyphenolic extract capsules (SPE).

Extract component	Basic seaweed extract	HMW seaweed extract fraction	Blend (SPE capsule)
	mg/175mg of extract	mg/50mg of extract	mg/400mg capsule
Phlorotannin	61.25	46.05	107.3
Iodine	0.48	0.02	0.5
Maltodextrin[1]	0	0	175
Minerals	39.38	13	52.38
Fucoxanthin	0	36.5	36.5
Laminarin as glucose	10.68	1.75	12.43
Fucoidan as fucose	0	<0.001	<0.001
Mannitol	29.23	5.9	35.13
Inorganic arsenic	<0.001	<0.001	<0.001
Cadmium (LD 0.15mg/kg)	<LD	<LD	<LD
Mercury (LD 0.016mg/kg)	<LD	<LD	<LD
Lead (LD 1.1mg/kg)	<LD	<LD	<0.001
Tin (LD 1.7mg/kg)	<LD	<LD	<LD

[1]maltodextrin was added to the capsule formulation as an excipient.

Table 2. Baseline characteristics of the study population (n=80).

Variable	Placebo treatment phase (n=80)	SPE treatment phase (n=80)
Age (y)	42.8 ± 7.2	42.9 ± 7.1
Gender (M/W)	20/20	19/21
Height (m)	1.71 ± 0.08	1.72 ± 0.10
Weight (kg)	88.9 ± 14.1	89.1 ± 17.3
BMI (kg/m^2)	30.3 ± 3.5	30.0 ± 4.4

No significant differences P>0.05, Paired T Test.

Table 3. Habitual nutrient intake of subjects during intervention study

Variable intake	Placebo treatment phase (n=77)[1]	SPE treatment phase (n=77)	% Change[2]	P value[3]
Energy (kcal/d)	1949 ± 590[4]	2057 ± 684	5	0.110
Protein (g/d)	79.4 ± 28.6	79.9 ± 25.3	0.5	0.782
Carbohydrate (g/d)	220.6 ± 72.1	231.3 ± 84.2	5	0.278
Total fat (g/d)	78.6 ± 26.5	85.2 ± 34.8	8	0.058
Saturated fat (g/d)	29.0 ± 10.2	30.7 ± 15.3	6	0.417
Monounsaturated fat (g/d)	24.4 ± 9.3	26.8 ± 12.4	9	0.112
Polyunsaturated fat (g/d)	12.1 ± 5.4	13.5 ± 6.4	10	0.059
Fibre (g/d)[5]	12.9 ± 6.2	13.3 ± 4.8	3	0.259
Vitamin C (mg/d)	71.8 ± 50.5	69.4 ± 47.1	-3	0.909
Vitamin E (mg/d)	7.17 ± 3.7	7.67 ± 4.0	7	0.279
Folate (µg/d)	223.9 ± 98.3	228.2 ± 85.5	2	0.370
Carotene (µg/d)	2821 ± 1900	2553 ± 1706	-11	0.185

[1]S66 did not complete a food diary in either phase. [2] Calculated from SPE phase – Placebo phase. [3] Mean treatment group values were not significantly different between phases, $P<0.05$ (Wilcoxin signed rank test). [4] Mean ± SD. [5]Calculated using the Englyst method.

Table 4. Effects of seaweed polyphenol extract on lymphocyte DNA damage, CRP and blood lipids.

	Total (n=78)				Overweight (n=42)				Obese (n=36)			
Blood marker	Average baseline value	Placebo treatment effect	SPE treatment effect	P value	Average baseline value	Placebo treatment effect	SPE treatment effect	P value	Average baseline value	Placebo treatment effect	SPE treatment effect	P value
DNA damage – basal (% Tail)	6.72 ± 2.48	0.74 ± 2.86	-0.41 ± 3.13	0.350	6.59 ± 2.80	0.32 ± 2.14	0.57 ± 3.24	0.129	6.91 ± 2.00	1.81 ± 4.50	0.15 ± 2.93	**0.044**
DNA damage - H_2O_2 (%Tail)	34.2 ± 7.00	-1.56 ± 6.60	-2.03 ± 6.40	0.390	35.2 ± 7.11	0.13 ± 2.19	0.76 ± 1.66	0.062	32.8 ± 6.69	1.26 ± 2.09	0.54 ± 1.54	0.111
CRP (mg/ml)	2.67 ± 3.9	0.01 ± 3.3	-0.83 ± 4.9	0.429	2.59 ± 4.69	0.00 ± 3.40	-1.32 ± 6.22	0.348	2.80 ± 2.56	-1.81 ± 11.82	0.72 ± 5.15	0.258
Cholesterol (mmol/l)	5.20 ± 0.77	-0.06 ± 0.57	-0.10 ± 0.57	0.256	5.18 ± 0.82	0.01 ± 0.55	-0.53 ± 0.54	0.201	5.24 ± 0.73	-0.13 ± 0.58	-0.15 ± 0.60	0.419
Triglycerides (mmol/l)	1.51 ± 0.94	0.01 ± 0.82	0.04 ± 0.96	0.385	1.34 ± 0.72	0.07 ± 0.79	-0.04 ± 0.48	0.278	1.75 ± 1.15	-0.06 ± 0.85	-0.02 ± 1.34	0.497
HDL (mmol/l)	1.37 ± 0.32	-0.01 ± 0.15	0.03 ± 0.15	0.187	1.46 ± 0.31	0.01 ± 0.16	-0.03 ± 0.15	0.150	1.25 ± 0.32	-0.04 ± 0.11	-0.04 ± 0.14	0.446
LDL (mmol/l)	3.16 ± 0.1	-0.08 ± 0.5	-0.06 ± 0.50	0.478	3.13 ± 0.69	-0.07 ± 0.57	0.01 ± 0.45	0.383	3.20 ± 0.67	-0.09 ± 0.56	-0.13 ± 0.56	0.412

Data is presented as treatment affects, calculated based on 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 (Seaweed phenolic extract capsule) and placebo control phase (maltodextrin). Significance level was set at P<0.05 (one-tailed T test).

Table 5. Phlorotannins metabolites tentatively identified in human urine samples from Group 1 (70 subjects) and Group 2 (8 subjects) after seaweed capsule consumption.

ID	VIP value	Rt (min)	Experimental Molecular Weight	Predicted Formula	Metabolite putative identification	Ratio[1]
Group 1 (72 subjects)						
652	2.06	25.6	205.9881	C6H6O6S	Pyrogallol/phloroglucinol sulfate	1.5
800	2.28	25.8	205.9881	C6H6O6S	Pyrogallol/phloroglucinol sulfate	1.2
1472	3.86	24.9	205.9881	C6H6O6S	Pyrogallol/phloroglucinol sulfate	1.1
1352	4.26	25.6	486.1727	C22H30O12	Hydroxytrifuhaol A-glucuronide	2.0
1453	3.83	25.4	486.1727	C22H30O12	Hydroxytrifuhaol A-glucuronide	1.9
1458	4.23	25.3	486.1727	C22H30O12	Hydroxytrifuhaol A-glucuronide	2.2
1483	4.39	25.3	486.1727	C22H30O12	Hydroxytrifuhaol A-glucuronide	2.2
1917	2.11	31.4	544.2881	C27H44O11	Dioxinodehydroeckol glucuronide	1.1
Group 2 (8 subjects)						
293	2.08	9.5	327.0951	C12H8O9S	C-O-C dimer of phloroglucinol-sulfate	1.2
702	2.20	24.1	248.0315	C12H8O6	C-O-C dimer of phloroglucinol	1.2
853	2.08	27.8	797.3186	C45H49O13	Fucophloroethol glucuronide	1.1
1293	2.28	34.2	330.1675	C12H10O9S	Diphlorethol sulfate	1.9
1356	2.31	34.1	330.1675	C12H10O9S	Diphlorethol sulfate	1.7
1633	2.07	35.1	544.2152	C27H44O11	Dioxinodehydroeckol glucuronide	1.2

ID, identification number. VIP (variable influence in projection) is a variable that summarizes the importance of X variables to the OPLS-DA model. Variables with values > 2 were the most influential in the model. All predicted formula derived with < 5 ppm mass accuracy data.

[1]Ratio: seaweed capsule consumption/placebo consumption.

FIGURE 1. CONSORT DIAGRAM. Progress of participants through the intervention study.

FIGURE 2. Comparison of the effects of SPE supplementation phase (☐) with that of a placebo phase (•) on total oxidative capacity (TOC) in the total study population (n=78), in overweight (n=42), and in obese (n=36). All values were measured in duplicate expressed as mean ± SD. TOC is represented as the mean of the individual difference values (after – before supplementation) in the SPE and placebo treatment phases.

FIGURE 3. Comparison of the effects of SPE supplementation phase (■) with that of a placebo phase (☐) on cytokine levels in the total study population (n=78), in overweight (n=42), and in obese (n=36). All values were measured in duplicate expressed as mean ± SD. Cytokines were measured as the mean of the individual difference value (after – before supplementation) in the SPE and placebo treatment phases. Change in mean treatment group values were not significantly different between treatment phases, P>0.05 (Paired T Test; one-tailed test). M.F.I.; Mean Fluorescence Intensity.

FIGURE 4. Principal Component Analysis (PCA) (A) and Hierachical Cluster Analysis Cluster (HCA) (B) of urinary profiles before (♦) and after (■) seaweed consumption by 80 participants. The HCA was calculated based on Eucledian distances and the Ward hierachical agglomerative method. The PC explained 23.14% of the total variance (PC-1 14.8% and PC-2 8.34 %).

FIGURE 5 A) OPLS-DA scores and B) loadings of the urine samples belong to group of participants 1 (70 participants). C) OPLS-DA scores and D) loading of urine samples belong to group of participants 2 (8 participants) before (♦) and after (■) seaweed ingestion. (Circles shown in the graph represent a confidence of 95%). LV1: latent variable 1; LV 2: latent variable 2. The cut off VIP value selected to be 2. For VIP scores identification see **Table 5** and Supplemental **Table 1**.
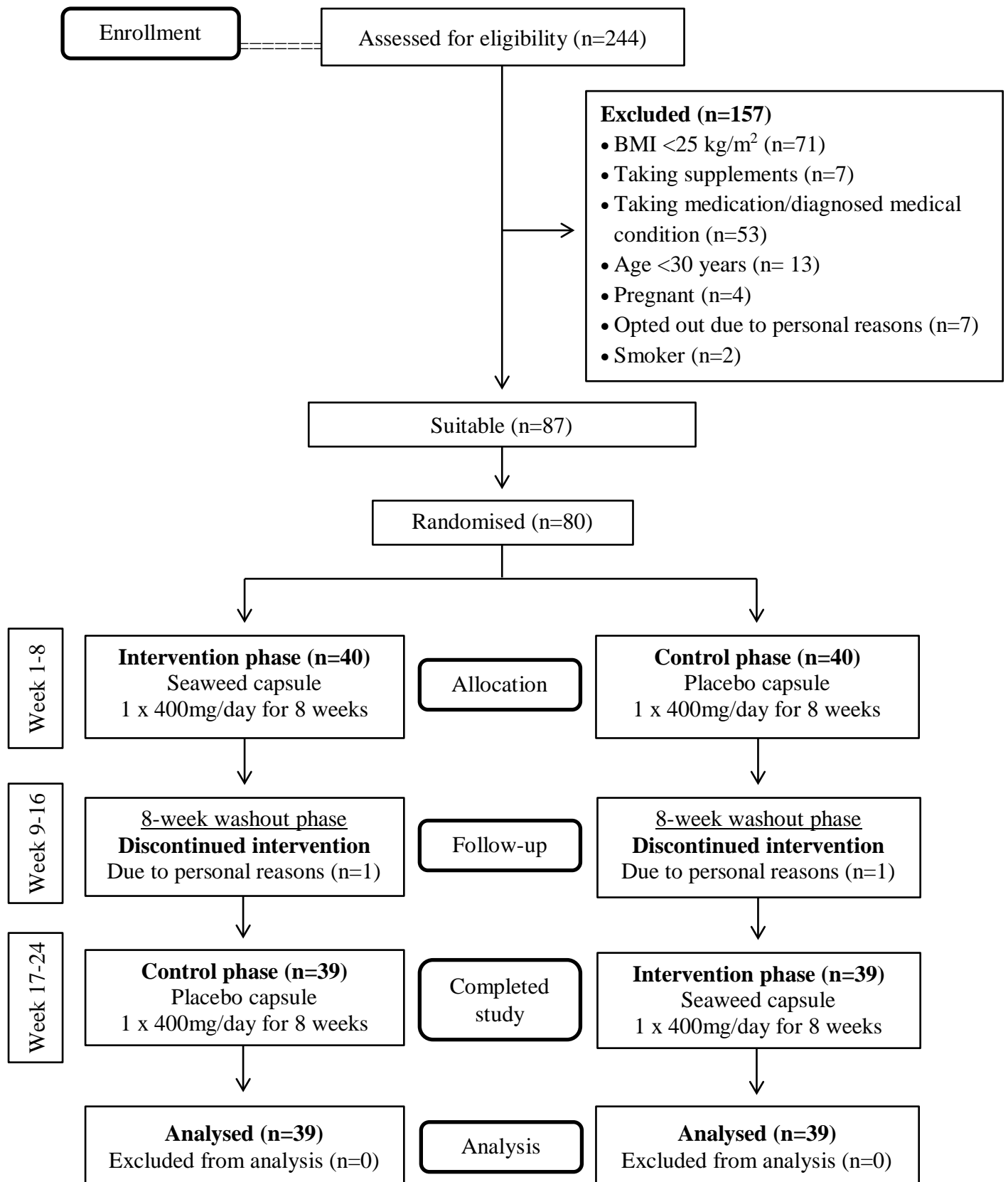
FIGURE 1.
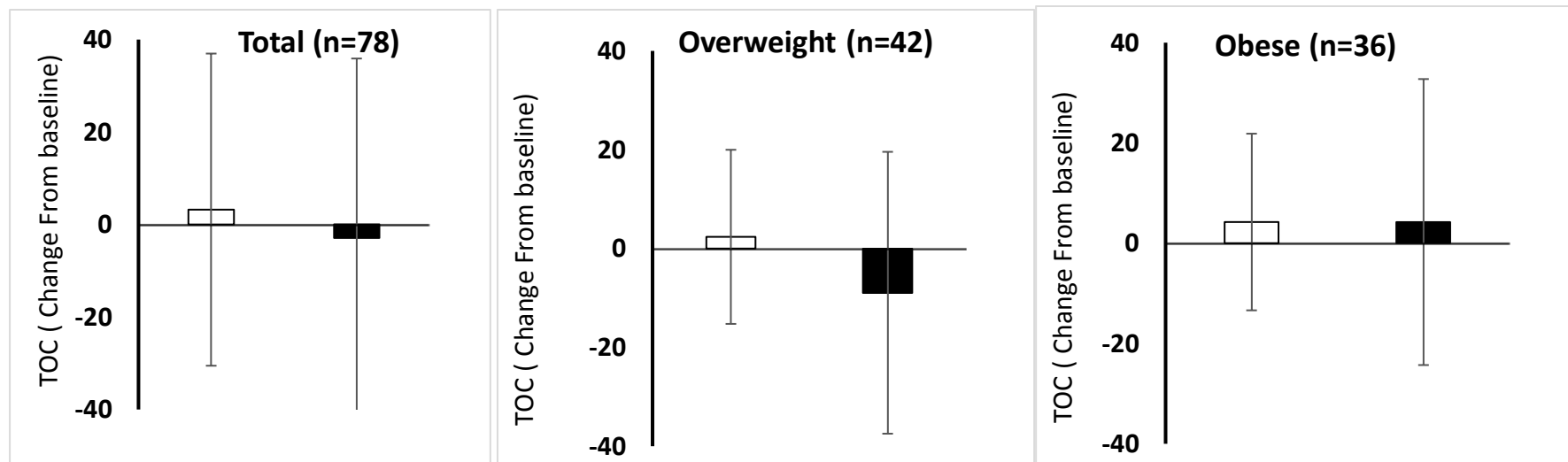
FIGURE 2.
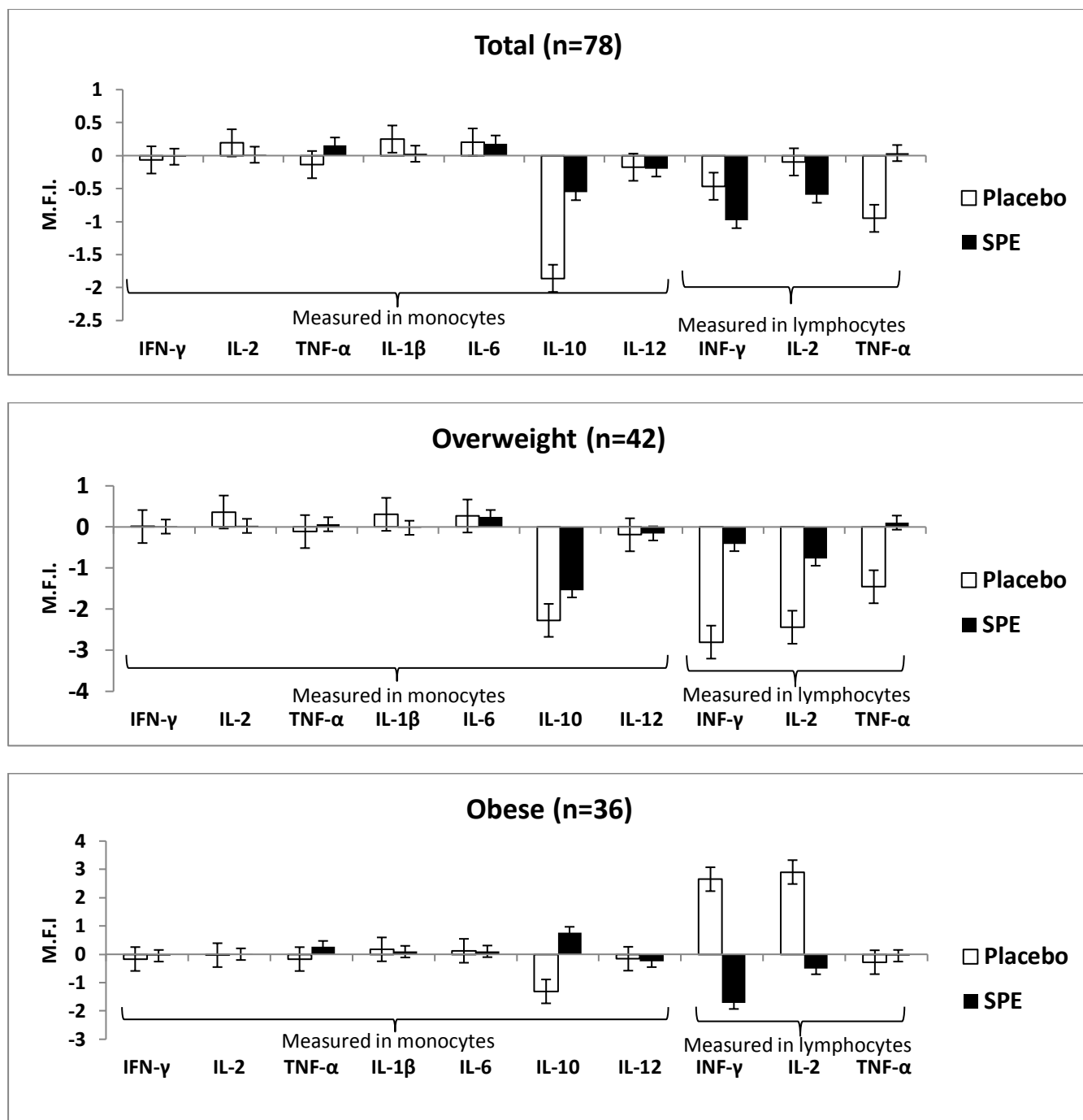
FIGURE 3.
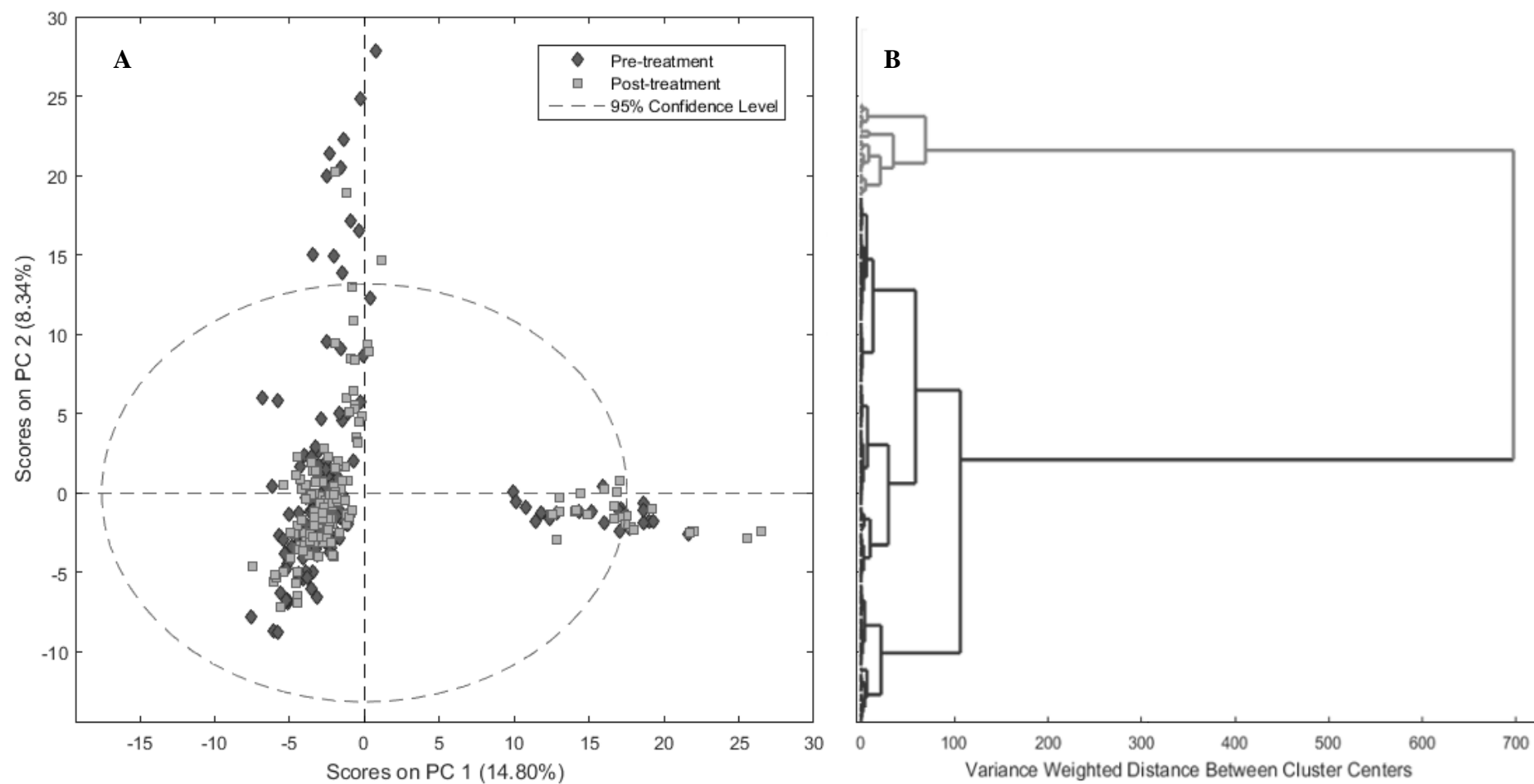
FIGURE 4.

Supplemental Information

Impact of a polyphenol-rich extract from *Ascophyllum nodosum* on DNA damage and antioxidant activity in an overweight/obese population

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Analysis of the SPE capsule by NP-HPLC and LC-MS analysis (as reported in Corona et al Br J Nutr. 2016 Apr 14;115(7):1240-53.)

NP-HPLC analysis: The phlorotannins in the food-grade SPE used to produce the capsule were analyzed by normal phase HPLC analysis as previously described (Corona G et al, BJN, 2016) 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.

LC-MS analysis: LC-MS analysis was conducted to analyse the food grade seaweed capsule as previously described (Corona G et al, BJN, 2016), and was carried out in the negative ion mode using LC-MS/MS utilizing electrospray ionisation (ESI). Characterization was achieved using 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) and HPLC conditions were as follows: injection volume: 1 μL; column temperature: 25 °C; binary mobile system: (A) 0.1 % aqueous formic acid and (B) 0.1 % of formic acid in acetonitrile; flow rate: 0.2 mL/min. A series of linear gradients were used for separation (min/%B): 0/10, 3/10, 15/40, 40/70, 50/70, 65/10. Mass spectrometry was performed in the negative ion mode (scan range, m/z 100-1000 Da; source temperature, 350 °C). All solvents used were LC-MS grade.

The SPE (supplemental figure 1) comprised a wide range of molecular weights (20-70 min), with abundance of very high molecular weight phlorotannins eluting at later retention time (50-70min) in our Normal-Phase method. Using a RP-HPLC separation method coupled to ESI-MS analysis in negative ion mode (supplemental figure 3) we were able to identify some phlorotannin oligomers such as hydroxytrifuhalol A, tetrafucol, fucodiphlorethol, C-O-C dimmer of phloroglucinol, 7-hydroxyeckol, diphloretol and difucol.
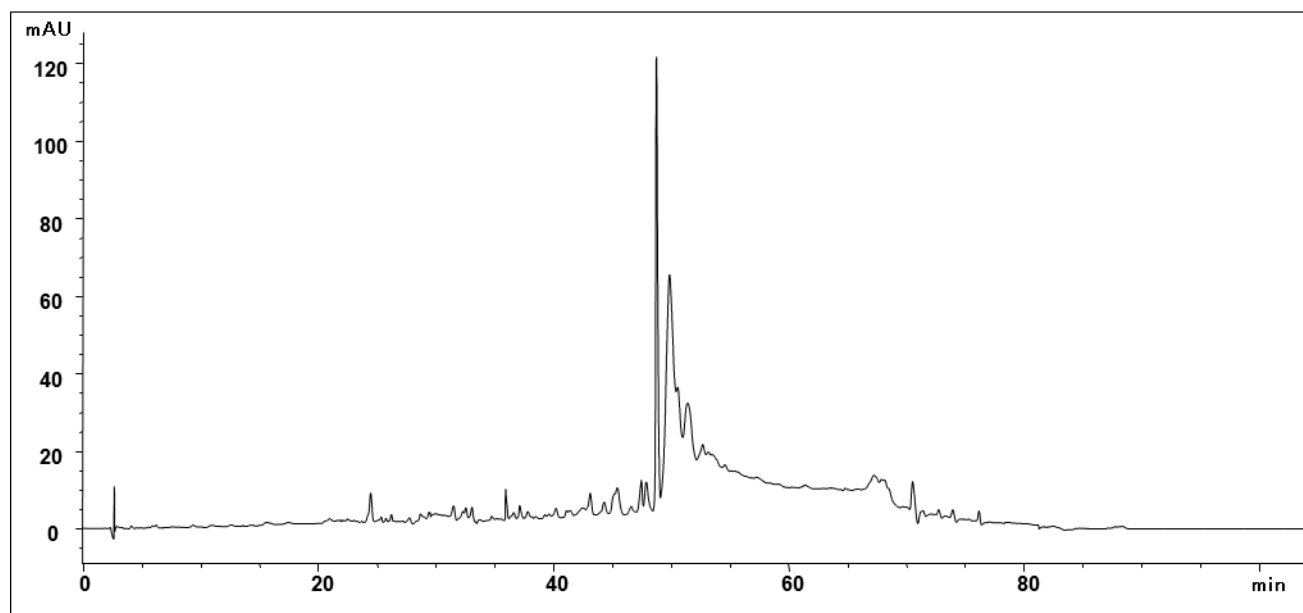
Supplemental Figure 1 . Chromatographic separation of phlorotannins contained in the seaweed extract by Normal-Phase HPLC with diode array detection (268nm).
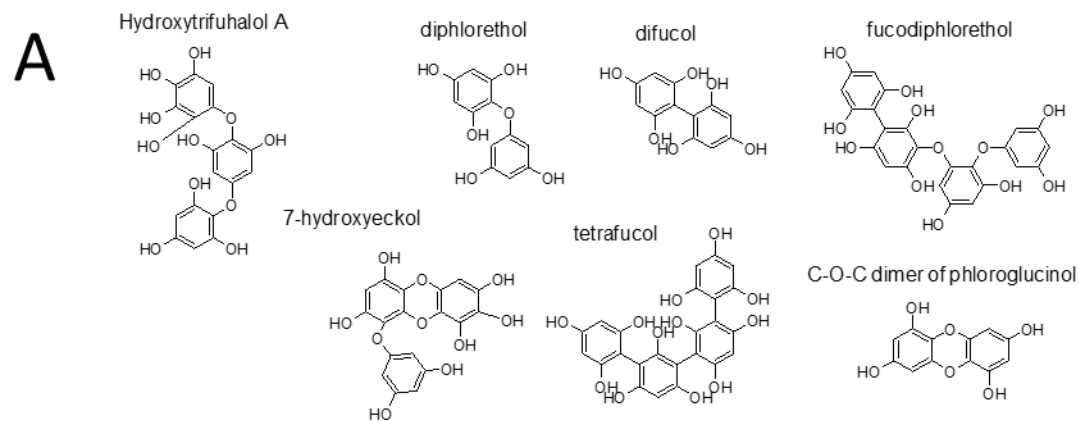
LC-MS analysis in negative ion mode of the seaweed extract phlorotannins

Peak N	RT (min)	[M-H]⁻ m/z		MS² m/z		tentative identification
1	2.7	405	387	191		hydroxytrifuhalol A
2	3.3	497	479	353	205	tetrafucol, fucodiphlorethol
3	5.6	247	203			C-O-C dimer of phloroglucinol
4	10.6	387	369	230		7-hydroxyeckol
5	43.7	249	181	113		diphlorethol / difucol
6	46.2	249	181	113		diphlorethol / difucol

Supplemental figure 2. Characterisation of phlorotannins in the seaweed extract. A: Structures of phlorotannins identified in the seaweed extract: B: Phlorotannins in the seaweed extracts identified by LC-MS analysis in negative ion mode

Extraction /Analysis of Plasma and Urine samples by HPLC DAD.

The HPLC-PDA described below has been removed from the original data due to the limitation of the HPLC DAD approach applied and replaced by analysis as decribed in paper. The data is now only available in supplemental information for context with existing papers on this topic using the same seaweed extract (SPE) .

1) Corona G, Ji Y, Anegboonlap P, Hotchkiss S, Gill C, Yaqoob P, Spencer JP, Rowland I.Gastrointestinal modifications and bioavailability of brown seaweed phlorotannins and effects on inflammatory markers. Br J Nutr. 2016 Apr 14;115(7):1240-53

2).Corona G, Coman MM, Guo Y, Hotchkiss S, Gill C, Yaqoob P, Spencer JP, Rowland I.Effect of simulated gastrointestinal digestion and fermentation on polyphenolic content and bioactivity of brown seaweed phlorotannin-rich extracts. Mol Nutr Food Res. 2017 Nov;61(11). doi: 10.1002/mnfr.201700223. Epub 2017 Aug 29

Extraction of (poly)phenols from urine and blood samples for HPLC DAD

Urine samples were prepared as follows: 10 µl of internal standard solution (resorcinol 200 µg/ml) were added to 250 µl urine. Samples were analysed with and without enzymatic treatment (37°C, 40 min), in the presence of 1500 IU of β-glucuronidase and 50 IU of sulfatases from *Helix pomatia* (Type H-1). 1 ml of methanol acidified with 0.5% acetic acid was added, samples were mixed and centrifuged for 15 min at 16,100 x g at 4°C, supernatants were transferred

to a new tube and dried on a speedvac. Dried samples were resuspended in 125 µl of mobile phase, completely dissolved, centrifuged and transferred to vials for HPLC-DAD.

Plasma samples were prepared as follows: 10 µl of internal standard solution (resorcinol 200 µg/ml) were added to 450 µl of plasma, then 50 µl of 1.2 M acetic acid were added and samples were mixed. Samples were analysed with and without enzymatic treatment (37°C, 40 min) in the presence of 1500 IU of β-glucuronidase and 50 IU of sulfatases from *H. pomatia* (Type H-1). 1 ml of 100% methanol acidified with 0.5% acetic acid was added and samples were centrifuged for 15 min at 16,100 x g at 4°C and supernatants were collected. This step was repeated 3 times, the last occasion with 50% methanol acidified with 0.5% acetic acid, and the supernatants were dried using a speedvac. The pellets were dissolved with 125 µl of mobile phase and transferred to vials for HPLC analysis.

Analysis of seaweed phenolics in blood and urine by HPLC-DAD analysis

Sample 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 analysed by reverse-phase HPLC using a Nova-Pak C18 column (4.6 x 250 mm) with 4µm particle size. The temperature of the column was maintained at 30ºC. The mobile phases consisted of a mixture of aqueous methanol 5% in 0.1% hydrochloric acid 5 M (A) and a mixture of aqueous acetonitrile 50 in 0.1% hydrochloric acid 5 M (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 280 nm and spectra of products obtained over the 200–600 nm range. Peaks were characterised by their retention time and spectra characteristics. A calibration curve of phloroglucinol was constructed using authentic standard (0.1–100 μg/ml) and in each case was found to be linear with correlation coefficients of 0.995. Metabolites were quantified as phloroglucinol equivalents.

HPLC-DAD analysis of the urine (Supplmental Table 2) and plasma (Supplmental Table 3) samples, with and without glucuronidase/sulfatase treatment, showed the presence of a variety of metabolites absent in the baselines (before the intervention) in both plasma and urine samples. 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) and their enzymatically released un-conjugated forms. There was substantial inter-individual variation in (poly)phenol s excreted by the participants. In 42 participants, no (poly)phenol s were detected in urine and in the remainder (n=36) the amounts excreted ranged from 0.5 to 11.8 mg/day (mean 8.2 ± 12.1 mg/d). Similarly, there was a large variation in plasma concentrations (42.1 ± 117.0 μg/ml),

with only 14 participants having detectable (poly)phenol s in their plasma, seven of which had no (poly)phenol s detected in their urine samples, in accordance with what was observed after acute consumption. It is worth clarifying that, despite inter-individual variability in polyphenols metabolism being common for many polyphenol classes, in the present case the observed variability could partially be the result of the methodological limitations with the HPLC-DAD analysis.

Supplemental Table 1

RP-HPLC analysis of seaweed metabolites in urine samples. Metabolites were detected in 36 out of 78 participants.

RT (min)	Metabolite	Mean (mg)	Stdev	Volunteers (n)	Enz (-)	Enz (+)
11.9	UM1	11.82	14.14	4	yes	yes
12.8	UM2	3.63	3.03	2	yes	yes
13.1	UM3	6.87	5.01	6	yes	yes
16.3	UM4	3.31	0.03	2	yes	yes
18.6	UM5	3.66	2.07	4	yes	no
21.7	UM6	6.17	3.16	4	yes	yes
24.5	UM7	3.46	2.02	4	no	yes

25.2	UM8	0.47	0.26	2	yes	no
25.9	UM9	2.26	1.18	3	yes	no
28.8	UM10	0.79	0.53	2	no	yes
35.0	UM11	1.57	0.95	3	yes	yes
36.4	UM12	1.02	0.49	4	yes	yes
39.5	UM13	1.18	1.02	4	yes	yes
41.5	UM14	0.95	0.92	4	no	yes
44.2	UM15	0.92	0.74	6	no	yes
48.0	UM16	2.38	2.14	4	yes	no
49.6	UM17	5.42	5.23	5	no	yes
53.8	UM18	0.90	1.17	3	no	yes
Group Mean Total		8.16	12.10	36		

Phenolics in urine were quantified as phloroglucinol equivalents using HPLC and expressed as mg/d in urine. Note; the row "Group Mean Total" represents the average of the sum (total excretion) of metabolites calculated for each individual volunteer.

Supplemental Table 2

RP-HPLC analysis of seaweed metabolites in plasma samples. Metabolites were detected in 14 out of 78 participants.

RT (min)	Metabolite	Mean (µg/ml)	Stdev	Volunteers (n)	Enz (-)	Enz (+)
8.5	PM1	148.12	91.12	2	no	yes
9.1	PM2	0.75	0.31	2	yes	no
11.0	PM3	23.71	12.69	2	yes	no
14.9	PM4	110.85	139.63	2	yes	no
31.0	PM5	2.56	1.54	3	yes	no
37.3	PM6	1.55	0.69	2	yes	yes
43.2	PM7	1.34	0.21	2	no	yes
45.3	PM8	0.64	0.18	2	no	yes
48.0	PM9	0.78	0.16	3	yes	no
58.7	PM10	2.62	0.56	2	yes	yes
Group Mean Total		42.08	117.06	14		

Phenolics in plasma were quantified as phloroglucinol equivalents using HPLC and expressed as µg/ml in plasma.

Online Supporting Material

Note; the row "Group Mean Total " represents the average of the sum (total excretion) of metabolites calculated for each individual volunteer.

Supplemental Table 3. List of urine metabolites correlated with the chronic intake of seaweed capsules in group 1 formed by 70 participants.

ID	VIP value	Experimental Molecular Weight	Metabolite tentative identification
41	5.8	332.18323	Unknown
67	6.0	332.18323	Unknown
165	5.2	182.99788	Saccharin
292	5.0	182.99797	Saccharin
298	5.1	176.06742	3-hydroxy-2-methoxyglutarate/glycerol-1,2-diacetate/3-isopropylmalate/2,3-dimethyl-3-hydroxyglutaric acid/2-isopropylmalic acid
328	5.6	693.32784	Unknown
372	5.1	182.9979	Saccharin
416	4.9	182.99769	Saccharin
457	4.6	234.01896	Ajoene/4-(2,2´-bithiophen-5-yl)-3-butyn-1-ol
464	5.8	183.0885	Epinephrine/tretraydrohippuric acid/normetanephrine
500	5.4	188.10399	Azelaic acid/butyl ethyl malonate/entre otros
505	4.9	188.10391	Azelaic acid/butyl ethyl malonate/entre otros
556	5.3	183.08848	Epinephrine/tretraydrohippuric acid/normetanephrine
557	4.5	188.10397	Azelaic acid/butyl ethyl malonate/entre otros
602	4.5	174.9926	Unknown
650	6.6	382.16221	Ibuprofen glucuronide/cyclocalopin B
652	3.8	205.98811	Pyrogallol-O-sulfate
800	4.2	205.98816	Pyrogallol-O-sulfate
801	5.2	182.99757	Saccharin
847	3.3	350.1569	Eremopetasin sulfoxide
940	4.2	692.32582	Pisumoside B
956	5.3	372.10485	Dihydroferulic acid-4-O-glucuronide
981	3.6	440.16758	Unknown

1025	4.0	351.09499	Indole-3-acetic-acid-O-glucuronide
1106	5.8	150.01492	Tartalic acid/2,3-dihydroxybutanedioic acid/2-2-trienylfuran
1130	3.7	440.16786	Unknown
1155	3.9	440.16724	Unknown
1213	5.0	542.27235	Cortolone-3-glucuronide
1217	4.3	134.05685	2,3-dihydroxyvaleric acid/deoxyribose/dihydroxy-isovalerate/glycerol acetate
1248	5.2	150.0146	Tartalic acid/2,3-dihydroxybutanedioic acid/2-2-trienylfuran
1262	4.4	134.05696	2,3-dihydroxyvaleric acid/deoxyribose/dihydroxy-isovalerate/glycerol acetate
1269	4.6	134.05687	2,3-dihydroxyvaleric acid/deoxyribose/dihydroxy-isovalerate/glycerol acetate
1303	4.5	134.05689	2,3-dihydroxyvaleric acid/deoxyribose/dihydroxy-isovalerate/glycerol acetate
1306	4.2	440.16771	Unknown
1315	6.1	150.01506	Tartalic acid/2,3-dihydroxybutanedioic acid/2-2-trienylfuran
1352	4.3	486.17277	Hydroxytrifuhaol A-glucuronide
1408	4.3	810.29457	Unknown
1429	4.5	356.11012	Hydroxy-methoxycinnamoyl-b-glucose/1-O-feruloylglucose/veranisatin B
1453	3.8	486.17314	Hydroxytrifuhaol A-glucuronide
1458	4.2	486.1734	Hydroxytrifuhaol A-glucuronide
1472	3.9	205.98794	Pyrogallol-O-sulfate
1483	4.4	486.17327	Hydroxytrifuhaol A-glucuronide
1493	5.0	310.97636	Unknown
1544	4.7	824.274	Unknown
1648	5.3	233.03502	Dopamine sulfate
1665	4.8	435.18873	Unknown
1679	4.7	219.08849	N-deschlorobenzoyl indomethacin/alpha-methoxy-1-H-indole-3-propanoic acid/nigellimine N-oxide/alpha-hydroxy-1H-indole-3-propanoic acid
1693	5.4	251.0786	N-carboxyacetyl-D-phenylalanine/N-phenylacetylaspartic acid/4-hydroxy-3-methoxy-cinnamoylglycine
1711	4.8	233.0349	Dopamine sulfate
1715	4.6	181.07286	Tyrosine
1725	4.2	298.10513	2-Phenylethanol glucuronide

1727	5.2	251.07833	N-carboxyacetyl-D-phenylalanine/N-phenylacetylaspartic acid/4-hydroxy-3-methoxy-cinnamoylglycine
1737	5.0	219.0888	N-deschlorobenzoyl indomethacin/alpha-methoxy-1-H-indole-3-propanoic acid/nigellimine N-oxide/alpha-hydroxy-1H-indole-3-propanoic acid
1751	5.3	414.15204	Ptelatoside A
1778	5.1	251.0785	N-carboxyacetyl-D-phenylalanine/N-phenylacetylaspartic acid/4-hydroxy-3-methoxy-cinnamoylglycine
1799	5.4	842.39386	Unknown
1806	3.5	338.947	Unknown
1828	6.1	364.17303	Unknown
1877	5.2	842.39393	Unknown
1917	5.0	544.28816	Dioxinodehydroeckol glucuronide
1924	4.7	360.1411	Dimethoxyphenyl-ethanediol O-glucoside/2-(4-hydroxy-3,5-dimethoxyphenyl)ethanol 4´-glucoside/ deoxyloganic acid
1935	4.7	223.11987	Cerulenin
1945	4.9	251.0785	N-carboxyacetyl-D-phenylalanine/N-phenylacetylaspartic acid/4-hydroxy-3-methoxy-cinnamoylglycine
1948	4.2	218.02395	tyrosol-sulfate/diphenyl disulfide
1953	4.1	378.05751	Unknown
1961	4.9	400.1728	Corchoionoside B
1970	4.7	223.11986	Cerulenin
1995	5.1	372.10472	Dihydroferulic acid-4-O-glucuronide
2038	5.3	360.14121	Dimethoxyphenyl-ethanediol O-glucoside/2-(4-hydroxy-3,5-dimethoxyphenyl)ethanol 4´-glucoside/ deoxyloganic acid
2040	5.5	526.27719	Unknown
2059	5.4	504.19905	Myricatomentoside I/chlorhexidine
2063	4.4	398.10594	Methyl 3,4-dihydroxy-5-prenylbenzoate-3-glucoside
2072	4.4	372.10487	Dihydroferulic acid-4-O-glucuronide
2080	5.4	324.06294	Sterigmatocystin/greviline A

2081	4.6	251.07794	N-carboxyacetyl-D-phenylalanine/N-phenylacetylaspartic acid/4-hydroxy-3-methoxy-cinnamoylglycine
2185	4.8	280.05956	Unknown
2186	4.8	280.05942	Unknown
2188	4.8	240.12161	Anserine/homocarnosine/balenine
2194	5.4	213.00886	Indoxyl sulfate/carmustine

All predicted formula derived with < 5 ppm mass accuracy data. VIP (variable influence in projection) is a variable that summarizes the importance of X variables to the OPLS-DA model. Variables with VIP values > 2 were the main contributors in the model. ID, identification number. Metabolites were searcher against two data based, (Metlin database; http://metlin.scripps.edu/ and HMDB: http://hmdb.ca). Searching metabolites do not include heavy metal and halogens.

Supplemental Table 4. List of plasma metabolites correlated with the chronic intake of seaweed capsules.

ID	VIP value	Experimental weight	Molecular Tentative identification
1315	2.1	150.01506	Tartalic acid/2,3-dihydroxybutanedioic acid/2-2-trienylfuran
1450	3.1	188.01341	p-cresol sulfate
246	2.2	195.95296	Unknown
114	2.4	195.953	Unknown
111	2.3	195.95304	Unknown
977	2.3	208.00368	Unknown
2066	2.1	210.03589	Galactaric acid/glucaric acid
797	2.0	213.00848	Indoxyl sulfate/carmustine
1948	2.0	218.02395	tyrosol-sulfate/diphenyl disulfide
1711	2.1	233.0349	Dopamine sulfate
1648	2.3	233.03502	Dopamine sulfate
2097	2.2	233.03511	Dopamine sulfate
587	2.0	236.10413	4-hydroxy-3-methoxy-5-(3-methylbut-2-en-1-yl)benzoic acid/carboxy-ibuprofen/ethyl vanillin isobutyrate
496	2.0	236.10422	4-hydroxy-3-methoxy-5-(3-methylbut-2-en-1-yl)benzoic acid/carboxy-ibuprofen/ethyl vanillin isobutyrate
892	2.1	246.0189	Unknown
2138	2.7	278.03987	Unknown
2186	3.2	280.05942	Unknown
2185	3.3	280.05956	Unknown
706	2.5	293.9301	Unknown
622	2.1	293.9301	Unknown
2119	3.2	324.12077	Unknown
2035	3.0	324.12114	Unknown
588	2.1	326.13624	4-(4´-hydroxyphenyl)2-butanone glucoside/ citrusin C/1-methoxy-3-(4-hydroxyphenyl)-2E-propenal 4´-glucoside

654	2.4	326.13646	4-(4´-hydroxyphenyl)2-butanone glucoside/ citrusin C/1-methoxy-3-(4-hydroxyphenyl)-2E-propenal 4´-glucoside
1293	2.3	330.16753	Diphlorethol sulfate
1356	2.3	330.16795	Diphlorethol sulfate
2163	2.0	336.0294	Unknown
2013	2.0	336.02953	Unknown
1958	2.2	347.14012	Unknown
2068	2.5	358.08919	Dihydrocaffeic acid -O-glucuronide
519	2.6	360.14168	Dimethoxyphenyl-ethanediol O-glucoside/2-(4-hydroxy-3,5-dimethoxyphenyl)ethanol 4´-glucoside/ deoxyloganic acid
430	2.2	360.14198	Dimethoxyphenyl-ethanediol O-glucoside/2-(4-hydroxy-3,5-dimethoxyphenyl)ethanol 4´-glucoside/ deoxyloganic acid
1540	2.3	380.14656	Gibberellin A75/8-oxodiacetoxyscirpenol
1357	2.0	382.16224	Ibuprofen glucuronide/cyclocalopin B
381	2.4	398.15706	Methyl 3,4-dihydroxy-5-prenylbenzoate-3-glucoside
364	2.0	398.15764	Methyl3,4-dihydroxy-5-prenylbenzoate-3-glucoside
234	2.0	412.13594	Methylpicraquassioside A
134	2.3	412.136	Methylpicraquassioside A
214	2.0	412.13618	Methylpicraquassioside A
154	2.0	412.13626	Methylpicraquassioside A
159	2.2	412.13637	Methylpicraquassioside A
164	2.2	412.13649	Methylpicraquassioside A
1751	2.0	414.15204	Ptelatoside A
2134	2.1	440.09245	3,5-dihydroxyphenyl-1-O-(6-O-galloyl-beta-D-glucopyranoside)
1633	2.1	544.21522	Dioxinodehydroeckol glucuronide
351	2.0	796.31532	Unknown
853	2.1	797.31862	Fucophloroethols glucuronide
1182	2.0	169.0363	2-furoylglycine/L-2-3,dihydrodipicolinate
1627	2.4	174.08776	Suberic acid/ 2-propylglutaric acid
753	2.1	174.99243	Unknown

1083	2.1	174.99252	Unknown
602	2.8	174.9926	Unknown
859	2.3	174.9926	Unknown
1023	2.1	174.9926	Unknown
769	2.0	174.99263	Unknown
2094	2.3	189.00848	Lanthionine ketimine/2-aminophenol sulphate
244	2.1	229.00336	Unknown
702	2.0	248.0315	C-O-C dimer of phloroglucinol
1918	2.8	259.99821	Caffeic acid 3-sulfate
887	2.2	261.03016	Unknown
1120	2.8	290.04494	4-hydroxy-5-(hydroxyphenyl)-valeric acid-sulfate
1677	2.1	303.05847	Unknown
1481	2.9	314.09898	2-hydroxyphenylacetic acid-O-glucuronide
1993	2.1	314.09898	2-hydroxyphenylacetic acid-O-glucuronide
293	2.1	327.09512	C-O-C dimer of phloroglucinol-sulfate
839	2.1	328.09828	Difucol/diphlorethol glucuronide
952	2.2	350.02393	Unknown
847	2.0	350.1569	Eremopetasin sulfoxide
1066	2.4	357.10545	HMBOA-GLc (known as 2-O-glucosyl-7-methoxy-1,4-benzoxazin-3-one
625	2.2	357.10548	HMBOA-GLc (known as 2-O-glucosyl-7-methoxy-1,4-benzoxazin-3-one
479	2.1	357.1056	HMBOA-GLc (known as 2-O-glucosyl-7-methoxy-1,4-benzoxazin-3-one
626	2.4	357.10573	HMBOA-GLc (known as 2-O-glucosyl-7-methoxy-1,4-benzoxazin-3-one
1966	2.0	358.08912	Dihydrocaffeic acid-O-glucuronide
1620	2.1	359.06694	Unknown
1351	2.3	362.15735	Unknown
870	2.4	362.15748	Unknown
1792	2.9	368.10989	3-O-feruloylquinic acid/O-caffeoyl-O-methylquinic acid

1758	2.4	370.08925	Feruloyl C-glucuronide/isoferulic acid-3-O-glucuronide/5-hydroxy-6-methoxycoumarin7-glucoside
1683	2.0	370.08926	Feruloyl C-glucuronide/isoferulic acid-3-O-glucuronide/5-hydroxy-6-methoxycoumarin7-glucoside
1788	2.3	370.08955	Feruloyl C-glucuronide/isoferulic acid-3-O-glucuronide/5-hydroxy-6-methoxycoumarin7-glucoside
1682	2.9	372.10469	Dihydroferulic acid-4-O-glucuronide
1757	2.8	372.10469	Dihydroferulic acid-4-O-glucuronide/veranisatin C
1634	2.6	372.10469	Dihydroferulic acid-4-O-glucuronide
1765	2.9	372.10475	Dihydroferulic acid-4-O-glucuronide
1719	2.9	372.10491	Dihydroferulic acid-4-O-glucuronide
1570	2.1	372.10499	Veranisatin C/dihydroferulic acid-glucuronide
1776	2.2	380.16774	3-Methyl-3-butenyl apiosyl-glucoside/prenyl arabinosyl-glucoside/ecabet
1496	2.8	384.05021	Unknown
2017	3.0	384.12615	Unknown
1512	2.0	388.17252	hydroxypropyl-2-methoxyphenoxyl-1,3-propanediol 1 xyloside/jasmonic acid derivative
1941	2.1	388.17283	hydroxypropyl-2-methoxyphenoxyl-1,3-propanediol 1 xyloside/jasmonic acid derivative
1638	2.7	388.17311	hydroxypropyl-2-methoxyphenoxyl-1,3-propanediol 1 xyloside/jasmonic acid derivative
1330	2.7	410.04463	Unknown
1768	2.8	410.0451	Unknown
1341	2.4	446.09862	Unknown
1551	2.3	446.09904	Unknown
1664	2.0	496.23067	Glaucarubin
1781	2.5	509.13896	trans-hydroxycyclohexyl glyburide (Benzenesulfonamides)

All predicted formula derived with < 5 ppm mass accuracy data. VIP (variable influence in projection) is a variable that summarizes the importance of X variables to the OPLS-DA model. Variables with VIP values > 2 were the main

contributors in the model. ID, identification number. Metabolites were searcher against two data based, (Metlin database; http://metlin.scripps.edu/ and HMDB: http://hmdb.ca). Searching metabolites do not include heavy metal and halogens.
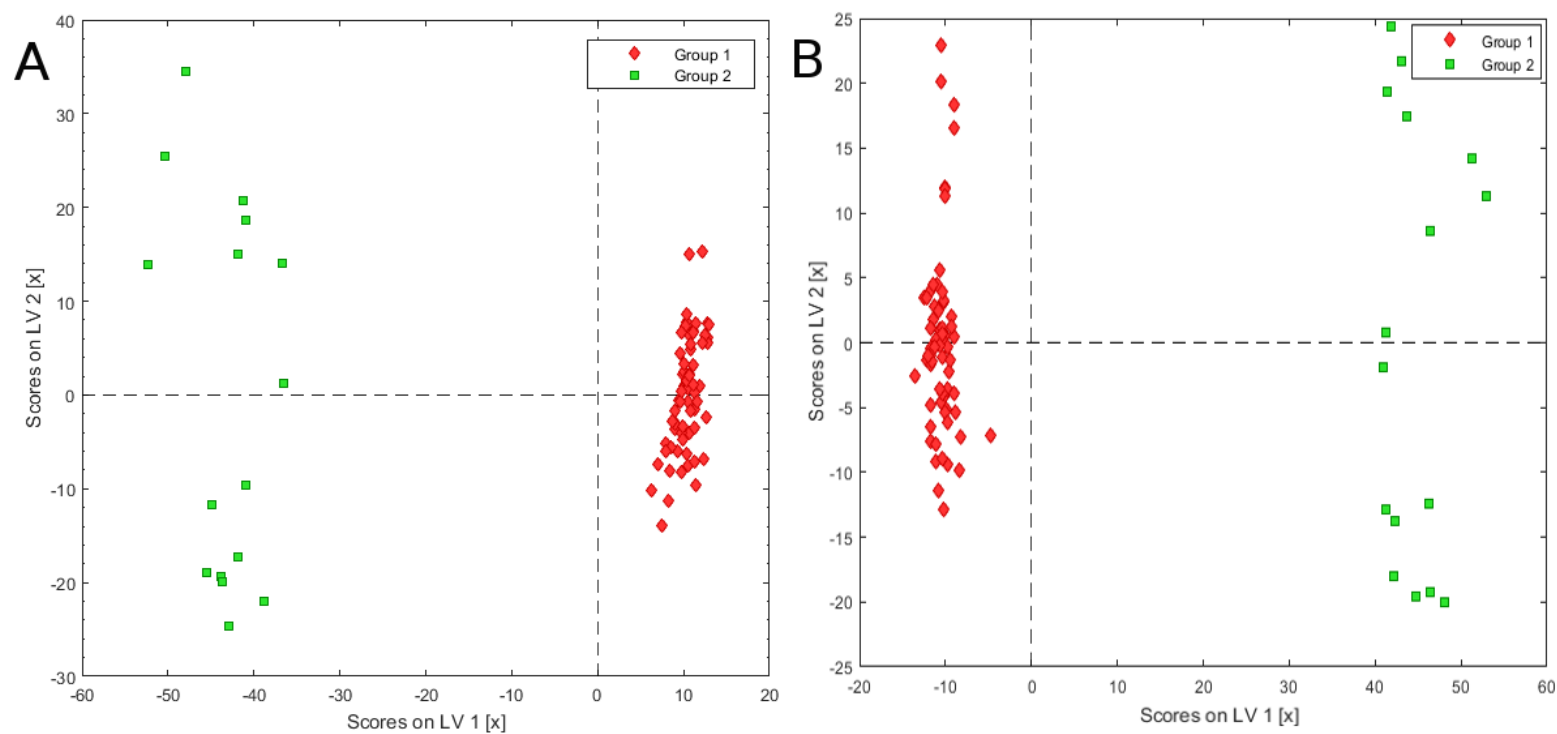
Supplemental Figure 3. OPLS-DA scores plots of the urine samples belong to group of participants 1 (70 volunteers-red) and group of subject 2 (8 participants-green) before (A) and after (B) seaweed consumption. LV1: latent variable 1; LV 2: latent variable 2.

Subject Id	Pyrogallol/Phloroglucinol-sulfate
S58	0.000
S61	0.000
S76	0.000
S78	0.000
S60	0.000
S70	0.003
S79	0.000
S83	0.000
S64	0.015
S40	0.047
S27	0.057
S66	0.064
S25	0.040
S65	0.085
S30	0.040
S71	0.000
S10	0.070
S33	0.128
S03	0.085
S59	0.111
S74	0.098
S19	0.156
S43	0.176
S39	0.085
S75	0.065
S01	0.184
S15	0.130
S53	0.125
S41	0.209
S36	0.225
S49	0.327
S82	0.328
S04	0.312
S46	0.347
S57	0.407
S81	0.373
S54	0.232
S35	0.204
S67	0.235
S16	0.447
S05	0.060
S23	0.547
S17	0.508
S08	0.302
S52	0.477
S29	0.407
S56	0.513

S02	0.532
S18	0.635
S38	0.337
S34	0.377
S31	0.531
S21	0.417
S24	0.453
S55	0.596
S22	0.500
S11	0.514
S37	0.287
S48	0.604
S42	0.436
S44	0.846
S63	0.535
S06	0.601
S12	0.366
S13	0.317
S62	0.555
S14	0.640
S20	0.843
S72	0.772
S32	0.682
S73	0.711
S26	0.533
S77	1.089
S07	0.622
S09	0.867
S51	1.158
S47	0.731
S50	1.470
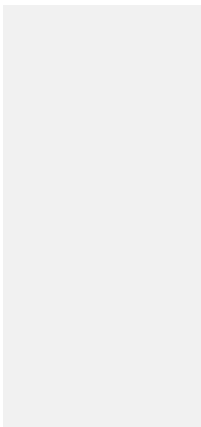
Pyrogallol/Phloroglucinol-sulfate	Pyrogallol/Phloroglucinol-sulfate
0.000	0.000
0.000	0.000
0.000	0.000
0.000	0.000
0.000	0.000
0.004	0.004
0.000	0.000
0.000	0.000
0.016	0.022
0.036	0.004
0.064	0.012
0.091	0.012
0.026	0.019
0.055	0.110
0.062	0.007
0.000	0.000
0.078	0.020
0.259	0.018
0.095	0.017
0.214	0.015
0.148	0.003
0.114	0.026
0.285	0.030
0.119	0.011
0.263	0.023
0.171	0.106
0.105	0.035
0.081	0.093
0.319	0.034
0.313	0.021
0.327	0.090
0.212	0.037
0.348	0.048
0.417	0.014
0.514	0.014
0.570	0.046
0.583	0.045
0.253	0.037
0.493	0.063
0.616	0.074
0.052	0.045
0.320	0.062
0.574	0.108
0.373	0.046
0.511	0.083
0.586	0.056
0.473	0.193

0.662	0.055
0.543	0.074
0.261	0.716
0.580	0.069
0.867	0.085
0.334	0.183
0.573	0.062
0.671	0.094
0.720	0.083
0.693	0.043
0.628	0.058
0.714	0.081
0.340	0.591
0.611	0.157
0.952	0.067
0.673	0.111
0.452	0.042
0.246	0.402
0.944	0.077
0.964	0.037
0.786	0.318
1.026	0.031
1.178	0.105
1.223	0.120
1.039	0.129
0.898	0.284
0.884	0.072
0.977	0.126
0.827	0.167
1.534	0.132
0.984	0.216

Participants
Hydroxytrifuhaol-A-glucuronide
0.000
0.000
0.000
0.000
0.000
0.000
0.000
0.000
0.000
0.029
0.034
0.023
0.062
0.017
0.068
0.000
0.090
0.019
0.094
0.063
0.076
0.073
0.031
0.117
0.093
0.069
0.146
0.156
0.105
0.105
0.076
0.095
0.112
0.095
0.074
0.062
0.123
0.206
0.149
0.064
0.331
0.127
0.070
0.217
0.142
0.120
0.060

0.110
0.065
0.042
0.195
0.041
0.202
0.171
0.103
0.142
0.188
0.216
0.120
0.130
0.089
0.120
0.180
0.375
0.382
0.162
0.165
0.081
0.127
0.086
0.086
0.287
0.078
0.323
0.249
0.181
0.117
0.378

Total mmoles excreted in urine

Hydroxytrifuhaol-A-glucuronide	Hydroxytrifuhaol-A-glucuronide
0.000	0.000
0.000	0.000
0.000	0.000
0.000	0.000
0.000	0.000
0.000	0.000
0.000	0.000
0.000	0.000
0.000	0.000
0.027	0.028
0.030	0.031
0.025	0.028
0.055	0.039
0.022	0.025
0.066	0.064
0.000	0.000
0.080	0.082
0.016	0.015
0.083	0.084
0.050	0.043
0.069	0.069
0.061	0.061
0.032	0.032
0.113	0.106
0.082	0.085
0.063	0.063
0.133	0.133
0.133	0.142
0.088	0.087
0.091	0.086
0.068	0.069
0.097	0.097
0.095	0.101
0.084	0.083
0.070	0.067
0.054	0.056
0.121	0.109
0.199	0.187
0.141	0.135
0.062	0.058
0.299	0.301
0.151	0.115
0.066	0.064
0.193	0.193
0.117	0.102
0.129	0.129
0.115	0.134

0.097	0.093
0.104	0.107
0.081	0.103
0.167	0.153
0.045	0.048
0.193	0.185
0.166	0.155
0.102	0.094
0.126	0.129
0.177	0.165
0.242	0.196
0.117	0.109
0.134	0.126
0.080	0.081
0.098	0.109
0.159	0.163
0.318	0.341
0.335	0.347
0.172	0.147
0.147	0.150
0.076	0.074
0.122	0.116
0.096	0.102
0.080	0.078
0.250	0.229
0.089	0.088
0.286	0.293
0.221	0.226
0.164	0.164
0.108	0.107
0.411	0.343

Hydroxytrifuhaol-A-glucuronide	Dioxinodehydroeckol-glucuronide (N=78)
0.000	0.000
0.000	0.000
0.000	0.001
0.000	0.000
0.000	0.000
0.000	0.000
0.000	0.000
0.000	0.000
0.000	0.001
0.029	0.000
0.029	0.000
0.028	0.000
0.035	0.001
0.026	0.000
0.059	0.000
0.000	0.000
0.037	0.000
0.017	0.000
0.038	0.001
0.028	0.000
0.066	0.000
0.069	0.000
0.032	0.000
0.099	0.001
0.071	0.000
0.057	0.000
0.128	0.001
0.143	0.000
0.056	0.001
0.060	0.000
0.006	0.000
0.105	0.000
0.090	0.000
0.066	0.000
0.065	0.000
0.055	0.001
0.013	0.000
0.167	0.000
0.121	0.000
0.059	0.000
0.304	0.000
0.107	0.001
0.059	0.000
0.151	0.000
0.050	0.000
0.122	0.000
0.127	0.000

0.082	0.000
0.113	0.001
0.116	0.000
0.115	0.001
0.047	0.000
0.187	0.001
0.151	0.001
0.094	0.001
0.095	0.000
0.018	0.000
0.194	0.000
0.107	0.000
0.125	0.001
0.076	0.000
0.105	0.000
0.120	0.001
0.182	0.000
0.160	0.002
0.149	0.000
0.116	0.000
0.070	0.000
0.110	0.000
0.108	0.001
0.079	0.000
0.132	0.001
0.089	0.001
0.217	0.001
0.103	0.000
0.162	0.000
0.102	0.001
0.338	0.000

C-O-C dimer of phloroglucinol-sulfat	C-O-C dimer of phloroglucinol
0.000	0.000
0.000	0.001
0.000	0.001
0.000	0.001
0.002	0.000
0.000	0.000
0.000	0.001
0.039	0.006
0.000	0.000
0.000	0.000
0.000	0.000
0.000	0.000
0.000	0.000
0.000	0.000
0.000	0.000
0.391	0.005
0.000	0.000
0.000	0.000
0.000	0.000
0.000	0.000
0.000	0.000
0.000	0.000
0.000	0.000
0.000	0.000
0.000	0.000
0.000	0.000
0.000	0.000
0.000	0.000
0.000	0.000
0.000	0.000
0.000	0.000
0.000	0.000
0.000	0.000
0.000	0.000
0.000	0.000
0.000	0.000
0.000	0.000
0.000	0.000
0.000	0.000
0.000	0.000
0.000	0.000
0.000	0.000
0.000	0.000
0.000	0.000
0.000	0.000
0.000	0.000
0.000	0.000
0.000	0.000

0.000	0.000
0.000	0.000
0.000	0.000
0.000	0.000
0.000	0.000
0.000	0.000
0.000	0.000
0.000	0.000
0.000	0.000
0.000	0.000
0.000	0.000
0.000	0.000
0.000	0.000
0.000	0.000
0.000	0.000
0.000	0.000
0.000	0.000
0.000	0.000
0.000	0.000
0.000	0.000
0.000	0.000
0.000	0.000
0.000	0.000
0.000	0.000
0.000	0.000
0.000	0.000
0.000	0.000
0.000	0.000
0.000	0.000
0.000	0.000
0.000	0.000

Fucophloroethol glucuronide	Diphlorethol sulfate
0.000	0.000
0.000	0.000
0.000	0.001
0.000	0.001
0.000	0.002
0.000	0.000
0.000	0.009
0.000	0.001
0.000	0.000
0.000	0.000
0.000	0.000
0.000	0.000
0.000	0.000
0.000	0.000
0.000	0.000
0.000	0.004
0.000	0.000
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0.000	0.000
0.000	0.000
0.000	0.000
0.000	0.000
0.000	0.000
0.000	0.000
0.000	0.000
0.000	0.000

Diphlorethol sulfate	Total metabolites (mmoles)
0.000	0.001
0.000	0.001
0.001	0.003
0.001	0.003
0.002	0.006
0.000	0.012
0.008	0.019
0.001	0.047
0.000	0.055
0.000	0.200
0.000	0.258
0.000	0.270
0.000	0.278
0.000	0.341
0.000	0.367
0.004	0.405
0.000	0.459
0.000	0.473
0.000	0.498
0.000	0.525
0.000	0.530
0.000	0.561
0.000	0.618
0.000	0.651
0.000	0.683
0.000	0.714
0.000	0.812
0.000	0.874
0.000	0.899
0.000	0.902
0.000	0.964
0.000	0.972
0.000	1.106
0.000	1.107
0.000	1.212
0.000	1.216
0.000	1.227
0.000	1.254
0.000	1.338
0.000	1.381
0.000	1.394
0.000	1.430
0.000	1.450
0.000	1.477
0.000	1.483
0.000	1.550
0.000	1.616

0.000	**1.632**
0.000	**1.642**
0.000	**1.657**
0.000	**1.660**
0.000	**1.665**
0.000	**1.701**
0.000	**1.733**
0.000	**1.756**
0.000	**1.796**
0.000	**1.797**
0.000	**1.821**
0.000	**1.852**
0.000	**1.883**
0.000	**1.942**
0.000	**1.986**
0.000	**2.007**
0.000	**2.078**
0.000	**2.192**
0.000	**2.206**
0.000	**2.220**
0.000	**2.249**
0.000	**2.305**
0.000	**2.357**
0.000	**2.377**
0.000	**2.600**
0.000	**2.617**
0.000	**2.699**
0.000	**2.769**
0.000	**2.825**
0.000	**2.832**
0.000	**4.140**

Mean	1.29
Sd	0.88
min	0.001
max	4.140

Low (<.5 mmole	High>2mmole
	1
	1
	1
	1
	1
	1
	1
	1
	1
	1
	1
	1
	1
	1
	1
	1
	1
	1
	1

1
1
1
1
1
1
1
1
1
1
1
1
1
1
1
1

Low (<.5 mmole	High>2mmole
19	16

Subject Id	Total meta	Low (<.5 mmole =1	High>2mmole =2
S58	0.00	1.00	
S61	0.00	1.00	
S76	0.00	1.00	
S78	0.00	1.00	
S60	0.01	1.00	
S70	0.01	1.00	
S79	0.02	1.00	
S83	0.05	1.00	
S64	0.05	1.00	
S40	0.20	1.00	
S27	0.26	1.00	
S66	0.27	1.00	
S25	0.28	1.00	
S65	0.34	1.00	
S30	0.37	1.00	
S71	0.40	1.00	
S10	0.46	1.00	
S33	0.47	1.00	
S03	0.50	1.00	
S59	0.53		
S74	0.53		
S19	0.56		
S43	0.62		
S39	0.65		
S75	0.68		
S01	0.71		
S15	0.81		
S53	0.87		
S41	0.90		
S36	0.90		
S49	0.96		
S82	0.97		
S04	1.11		
S46	1.11		
S57	1.21		
S81	1.22		
S54	1.23		
S35	1.25		
S67	1.34		
S16	1.38		
S05	1.39		
S23	1.43		
S17	1.45		
S08	1.48		
S52	1.48		
S29	1.55		
S56	1.62		
S02	1.63		
S18	1.64		

S38	1.66	
S34	1.66	
S31	1.67	
S21	1.70	
S24	1.73	
S55	1.76	
S22	1.80	
S11	1.80	
S37	1.82	
S48	1.85	
S42	1.88	
S44	1.94	
S63	1.99	
S06	2.01	2.00
S12	2.08	2.00
S13	2.19	2.00
S62	2.21	2.00
S14	2.22	2.00
S20	2.25	2.00
S72	2.31	2.00
S32	2.36	2.00
S73	2.38	2.00
S26	2.60	2.00
S77	2.62	2.00
S07	2.70	2.00
S09	2.77	2.00
S51	2.82	2.00
S47	2.83	2.00
S50	4.14	2.00