NATURAL PRODUCT COMMUNICATIONS

An International Journal for Communications and Reviews Covering all Aspects of Natural Products Research

Volume 13. Issue 5. Pages 513-656. 2018
ISSN 1934-578X (printed); ISSN 1555-9475 (online)
www.naturalproduct.us
Composition, Anti-inflammatory Activity, and Bioaccessibility of Green Seaweeds from Fish Pond Aquaculture

Andrea Ripolb,c, Carlos Cardosoc,d, Cláudia Afonsob,c, João Varelaa, Hugo Quental-Ferreirab,d, Pedro Pousão-Ferreirab,d and Narcisa M. Bandarrabc

aCentre of Marine Sciences, University of Algarve, Campus de Gambelas, 8005-139 Faro, Portugal
bDivision of Aquaculture and Upgrading (DivAV), Portuguese Institute for the Sea and Atmosphere (IPMA, IP), Rua Alfredo Magalhães Ramalho, 6, 1495-006 Lisbon, Portugal
cCIIMAR, Interdisciplinary Centre of Marine and Environmental Research, University of Porto, Rua dos Bragas 289, 4050-123 Porto, Portugal
dAquaculture Research Station, Olhão (EPPO), Portuguese Institute for the Sea and Atmosphere (IPMA, IP), Avenida 5 de Outubro, 8700-305 Olhão, Portugal
carlos.cardoso@ipma.pt

Received: December 2nd, 2017; Accepted: March 9th, 2018

Seaweeds are experiencing a growing market interest and their production has increased in the Asia-Pacific region. Though most seaweed is harvested offshore, they can also be produced in separate ponds or as co-products in fish farming [1], for instance, in meagre (Argyrosomus regius) farming in earth ponds. Particularly, fish pond aquaculture production systems are a new and promising scientific field that brings together fish farming and production of seaweeds and other marine organisms [1]. This is environmentally valuable and may usher in economic advantages. The composition and economic value of seaweeds may vary between species and, for a given species, parameters depend on abiotic/biotic conditions [2,3]. Hence, it is worthwhile to study the composition and properties of seaweeds from systems of fish pond aquaculture where integrated multi-trophic aquaculture can be implemented. Regarding green seaweed nutritional composition, moisture content is typically high, protein levels are significant, and lipid content is low, even on a dry matter basis [4,5]. Though fatty acid profiles may vary, they are usually rich in polyunsaturated fatty acids (PUFA), with a typically high level of some ω6 PUFA, such as 16:4ω3 and 18:4ω3, which are not so abundant in other organisms [4]. Moreover, green seaweeds have been reported to display high average carbohydrate concentrations [6]. Seaweed polysaccharides are a potential source of soluble and insoluble dietary fibers, many of which exhibit high water holding capacity. Soluble dietary fibers demonstrate an ability to increase viscosity, form gels, and/or act as emulsifiers [7].

Concerning biological properties, there are reports of anti-inflammatory activity measured in bioassays with mice [8]. Namely, methanol extracts of Ulva linza, at a concentration of 40 mg/mL, showed strong suppression of edema, with a relative inhibition of 84 %, and suppression of erythema, with an inhibition of 70 % [8]. Given the aforementioned components and bioactivities of green seaweeds, they may be worthy of further research aimed at nutritional and pharmacological applications. However, it must be taken into account that the absorbable quantity of a compound in the gastrointestinal (GI) tract is not accurately predicted by its total content in the seaweed. Bioaccessibility corresponds to the share of the initial content that is rendered free from the seaweed matrix into the GI tract [9]. Thus, determining bioaccessibility may contribute to the assessment of the effective nutraceutical/pharmacological potential of a specific species. A bioaccessibility study requires the utilization of an adequate in vitro digestion model that reliably simulates human digestion. Indeed, several methodologies have been developed [10], one being a static model with a digestive compartment distinction and complete digestive juices, including enzymes in all steps; this is one of the more accurate and robust models [10,11]. In recent years, these in vitro techniques for assessing human bioaccessibility have been improved through optimisation of reagents and enzyme proportions [9]. Therefore, experimental work was carried out in order to determine the nutritional composition, biological activities, and critical bioaccessibility effects on these properties for a significant group of green seaweeds grown under fish pond aquaculture conditions.

The proximate composition of the studied green seaweed species is displayed in Table 1. It was observed that there were differences between species. In particular, U. prolifera and C. linum had very high moisture content. The dry matter of these species (as well as of the other species) was mainly composed of protein and carbohydrates. The differences between wet weight ash, fat, protein, and carbohydrate contents changed when dry weight was considered, due to differences in the moisture content of the algae.
The ash content of *U. prolifera* and *C. linum* on both a wet and dry basis was lower than that of the other three species. This was not observed for protein, since *C. linum* had the highest protein content based on dry matter. *C. linum* had the lowest carbohydrate content. Fat levels were always very low, not surpassing 3% w/dw.

These results are similar to those reported for green seaweeds in previous studies [12-15] in that moisture content is very high (>80% wet weight) and lipid content is very low, even on a dry weight basis (<5% dry weight). Protein content on a dry weight basis was lower than that of the other three species. This was not observed for protein, since *C. linum* had the highest protein content based on dry matter. *C. linum* had the lowest carbohydrate content. Fat levels were always very low, not surpassing 3% w/dw.

These results are similar to those reported for green seaweeds in previous studies [12-15] in that moisture content is very high (>80% wet weight) and lipid content is very low, even on a dry weight basis (<5% dry weight). Protein content on a dry weight basis was significantly higher than the values reported previously (up to 2-fold). In *C. linum*, protein content was clearly higher than values found in the literature [14]. The main nutritional value of the studied green seaweeds lies in their protein and carbohydrate contents.

The FA composition of the studied species is presented in Table 2.

### Table 1: Proximate crude composition (g/100 g wet weight and for ash, protein, fat, and carbohydrate; mg/100 g dry weight) in the five studied green seaweed species

<table>
<thead>
<tr>
<th>Parameter</th>
<th><em>R. riparium</em></th>
<th><em>U. lactuca</em></th>
<th><em>U. prolifera</em></th>
<th><em>C. linum</em></th>
<th><em>U. intestinalis</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Moisture (g/100 g)</td>
<td>7.9 ± 0.2 a</td>
<td>9.1 ± 0.1 b</td>
<td>6.8 ± 0.0 a</td>
<td>7.5 ± 0.1 a</td>
<td>7.4 ± 0.0 b</td>
</tr>
<tr>
<td>Ash (g/100 g)</td>
<td>0.1 ± 0.0 a</td>
<td>0.1 ± 0.0 a</td>
<td>0.1 ± 0.0 a</td>
<td>0.1 ± 0.0 a</td>
<td>0.1 ± 0.0 a</td>
</tr>
<tr>
<td>Ash (%)</td>
<td>2.7 ± 0.1 a</td>
<td>2.1 ± 0.0 a</td>
<td>1.9 ± 0.0 b</td>
<td>2.5 ± 0.2 a</td>
<td>2.3 ± 0.0 a</td>
</tr>
<tr>
<td>Protein (g/100 g)</td>
<td>3.4 ± 0.1 a</td>
<td>2.8 ± 0.1 b</td>
<td>3.6 ± 0.0 a</td>
<td>3.2 ± 0.0 a</td>
<td>3.0 ± 0.0 a</td>
</tr>
<tr>
<td>Carbohydrate (%)</td>
<td>3.4 ± 0.0 a</td>
<td>3.2 ± 0.0 a</td>
<td>4.0 ± 0.1 b</td>
<td>3.6 ± 0.0 a</td>
<td>3.3 ± 0.0 a</td>
</tr>
<tr>
<td>Fat (g/100 g)</td>
<td>0.2 ± 0.0 a</td>
<td>0.2 ± 0.0 a</td>
<td>0.3 ± 0.0 ab</td>
<td>0.1 ± 0.0 a</td>
<td>0.3 ± 0.0 a</td>
</tr>
<tr>
<td>Fat (%)</td>
<td>2.9 ± 0.1 a</td>
<td>2.4 ± 0.0 a</td>
<td>3.6 ± 0.1 b</td>
<td>2.4 ± 0.0 a</td>
<td>3.0 ± 0.0 a</td>
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<tr>
<td>Carbohydrate (%)</td>
<td>3.4 ± 0.0 a</td>
<td>3.2 ± 0.0 a</td>
<td>4.0 ± 0.1 b</td>
<td>3.6 ± 0.0 a</td>
<td>3.3 ± 0.0 a</td>
</tr>
</tbody>
</table>

Values are presented as average ± standard deviation (*n* = 3). Different letters within a row correspond to statistical differences (*p* < 0.05).

A global comparison between the green seaweed species enabled us to highlight two main aspects: *U. lactuca* and *U. intestinalis* fatty acid profiles were very similar; all other profiles were quite different. Whereas *U. prolifera* was very rich in 18:3 n6 PUFA, *R. riparium* was much richer in 18:3 n3 PUFA. However, concerning the 18:3/18:2 n6 ratio, the highest value was found for *C. linum*. On the other hand, this being the most abundant FA in all profiles except for that of *U. prolifera*. Indeed, it has been claimed that palmitic acid is predominant in seaweeds [16]. Another common feature of green seaweeds found in the current study was a high C18/C20 PUFA ratio [12]. In addition, the abundance of 18:3 n3 is another distinctive trait of green seaweeds observed in the species under study [17]. Moreover, this trait is not found in red or brown seaweeds. However, even though n-6 PUFA is considered characteristic of the order Ulvales, reaching 10-20% of the total FAs [12], its content in the studied Ulvales (genus *Ulua*) was low. The DHA levels were low in all species, thus agreeing with other studies on the FA composition of green seaweeds, such as those on the genus *Ulua* [18].

On the other hand, there are differences between species due to particular features. This is corroborated by relevant literature [12]. At first it may seem that lipidomics with the total lipid fraction would be a useful tool in distinguishing green seaweed species. However, there are important differences between the same species collected from different places and grown under different conditions. For instance, whereas *U. lactuca* collected along the North California coast in November had 11% α-linolenic acid, 22% stearidonic acid, 18% oleic acid, 15% palmitic acid and 18% linolenic acid, in Portugal harvested in early Summer, as reported in the current study, which had less than 1% α-linolenic acid + stearidonic acid. Indeed, it has been claimed that palmitic acid is predominant in seaweeds [16]. Moreover, this trait is not found in red or brown seaweeds. However, even though n-6 PUFA is considered characteristic of the order Ulvales, reaching 10-20% of the total FAs [12], its content in the studied Ulvales (genus *Ulua*) was low. The DHA levels were low in all species, thus agreeing with other studies on the FA composition of green seaweeds, such as those on the genus *Ulua* [18].

In a more detailed analysis of each FA it was observed that long chain 18:3 n3 PUFA (eicosapentaenoic, 20:5 n3, EPA, and docosahexaenoic acid, 22:6 n3, DHA) levels were always low in all seaweeds. The most abundant 18:3 n3 PUFA in *R. riparium* and *C. linum* was α-linolenic acid (18:3 n3). In the other three species, C16 18:3 FAs were the most abundant n3 PUFA. *U. prolifera* is remarkable for its high content of linoleic acid (18:2 n6), 22.0 ± 0.8%, thereby diverging from the other species of *Ulva*. *C. linum* was found to be an abundant source of 18:1, while this FA was only present in relatively low concentrations in *U. lactuca* and *U. intestinalis*. Myristic (14:0) and palmitic (16:0) acids were the main SFA in all species. Stearic acid (18:0) was only present in minute levels in the analyzed profiles. Although *C. linum* was richest in SFA, it had the lowest myristic acid content. On the other hand, its palmitic acid level was the highest of all species, clearly contrasting with the remaining seaweed species.

The FA profile had some similarities with those of other green seaweeds [4]. Nevertheless, the obtained 18:2/18:3 ratios were lower than in this study in *U. armoricana*. 6.5. *C. linum* was the one that exhibited a FA composition nearest to this reported profile, including its low linoleic acid and very high palmitic acid contents. High palmitic acid contents were also found in all other seaweeds, this being the most abundant FA in all profiles except for that of *U. prolifera*. Indeed, it has been claimed that palmitic acid is predominant in seaweeds [16]. Another common feature of green seaweeds found in the current study was a high C18/C20 PUFA ratio [12]. In addition, the abundance of 18:3 n3 is another distinctive trait of green seaweeds observed in the species under study [17]. Moreover, this trait is not found in red or brown seaweeds. However, even though α-linolenic acid is considered characteristic of the order Ulvales, reaching 10-20% of the total FAs [12], its content in the studied Ulvales (genus *Ulua*) was low. The DHA levels were low in all species, thus agreeing with other studies on the FA composition of green seaweeds, such as those on the genus *Ulua* [18].
The performed experimental work represented a first step in the bioprospection of green seaweeds from fish pond aquaculture and integrated aquaculture production and it has identified strengths and weaknesses for each species. Moisture content of the studied seaweed species was very high, exceeding 87%. The dry matter was mainly composed of protein and carbohydrates. Lipid content was very low (<3 g/100 g dry weight) with almost no difference between species. However, there were differences between lipid fractions, since fatty acid profiles varied considerably between the five seaweed species. The anti-inflammatory activity was more remarkable in *U. prolifera* and *C. linum*. Indeed, significant COX-2 activity inhibition was found in the extracts at 100 µg/mL.

Apparently, the compounds causing this anti-inflammatory activity were not rendered bioaccessible. Future work should focus on the extraction of the bioactive compounds for nutraceutical or even pharmaceutical applications as well as explore the preparation of tisanes after destruction of the cell walls and analogous products as strategies to render the bioactive compounds more bioaccessible.

### Experimental

**Cultivation conditions:** At the Aquaculture Research Station, Olhão (EPPO), earth ponds with 0.2 ha and 2500 m³ in volume were used for meagre (*Argyrosomus regius*) experimental grow-out from 10 g to 1 kg and, in some tanks, till 2.5 kg in fish weight—fish density varied between 0.1 and 2.0 kg/m³. All ponds had constant water renovation, with a daily average of 30%, using pumped water from a reservoir connected directly to the Ria Formosa Lagoon. Dry feed was distributed to fish daily, starting with 2.3 (Winter, cold water, low feed consumption by the fish) and increasing progressively to 44 kg/day (Summer, warm water, high feed consumption by the fish), thereby reaching a total of 5,125 kg. Regarding the daily average of feed per fish kg, it ranged between 5 and 100 g. Seaweed grew spontaneously under daylight without any nutrient addition (besides those nutrients derived from fish feeding) or any other human intervention. No algicide (such as copper sulfate) was used during the grow-out and the presence of seaweed-feeders like gilthead seabream, *Sparus aurata*, was low (less than 500 specimens per pond). Seaweed biomass in the ponds was allowed to grow naturally until covering around 20% of water surface area and was collected weekly.

**Samples:** Green seaweeds were identified by a specialist on the basis of their morphological features [30]. Harvest was in the summer (July). Samples of five species of green seaweeds (*Chaetomorpha linum* (O.F. Müller) Kützing, *Rhizoclonium riparium* (Roth) Harvey, *Ulva intestinalis* Linnaeus, *U. lactuca* Linnaeus, *U. prolifera* O.F. Müller) were collected manually with a net from near the pond surface and transported immediately in seawater to a nearby lab (<100 m). Each sample was thoroughly washed with seawater to eliminate any biofouling organisms. After washing, the frond samples were kept moist inside a 20 L bucket in a refrigeration room and transported under refrigeration to the IPMA Lisbon Lab. Seaweeds were then finely minced. The processed biological material was frozen, freeze-dried, and stored at −20°C.

### In vitro digestion model:

An *in vitro* digestion model was chosen for the determination of bioaccessibility in each of the 5 seaweed species. Such a model comprises 3 sections, which enable the simulation of digestion in 3 different parts of the GI tract: mouth, stomach, and small intestine [9].

The composition of digestive juices (saliva, gastric, duodenal and bile) was the same as that described by Versantvoort *et al.* [11].
KCl, NaH₂PO₄, Na₂SO₄, NaCl, NaHCO₃, HCl, CaCl₂-2H₂O, KH₂PO₄ and MgCl₂ used for preparation of the digestive fluids, were obtained from Merck (Darmstadt, Germany). NH₄Cl was obtained from Fluka (Buchs, Switzerland) and all other chemicals from Sigma (St. Louis, MO, USA). In the case of duodenal juice, trypsin and α-chymotrypsin from Sigma (St. Louis, MO, USA) were also added. The quantities of these 2 enzymes (0.08 g trypsin and 0.87 g α-chymotrypsin in 500 mL of duodenal juice) were estimated on the basis of the work of Gattelier and Santé-Lhoustelier [31].

Briefly, 1.5 g homogenised and hydrated (with water to ensure a total of 1.5 g) seaweed was weighed. For the bioaccessibility blank, 1.5 mL of Milli-Q water was used. Each sample was mixed with 4 mL of artificial saliva at pH 6.8 ± 0.2 for 5 min, then 8 mL of artificial gastric juice (pH 1.3 ± 0.02 at 37 ± 2 ºC) was added to the sample-saliva mixture, and the pH lowered to 2.0 ± 0.1. The mixing lasted 2 h in a “head-over-heels” movement (37 rpm at 37 ± 2 ºC). Finally, 8 mL of artificial duodenal juice (pH 8.1 ± 0.2 at 37 ± 2 ºC), 4 mL of bile (pH 8.2 ± 0.2 at 37 ± 2 ºC), and 1.33 mL of HCO₃⁻ solution (1 M) were added. The pH of the mixture was set at 6.5 ± 0.5 and agitation for 2 h was identical to gastric conditions. The mixture generated in the in vitro model was subjected to centrifugation at 2750 × g for 5 min, thus yielding a non-digested portion and the bioaccessible fraction.

**Calculation of bioaccessibility:** The percentage (%) of each seaweed constituent (C) in the bioaccessible and non-digested fractions was estimated as follows:

\[
\% C \text{ bioaccessible} = \frac{[C]_{\text{bioaccessible}} \times 100}{[S]}
\]

and

\[
\% C \text{ non-digested} = \frac{[C]_{\text{non-digested}} \times 100}{[S]}
\]

Being:

- 

[C] = Concentration of constituent.

[S] = [C] in the bioaccessible fraction + [C] in the non-digested fraction.

**Proximate composition:** The moisture and ash contents were determined according to AOAC methods [32]. The protein level was quantified according to the Dumas method [33] and a conversion factor of nitrogen into protein specific to seaweed of 5 [34] was used. Crude fat was determined following the Folch extraction method [35]. Carbohydrate content was determined by difference between 100 % and the sum of the moisture, protein, crude fat, and ash contents.

**Fatty acid profile:** Fatty acid methyl esters (FAMEs) were prepared from freeze-dried seaweed by acid-catalyzed transesterification using the methodology described by Bandarra et al. [36]. Samples were injected into a Varian Star 3800 CP gas chromatograph (Walnut Creek, CA, USA), equipped with an auto sampler with a flame ionization detector at 250 ºC. A capillary DB-Wax capillary column (30 m × 0.25 mm ID × 0.25 μm film thickness; J&W Scientific, Agilent, Santa Clara, CA, USA) was used. Adequate separation was obtained over a 40-min period, with 5 min at 180 ºC, followed by an increase of 4 ºC/min until 220 ºC, and kept at this temperature for 25 min. FAMES were identified by comparing their retention times with those of Sigma–Aldrich standards (PUFA-3, Menhaden oil, and PUFA-1, Marine source from Supelco Analytical).

**Anti-inflammatory activity:**

**Extract preparation for in vitro anti-inflammatory activity:** For each seaweed and each respective bioaccessible fraction (see in vitro digestion model), an aqueous extract was prepared with the purpose of attaining a fraction with anti-inflammatory properties to be tested in vitro.

Accordingly, approximately 200 mg of freeze-dried seaweed was weighed or 5 mL bioaccessible fraction was measured and homogenized with 2 mL of Milli-Q water using a model Polytron PT 6100 homogenizer (Kinematica, Luzern, Switzerland) at a velocity of 30,000 rpm during 1 min. Afterwards, the mixture was subjected to a thermal treatment (at 80 ºC for 1 h). Both the seaweed and bioaccessible extraction mixtures were centrifuged (3,000 × g at 4 ºC during 10 min) and the respective supernatant was evaporated using a vacuum rotary evaporator with the water bath temperature at 65 ºC and an inert gas (nitrogen) stream.

**Cyclooxygenase (COX-2) inhibition assay:** The cyclooxygenase (COX-2) inhibition assay is a practical and quick screening method for assessing anti-inflammatory activity. The prepared extracts were dissolved in 100 % dimethyl sulfoxide (DMSO) to prepare a stock preparation with a concentration of 10 mg/mL. The extract was tested at 1 mg/mL and 100 μg/mL using a commercial cyclooxygenase (COX) inhibitory screening assay kit (Cayman test-kit 560131 (Cayman Chemical Company, Ann Arbor, MI, USA)). The COX inhibitor screening assay directly measures the amount of Prostaglandin F2α generated from arachidonic acid (AA) in the cyclooxygenase reaction. Ten μL of each test extract in DMSO or only DMSO was used. The reaction was initiated by addition of 10 μL 10 mM AA and each reaction tube was incubated at 37 ºC for 2 min. Reaction was terminated by addition of 50 μL 1 N HCl and saturated stannous chloride. Assays were performed using 100 units of human recombinant COX-2. An aliquot was removed and the prostanoïd produced was quantified spectrophotometrically (412 nm) via enzyme immunoassay (Enzyme-Linked ImmunoSorbent Assay, ELISA) after 18 h incubation at room temperature, washing, addition of Ellman’s reagent, and a further 90 min incubation. Interference by solutions and digestive enzymes used in the bioaccessibility method was taken into account by the following formula:

\[
\text{COX-2 Inh.}_{S,corr} = \text{COX-2 Inh.}_{S} - \text{COX-2 Inh.}_{\text{blank}}
\]

Where,

- COX-2 Inh.ₜₜ = Corrected COX-2 inhibition in the bioaccessible fraction samples;
- COX-2 Inh.ₜₜ = COX-2 inhibition measured in the bioaccessible fraction samples;
- COX-2 Inh.ₜₜ = COX-2 inhibition of the bioaccessibility blank.

**Statistical analysis:** Analyses were made in triplicate. In order to test normality and variance homogeneity, the Kolmogorov-Smirnov’s test and Levene’s F-test, respectively, were used. Data fulfilled both of these parametric tests’ assumptions. The seaweed species as well as the contrast between initial and bioaccessible contents were the 2 studied factors. The parametric test, Tukey HSD, was made with STATISTICA 6, 2003 version (StatSoft, Inc., Tulsa, OK, USA). For all statistical tests significance level (α) was 0.05.

**Acknowledgments** - This work was supported by the following Post Doctoral Grants: Ref.: SFRH/BPD/102689/2014 (“Fundação para a Ciência e a Tecnologia”, FCT) for the author Carlos Cardoso; Ref.: SFRH/BPD/64951/2009 (FCT) and DIVERSIAQUA (MAR2020) for the author Cláudia Afonso; and the CCMAR/Multi/04326/2013 grant for the authors Andrea Ripol and João Varela. Moreover, experimental work was funded by the
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