BIOACTIVE POLYSACCHARIDES EXTRACTS FROM SARGASSUM MUTICUM BY HIGH HYDROSTATIC PRESSURE

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ABSTRACT

Sargassum muticum is an important source of bioactive polysaccharides; hence, high hydrostatic pressure (HHP) was used to improve their extraction efficiency. Response surface methodology and a Box-Behnken full factorial design were employed to assess and optimize the effects of extraction conditions on the yield, total sugars, total sulfated sugars and antioxidant activity of S. muticum extracts. The extraction yield ranged between 32 and 40.4% independently of the extraction conditions or seaweed solid/liquid ratio resulting in average increases of 3.6 to 4.8-fold for total sugars and sulfated sugars, as compared to conventional extraction. Extracts displayed improved antioxidant activities, yet maximum values were achieved under different optimum conditions of HHP processing, for example, 5–5.5 min, 300 MPa and 1 g of dry seaweed for yield. In conclusion, the optimal HHP technology conditions described in this paper enables to obtain enriched bioactive polysaccharide S. muticum extracts.

PRACTICAL APPLICATIONS

High hydrostatic pressure (HHP) is a suitable method used to improve the extraction of natural ingredients with added value both in terms of nutritional value as in biological properties from different natural sources. In this study it was intended to evaluate the potential and the effectiveness of HHP to obtain extracts concentrated in bioactive polysaccharides from the brown seaweed Sargassum muticum. A new and possible strategy to valorize this invasive brown seaweed rich in sulfated polysaccharides with several health benefits associated. The main results have demonstrated that HHP increased extractability (higher extraction yields) and bioactivity from the seaweed S. muticum, providing extracts with higher content in polysaccharides which can be used as ingredients to develop novel functional foods.

INTRODUCTION

Sargassum muticum (Yendo) Fensholt is a brown edible seaweed that can be found along the European waters and on the West Coast of North America (Milledge et al. 2015). As invasive seaweed of difficult eradication it causes negative impacts on ecology, fishing and recreational activities (Balboa et al. 2013; Milledge et al. 2015). Despite these less positive aspects, the fact that S. muticum is available in large quantities (high biomass) and in itself holds important constituents of good nutritional, or even bioactive potential, has led to the development of strategies to valorize such seaweed compounds. Previous studies revealed that S. muticum extracts possess various potential biological proprieties, including antioxidant, anti-proliferative, anti-angiogenesis, antimicrobial and anti-inflammatory activities (Kim et al. 2007; Yoon et al. 2010; Namvar et al. 2013; Rodrigues et al. 2015a). Brown seaweeds are rich in sulfated polysaccharides.
namely fucoidans and laminaran which have several health benefits associated such as antioxidant activity (Wijesekara et al. 2011; Freitas et al. 2015; Kadam et al. 2015).

In general, these compounds may be extracted and/or concentrated and used in different food or nutraceutical applications. Many extraction techniques are available for such purpose yet are not always of favorable application due to associated environmental pollution and costs. Hence, improved extraction techniques are continuously being sought in terms of shortening of operating times, reduction of organic solvent consumption and increase in extraction efficiency. High hydrostatic pressure (HHP) processing has been considered an emerging nonthermal food processing technique that has shown great promise in food and pharmaceutical industries as well as in biotechnological research (Huang et al. 2013). HHP is able to increase the mass transfer rate by changing the solid/liquid ratio gradient and diffusivity, causing damage to the cell membrane and increasing its permeability thus enhancing permeation of the extraction solvent into the cells (Prasad et al. 2012). Since HHP is performed at room temperature it prevents thermal degradation and loss of biological properties of the extracts being prepared (Huang et al. 2013). Several research studies have revealed HHP to be a technique that enables shorter processing periods, less costs, higher processing safety and increment of extraction yields (Prasad et al. 2012).

In fact, according to Huang et al. (2013) there are numerous studies that endorse the use of high pressure as a suitable method to increase the extraction of natural ingredients with added value in terms of nutritional value and biological properties from food and medicinal herb matrices. For example, HHP treatment combined with enzymes increased the extractability and bioactivity of fermented rice bran (Kim and Han 2012). Higher levels of Xanthohumol content in beer wort was obtained by Santos et al. (2013), using HHP treatments opening the possibility to produce healthier beer with higher amounts of Xanthohumol, a bioactive compound with anti-carcigenic, antioxidant, anti-inflammatory and anti-infective activities. In addition, some studies have suggested that HHP processing may enhance the antioxidant activity of strawberry, blackberry, tomato and carrot purées (Patras et al. 2009a,b). The use of high pressure for extraction of compounds with biological properties from seaweeds can be an alternative way to the conventional extraction methods. Therefore, the main objectives of this paper were to assess the potential and effectiveness of HHP to obtain extracts concentrated in bioactive polysaccharides from the brown seaweed S. muticum and to optimize extraction parameters, namely, extraction time, pressure and seaweed solid/liquid ratio using a three level Box–Behnken design. Moreover, to our best of knowledge this is the first study applying HHP to extract bioactive polysaccharides from S. muticum. Consequently, an additional objective was to test the total antioxidant capacity and hydroxyl-radical scavenging activity of S. muticum polysaccharides extracts obtained via HHP extraction.

MATERIALS AND METHODS

High Hydrostatic Pressure Extraction of Bioactive Polysaccharides From S. muticum

Specimens of the brown seaweed (Heterokontophyta, Phaeophyceae) Sargassum muticum (Fucales) Sargassaceae family were harvested in April 2012 from Buarcos bay (Figueira da Foz, Portugal). The identification of seaweeds was based on AlgaeBase (Guiry and Guiry 2013). The seaweeds were first washed with running tap water and then with deionized water and then dried in an oven at 60°C. The dried seaweeds were milled to less than 1.0 mm particle size and stored in a dark desiccator until further use.

Prior to high hydrostatic pressure (HHP) assisted extraction, dried and milled seaweed was weighed and dispersed in 50 mL of deionized water and placed in an agitating water bath at room temperature for 24 h. Subsequently, samples were transferred to polyethylene bags which were heat sealed under vacuum (Albipack Packaging Solutions, Aveiro, Portugal). The bags containing the water extracts were subject to HHP under different time periods and pressures according to the established experimental design (Table 1). The HHP-assisted extraction was performed in a hydrostatic press (Hyperbaric 55, Hyperbaric, Burgos, Spain), which has a pressure vessel of 200 mm inner diameter and 2,000 mm length with a maximum operation pressure of 600 MPa. It is connected to a refrigeration unit (RMA KH 40 LT, Ferroli, Burgos, Spain) that enables the control of the input water temperature used as a pressurizing fluid at room temperature (20–22°C).

After the HHP processing step, the water extracts were removed from the bags, centrifuged at 5,000 g for 10 min at 4°C (centrifuge Medifriger BL-S, JP Selecta, Spain), the obtained supernatants were filtered through a glass filter funnel (porosity 1) and the resulting extracts frozen at −80°C until freeze-drying.

Design of Experiments

The design was constructed and analyzed using Minitab 17 (Minitab Statistical Software, Pennsylvania State University, State College, PA) and the response surface plots were plotted by SigmaPlot 13 Trial version (USA/Canada).

As shown in Table 1, a Box–Behnken design with three independent variables at three levels, namely extraction time (X1: 5, 17.5 and 30 min), extraction pressure (X2: 300, 450 and 600 MPa), and seaweed solid/liquid ratio (X3: 1, 3...
### Table 1. Box-Behnken Matrix Along with Experimental and Predicted Values of the Responses for the HHP Extraction of *S. muticum*

<table>
<thead>
<tr>
<th>Run No.</th>
<th>Time (min)</th>
<th>Pressure (MPa)</th>
<th>Seaweed conc. (g/50 mL)</th>
<th>Coded levels</th>
<th>Responses</th>
<th>Yield</th>
<th>Sugars</th>
<th>Sulfated sugars</th>
<th>ABTS</th>
<th>OH⁻</th>
<th>% Scavenging</th>
<th>% Scavenging</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>0 (17.5)</td>
<td>+1 (600)</td>
<td>−1 (1)</td>
<td>X₁</td>
<td>Observed</td>
<td>39.2</td>
<td>39.1</td>
<td>39.7 ± 0.7</td>
<td>43</td>
<td>3</td>
<td>28.9 ± 0.3</td>
<td>34.8 ± 0.3</td>
</tr>
<tr>
<td>2</td>
<td>0 (17.5)</td>
<td>0 (450)</td>
<td>0 (3)</td>
<td>X₂</td>
<td>Predicted</td>
<td>36.3</td>
<td>36.3</td>
<td>36.3 ± 0.3</td>
<td>43</td>
<td>3</td>
<td>28.9 ± 0.3</td>
<td>34.8 ± 0.3</td>
</tr>
<tr>
<td>3</td>
<td>+1 (30)</td>
<td>+1 (600)</td>
<td>0 (3)</td>
<td>X₃</td>
<td>Observed</td>
<td>35.7</td>
<td>35.8</td>
<td>35.7 ± 0.4</td>
<td>41.9</td>
<td>0.4</td>
<td>27 ± 1</td>
<td>38.0 ± 0.6</td>
</tr>
<tr>
<td>4</td>
<td>+1 (30)</td>
<td>0 (450)</td>
<td>+1 (5)</td>
<td></td>
<td>Predicted</td>
<td>32.4</td>
<td>32.5</td>
<td>32.5 ± 0.2</td>
<td>37.3</td>
<td>0.2</td>
<td>26.7 ± 0.7</td>
<td>38.0 ± 0.3</td>
</tr>
<tr>
<td>5</td>
<td>0 (17.5)</td>
<td>0 (450)</td>
<td>+1 (5)</td>
<td></td>
<td>Observed</td>
<td>33.8</td>
<td>33.6</td>
<td>33.8 ± 0.5</td>
<td>42.2</td>
<td>0.5</td>
<td>26.2 ± 0.6</td>
<td>37.9 ± 0.3</td>
</tr>
<tr>
<td>6</td>
<td>0 (17.5)</td>
<td>0 (450)</td>
<td>0 (3)</td>
<td></td>
<td>Predicted</td>
<td>36.2</td>
<td>36.3</td>
<td>36.3 ± 0.2</td>
<td>43</td>
<td>1</td>
<td>29.3 ± 0.6</td>
<td>37.1 ± 0.1</td>
</tr>
<tr>
<td>7</td>
<td>−1 (5)</td>
<td>0 (450)</td>
<td>−1 (1)</td>
<td></td>
<td>Observed</td>
<td>40.2</td>
<td>40.1</td>
<td>40.2 ± 0.1</td>
<td>43</td>
<td>1</td>
<td>33 ± 1</td>
<td>31.4 ± 0.3</td>
</tr>
<tr>
<td>8</td>
<td>0 (17.5)</td>
<td>0 (450)</td>
<td>0 (3)</td>
<td></td>
<td>Predicted</td>
<td>36.6</td>
<td>36.3</td>
<td>36.6 ± 0.3</td>
<td>43</td>
<td>1</td>
<td>29 ± 1</td>
<td>38.0 ± 0.1</td>
</tr>
<tr>
<td>9</td>
<td>0 (17.5)</td>
<td>−1 (300)</td>
<td>−1 (1)</td>
<td></td>
<td>Observed</td>
<td>40.4</td>
<td>40.6</td>
<td>40.4 ± 0.2</td>
<td>46</td>
<td>2</td>
<td>30 ± 2</td>
<td>32.3 ± 0.6</td>
</tr>
<tr>
<td>10</td>
<td>−1 (5)</td>
<td>−1 (300)</td>
<td>0 (3)</td>
<td></td>
<td>Predicted</td>
<td>36.5</td>
<td>36.4</td>
<td>36.5 ± 0.4</td>
<td>46</td>
<td>2</td>
<td>29 ± 5</td>
<td>38.2 ± 0.9</td>
</tr>
<tr>
<td>11</td>
<td>0 (17.5)</td>
<td>0 (450)</td>
<td>0 (3)</td>
<td></td>
<td>Observed</td>
<td>36.6</td>
<td>36.3</td>
<td>36.6 ± 0.5</td>
<td>43</td>
<td>1</td>
<td>30.9 ± 0.2</td>
<td>36.0 ± 0.7</td>
</tr>
<tr>
<td>12</td>
<td>+1 (30)</td>
<td>0 (450)</td>
<td>−1 (1)</td>
<td></td>
<td>Predicted</td>
<td>38.7</td>
<td>38.6</td>
<td>38.7 ± 0.7</td>
<td>41.9</td>
<td>0.7</td>
<td>28 ± 1</td>
<td>35.7 ± 0.8</td>
</tr>
<tr>
<td>13</td>
<td>+1 (30)</td>
<td>−1 (300)</td>
<td>0 (3)</td>
<td></td>
<td>Observed</td>
<td>36.5</td>
<td>36.4</td>
<td>36.5 ± 0.8</td>
<td>34</td>
<td>2</td>
<td>28 ± 1</td>
<td>34 ± 1</td>
</tr>
<tr>
<td>14</td>
<td>−1 (5)</td>
<td>0 (450)</td>
<td>+1 (5)</td>
<td></td>
<td>Predicted</td>
<td>31.9</td>
<td>32.0</td>
<td>31.9 ± 0.3</td>
<td>43</td>
<td>4</td>
<td>28.5 ± 0.8</td>
<td>34.9 ± 1</td>
</tr>
<tr>
<td>15</td>
<td>0 (17.5)</td>
<td>0 (450)</td>
<td>0 (3)</td>
<td></td>
<td>Observed</td>
<td>36.0</td>
<td>36.3</td>
<td>36.0 ± 0.4</td>
<td>43</td>
<td>2</td>
<td>28.9 ± 0.9</td>
<td>38.3 ± 0.2</td>
</tr>
<tr>
<td>16</td>
<td>0 (17.5)</td>
<td>−1 (300)</td>
<td>+1 (5)</td>
<td></td>
<td>Predicted</td>
<td>32.0</td>
<td>32.0</td>
<td>32.0 ± 0.2</td>
<td>38</td>
<td>1</td>
<td>27.5 ± 0.9</td>
<td>35 ± 1</td>
</tr>
<tr>
<td>17</td>
<td>−1 (5)</td>
<td>+1 (600)</td>
<td>0 (3)</td>
<td></td>
<td>Observed</td>
<td>36.8</td>
<td>36.9</td>
<td>36.8 ± 0.3</td>
<td>36.8</td>
<td>0.5</td>
<td>26.9 ± 0.6</td>
<td>33.2 ± 0.5</td>
</tr>
</tbody>
</table>

Responses values for total sugars, sulfated sugars, ABTS and OH⁻ are expressed as mean ± standard deviation.
and 5 g of dry seaweed in 50 mL of deionized water) was used for optimization (Box and Behnken 1960). The range of values for the three independent variables, presented in Table 1, were based on the results of preliminary experiments (data not shown); moreover, in the case of extraction pressure the full possible range enabled by the equipment was used in order to enhance the maximum rupture of seaweed cell walls. Extraction yield, total sugars, total sulfated sugars, total antioxidant capacity and hydroxyl-radical scavenging activity were selected as the response for the combination of the independent variables. The response surface design consisted in 17 runs in randomized order, to minimize the effects of unexpected variability in the observed responses, with five replicates in the center point to estimate the pure error sum of squares (Table 1). The three variables were designated as $X_1$, $X_2$, $X_3$ and prescribed into three levels, coded $+1$, 0, $-1$ for high, intermediate and low values, respectively. A full quadratic model was used to fit the data according to Eq. (1):

$$Y = \beta_0 + \sum_{i=1}^{3} \beta_i X_i + \sum_{i=1}^{3} \sum_{j=1}^{3} \beta_{ij} X_i X_j$$

(1)

where $Y$ is the predicted response, $\beta_0$, $\beta_i$, $\beta_{ij}$ are coefficients in the intercept, linear, quadratic and interaction terms, respectively. $X_i$ and $X_j$ are the independent variables.

Analysis of variance (ANOVA) for response surface quadratic model validation was performed, and the test for significance of each term to test for goodness of fit was conducted at $P < 0.05$.

### Yield and Determination of Total and Sulfated Sugars Content

The extraction yield was calculated as weight percentage of lyophilized extract ($M$) to the dried seaweed submitted to extraction ($W$) as given in Eq. (2):

$$\text{Extraction yield} \% = \frac{M}{W} \times 100$$

(2)

Total sugars content was determined by the phenol-H$_2$SO$_4$ method (Dubois et al. 1956) with modifications using glucose (0–200 µg/mL) as a standard; 400 µL of extract solution (2 mg/mL) was mixed with an equivalent amount of 5% phenol solution and 2 mL of concentrated H$_2$SO$_4$. After homogenization, the mixture was kept for 30 min at room temperature. Absorbance was measured at 490 nm. The content of sulfate groups was determined by turbidity through the barium chloride–gelatin method (Dodgson 1961) using Na$_2$SO$_4$ as a standard (0–200 µg/mL).

### Total Antioxidant Capacity

Total antioxidant capacity of extracts’ solutions was measured according to the method described by Gião et al. (2007). This method is able to quantify both water and lipid-soluble antioxidants, as pure compounds or in crude extracts via direct production of the ABTS$^+$ chromophore (blue/green) by reaction of ABTS and potassium persulfate. To 2 mL of diluted ABTS$^+$ solution it was added 120 µL of extract solution (2 mg lyophilized solids/mL) and absorbance at 734 nm was measured ($A_{\text{sample}}$). Three replicates were performed using ascorbic acid as standard (0–100 µg/mL), and the results were expressed as equivalent concentration of ascorbic acid ($\mu$g ascorbic acid equiv/mL). The percentage of scavenging activity was also determined using the following Eq. (3):

$$\text{Scavenging} \% = \left(1 - \frac{A_{\text{sample}}}{A_{\text{ABTS}+}} \right) \times 100$$

(3)

For each sample the initial absorbance of 2 mL of diluted ABTS$^+$ was measured ($A_{\text{ABTS}+}$).

### Hydroxyl-Radical Scavenging Activity

Hydroxyl-radical (OH·) scavenging activity was measured according to the method described by Sudha et al. (2011) based on Smirnoff and Cumbes (1989). An aliquot (1 mL) of each extract (2 mg lyophilized solids/mL) was added to 2 mL of reaction mixture containing 1 mL of 1.5 mM FeSO$_4$, 0.7 mL of 6 mM hydrogen peroxide and 0.3 mL of 20 mM of sodium salicylate. After incubation for 1 h at 37°C, the absorbance of the hydroxylated salicylate complex was measured at 562 nm ($A_{\text{sample}}$). Three replicates were performed using ascorbic acid as standard (0–100 µg/mL), and results were expressed as equivalent concentration of ascorbic acid equivalent ($\mu$g ascorbic acid equiv/mL) whereas the percentage of scavenging activity of hydroxyl radical was also calculated using the following Eq. (4):

$$\text{Scavenging} \% = \left(1 - \frac{A_{\text{sample}}}{A_{\text{control}}} \right) \times 100$$

(4)

For each sample, the absorbance of 2 mL reaction mixture plus 1 mL of deionized water was measured as the control ($A_{\text{control}}$) whereas 2 mL of reaction mixture, with sodium salicylate substituted by water plus 1 mL of extract was measured as blank ($A_{\text{blank}}$).

### RESULTS AND DISCUSSION

#### Overview of the Efficiency of HHP-Assisted Extraction of *S. muticum*

Table 1 shows the extraction yield, total sugar and total sulfated sugar contents, and antioxidant activity (total
antioxidant capacity and hydroxyl-radical scavenging activity) of *S. muticum* extracts prepared under HHP extraction according to Box-Behnken experimental design. Yield values of the extracts ranged between 32 and 40.4 g/100 g dry seaweed independently of the extraction conditions or seaweed solid/liquid ratio used in the extraction. These values represent a higher yield (i.e., an increment between 36 and 72%) than the *S. muticum* extracts obtained by hot water assisted extraction (HWE) or by ultrasound-assisted extraction (UEA) (23.5–24.0%; Rodrigues et al. 2015a). These results demonstrated that high hydrostatic pressure improved overall extraction efficiency from *S. muticum*. According to Briones-Labarca et al. (2015) higher yields were obtained with HHP-assisted extraction from Chilean papaya than UAE and conventional extractions (CE) with organic solvents. Specifically, *S. muticum* extracts from HHP treatment showed a significantly higher level of total sugar and sulfated sugar contents; 142–173 mg/glyophilized extract of total sugars were obtained representing a 3.8 to 4.6-fold in comparison to HWE or to UAE of *S. muticum* (37.8–41 mg/glyophilized extract; Rodrigues et al. 2015a). In terms of sulfated sugars a similar trend was observed; 34–46 mg/glyophilized extract were obtained representing an increment of 3.6 to 4.8-fold in comparison to HWE or to UAE of *S. muticum* (9.5–10.1 mg/glyophilized extract; Rodrigues et al. 2015a). Sulfated polysaccharides in brown algae are mainly fucans comprising families of polydisperse molecules based on sulfated L-fucose, whereas heterofucans are designated fucoidans (Freitas et al. 2015; Rodrigues et al. 2015b). To our knowledge, this is the first study applying HHP in preparation of *S. muticum* extracts and there are no published reports regarding the application of this technology to seaweeds so any comparative discussion can only be done in relation to other studies that also employed natural sources including medicinal plants, fruits or mushrooms. For example, Prasad et al. (2010) evaluated the effects of ultra-high-pressure-assisted extraction on longan fruit pericarp at 200, 300, 400 and 500 MPa; highest extraction yields with high phenolic contents and highest antioxidant activities were achieved with 500 MPa. In turn, Vega-Gálvez et al. (2014) evaluated the effects of HHP at 300, 400 and 500 MPa through 1, 3 and 5 min on nutritional and antioxidant properties of cape gooseberry pulp after the HHP treatment and after 60 days of storage. Maximum values of total phenolic content and antioxidant activity were observed at 500 MPa for 5 min whereas after 60 days of storage the treatments above 300 MPa for 5 min resulted in higher antioxidant capacity indicating the effectiveness of HHP treatments for the production of functional compounds based on gooseberry pulp.

An unexpected result was that reported for evaluation of total antioxidant activity. Percentages of inhibition of the ABTS radical oscillated between 26 and 33% whereas for the hydroxyl-radical scavenging activity ranged between 31 and 38%, independently of the extraction conditions or seaweed solid/liquid ratio used in the extraction process (Table 1). Surprisingly, these values are lower than those observed for HWE and UAE extracts from *S. muticum* (Rodrigues et al. 2015a). According to several authors, application of high extraction temperatures and pressures used in pressurized liquid extraction did not increase antioxidant activities for several seaweeds extracts and species in comparison to solid/liquid extraction (Tierney et al. 2013; Heffernan et al. 2014). Since a higher concentration of sulfated sugars was observed in HHP-assisted extracts higher antioxidant activities were likewise expected. Despite this nonrelationship, the results in Table 1 imply that the activity of *S. muticum* extracts still have an important scavenging power on hydroxyl radicals. According to Wijesekara et al. (2011) sulfated polysaccharides not only function as dietary fiber but also contribute to antioxidant activity of seaweeds; heterofucans from *Sargassum filipendula* displayed considerable antioxidant activity expressed as 90.7 ascorbic acid equivalent (Costa et al. 2011). A significant correlation between sulfate content and antioxidant activities was observed in different fractions of the brown *Saccharina latissima* (Jiménez-Escrig et al. 2015).

**Model Fitting for HHP-Assisted Extraction**

The results of the 17 experiments including five replicates at the center point generated from three important parameters, extraction time (X₁), extraction pressure (X₂) and seaweed solid/liquid ratio (X₃) in the Box-Behnken design are listed in Table 1 along with the predicted values, within the limits of the experimental factors, by the response surface regression. The second order quadratic models expressed as a function of time, pressure and seaweed solid/liquid ratio are displayed in Tables (2–4) for each measured response, namely, extraction yield, total sugar content, total sulfated sugars content, total antioxidant capacity and hydroxyl-radical scavenging activity. The goodness of fit of the models can be checked by the coefficients $R^2$ and adj $R^2$. $R^2$ values close to 1.0 implies better accuracy of the model; however, according to Garai and Kumar (2013) the incorporation of a large number of insignificant variables in the model may result in high $R^2$ value, but the model may not be able to predict adequate responses. Therefore, the term adj $R^2$ (which corrects $R^2$) should also be considered and must be ideally close to $R^2$. The predictive model for extraction yield, total sugar and total sulfated sugar contents, total antioxidant capacity and hydroxyl-radical scavenging activity had a coefficient $R^2$ of 0.996, 0.999, 0.998, 0.990 and 0.943, respectively (Tables (2–4)). High values of $R^2$ with close values of adj $R^2$, were observed except for hydroxyl-radical scavenging activity (Tables (2–4)), evidenced good correlation between experimental and predicted values with more than 94%
variability of the responses explained by the model. Statistical significance and adequacy of the model was tested by analysis of variance and results are shown in Tables (2–4). All quadratic models used in the experiments were found to be highly significant, as evident from Fisher’s F-test with high values of calculated F values and very low probability values ($P \leq 0.001$). In addition lack of fit was found to be nonsignificant ($P > 0.05$) suggesting that the model equation for each measured response was adequate to predict the respective values under any sets of combination within the range of experimental variables (Tables (2–4)).

**Effect of Time, Pressure and Seaweed Solid/Liquid Ratio on the S. muticum Extracts Analyzed Responses and Optima Values**

Extraction yield was significantly affected ($P < 0.05$) by pressure ($X_2$) and seaweed solid/liquid ratio ($X_3$), interaction effect of time and seaweed solid/liquid ratio ($X_1X_3$) as well of pressure and seaweed solid/liquid ratio ($X_2X_3$) (Table 2). Results showed that the extraction yield ranged from 32 to 40 g/100 g dry seaweed and the highest values (40 g/100 g DW) occurred at 5 min ($X_1$), 450 MPa ($X_2$), 1 g ($X_3$) and at 17.5 min ($X_1$) 300 MPa ($X_2$), 1 g ($X_3$). The 3D response surface plots and 2D contour plots are the graphical representations of regression model, helping to understand the main and the interaction effects between the independent variables (measured responses). The 3D response surfaces for extraction yield are shown in Fig. 1a–c, respectively. Extraction yield decreased with increased seaweed solid/liquid ratio (Fig. 1b,c; also reflected in the negative value of the $\beta$-coefficient value of the linear term) which could be due to the higher ratio of solvent in relation to seaweed mass. No clear effect is visualized for time and pressure, although this last parameter was statistically significant ($P = 0.021$). Indeed, pressure was expected to be one of the most significant extraction factors affecting response variables as reported by other authors in plant extractions (Xi and Wang 2013), due to its capacity to destroy cell structures and membranes which greatly facilitates mass transfer of solvents into raw materials and the soluble constituents into the solvents (Huang et al. 2013). However, this behavior will depend on the type of cells; 490 MPa and a liquid/solid ratio of 20 mL/g were able to extract maximum phenolic content from green tea (Xi and Wang 2013). According to Heffernan et al. (2014), pressurized liquid extraction was not in general responsible for higher extraction yields for several seaweeds species in comparison to solid/liquid extraction. The pattern of Fig. 1b,c are commonly known as stationary ridge pattern, since the extraction yield did not changed considerably with extraction time (Fig. 1b) and with the extraction pressure (Fig. 1c). The maximum predicted extraction yield could be achieved with the following HHP conditions: extraction time ($X_1$), 5.5 min; extraction pressure ($X_2$), 300 MPa; and seaweed solid/liquid ratio ($X_3$):1 g/50 mL (Table 5). Under these optima conditions the predictive response for extraction yield was of 40.9 g/100 g dry seaweed. The use of HHP assisted extraction has shown great advantages in terms of extraction yields; higher extraction yields with high phenolic contents was reported by Prasad et al. (2010) in comparison to conventional extraction (CE) techniques. Shorter extraction times and higher extraction yields of bioactive compounds are reported by Briones-Labarca et al. (2015) by HHP assisted extraction than by UAE and CE.

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**TABLE 2. ANALYSIS OF VARIANCE (ANOVA) FOR THE RESPONSE SURFACE QUADRATIC MODEL FOR EXTRACTION YIELD OF HHP S. MUTICUM EXTRACTS AS A FUNCTION OF THE INDEPENDENT VARIABLES**

<table>
<thead>
<tr>
<th>Source</th>
<th>Coefficients</th>
<th>Standard errors</th>
<th>$T$ value</th>
<th>$R^2$</th>
<th>Adj $R^2$</th>
<th>Degree of freedom</th>
<th>Mean square</th>
<th>$F$ value</th>
<th>$P$ value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Model</td>
<td>46.6789</td>
<td>1.43713</td>
<td>32.481</td>
<td>99.55%</td>
<td>98.97%</td>
<td>9</td>
<td>11.8093</td>
<td>171.99</td>
<td>0.000</td>
</tr>
<tr>
<td>$X_1$</td>
<td>0.0433</td>
<td>0.04593</td>
<td>0.943</td>
<td></td>
<td></td>
<td>1</td>
<td>0.0611</td>
<td>0.89</td>
<td>0.377</td>
</tr>
<tr>
<td>$X_2$</td>
<td>-0.0162</td>
<td>0.00545</td>
<td>-2.966</td>
<td></td>
<td></td>
<td>1</td>
<td>0.6039</td>
<td>8.80</td>
<td>0.021</td>
</tr>
<tr>
<td>$X_3$</td>
<td>-2.8272</td>
<td>0.29304</td>
<td>-9.648</td>
<td></td>
<td></td>
<td>1</td>
<td>6.3912</td>
<td>93.08</td>
<td>0.000</td>
</tr>
<tr>
<td>$X_1^2$</td>
<td>-0.0016</td>
<td>0.00082</td>
<td>-1.937</td>
<td></td>
<td></td>
<td>1</td>
<td>0.2577</td>
<td>3.75</td>
<td>0.094</td>
</tr>
<tr>
<td>$X_2^2$</td>
<td>0.0000</td>
<td>0.00001</td>
<td>2.195</td>
<td></td>
<td></td>
<td>1</td>
<td>0.3307</td>
<td>4.82</td>
<td>0.064</td>
</tr>
<tr>
<td>$XX_1$</td>
<td>-0.0745</td>
<td>0.03193</td>
<td>-2.335</td>
<td></td>
<td></td>
<td>1</td>
<td>0.3742</td>
<td>5.45</td>
<td>0.052</td>
</tr>
<tr>
<td>$XX_2$</td>
<td>-0.0002</td>
<td>0.00007</td>
<td>-2.168</td>
<td></td>
<td></td>
<td>1</td>
<td>0.3229</td>
<td>4.70</td>
<td>0.067</td>
</tr>
<tr>
<td>$XX_3$</td>
<td>0.0195</td>
<td>0.00524</td>
<td>3.729</td>
<td></td>
<td></td>
<td>1</td>
<td>0.9547</td>
<td>13.90</td>
<td>0.007</td>
</tr>
<tr>
<td>$X_1X_2$</td>
<td>0.0026</td>
<td>0.00044</td>
<td>5.876</td>
<td></td>
<td></td>
<td>1</td>
<td>2.3707</td>
<td>34.53</td>
<td>0.001</td>
</tr>
<tr>
<td>Residual</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>7</td>
<td>0.0688</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lack of fit</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>3</td>
<td>0.0782</td>
<td>1.27</td>
<td>0.397</td>
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<tr>
<td>Pure error</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>4</td>
<td>0.0615</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>16</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

$X_1$, time (min); $X_2$, pressure (MPa); $X_3$, mass (g).
For total sugars, all linear, quadratic and interaction effects were significantly affected ($P < 0.05$), except for the quadratic term of seaweed solid/liquid ratio ($X_3^2$). The response surface plots in Fig. 2a–c show that total sugar content increased with increased pressure and time (in agreement with the positive values of the corresponding linear terms). The increase of total sugar content ($>170 \text{ mgtotal sugar/mglyophilized extract}$) is mostly evident with the variable pressure (Fig. 2a,c) for values higher than 450 MPa. The influence of seaweed solid/liquid ratio is more complex and dependent of the independent variables; in Fig. 2b the amount of total sugars fluctuated with time but decrease with seaweed solid/liquid ratio whereas in Fig. 2c an increase is observable for both seaweed solid/liquid ratio and pressure. It is clear that significant interactions occurred and influenced the amount of total sugars extracted. According to the model, the maximum predicted total sugar content could be achieved with the following HHP conditions: 

- Extraction time ($X_1$), 5.0 min; 
- Extraction pressure ($X_2$), 600 MPa; and 
- Seaweed solid/liquid ratio ($X_3$), 1 g/50 mL (Table 5). Under these optima conditions the predictive response for yield extraction was of 181.5 mg glucose equiv/g glyoph extract.

The amount of sulfated sugars was significantly affected ($P < 0.05$) by extraction time ($X_1$), seaweed solid/liquid ratio, quadratic terms of extraction time and pressure ($X_2^1$, $X_2^2$) and all interactions. No significant influence by the extraction pressure was observed with highest values occurring at 300 MPa (Fig. 2d,f). The lowest pressure and lowest extraction time was enough to extract the maximum of sulfated sugars which is in accordance to the maximum predicted values at extraction time ($X_1$), 5.0 min; extraction pressure ($X_2$), 300 MPa; and seaweed solid/liquid ratio ($X_3$), 1 g/50 mL (Table 5). Under these optima conditions the predictive response for extraction yield was of 48.8 mg Na$_2$SO$_4$ acid equiv/g glyoph extract. A pressure level of 300 MPa seems to be enough to cause rupture of *S. muticum* cells extracting...
soluble compounds such as sulfated sugars enabling maximum extraction yield; this happens when compression level exceeds the deformation limit of the cells leading for example to formation of cracks (Huang et al. 2013). According to Kim et al. (2014), HHP assisted extraction with 100–300 MPa was not able to increase recovery of solids.
polyphenols, total sugars and reducing sugars in the extracts of cactus cladodes (Opuntia humifusa Raf.). Pressurized liquid extraction for the majority of solvent combinations was not able to increase phenolic contents for four seaweed species (Heffernan et al. 2014).

In terms of total antioxidant capacity all linear, quadratic and interaction effects were significantly affected ($P < 0.05$) with exception of the quadratic term of extraction time ($X_2^2$) and interaction of extraction time and seaweed solid/liquid ratio. Results showed that the total antioxidant capacity values ranged from 26 to 33% of scavenging, decreasing with increased seaweed solid/liquid ratio (Fig. 3b,c) and varying with extraction pressure and time. The maximum values for total antioxidant capacity was observed under the experimental conditions of 5 min ($X_1$), 450 MPa ($X_2$), 1 g ($X_3$) and 17.5 min ($X_1$) 300 MPa ($X_2$), 1 g ($X_3$) which are the same conditions as those that enabled achievement of the highest extraction yield values. Similar optima values as those for extraction yield and sulfated sugars were predicted by the model, i.e., extraction time ($X_1$), 5.0 min; extraction pressure ($X_2$), 300 MPa; and seaweed solid/liquid ratio ($X_3$), 1 g/50 mL (Table 5). Under these optima conditions the predictive response for total antioxidant capacity was of 34.3% of scavenging.

Hydroxyl radical scavenging activity was significantly affected ($P < 0.05$) by pressure ($X_2$) and seaweed solid/liquid ratio ($X_3$) as well as by the quadratic terms of time, pressure and solid/liquid ratio and also by the interaction effect of extraction time and pressure. In all 3D response surface plots (Fig. 3d,f) the interaction effects between the independent variables are visible evidenced by the fluctuation of hydroxyl radical scavenging activity along the different axis; Higher values of hydroxyl radical scavenging activity were observed between 3 and 4 g/50 mL, pressure higher than 450

![FIG. 2. RESPONSE SURFACE AND CONTOUR PLOTS SHOWING THE EFFECT OF TWO INDEPENDENT VARIABLES](a, d) Pressure and time, (b, e) seaweed solid/liquid ratio and time and (c, f) seaweed solid/liquid ratio and pressure (a–c) on the total sugars and (d–f) on total sulfated sugars for the HHP extraction of S. muticum.)

### Table 5. Optima Extraction Conditions and Predictive Values for Each Measured Response

<table>
<thead>
<tr>
<th>Responses</th>
<th>$X_1$ (time, min)</th>
<th>$X_2$ (pressure, MPa)</th>
<th>$X_3$ (mass, g/50 mL)</th>
<th>Predictive value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yield (g/100 g dry seaweed)</td>
<td>5.5</td>
<td>300</td>
<td>1</td>
<td>40.9</td>
</tr>
<tr>
<td>Total sugar (mg glucose equiv/glucose extract)</td>
<td>5</td>
<td>600</td>
<td>1</td>
<td>181.5</td>
</tr>
<tr>
<td>Total sulfated sugars (mg Na₂SO₄ acid equiv/glucose extract)</td>
<td>5</td>
<td>300</td>
<td>1</td>
<td>48.8</td>
</tr>
<tr>
<td>Total antioxidant capacity (% scavenging)</td>
<td>5</td>
<td>300</td>
<td>1</td>
<td>34.3</td>
</tr>
<tr>
<td>Hydroxyl radical scavenging activity (% scavenging)</td>
<td>29</td>
<td>552</td>
<td>3.7</td>
<td>39.2</td>
</tr>
</tbody>
</table>
MPa and a longer period of extraction time (>20 min.)
According to the model, the maximum predicted hydroxyl
radical scavenging activity could be achieved with the fol-
lowing HHP conditions: extraction time \( (X_1) \), 29.0 min;
extraction pressure \( (X_2) \), 552 MPa; and seaweed solid/liquid
ratio \( (X_3) \), 3.66 g/50 mL (Table 5). Under these optima condi-
tions the predictive response for hydroxyl radical scaveng-
ing activity was of 39.2 percentage of scavenging.

CONCLUSION

In this study, response surface methodology was applied for
optimizing high hydrostatic pressure (HHP) assisted extrac-
tion of bioactive polysaccharides from \( S. \ muticum \). This
technology improved extractability (higher extraction
yields) and bioactivity from brown seaweeds such as \( S. \ muticum \)
providing extracts concentrated in polysaccharides
which can be explored as an ingredient source in functional
foods.

The maximum values of all responses were determined
under different optimum conditions of HHP processing:
5–5.5 min, 300 MPa and 1 g/50 mL of dry seaweed for
extraction yield, sulfated sugar content and total antioxi-
dant capacity; same conditions but with 600 MPa for total
sugar content and 29 min, 552 MPa and 3.7 g of dry
seaweed for hydroxyl-radical scavenging activity. Overall,
the results demonstrated that the minimum processing
tested values for extraction time, extraction pressure and
seaweed solid/liquid ratio are a set of experimental condi-
tions that enable achieving maximum values for three of
the important measured responses namely higher extrac-
tion yields, sulfated sugar content and total antioxidant
capacity (well correlated between each other) which dem-
onstrate the potential efficiency of HHP to extract comp-
ounds from brown seaweeds such as \( S. \ muticum \), an
invasive seaweed, in a few minutes. The experimental con-
ditions allow a fast and cost-saving process in extraction of
bioactive polysaccharides. Further studies on the chemical
structures and other biological functions of polysaccharides
are in progress for further incorporation into functional
food development.

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FIG. 3. RESPONSE SURFACE AND CONTOUR PLOTS SHOWING THE EFFECT OF TWO INDEPENDENT VARIABLES
(a, d) Pressure and time, (b, e) seaweed solid/liquid ratio and time and (c, f) seaweed solid/liquid ratio and pressure (a–c) on the total antioxidant
capacity and (d–f) on hydroxyl radical scavenging activity for the HHP extraction of \( S. \ muticum \).
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