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In vitro inhibition of starch digestive enzymes by ultrasound-assisted extracted polyphenols from *Ascophyllum nodosum* seaweeds

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Abstract: Seaweeds are gaining importance due to their antidiabetic characteristics. This study investigated the inhibitory effects of aqueous *Ascophyllum nodosum* extracts, obtained by ultrasound-assisted extraction with different sonication powers (70–90 W/cm^2) and subjected to resin purification, against α-amylase and α-glucosidase enzymes. Different inhibition methodologies were carried out, preincubating the extract either with the enzyme or the substrate. Chemical characterization, in terms of proximate analysis, antioxidant capacity (2,2-diphenyl-1-picryl-hydrazyl-hydrate [DPPH] and FRAP), and polyphenols characteristics (reversed-phase high-performance liquid chromatography [RP-HPLC] and ^{1}H-NMR) were carried out to explain inhibitory activities of extracts. Sonication power did not influence the proximal composition nor antiradical activity of extracts, but increasing sonication power increased inhibition capacity (>15%) against both starch digestive enzymes. The extract purification largely improved the inhibition efficiency decreasing the IC_{50} of α-amylase and α-glucosidase by 3.0 and 6.1 times, respectively. Seaweed extracts showed greater inhibition effect when they were preincubated with the enzyme instead of the substrate. RP-HPLC together with ^{1}H-NMR spectra allowed relating the presence of uronic acids–polyphenols complexes and quinones in the extracts with the different inhibitory capacities of samples.

KEYWORDS
α-amylase, α-glucosidase, gelled starch, HPLC, ^{1}H-NMR, wheat

Practical Application: The study confirms that ultrasound-assisted extracts from *Ascophyllum nodosum* can be used to inhibit digestive enzymes. This opens the alternative to be used in foods for modulating glycemic index.

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1 | INTRODUCTION

In the last decade, carbohydrates digestion has attracted much attention due to its direct relationship with postprandial glycemic response, which in turn has been associated with an increased risk of developing metabolic diseases (Dall'Asta et al., 2020). Digestive carbohydrates are mainly present in human diets as starch, sucrose, free glucose fructose, and oligosaccharides. One strategy to manage high blood glucose levels is the inhibition of α-amylase (AM) and α-glucosidase (AG), digestive enzymes involved in the breakdown of starch and disaccharides to glucose (Ríos et al., 2015).

Some antidiabetic drugs such as acarbose are both α-amylase and α-glucosidase enzymes inhibitors and allow the control of postprandial glucose in diabetic patients (Zhao et al., 2021). However, current research is focused on discovering natural inhibitors of α-amylase and α-glucosidase enzymes involving plant- and seaweed-derived compounds such as polyphenols because of their health beneficial features (Kan et al., 2020).

Polyphenols are secondary metabolites from plants, lichens, or seaweeds (Koivikko et al., 2007). The potential industrial use of such polyphenols depends on both the concentration and the availability of the biomass, and brown seaweeds contain large concentrations of these interesting compounds (Jacobsen et al., 2019). Particularly, the commercial interest of brown macroalgae is well-known, especially for species such as *Ascophyllum nodosum* or *Fucus vesiculosus*, mainly used for extracting alginate, laminarin, or fertilizers production (Audibert et al., 2010). Likewise, *A. nodosum* is being considered as a valuable source of polyphenols to be used in drugs and the food industry (Leandro et al., 2020). The composition and polyphenols features of *A. nodosum* extracts were recently determined employing liquid chromatography-mass spectroscopic techniques evidenced the presence of high structural diversity of phlorotannins (Allwood et al., 2020). Those authors confirmed the presence of dibenzodioxin linked phlorotannins, sulphated phlorotannins, and phenolic acids. Moreover, Apostolidis and Lee (2010) showed the pharmaceutical potentiality of *A. nodosum* against hyperglycemia.

Nevertheless, the extraction procedures and conditions employed to produce polyphenols extracts strongly affect their inhibition capacity against α-amylase and α-glucosidase (Alu'datt et al., 2017). Ultrasound-assisted extraction (UAE) is an efficient method for polyphenols extraction, since it is a low-cost equipment and an easy-procedure methodology (Kadam et al., 2015), without affecting heat-sensitive polyphenols (Moreira et al., 2016). Usually, aqueous mixtures of methanol, ethanol, acetone, acids among others are employed (Koivikko et al., 2005; Liu et al., 2016). Following the main principle of green chemistry (EPA, 2012), replacement of organo-solvents by water as unique extraction solvent is promoted; hence, it has been demonstrated that water is efficient for polyphenols extraction (Leyton et al., 2016) and additionally its use reduces subsequent purification stages and waste residue generation. In fact, several authors have found relevant phytochemical features of aqueous extracts from *A. nodosum* seaweed employing UAE technology (Kadam et al., 2015). Considering the efficiency of this technique, initial hypothesis is that aqueous polyphenolic extracts obtained *A. nodosum* through UAE might have prominent inhibition capacities against digestive enzymes.

In a previous work (Gisbert et al., 2021), it was found that aqueous UAE for only 2 min employing high sonication powers (>70 W/cm^2) was effective for the extraction of polyphenols, but secondary compounds (mainly, carbohydrates and, specifically, uronic acids [UA]) were also co-extracted diminishing the selectivity. Thus, it is convenient to determine the chemical characterization of these raw extracts and to evaluate the effect of additional purification operations on the bioactive activities and inhibition capacities after the secondary compounds removal, resulting enriched extracts (Gonçalves-Fernández et al., 2019). The bioactivity and inhibitory characterization of raw and purified extracts will extend their further food applications.

The aims of this study were to analyze and identify the in vitro inhibitory effect against digestive enzymes of different aqueous polyphenols extracts from *A. nodosum* using UAE and the effect on inhibition after secondary compounds removal employing resin purification. For these purposes, seaweeds extracts obtained under different sonication conditions and purified extracts were chemically characterized determining the proximate composition and the corresponding polyphenols features by means of RP-HPLC and ^{1}H-NMR techniques. To understand the inhibitory mechanism of the extracts against α-amylase or α-glucosidase, studies were conducted adding the polyphenol either to the enzymes or the substrates (starch or maltose, respectively).

2 | MATERIALS AND METHODS

2.1 | Materials

Type VI-B α-amylase from porcine pancreas (EC 3.2.1.1) (8 U/mg), type I α-glucosidase from *Saccharomyces cerevisiae* (EC 3.2.1.20) (11 U/mg), native wheat starch (S5127),

D (+)-maltose, acarbose, 3,5-dinitrosalicylic acid (DNS), Amberlite XAD16 resin (surface area 800 m^2/g, pore diameter 10 nm), phloroglucinol, resorcinol, glucose, and D (+)-glucuronic acid were obtained from Sigma Aldrich (Sigma Chemical, St. Louis, MO, USA). D-Glucose assay kit (GOD/POD) was obtained from Megazyme (K-GLUC 08/18; Megazyme International Ireland Ltd., Bray, Co. Wicklow, Ireland). All chemical reagents used were of analytical grade.

2.2 | Seaweed sampling

Fresh *A. nodosum* seaweed from Galicia's coasts (NW of Spain) harvested on November 2019, supplied by Mar de Ardora S.L. (Ortigueira, Spain), were dried in a hot air convective dryer (Angelantoni, Challenge 250, Massa Martana, Italy) at 50°C, with a constant relative humidity of 30% and air velocity at 2 m/s. Dried algae was ground in an ultra-centrifugal mill (Retsch GmbH, ZM200, Haan, Germany) and sieved using a vibratory sieve with average particle size of 276 ± 8 μm. Milled seaweeds were hydrated for 15 min with double distilled water with a liquid–solid ratio of 20 g of water per g of dried seaweed (g_W/g_{DS}) before sonication. UAE was carried out with a 1000 W ultrasound processor (Hielscher, UIP-1000 hdT, Teltow, Germany) in a jacked chamber cooled by a cold-water bath to maintain blend temperature under 30°C. Continuous UAE operation was performed controlling the solid–liquid dispersion flow with a peristaltic pump (Cole Parmer Masterflex™, Vernon Hills, IL, USA). Extracts were sonicated for 2 min at 70 (E70), 80 (E80), and 90 W/cm^2 (E90) of sonication power, following the conditions previously determined (Gisbert et al., 2021). Solid residue was removed from liquid phase by centrifugation at 12,500 × *g* for 10 min.

E90 sample was further purified in a (2.5 × 47 cm) Bio-Rad column (Bio-Rad, Hercules, CA, USA) filled with Amberlite XAD16 resin. Working flux was set at 110 ml/h using a peristaltic pump. Aqueous extract (100 ml) was poured into the column and washed with 300 ml of distilled water. Then, the column was flushed with 200 ml of ethanol 70% (v/v) to obtain a polyphenols-enriched fraction (P90). All extracts and purified fraction were freeze-dried (FD) at −55°C and 50 Pa with a Lyoquest-55 freeze dryer (Telstar Technologies, Terrassa, Spain).

2.3 | Chemical analysis

Protein and fat content in seaweed extracts were determined by standard AOAC methods (AOAC, 2000). The fat content was determined by Soxhlet extraction and proteins were analyzed with a Elementar rapid N exceed nitrogen analyzer (Elementar, Langenselbold, Germany) using 5.0 as a conversion factor of nitrogen into proteins (Angell et al., 2016). Ash content was analyzed by burning a weighed sample in a muffle furnace at 550°C for 6 h. Data were expressed as percentage on a dry weight (DW). Carbohydrate content (CHOS) was evaluated by the Dubois method (Dubois et al., 1956), and results were given in mg of glucose equivalents per mg of lyophilized extract (mg_{GE}/g_{DW}). Total polyphenol content (TPC) was determined following Folin–Ciocalteau reaction, measured spectrophotometrically at 765 nm, and results expressed in mg of phloroglucinol equivalents per mg of DW extract (g_{PE}/g_{DW}). Uronic acid content (UA) determination was carried out at 520 nm (Blumenkrantz & Asboe-Hansen, 1973), and the contents were given in mg of glucose equivalents per mg of DW extract (g_{GE}/g_{DW}). DPPH scavenging activity (%) was determined at 515 nm (Brand-Williams et al., 1995). The iron cation reduction capacity (FRAP) of the extracts was measured at 593 nm after tested samples incubation in darkness for 30 min, and results were expressed as ug of trolox equivalents per mg of dry weight of DW extract (ug_{TE}/mg_{DW}).

2.4 | Inhibition assays of α-amylase and α-glucosidase

The inhibition assay of α-amylase from porcine pancreas was analyzed following the methodology described by Aleixandre et al. (2021), with minor modifications. Briefly, wheat starch solution (6.25 mg/ml) was prepared in sodium phosphate buffer (0.02 M, pH 6.9 containing 6 mM NaCl), followed by a gelatinization in a water bath at 100°C for 20 min. Reaction media included 50 μg of freeze-dried seaweed extracts dissolved in ethanol (20% v/v) to improve their solubility, 50 μl of α-amylase (50 U/ml), and 400 μl of gelatinized wheat starch. Three methodologies were carried out to evaluate extracts inhibition (Figure 1): (i) ($M1_{AM}$) enzyme and polyphenol solutions were mixed and preincubated in an Eppendorf Thermomixer Compact at 37°C for 10 min at a speed of 600 rpm. Then, gelatinized starch was added, and reaction mixture was incubated at the same conditions. (ii) ($M2_{AM}$) Gelatinized starch and polyphenol extracts were mixed and preincubated at 37°C for 10 min before adding the enzyme, and then the mixture was incubated at 37°C. (iii) ($M3_{AM}$) Starch was gelatinized in the presence of the extract, and then cooled at 25°C for 10 min till reaching 37°C. The enzyme solution was then added, and the mixture was incubated 37°C for 10 min. To stop the reaction in the three methods ($M1_{AM}$, $M2_{AM}$, and $M3_{AM}$), 500 μl of 3,5-dinitrosalicylic acid (DNS) color reagent was added, and the mixture was boiled in a

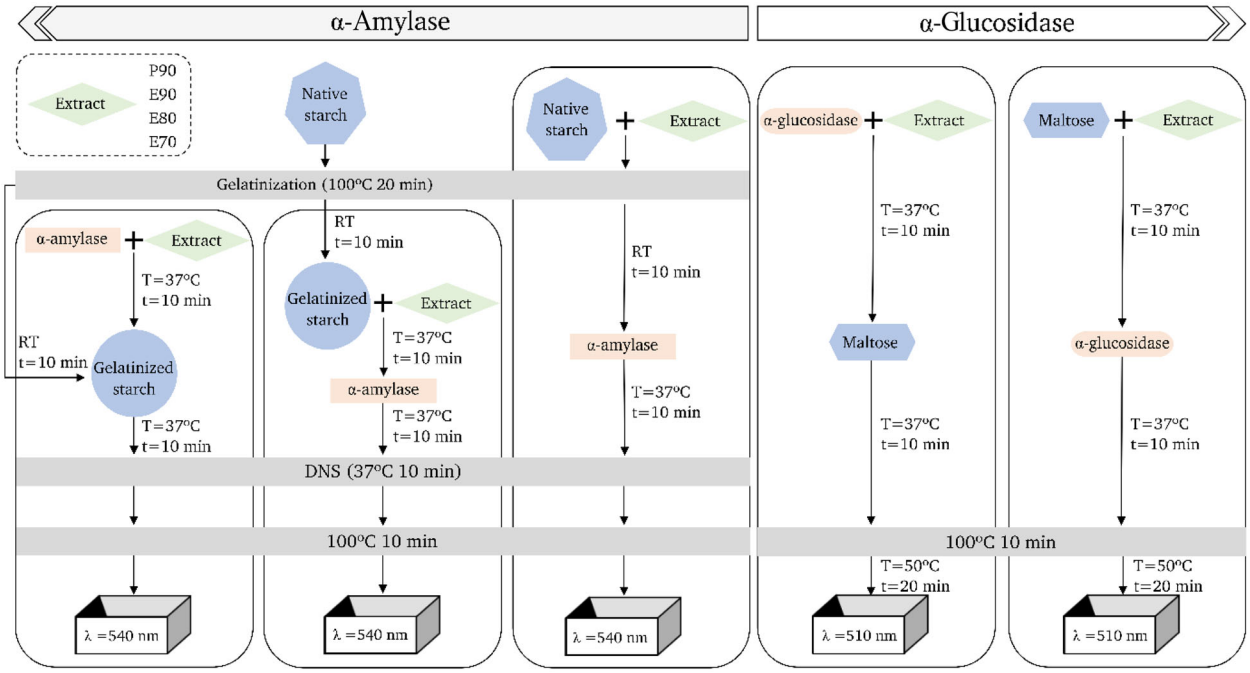

FIGURE 1 Methodology scheme of inhibitory assays (M1, M2, and M3) of α-amylase (AM) and α-glucosidase (AG) digestive enzymes against aqueous polyphenols extracts (E70, E80, E90, and P90) from *A. nodosum* seaweed

water bath for 10 min. Samples were diluted in distilled water (1:10), and their absorbances measured at 540 nm in a microplate reader (Epoch Biotek Instruments, Winooski, VT, USA).

The α-glucosidase from *Saccharomyces cerevisiae* activity was measured using maltose (10 mg/ml) dissolved in sodium phosphate buffer (0.1 M, pH 6.9). Reaction mixture consisted of 50 µl of seaweed extracts, 50 µl of α-glucosidase (10 U/ml), and 400 µl of maltose, but two different methodologies ($M1_{AG}$ and $M2_{AG}$) were tested (Figure 1). In $M1_{AG}$ method, enzyme and polyphenol were initially preincubated at 37°C for 10 min, and then maltose was added to initiate the enzymatic reaction at 37°C. For $M2_{AG}$ methodology, maltose and seaweed extract were initially preincubated at 37°C for 10 min, and the enzyme was then added. In both, $M1_{AG}$ and $M2_{AG}$, enzymatic reaction was stopped by boiling samples in a water bath for 10 min. Absorbance was measured at 510 nm using the above-mentioned GOD/POD kit.

Solutions without seaweed extracts and without enzymes were analyzed as control and blank, respectively. The inhibition rate of seaweed extracts was calculated by Equation 1:

$$\%\,\text{Enzyme inhibition} = \left[1 - \frac{\left(Abs_{\text{sample}} - Abs_{\text{blank sample}}\right)}{Abs_{\text{control}} - Abs_{\text{blank control}}}\right] 100, \quad (1)$$

where Abs_{sample} was the absorbance of sample with substrate and enzyme, $Abs_{\text{blank sample}}$ was the absorbance obtained without enzyme, Abs_{control} was the absorbance without extracts sample, and $Abs_{\text{blank control}}$ was the absorbance of substrate. The IC_{50} value is the sample concentration required for 50% inhibition of the α-amylase or α-glucosidase activity. Acarbose was used as a positive control.

2.5 | Chromatographic separation

P90, E90, E80, and E70 chromatographic profiles were obtained with an HPLC system (Jasco, Tokyo, Japan) equipped with a PU-980 pump, an UV-1575 detector, and a degasser Populaire DP4003. Data were obtained and processed with PowerChrom 2.5 (eDaq Technologies, NZ, Australia) software and MATLAB R2019b software (MathWorks Inc., USA). Chromatographic separation was performed on a Kromasil C-18 semi-preparative column (8 × 250 mm) at 30°C. A gradient elution using water (A) and methanol (B) consisted of 0–3 min (99% A, 1% B), 30 min (5% A, 95% B), and 40 min (99% A, 1% B). Freeze-dried samples (2 mg/ml) were diluted in EtOH 20% (v/v) and filtered through 0.45 µm syringe filters. Injection volume was 50 µl, detection wavelength 266 nm, and solvent flow rate 0.4 ml/min. Polyphenols content was calculated with standard molecules of phloroglucinol and resorcinol; additionally, glucose and glucuronic acid standards were injected to check for possible interference of UA and sugars (Gonçalves-Fernández et al., 2019). To ensure the

reproducibility of the assays, a minimum of four injections of each extract were carried out.

2.6 | ^{1}H-NMR

Proton nuclear magnetic resonance spectroscopy (^{1}H-NMR) spectra were collected with Bruker NEO 750 spectrophotometer operated with a 17.61 T (750 MHz resonance ^{1}H) magnetic field strength. Phenolic extracts were blended with methanol-d_4. ^{1}H-RMN spectra treatment was carried out with MestreNova software (Mestrelab Research, Santiago de Compostela, Spain).

2.7 | Statistical analyses

All measurements were carried out at least in duplicate and presented as mean ± SD. Statistical analysis was carried out by IBM SPSS statistics 24 (SPSS Inc., Chicago, IL, USA) software. A one-way analysis of variance (ANOVA) was assessed based on confidence interval of 95% ($p < 0.05$) using a Duncan test.

3 | RESULTS AND DISCUSSION

3.1 | Seaweed extracts chemical composition

It was previously reported (Gisbert et al., 2021) that aqueous UAE was effective for the extraction of polyphenols, but other compounds like carbohydrates and particularly UA were also co-extracted. Because of that proximate composition, antioxidant activities and UA content were selected to evaluate the impact of UAE and further purification. The moisture, protein, mineral, fat, CHOS, total polyphenol (TPC), UA content, and antioxidant activities (DPPH and FRAP methods) were determined from *A. nodosum* ultrasound-assisted crude (E90, E80 and E70), and purified (P90) extracts (Table 1). Sonication power did not modify significantly ($p > 0.05$) the proximate composition of the extracts, since protein, mineral, and fat content were around 7.47 ± 0.10, 29.75 ± 0.25, and 4.13 ± 0.12 (%, DW), respectively, CHOS (0.10 ± 0.03) and UA (0.20 ± 0.06 mg_{GE}/mg_{DW}) content. TPC (mg_{PE}/mg_{DW}), CHOS (mg_{GE}/mg_{DW}), and UA (mg_{GE}/mg_{DW}) were also invariant with sonication power with values of 0.21 ± 0.04, 0.17 ± 0.02, and 0.17 ± 0.04, respectively. Finally, moisture (%) varied in a narrow range 8.56 ± 0.26 (E80) up to 10.13 ± 0.59 (E70). No significant differences ($p > 0.05$) on antioxidant activities were observed between extracts obtained under different conditions with values of 39.38 ± 1.02% of DPPH scavenging activity and 1.11 ± 0.04 $\mu g_{TE}/mg_{DW}$ of Fe^{2+} reducing power. Kadam et al. (2015) working with *A. nodosum* aqueous extracts obtained from UAE (35.6 W/cm^2 for 15 min) reported slightly lower TPC values (0.16 mg_{PE}/g_{DW}) with a DPPH decay value of 61.46%.

Nevertheless, purification of the extracts significantly decreased protein (0.93-fold), mineral (0.28-fold), fat (0.73-fold), and UA (0.27-fold) content and increased CHOS (1.65-fold) and TPC (2.21-fold) content. Simultaneously, an interestingly relevant increase of DPPH decay (1.29-fold) and FRAP (1.61-fold) content was determined in the purified extract.

According to Wang et al. (2016), polyphenols and UA could be partially forming complexes. The complexation effects on extracts bioactivity are still not elucidated. Hydrogen bridges or hydrophobic interactions (Koivikko et al., 2005) and covalent bonds of ether, ester, and hemiacetal bonds (Salgado et al., 2009) have been proposed. The bonds of these complexes could affect their structural properties and promote or avoid some chemical interactions. However, the presence of these complexes was not discriminated by common TPC and UA analyses and antioxidant activities (DPPH and FRAP; Table 1). Purification step reduced UA content and increased bioactivity of extracts. UA/TPC ratio was employed as a measure of cleaning of the extracts, since CHOs values varied in a narrow range (from 0.11 to 0.17 mg_{GE}/mg_{DW}) after purification. In fact, UA/TPC ratio decreased significantly from 0.96 ± 0.08 (E90, E80 and E70) up to 0.12 ± 0.02 (P90) after purification with Amberlite XAD-16. These results indicated that this resin was an efficient adsorbent for the removal of UA in the extracts, improving bioactive features (TPC, DPPH decay, and FRAP) of purified extract. The reduction of UA/TPC ratio could help to explain further enzymatic inhibitory differences of tested extracts.

3.2 | Inhibition effect of seaweed extracts against α-amylase and α-glucosidase enzymes

A comparative study was conducted to determine the capability of the four different *A. nodosum* extracts (E90, E80, and E70 and purified P90) to inhibit α-amylase and α-glucosidase activity three methods (M1 = preincubation of extract + enzyme, M2 = preincubation of extract + gelatinized starch, and M3 = gelatinization of starch + extract). Figures 2 and 3 show the α-amylase and α-glucosidase inhibition, respectively, induced by the different extracts compared to acarbose, which was taken as standard inhibitor (Table 2). In M1 method (Figures 2a and 3a), increasing the UAE sonication power significantly decreased the IC50 meaning that the inhibition capacity

TABLE 1 Chemical composition of freeze-dried (E90, E80, and E70) and purified (P90) extracts obtained by ultrasound-assisted extraction (UAE) from Ascophyllum nodosum brown edible seaweed

									p-Value	
	P90		**E90**		**E80**		**E70**		**Purification**	**Sonication**
Moisture (%)	16.39 ± 0.12	c	8.78 ± 0.15	a	8.56 ± 0.26	a	10.13 ± 0.59	b	0	0.2383
Protein (%, DW)	6.96 ± 0.30	a	7.48 ± 0.02	b	7.37 ± 0.13	b	7.57 ± 0.11	b	0.0420	0.5649
Mineral (%, DW)	8.19 ± 0.17	a	29.49 ± 0.20	b	29.78 ± 0.09	b	29.99 ± 0.40	b	0	0.2383
Fat (%, DW)	3.02 ± 0.04		4.19 ± 0.04		3.99 ± 0.87		4.20 ± 0.13		0.0577	0.8729
CHOS (mg_{GE}/mg DW)	0.17 ± 0.04	b	0.11 ± 0.02	a	0.10 ± 0.01	a	0.10 ± 0.02	a	0.0198	0.8193
TPC (mg_{PE}/mg DW)	0.42 ± 0.04	b	0.20 ± 0.03	a	0.19 ± 0.03	a	0.18 ± 0.03	a	0.0090	0.9131
UA (mg_{GE}/mg DW)	0.05 ± 0.01	a	0.21 ± 0.04	b	0.17 ± 0.02	b	0.17 ± 0.04	b	0.0205	0.6025
DPPH decay (%)	50.59 ± 2.16	a	39.59 ± 0.56	b	39.97 ± 0.46	b	38.57 ± 0.34	b	0.0041	0.5651
FRAP (μg $_{TE}$/mg DW)	1.79 ± 0.05	a	1.11 ± 0.01	b	1.10 ± 0.11	b	1.12 ± 0.04	b	0.0017	0.9759

Note; Means within a raw followed with different letter are significantly different ($p < 0.05$).
Abbreviations: CHOS, Carbohydrate; DW, dry weight; TPC, total polyphenol content; UV, uronic acid.

TABLE 2 IC_{50} values of seaweed extracts against α-amylase and α-glucosidase of the different analyzed methodologies: M1 = preincubation of extract + enzyme, M2 = preincubation of extract + gelatinized starch, M3 = gelatinization of starch with extract

	IC_{50} against α-amylase (AM) (μg/ml)						**IC_{50} against α-glucosidase (AG) (μg/ml)**			
	$M1_{AM}$		**$M2_{AM}$**		**$M3_{AM}$**		**$M1_{AG}$**		**$M2_{AG}$**	
Acarbose	11.51 ± 0.63	a	56.07 ± 3.00	a	142.82 ± 12.09	a	0.16 ± 0.01		0.13 ± 0.01	
P90	40.03 ± 1.11	b	74.03 ± 2.58	b	810.48 ± 24.35	b	3.20 ± 0.14	a	3.62 ± 0.23	a
E90	119.55 ± 0.73	c	309.16 ± 4.13	c	4066.32 ± 50.09	c	19.49 ± 0.75	b	26.15 ± 0.76	b
E80	128.81 ± 2.91	d	342.59 ± 0.69	d	4451.00 ± 49.18	d	20.26 ± 0.32	b	28.09 ± 0.04	c
E70	152.97 ± 4.35	e	439.82 ± 9.83	e	4777.66 ± 42.53	e	29.19 ± 0.37	c	30.36 ± 0.70	d
p-Value										
Purification	0.0001		0.0001		0.0001		0.0001		0.0001	
Sonication	0.0001		0.0001		0.0003		0.0001		0.0025	

Note: Means within a column followed with different letter are significantly different ($p < 0.05$).

of the extracts increased (up to 35% and 15% for α-amylase and α-glucosidase), despite extracts showed similar TPC and DPPH values. The extract purification with Amberlite (P90) largely improved the inhibition efficiency decreasing the IC_{50} of α-amylase and α-glucosidase by 3.0 and 6.1 times, respectively. This result might be explained by its higher TPC (Table 1) compared with those measured in the extracts. Comparing the effect of tested extracts, higher extract concentrations were required to inhibit α-amylase compared to α-glucosidase. In relation to acarbose, a specific competitive inhibitor, the results were lower; IC_{50} values (40.0 and 3.2 μg/ml) of P90 were four times higher than that of acarbose on α-amylase and 20 times higher for α-glucosidase (Table 2). These results support the findings described by Apostolidis and Lee (2010) when analyzing an aqueous extract from *A. nodosum*, observing higher inhibition activity against yeast α-glucosidase than against porcine pancreas α-amylase. Conversely, Pantidos et al. (2014) found that a tannin-rich fraction of *A. nodosum* obtained with different solvents was more effective inhibiting porcine pancreas α-amylase than rat intestinal α-glucosidase. Divergences might be ascribed to the different composition of the extracts, which depends on the extraction method. J. Zhang et al. (2007) determined an IC_{50} = 77 μg/ml against α-glucosidase working with water-ethanol *A. nodosum* extracts. Although, Liu et al. (2016) determined IC_{50} from 8.9 to 36.3 μg/ml against α-glucosidase from ethanolic extracts of *A. nodosum* in the same range than those found in this work. Nevertheless, discussion of results based on previously published data becomes complicated due to the wide range of experimental conditions used which makes it very difficult to compare IC_{50} values, which are specific for the enzyme type, the substrate used, and the reaction conditions.

Given the influence of the procedure carried out to analyze enzyme inhibition, besides the sonication power during extraction and purification step, two additional methodologies (M2 and M3), were used to identify the effect of polyphenols interaction with the enzymes on the level of inhibition. The most effective inhibition was

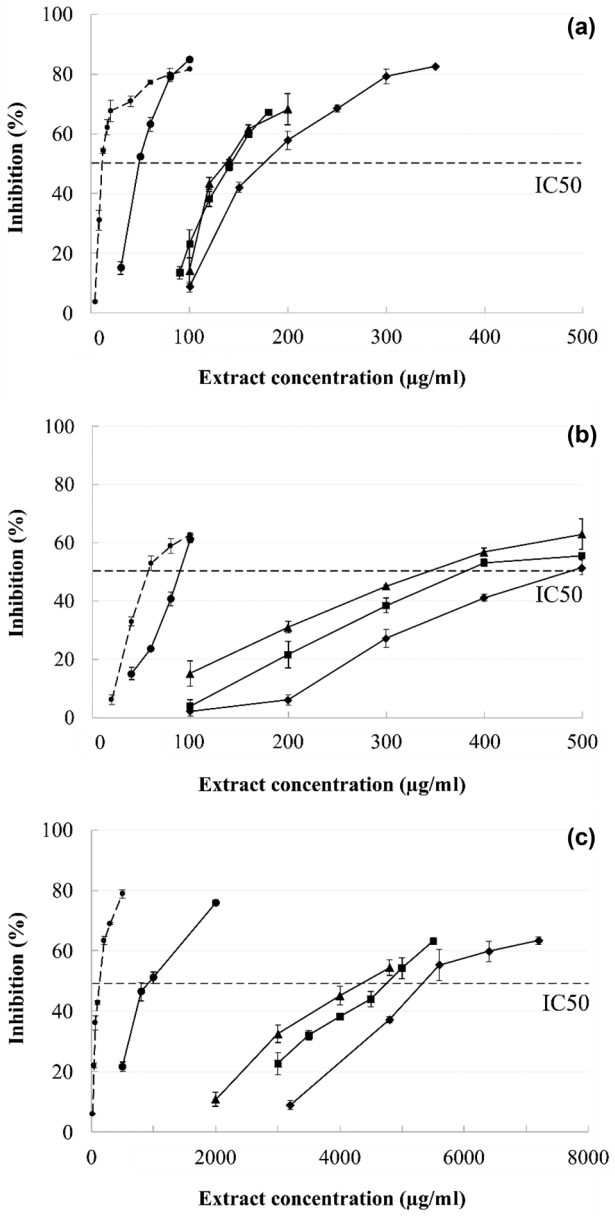

FIGURE 2 Inhibitory capacity of acarbose (–), purified (● P90), and crude (▲ E90, ■ E80, ◆ E70) seaweed extracts against α-amylase (A = $M1_{AM}$, B = $M2_{AM}$, and C = $M3_{AM}$)

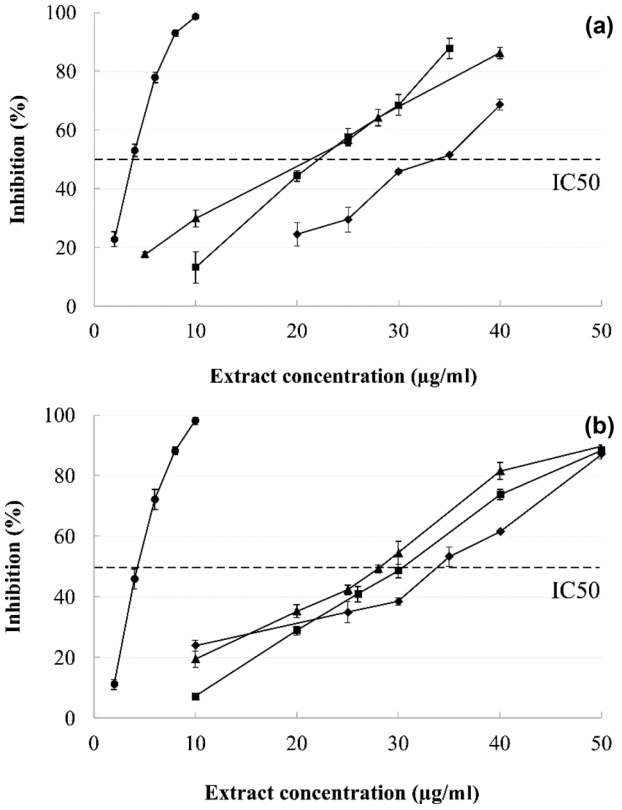

FIGURE 3 Inhibitory capacity of purified (● P90) and crude (▲ E90, ■ E80, ◆ E70) seaweed extracts against and α-glucosidase (A = $M1_{AG}$ and B = $M2_{AG}$)

obtained when the extracts were previously incubated with the enzyme (M1) (Figures 2a and 3a), where the interaction between polyphenols and enzyme was favored before substrate (starch or maltose) addition for α-amylase ($M1_{AM}$) or α-glucosidase ($M1_{AG}$), respectively. Conversely, higher IC_{50} values were required with M2 when the extract was previously mixed with gelatinized starch for α-amylase ($M2_{AM}$) (IC_{50} = 74.03, 309.16, 342.59, and 439.82 µg/ml for P90, E90, E80, and E70, respectively) or with maltose for α-glucosidase ($M2_{AG}$) (IC_{50} = 3.62, 26.15, 28.09, and 30.36 µg/ml for P90, E90, E80, and E70, respectively) (Figures 2b and 3b). These observations suggest that in the M2 method, the substrate hinders the polyphenol accessibility to the enzymes, increasing IC_{50} values. Pantidos et al. (2014) also observed lower effectiveness of a tannin-rich fraction, as porcine pancreas α-amylase inhibitor, when it was preincubated with gelatinized potato starch. Lordan et al. (2013) described porcine pancreas α-amylase inhibition (IC_{50} = 0.05 mg/ml) with *A. nodosum* extracts obtained from a three-stage process of at least 3 h duration, like the one obtained with P90 extract in M2. Similar to the previously mentioned polyphenol-enzyme binding, phenolic compounds can bind starch by noncovalent interactions, modulating starch digestion kinetics (Giuberti et al., 2020). This lower inhibition effect when the extract was preincubated with the starch highlights the significance of the hydrolysis kinetics during in vitro analysis and subsequently to the in vivo studies, where pancreatic α-amylase and intestinal α-glucosidase are secreted into the gut lumen, where they would meet the seaweed extract and the starch mixture.

Furthermore, the inhibitory effect against α-amylase dramatically decreased when extracts were added to native starch, and the blend was subjected to high temperatures to gelatinize the starch ($M3_{AM}$) (Figure 2c). IC_{50} values varied between 810.48 (P90) and 4777.66 (E70) µg/ml.

Nevertheless, in this case, besides the starch impediment previously mentioned for $M2_{AM}$, polyphenols and starch interaction during the gelatinization process or polyphenols stability might be considered. Wu et al. (2011) described the existence of hydrogen bonding interaction between tea polyphenols and rice starch during gelatinization. On the other hand, high temperature can affect the stability of polyphenols and antioxidant activity (Moreira et al., 2016), and therefore may affect also its ability as enzyme inhibitors. Betoret and Rosell (2020) analyzed the effect of temperature (70, 80, and 90°C for 20 min) on phenolic compounds of *Brassica napobrassica* blended with maize and rice starches. These authors reported the protective role of starch with phenolic compounds in which the high apparent viscosity might contribute to protect the bioactive compounds. Authors linked the changes of bioactive compounds after thermal treatments with thermal degradation, matrix un-structuring effect and interaction with other ingredients, protecting them from degradation. Nevertheless, to better understand the interactions between *A. nodosum* bioactive compounds and digestive enzymes additional techniques, such as scanning electron microscopy (SEM), Fourier transform infrared spectrophotometry (FT-IR) or X-ray diffraction (XRD), would provide valuable information.

3.3 | Seaweed freeze-dried extracts characterization: Chromatography (RP-HPLC-UV) and nuclear magnetic resonance (^{1}H-NMR)

Crude (E90, E80, and E70) and purified (P90) polyphenols extracts were chromatographically analyzed using phloroglucinol and resorcinol as standards. Chromatograms showed a unique peak (Figure 4) around 60 min of retention time, with no signals around 40 min (spectra not shown) that was the retention time of the standards used, concluding that the compounds detected had higher molecular mass than standards. This result was expected since phloroglucinol does not accumulate in the *A. nodosum* tissues, owing to the rapid polymerization reactions (Tierney et al., 2014). Standardized phlorotannins chromatographic characterization methodology is limited since commercial standards are scarce (Koivikko et al., 2007). Very small peak signals were observed during 20–40 min of retention time show the presence of polysaccharides, in accordance with additional carbohydrates and UA standards (glucose and D (+)-glucuronic acid). No significant differences on chromatographic spectra of crude extracts (E90, E80, and E70) were observed, indicating phlorotannin structure differences were not detectable with RP-HPLC-UV technique. The increase in TPC value of P90, measured by Folin–Ciocalteau, was also confirmed by high-performance liquid chromatography (HPLC) analysis, where 50 µl of 2.5 mg_{FD}/ml of P90 presented a similar peak than 50 µl of 5.0 mg_{FD}/ml from E90 extract.

Chromatographic results indicated that all UAE extracts contain polyphenols with high polymer size without significant presence of monomers or small size oligomers (expected at shorter times). Hence, differences in enzyme inhibition activities among extracts were not explained by HPLC results. Other authors have previously reported that HPLC method was ineffective for separating large polymeric phlorotannins from *A. nodosum* (Tierney et al., 2014) and needs to be complemented with MS and/or NMR techniques (Koivikko et al., 2007).

An adequate method for identifying and quantifying purified polyphenols is ^{1}H-NMR, but it is only qualitative in complex mixtures, since isomers appear at approximately the same chemical shifts. Nonetheless, ^{1}H-NMR analysis could give a proximate insight of overall structure of the assayed extracts and understand inhibition results. Figure 5 shows ^{1}H-NMR spectra of purified (P90) and crude UAE (E90, E80, and E70) extracts from *A. nodosum* seaweed. Three different ranges could be identified at 5.20–5.40 (A), 5.75–6.40 (B), and 6.40–6.55 (C) ppm in tested samples, which agree with seaweed's polyphenols signals reported between 5.0 and 6.5 ppm (Audibert et al., 2010; Susano et al., 2021). In addition, ^{1}H-NMR spectra of crude UAE extracts showed below 5.6 ppm a notorious increasing signal associated with the presence of UA and carbohydrates (these last, at low ppm, data not shown) (Q. Zhang et al., 2004).

Polyphenols extracted from brown seaweed are composed of phloroglucinol moieties linked by a mix of aryl–aryl and aryl–ether bonds (Choi et al., 2014). Hydrogens located near to aryl–ether bonds showed values around 6.50 ppm (C-region); meanwhile, the aryl–aryl bonds signal around 5.70–6.30 ppm (B-region) (Choi et al., 2014; Kim et al., 2019). This was also corroborated by phloroglucinol standard molecule spectra (data not shown) that showed a peak at 5.85 ppm from aryl-aryl bonds. A-region signal has been related to the presence of quinones derived from partial oxidation of polyphenols (Dobado et al., 2011). In fact, this signal dramatically decreased with sonication power (and disappeared in P90). This trend could be associated with the use of high power that increased the lixiviation of less oxidated polyphenols from inner cell-structures (i.e., physodes) (Koivikko et al., 2007) and, oppositely, when low power is employed surface polyphenols from cell-wall (more exposed to oxygen) were mainly extracted. All extracts presented B-region signals and sonication power seemed to only change overall shape. Several peaks observed in this region evidenced the pres-

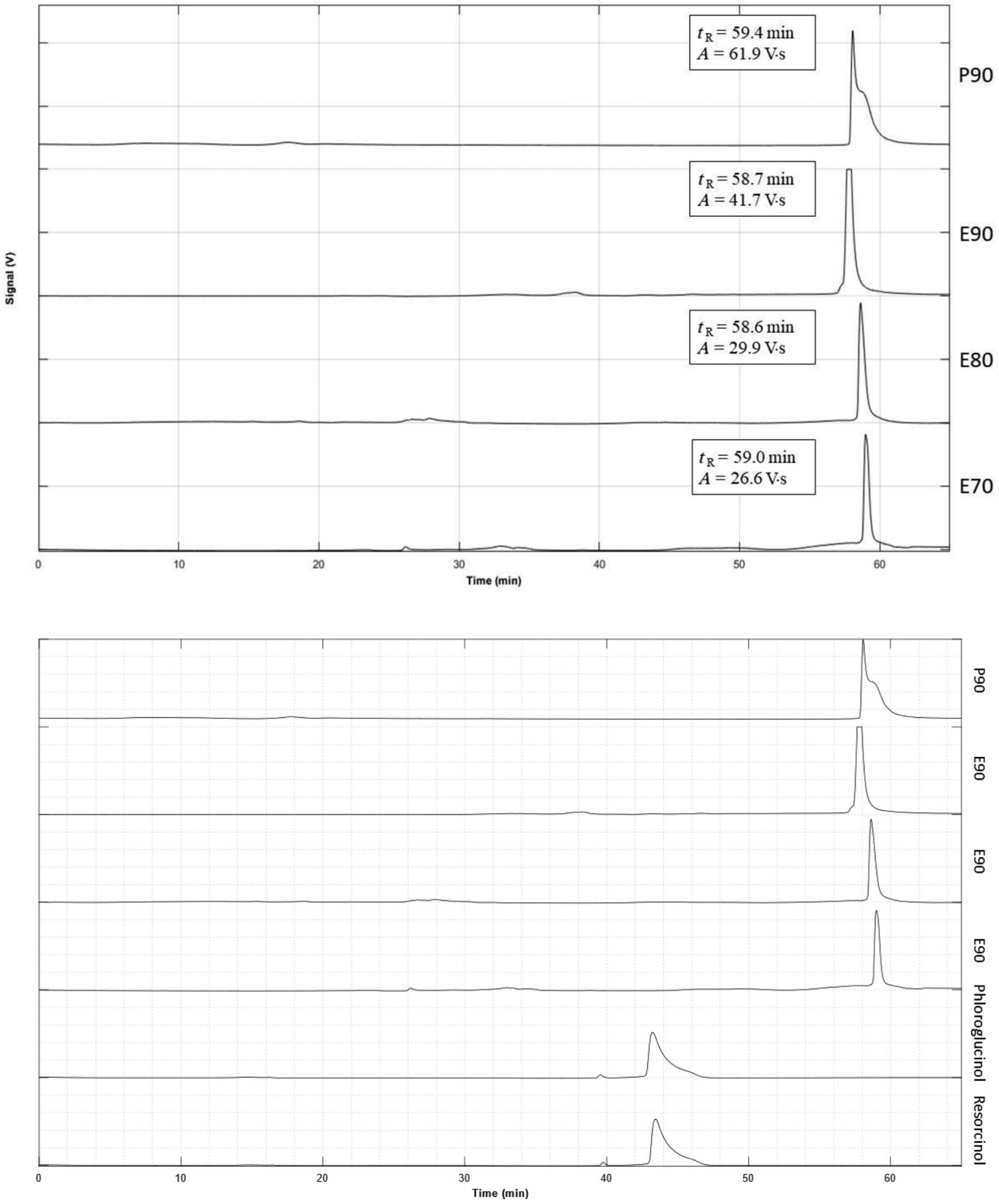

FIGURE 4 RP-HPLC-UV (Reverse Phase-High Performance Liquid Chromatography using UV detector) profiles of purified (P90, 50 μl of 2.5 mg_{FD}/ml) and crude (E90, E80, E70, injected 50 μl of 5.0 mg_{FD}/ml) aqueous extracts obtained by ultrasound-assisted extraction (UAE) from *A. nodosum* seaweed

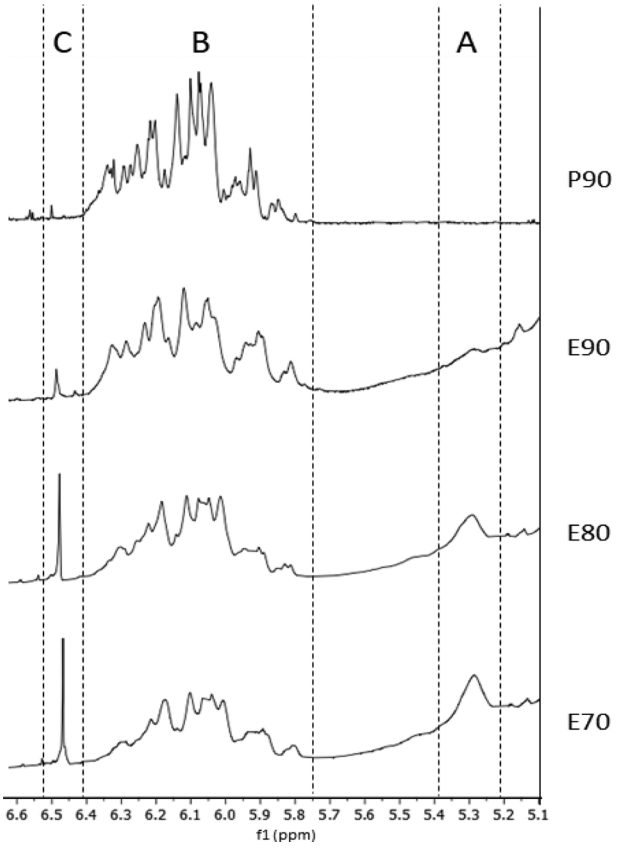

FIGURE 5 ^{1}H-NMR spectra of freeze-dried extracts purified (P90) and crude (E90, E80, and E70) obtained by ultrasound-assisted extraction (UAE) from *A. nodosum* seaweed

ence of different isomers of phlorotannins extracted during UAE treatment.

Prominent signal in C-region for E70 and E80 was observed in comparison to low signal in E90, meaning that C-region signal decreased with increasing sonication power. After Amberlite purification process (P90), this signal practically disappeared. Chemical composition of extracts (Table 1) showed a noticeable reduction of UA/TPC ratio for P90 regarding E90. Based on these results, it is hypothesized that signals of C-region could be indicative of the presence of some polyphenolic complexes, mainly with UA. The gradual signal reduction with increasing sonication power during UAE could be related to the disruption of these complexes that could increase polyphenol availability for enzymes inhibition. Then, NMR spectra and in vitro inhibitory activities suggested that polyphenols–UA complexes are present on the extracts. The observed trend of digestive enzyme inhibitory activities of the extracts (Table 2) could be explained due to the greater presence of "free" polyphenols (not complexed) that interacted more easily with α-amylase and α-glucosidase enzymes during in vitro inhibitory assays.

4 | CONCLUSION

Aqueous *A. nodosum* extracts obtained by UAE have shown to be highly effective inhibitors against α-amylase and particularly, α-glucosidase. Further purification of the UAE extract allowed increasing polyphenol content and reducing UA content. IC_{50} values of both enzymes progressively decreased with increasing sonication power applied during extraction. Purification of extracts increased inhibition against both digestive enzymes due to its higher polyphenols content. Aqueous seaweeds extracts were more effective inhibitors when they were added directly to the enzyme previously to the substrates (starch or maltose). The mixing of extracts with substrates (gelatinized or ungelatinized starch, or maltose) prior to the enzymatic reaction reduced the inhibitory effect of the extracts, being especially dramatic for the α-amylase inhibition. Antioxidant activities and chromatographic profiles of *A. nodosum* extracts did not explain the different inhibitions of α-amylase and α-glucosidase enzymes. Relevant differences could be observed in the ^{1}H-NMR spectra associated with the presence of UA–polyphenols complexes (C-region) and quinones (A-region) that could be related to the measured inhibitory capacities trends of tested extracts. In conclusion, polyphenols-enriched extracts from brown seaweeds with notorious inhibitory capacities against digestive enzymes might be suitable to be used as regulators of postprandial glucose in diabetic patients employing UAE with water (green-solvent). Nevertheless, further studies on heavy metal content will be conducted in the future to confirm their safety.

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AUTHOR CONTRIBUTIONS

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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