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Effects of *Undaria pinnatifida, Himanthalia elongata* and *Porphyra umbilicalis* extracts on *in vitro* α-glucosidase activity and glucose diffusion

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Abstract

Background: **Seaweeds are good sources of dietary fibre, which can influence glucose uptake and glycemic control.**

Objective: **To investigate and compare the in vitro inhibitory activity of different extracts from *Undaria pinnatifida* (Wakame), *Himanthalia elongata* (Sea spaghetti) and *Porphyra umbilicalis* (Nori) on α-glucosidase activity and glucose diffusion.**

Methods: **The *in vitro* effects chloroform-, ethanol- and water-soluble extracts of the three algae were assayed on α-glucosidase activity and glucose diffusion through membrane. Principal Components Analysis (PCA) was applied to identify patterns in the data and to discriminate which extract will show the most proper effect.**

Results: **Only water extracts of Sea spaghetti possessed significant *in vitro* inhibitory effects on α-glucosidase activity (26.2% less mmol/L glucose production than control, $p < 0.05$) at 75 min. PCA distinguished Sea spaghetti effects, supporting that soluble fibre and polyphenols were involved. After 6 h, Ethanol-Sea spaghetti and water-Wakame extracts exerted the highest inhibitory effects on glucose diffusion (65.0% and 60.2% *vs* control, respectively). This extracts displayed the lowest slopes for glucose diffusion-time lineal adjustments (68.2% and 62.8% *vs* control, respectively).**

Conclusions: **The seaweed hypoglycemic effects appear multi-faceted and not necessarily concatenated. According to present results, ethanol and water extracts of Sea spaghetti, and water extracts of Wakame could be useful for the development of functional foods with specific hypoglycemic properties.**

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Key words: *Glucose diffusion. α-Glucosidase. Glycemia control. Seaweed extracts.*

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EFECTOS DE EXTRACTOS DE *UNDARIA PINNATIFIDA, HIMANTALIA ELONGATA* Y *PORFIRA UMBILICALIS* SOBRE LA ACTIVIDAD α-GLUCOSIDASA Y LA DIFUSION DE LA GLUCOSA *IN VITRO*

Resumen

Antecedentes: **Las algas son importante fuente alimentaria de fibra dietética y puede influir sobre la absorción de glucosa y el control glucémico.**

Objetivo: **Evaluar y comparar *in vitro* los efectos de diferentes extractos de las algas *Undaria pinnatifida* (Wakame), *Himanthalia elongata* (Espagueti de mar) y *Porphyra umbilicalis* (Nori) sobre la actividad enzimática α-glucosidasa y la difusión de glucosa.**

Métodos: **Se estudiaron los efectos de los extractos clorofórmicos, etanólicos y acuosos de las tres algas sobre la actividad α-glucosidasa y la difusión de glucosa a través de una membrana de diálisis. Se aplicó a los resultados un análisis de los componentes principales (PCA) para identificar posibles patrones de composición y seleccionar el extracto que mejores propiedades posea.**

Resultados: **El extracto acuoso de Espagueti de mar inhibió de forma significativa la actividad α-glucosidasa (26,2% menos liberación de glucosa, $p < 0,05$). El PCA sugiere que la fibra soluble y los polifenoles son los responsables de tal efecto. Respecto a la difusión de glucosa, el extracto etanólico de Espagueti de mar y el acuoso de Wakame mostraron un mayor efecto inhibidor después de 6 horas (65% y 60,2% *vs* control, respectivamente) y las menores pendientes en los ajustes lineales difusión de glucosa-tiempo (68,2% y 62,8% *vs* control respectivamente).**

Conclusiones: **Los resultados de los diferentes extractos sugieren que los efectos hipogluceminates de las algas son variados y no están necesariamente concatenados. Los extractos acuosos y etanólicos de Espagueti de mar y los acuosos de Wakame parecen los más adecuados para el desarrollo de alimentos funcionales con propiedades hipoglucemiantes.**

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Palabras clave: *Difusión de glucosa. α-Glucosidasa. Control glucémico. Extractos de algas.*

Abbreviations

T2DM: Type 2 Diabetes Mellitus.
MS: Metabolic syndrome.
SDF: Soluble dietary fibre.
PCA: Principal component analysis.
AUC: Area under the curve.
Pr: proteins.
SPP: soluble polyphenols.
GDM: glucose concentration for glucose diffusion at medium time.
IGDM: percentage inhibition for glucose diffusion at medium time.
GDF: glucose concentration for glucose diffusion at final time.
IGDF: percentage inhibition for glucose diffusion at final time.
AGM: glucose concentration for α-glucosidase activity at medium time.
IAGM: percentage inhibition for α-glucosidase activity at medium time.
AGF: glucose concentration for α-glucosidase activity at final time.
IAGF: percentage inhibition for α-glucosidase activity at final time.
GAE: Gallic acid equivalents.

Introduction

Growing evidence suggests that abnormal increases in postprandial glycemia are a key risk factor for Type 2 diabetes (T2DM) and metabolic syndrome (MS)[1]. Reducing postprandial hyperglycemia through inhibiting carbohydrate-digestive enzymes and/or delaying glucose absorption in the small intestine, are central aspects in the blood glucose level control[2]. α-glucosidase inhibitors, such as acarbose, are prescribed for T2DM patients to control their blood glucose levels[3,4]. Several authors have reported the positive influence of fibre on glycemic control in T2DM patients[5].

Seaweeds are good sources of total and soluble/viscous dietary fibre, which can influence satiety and glucose uptake[6]. Furthermore, they contain fibre-associated bioactive compounds, such as polyphenols[7-9]. Iwai et al.[3] found some antidiabetic effects, based on the α-glucosidase (EC 3.2.1) inhibitory activity, of the phlorotannins present in the brown alga *Ecklonia stolonifera*. Fucoxanthin (the main carotenoid in brown seaweed) has been found to help lower bodyweight[10]. Cofrades et al.[11] have concluded that *Porphyra, Undaria,* and *Himanthalia* offer considerable potential as functional food ingredients, which can produce many different kinds of biological activities, and also for their high antioxidant capacity and potential effects on glycemia. Thus, these three algae have been employed as ingredients in functional meat product[12,13]. In order to search for the health benefits of seaweeds, the use of several *in vitro* models to screen for highly potential activities of different seaweed extracts could be a useful approach[14]. The measurement of glucose movement across dialysis membrane into external solution is considered a convenient model for assessing factors affecting glucose absorption *in vitro*[15]. However, few studies have examined the effect of seaweed extracts on glucose diffusion. Similarly, decreases in the α-glucosidase activity can be used as a marker of inhibitory effects on carbohydrate digestion[16].

Algal extracts present a very complex composition, thus, their compounds' identification results difficult and tedious. Extracting solvents will determine extracts composition. Soluble dietary fibre (SDF) is mostly found in water-soluble extracts[9,17,18] while pigments and non-polar fats in chloroform extracts[19], and polyphenols and polar fats in ethanol extracts[20]. Consequently, the physiological properties/effects of algal extracts (e.g. antihyperglycemic) could be related, at least indirectly, to major compounds and bioactive compounds present in them. Principal Component Analysis (PCA) would help to easily understand which seaweed extracts would exert better antihyperglycemic properties and the bioactive compound candidates involved.

As, natural ingredients from seaweeds can exert potential benefits on glucose metabolism and diabetes[2,3,6], in this paper we hypothesized that chloroform-, ethanol- and water-soluble algal extracts would possess different effects on glucose diffusion and α-glucosidase activity. Thus, taking into account the aforementioned information, present study aims to obtain ethanol-, chloroform-, and water-soluble extracts from three commonly consumed algae *Undaria pinnatifida* (Wakame), *Himanthalia elongata* (Sea spaghetti) and *Porphyra umbilicalis* (Nori) in order to investigate which extract shows the highest *in vitro* effects on α-glucosidase inhibition and glucose absorption.

Material and methods

Seaweeds samples and preparation of the seaweed extracts

Freeze-dried algae *Undaria pinnatifida*, *Porphyra umbilicalis*, and *Himanthalia elongata* were obtained from Algamar (Pontevedra, Spain). These seaweeds were finely powdered and stored at room temperature (20 ± 2 ºC) in opaque screw-top jars until analysis. Extraction of seaweed was carried out according to the method reported by Gray and Flatt[21] using high polarity solvents such as water and ethanol; and medium polarity solvents such as chloroform. Then 1 g of the powdered sample were placed for infusion in 40 mL of boiling distilled water, ethanol (95% v/v) or chloroform, depending on the extract required, and stirred thoroughly. The mixture was transferred into a 50 mL centrifuge tube and placed onto a roller mixer for 15

min. The tubes were closed with Teflon caps and centrifuged for 10 min at 4,000 rpm (2,500 × *g*) at 4º C and each supernatant was collected into bijoux tubes. Aliquots of extracts (5 mL) were dried under vacuum (Savant SPD121P Speed Vac, Labcare, Buckinghamshire, England), stored at -20 ºC. For activity analysi,s dried seaweed extracts were reconstituted with PBS buffer (0.002 M KH_2PO_4 and 0.14 M NaCl) pH 7.4 at 4 ºC just before use.

Total phenolic content

Phenolic contents of extracts were estimated by the method of Taga et al.[22] Briefly, 100 µL aliquot of sample was mixed with 2.0 mL of 2% Na_2CO_3 and allowed to stand for 2 min at room temperature. After incubation, 100 µL of 50% Folin-Ciocalteau's phenol reagent was added, and the reaction mixture was mixed thoroughly and allowed to stand for 30 min at room temperature in the dark. Absorbance of all the sample solutions was measured at 720 nm using spectrophotometer (Shimadzu, UV-160, Japan). Phenolic contents are expressed as Gallic acid equivalent per gram (GE g^{-1}). All tests were carried out in triplicate and the results were presented as means ± SD.

α-*glucosidase activity measurement*

α-glucosidase, as maltase activity, was tested measuring the production of glucose from maltose solution according to the Mai et al. method[23]. Rat intestinal acetone powder (Sigma-Aldrich, St Louis, MO, USA) was homogenised in a maleate buffer (pH 6.0). The homogenate was centrifuged at 3,000 rpm 10 min and the supernatant obtained was used as crude enzyme solution for assay. In short, a mix of 300 µL of maltose (20 µg/L) and 150 µL of the different dried seaweed extracts reconstituted with PBS buffer to the concentration of 50 g/L were pre-incubated at 37 ºC for 5 min. Afterwards, 150 µL of crude enzyme solution was immediately added, and the reaction mixture vortexed and incubated at 37 ºC. PBS buffer was used as a blank control. Acarbose, 1 mg/L in PBS buffer, was used as positive control as it has being referenced as α-glucosidase inhibitor (Anderson et al., 2004; Iwai, 2008). Glucose concentrations were measured at 15, 30, 45, 60 and 75 min on a Microstat P-GM7 portable analyser (Analox Instruments USA Inc, Lunenburg MA). α-glucosidase activity was expressed as mmol/L glucose produced *per* min. All tests were carried out in triplicate and the results were presented as means ± SD.

Glucose diffusion measurement

An adaptation of the Gallagher et al.[15] method was used to evaluate the effects of the different seaweed extracts on *in vitro* glucose diffusion. The seaweed extracts used have a concentration of 25 g/L. Briefly, the model used in the present experiments consisted of a dialysis tube (6 cm × 10 mm; dialysis tubing cellulose membrane, Sigma-Aldrich, Steinheim, Germany) into which 1,750 µL of D-glucose (0.22 M) and 250 µL of the different dried seaweed extracts reconstituted with PBS buffer to the concentration of 25 g/L were added. The dialysis bag was sealed at each end and placed in a tube containing 100 mL 0.9 g/L NaCl. Glucose on external NaCl solution was measured each 30 min for 6 h. Blank control tests were conducted with PBS buffer in the absence of seaweed samples. Glucose concentrations were measured using a Microstat P-GM7 portable analyser (Analox Instruments USA inc, Lunenburg MA, USA). All tests were carried out in triplicate and the results presented as means ± SD. To correspond with the *in vivo* glucose tolerance oral test (American Diabetes Association, 2011), the areas under the curve (AUC) of glucose were plotted after 180 min and 360 min taking into account glucose concentration diffused at each 30 min intervals.

Principal component analysis (PCA)

PCA was applied to identify patterns in the data and to highlight similarities/differences and used a statistical multivariate analysis to discriminate which extract will show the most proper effect. Since chloroform-extracts were largely devoid of activity (see below), PCA was only conducted on the water and ethanol extracts to investigate the relation between the soluble bioactive constituents of seaweeds and the glucose diffusion and α-glucosidase activity. Twelve different variables were tested, four related with composition: (proteins (Pr), ash, soluble polyphenols (SPP) and soluble dietary fibre (SDF), and eight about evaluated parameters: glucose concentration and percentage inhibition for glucose diffusion at medium (GDM and IGDM) and final times (GDF and IGDF) and for α-glucosidase activity and percentage inhibition for α-glucosidase activity at medium (AGM and IAGM) and final times (AGF and IAGF).

Statistical analysis

Glucose concentrations were plotted against incubation time and the linear adjustments were tested using the SPSS statistical package (version 19.0). Linear adjustments for the different extracts and control were compared using SAS 9.2 statistical package. Statistical comparisons between seaweed extracts were assessed by one way ANOVA followed by the Bonferroni *post hoc* test. The multivariate PCA analysis was applied to summarise the information in a reduced number of principal components, that explain the percentage of the total variance, selecting those values with eigen-

values >1.0. Then, the factors were rotated, using Varimax method, to obtain the expected weight for each extraction factor. The PCA scatter plot would inform that parameter located further to the left along principal compounds shows the highest glucosidase inhibition percentage while those contributing with relatively low percentage to the variance on glucose diffusion data, will be placed in upper side and negative part of the plot. Differences were considered significant at $p < 0.05$.

Results

Seaweeds composition

Table I summarizes composition of some relevant compounds for the three seaweeds tested as well as their swelling, water and oil retention capacities. Nori showed the highest protein content but lowest swelling capacity, water and oil retention capacities. See spaghetti displayed the highest total fibre and SDF content. Nori has lower ash content than the other seaweeds (120 g/kg dry matter (dm) *vs* 370 g/kg dm of Wakame and 300 g/kg dm of Sea spaghetti), mainly due to its lower sodium, potassium, calcium, and magnesium contents (data not shown).

The phenolic contents in all extracts were significantly different among species ($p < 0.05$). All type of Sea spaghetti extracts showed higher phenolic content than their Wakame and Nori counterparts. In comparison to other solvents, ethanol extracts of Wakame and Sea spaghetti showed the highest phenolic content (25.12 and 34.43 mg Gallic acid equivalents (GAE)/g, respectively) (table II).

α-*Glucosidase activity*

Table III shows the effect of blank control, acarbose, chloroform, ethanol, and water extracts from seaweeds on α-glucosidase activities *in vitro* at different time intervals. These activities were differently affected by the different extracts (at least, $p = 0.007$). Water-Sea spaghetti extract induced significant decrease in α-glucosidase after 30 min (at least, $p < 0.05$) with respect to blank control and to the other water extracts. Ethanol-Nori and Ehanol-Sea spaghetti extracts increased α-glucosidase activity with respect to blank control at 15 min ($p < 0.05$). Acarbose showed throughout the whole experiment (on α-glucosidase and glucose diffusion models $p < 0.001$) a potent inhibitory effect on α-glucosidase activity (all extracts, $p < 0.001$).

Figure 1 summarizes inhibitory effects of the different water extracts on α-glucosidase activity at different time intervals expressed as percentage of inhibition. Acarbose was stated at 100% inhibition level while blank control at 0%. Water Sea spaghetti induced a steady state inhibition (about 70%; $p < 0.001$) with respect to its blank control after 30 min experiment.

Table I
Composition, dietary fibre, and selected physico-chemical properties of Porphyra umbilicalis *(Nori),* Undaria pinnatifida *(Wakame) and* Himanthalia elongata *(Sea spaguetti)*

	Nori	*Wakame*	*Sea spaghetti*
Ash (g/kg dm)	120[1]	370[1]	300[1]
Protein (g/kg dm)	365[1]	112[1]	48[1]
Total fibre (g/kg dm)	370[1]	420[1]	510[1]
Soluble fibre (g/kg dm)	220[1]	140[1]	250[1]
Swelling capacity (mL/g dm)	6.08[2]	10.53[2]	10.97[3]
Water retention capacity (g/g dm)	5.12[2]	10.96[2]	7.26[3]
Oil retention capacity (g/g dm)	1.04[2]	0.96[2]	1.61[3]

dm, dry matter; [1]according to Cofrades et al.[11]; [2]according to Rupérez and Saura-Calixto[18]; [3]according to Gómez-Ordóñez et al.[24]

Table II
Total phenolic content (mg gallic acid equivalents/g extract) of water, ethanol and chloroform extracts from Porphyra umbilicalis *(Nori),* Undaria pinnatifida *(Wakame) and* Himanthalia elongata *(Sea spaghetti)*

Seaweeds	*Water extracts*	*Ethanol extracts*	*Chloroform extracts*
Nori	4.41 ± 0.11[a]	8.43 ± 1.02[a]	3.21 ± 1.12[a]
Wakame	11.12 ± 1.13[b]	25.12 ± 1.34[b]	7.24 ± 0.78[b]
Sea spaghetti	18.45 ± 2.04[c]	34.43 ± 2.11[c]	11.56 ± 1.24[c]

All values are mean ± SD of three determinations. Different superscript letters within the same column indicate significant differences between samples at the level of $p < 0.05$.

Table III
Effect of chloroform, ethanol and water extracts from Porphyra umbilicalis *(Nori),* Undaria pinnatifida *(Wakame) and* Himanthalia elongata *(Sea spaghetti) on α-glucosidase activity in vitro*[1]

Time (min)	*15*	*30*	*45*	*60*	*75*
Blank control	1.3 ± 0.2^{b}	2.5 ± 0.05^{c}	5.5 ± 0.3^{c}	7.7 ± 0.3^{ab}	9.2 ± 0.4^{a}
Ch-Nori	1.8 ± 0.1^{abc}	3.9 ± 0.5^{b}	6.1 ± 0.2^{abc}	7.6 ± 0.5^{ab}	9.4 ± 0.4^{a}
Ch-Wakame	1.5 ± 0.4^{bc}	3.5 ± 0.2^{b}	6.1 ± 0.3^{abc}	7.3 ± 0.0^{b}	9.3 ± 0.4^{a}
Ch-Sea spaghetti	2.2 ± 0.1^{a}	4.4 ± 0.2^{a}	6.4 ± 0.2^{b}	8.2 ± 0.3^{a}	9.7 ± 0.1^{a}
Acarbose	0.2 ± 0.1^{d}	1.5 ± 0.1^{d}	1.2 ± 0.2^{d}	0.8 ± 0.3^{c}	1.4 ± 0.5^{b}
ANOVA	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
Blank control	1.6 ± 0.2^{b}	2.9 ± 0.5^{ab}	5.7 ± 0.0^{ab}	7.7 ± 0.4^{b}	9.4 ± 0.0^{a}
Eth-Nori	2.1 ± 0.2^{a}	3.5 ± 0.9^{a}	6.0 ± 0.3^{a}	8.8 ± 0.0^{a}	10.1 ± 0.9^{a}
Eth-Wakame	1.4 ± 0.3^{b}	3.6 ± 0.3^{a}	5.1 ± 0.1^{ab}	8.1 ± 0.1^{b}	10.0 ± 0.3^{a}
Eth-Sea spaghetti	2.1 ± 0.1^{a}	4.0 ± 0.2^{a}	6.7 ± 0.1^{a}	8.5 ± 0.3^{ab}	10.4 ± 0.05^{a}
Acarbose	0.3 ± 0.0^{c}	0.9 ± 0.2^{c}	1.0 ± 0.4^{c}	0.5 ± 0.2^{c}	0.8 ± 0.0^{b}
ANOVA	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
Blank control	1.1 ± 0.2^{b}	3.3 ± 0.1^{b}	4.0 ± 1.4^{b}	7.0 ± 0.2^{a}	8.7 ± 0.0^{a}
W-Nori	1.7 ± 0.1^{a}	4.0 ± 0.2^{a}	6.4 ± 0.1^{a}	8.2 ± 0.2^{a}	10.4 ± 0.3^{b}
W-Wakame	1.7 ± 0.1^{a}	3.9 ± 0.0^{a}	5.7 ± 0.0^{ab}	8.3 ± 0.0^{a}	9.4 ± 0.5^{ab}
W-Sea spaghetti	1.3 ± 0.4^{b}	1.7 ± 0.3^{c}	2.2 ± 0.2^{c}	2.7 ± 0.9^{b}	3.3 ± 0.6^{c}
Acarbose	0.7 ± 0.3^{c}	1.0 ± 0.1^{d}	1.6 ± 0.2^{c}	1.4 ± 0.5^{b}	0.9 ± 0.2^{d}
ANOVA	0.007	<0.001	< 0.001	< 0.001	< 0.001

[1]Determined as glucose released (mmol/L glucose) from maltose. Mean ± SD of three determinations. Values within a column with unlike superscript letters were significantly different ($p < 0.05$). Ch, chloroform; Eth, ethanol; W, Water.

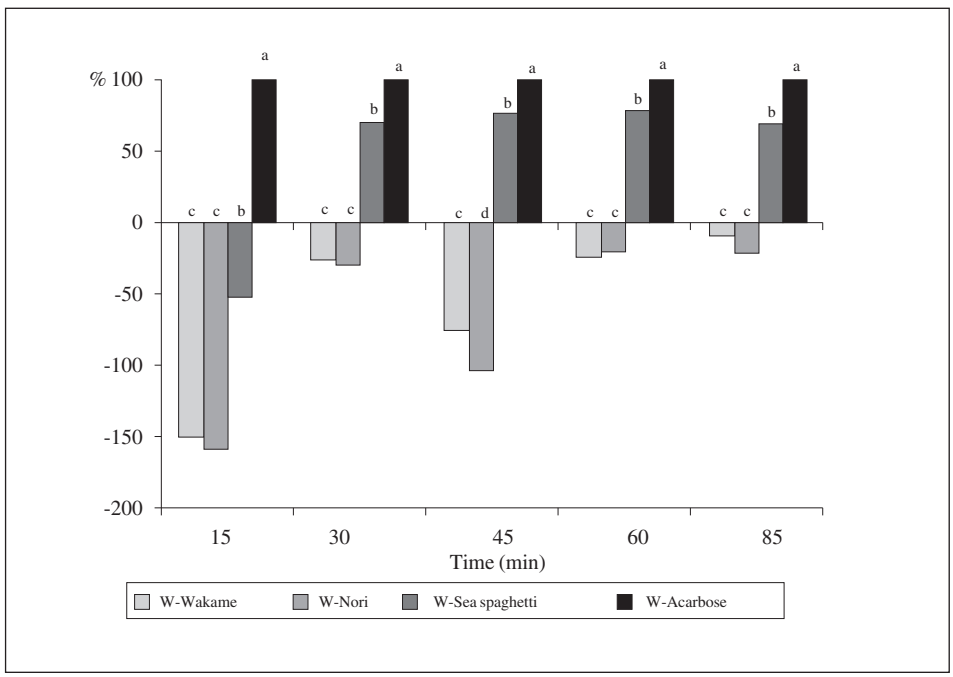

Fig. 1.—α-Glucosidase inhibition (%) of water extracts from seaweeds in comparison to blank control at different experimental times. Bars bearing different letters were significantly different ($p < 0.05$, Bonferroni test). (W-, water extract). Notice that acarbose inhibition was stated at +100% level while negative values mean an increase of α-glucosidase activity.

In vitro *glucose diffusion*

Table IV shows the effects of the different seaweed extracts tested upon glucose diffusion *in vitro*. The glucose diffusion at the different times assayed was significantly different between chloroform extracts (at least $p = 0.046$), ethanol extracts (at least $P = 0.036$), and water extracts (at least $p = 0.005$). During the first 60 min, two chloroform extracts (chloroform-Wakame and chloroform-Sea spaghetti), two ethanol extracts (ethanol-Wakame and ethanol-Nori), and two water extracts (water-Nori and water-Sea spaghetti) increased (at least $p < 0.05$), while ethanol-Sea spaghetti decreased (at least $p < 0.05$) the glucose

Table IV
Effect of chloroform, ethanol and water extracts from Porphyra umbilicalis *(Nori),* Undaria pinnatifida *(Wakame) and* Himanthalia elongata *(Sea spaghetti) on glucose diffussion in vitro*

Time (min)	*30*	*60*	*90*	*120*	*150*	*180*	*210*	*240*	*270*	*300*	*330*	*360*
Blank control	1.4 ± 0.2^{b}	1.5 ± 0.1^{c}	2.7 ± 1.2^{a}	3.7 ± 0.2^{a}	4.1 ± 0.1^{ab}	4.5 ± 0.1^{b}	4.9 ± 0.3^{c}	5.5 ± 0.2^{d}	6.2 ± 0.2^{c}	6.7 ± 0.1^{c}	7.9 ± 0.3^{a}	8.8 ± 0.3^{a}
Ch-Nori	2.0 ± 0.1^{a}	1.9 ± 0.3^{c}	2.7 ± 0.2^{a}	4.3 ± 0.6^{a}	5.0 ± 0.1^{a}	5.4 ± 0.2^{a}	5.9 ± 0.1^{b}	5.9 ± 0.0^{c}	6.1 ± 0.1^{c}	6.5 ± 0.1^{d}	6.7 ± 0.2^{b}	6.9 ± 0.1^{b}
Ch-Wakame	2.2 ± 0.1^{a}	3.5 ± 0.4^{a}	4.2 ± 0.1^{a}	4.7 ± 0.3^{a}	5.0 ± 0.2^{a}	5.5 ± 0.2^{a}	6.6 ± 0.1^{a}	6.9 ± 0.1^{a}	7.2 ± 0.2^{a}	7.6 ± 0.1^{a}	8.1 ± 0.2^{a}	8.4 ± 0.3^{a}
Ch-Sea spaghetti	2.1 ± 0.1^{a}	2.7 ± 0.1^{b}	3.6 ± 0.2^{a}	4.4 ± 0.0^{a}	3.3 ± 1.2^{b}	4.6 ± 0.1^{b}	5.3 ± 0.3^{bc}	6.5 ± 0.2^{b}	6.7 ± 0.1^{b}	7.1 ± 0.1^{b}	7.7 ± 0.1^{a}	8.8 ± 0.1^{a}
ANOVA	< 0.001	< 0.001	0.041	0.046	0.019	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
Blank control	1.4 ± 0.2^{b}	1.5 ± 0.1^{c}	2.7 ± 1.2^{a}	3.7 ± 0.2^{a}	4.1 ± 0.1^{ab}	4.5 ± 0.1^{b}	4.9 ± 0.3^{c}	5.5 ± 0.2^{d}	6.2 ± 0.2^{c}	6.7 ± 0.1^{c}	7.9 ± 0.3^{a}	8.8 ± 0.3^{a}
Eth-Nori	1.7 ± 0.1^{a}	1.8 ± 0.1^{b}	3.1 ± 0.6^{a}	2.5 ± 0.9^{ab}	1.7 ± 0.1^{b}	3.9 ± 0.1^{b}	4.1 ± 0.1^{b}	4.6 ± 0.1^{b}	5.2 ± 0.2^{b}	5.5 ± 0.1^{c}	6.3 ± 0.1^{b}	7.0 ± 0.1^{b}
Eth-Wakame	1.75 ± 0.1^{a}	2.2 ± 0.1^{a}	2.9 ± 0.0^{a}	3.3 ± 0.2^{a}	4.0 ± 0.1^{a}	4.7 ± 0.1^{a}	5.1 ± 0.1^{a}	5.4 ± 0.2^{a}	5.9 ± 0.5^{a}	6.2 ± 0.2^{b}	7.7 ± 0.2^{a}	8.6 ± 0.1^{a}
Eth-Sea spaghetti	1.0 ± 0.1^{c}	1.1 ± 0.1^{d}	1.3 ± 0.2^{a}	1.4 ± 0.2^{b}	1.7 ± 0.1^{b}	2.1 ± 0.1^{c}	2.3 ± 0.1^{c}	2.4 ± 0.1^{c}	2.5 ± 0.1^{c}	2.7 ± 0.1^{d}	2.7 ± 0.1^{c}	3.1 ± 0.1^{c}
ANOVA	< 0.001	< 0.001	0.036	0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
Blank control	1.4 ± 0.2^{b}	1.5 ± 0.1^{c}	2.7 ± 1.2^{b}	3.7 ± 0.2^{bc}	4.1 ± 0.1^{ab}	4.5 ± 0.1^{b}	4.9 ± 0.3^{c}	5.5 ± 0.2^{b}	6.2 ± 0.2^{c}	6.7 ± 0.1^{c}	7.9 ± 0.3^{a}	8.8 ± 0.3^{a}
W-Nori	2.4 ± 0.4^{a}	3.0 ± 0.1^{b}	3.5 ± 0.5^{b}	4.2 ± 0.1^{b}	4.0 ± 0.1^{b}	3.9 ± 0.1^{c}	4.3 ± 0.3^{c}	4.6 ± 0.1^{c}	5.2 ± 0.1^{c}	5.6 ± 0.1^{c}	6.8 ± 0.1^{c}	7.7 ± 0.3^{b}
W-Wakame	1.7 ± 0.3^{b}	1.8 ± 0.1^{c}	2.0 ± 0.1^{b}	2.8 ± 0.8^{c}	2.5 ± 0.7^{c}	1.8 ± 0.1^{d}	2.0 ± 0.1^{d}	2.3 ± 0.1^{d}	2.7 ± 0.1^{d}	2.9 ± 0.1^{d}	3.1 ± 0.1^{d}	3.6 ± 0.1^{c}
W-Sea spaghetti	1.7 ± 0.1^{bc}	4.6 ± 1.0^{a}	6.1 ± 0.5^{a}	8.0 ± 0.0^{a}	7.7 ± 0.3^{a}	8.0 ± 0.1^{a}	8.3 ± 0.2^{a}	8.6 ± 0.1^{a}	9.1 ± 0.1^{a}	9.5 ± 0.1^{a}	10.6 ± 0.1^{a}	11.8 ± 0.8^{a}
ANOVA	0.005	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001

Mean (mmol L^{-1} glucose) ± SD of three determinations. Values in the same column bearing different [small] letters were significantly different ($P < 0.05$). Ch, chloroform; Eth, ethanol; W, Water.

diffusion in comparison to its respective blank control. Significant decreases in the glucose diffusion with respect to the blank control were observed for corresponding extracts of chloroform-Nori after 300 min ($p < 0.05$), ethanol-Nori after 150 min (all $P \leq 0.001$) and ethanol-Sea spaghetti after 120 min (at least, $p = 0.002$), water-Wakame after 150 min (at least, $P < 0.05$) and water-Nori after 180 min (at least $p = 0.05$). The water-Sea spaghetti increased glucose diffusion *vs*. the blank control at any time tested ($p \leq 0.001$).

Table V shows the linear adjustments of glucose diffusion and time through the experiment for the different seaweed extracts. Blank control and all extracts in chloroform, ethanol and water were linearly adjusted ($r^2 > 0.882$; $p < 0.001$). Significant differences were observed between linear adjustments of blank control *vs*. chloroform-Nori, and between those of chloroform-Nori *vs* chloroform-Wakame (at least, $p < 0.05$). Significant differences between linear adjustments of ethanol-Nori and ethanol-Sea spaghetti *vs* the blank control, and among all ethanol algal extracts (all $p < 0.001$) were observed. Linear adjustments significantly differed between water-Wakame and water-Nori *vs* blank control; and among all water algal extracts (all $p < 0.001$).

Figure 2a shows that AUC from the extracts were significantly different ($p < 0.001$; ANOVA test) except for chloroform extracts at 180 min. Ethanol-Sea spaghetti at 180 (fig. 2a) and 360 min (fig. 2b) and water-Wakame at 360 min (fig. 2b) displayed significantly lower AUC values than the other extracts (at least, $p < 0.05$). Water-Sea spaghetti showed higher AUC ($p < 0.05$) than their counterparts (figs. 2a and 2b).

Principal components analysis

Since chloroform extracts were largely devoid of activity (tables III and IV), PCA was conducted only on the water and ethanol extracts to investigate the relation between the soluble bioactive constituents of seaweeds and the glucose diffusion and α-glucosidase activity (table VI and figs. 3 and 4). PCA was applied to identify patterns in our data and to highlight similarities/differences between extracts. Different variables were measured: four related with composition (Pr, ash, soluble SPP, and SDF) and eight derived from the combination of two extract types [in water and in ethanol], two experiments [glucose diffusion (GD) and α-glucosidase activity (AG) both expressed as glucose concentration and inhibition percentage] at two times [medium (M) and final times (F)].

The PCA scatter plot (fig. 3) indicates that entities located to the left had the highest α-glucosidase inhibition percentage while those with relatively low inhibition percentage were located to the top and negative part of the plot. The first three components in the PCA explained 96.2% of the total variance in the data set (eigenvalues = 7.023, 3.114, and 1.407, respectively). The first component (PC-1) accounted for 58.5% of the variance and correlated with the variance of glucose diffusion at medium time, and with the variance of α-glucosidase inhibition at both medium and final time. PC-1 also correlated with the variance of protein and ash (positively) and polyphenol (negatively) constituents. The second component (PC-2) accounted for 22.9% of the total variance and correlated with the variance of glucose diffusion both at medium and final

Table V

Linear regressions of glucose diffusion rate (mmol L^{-1} min^{-1}) by chloroform, ethanol and water extracts from Porphyra umbilicalis *(Nori),* Undaria pinnatifida *(Wakame) and* Himanthalia elongata *(Sea spaghetti)*

Linear regression	*Intercept ± SE*	*Slope ± SE*	*R^2; (β-coefficient)*	*Linear adjustment*	*p**
Blank control	0.449 ± 0.190	0.022 ± 0.001	0.962; (0.981)	<0.001	Nori *vs* blank control (p = 0.036)
Ch-Nori	1.342 ± 0.280	0.018 ± 0.001	0.882; (0.939)	<0.001	
Ch-Wakame	1.699 ± 0.251	0.020 ± 0.001	0.924; (0.961)	<0.001	Wakame *vs* Nori (p = 0.040)
Ch-Sea spaghetti	1.067 ± 0.244	0.021 ± 0.001	0.932; (0.965)	<0.001	
Blank control	0.449 ± 0.190	0.022 ± 0.001	0.962; (0.981)	<0.001	Nori *vs* blank control (p < 0.001);
Eth-Nori	0.570 ± 0.243	0.017 ± 0.001	0.902; (0.950)	<0.001	Sea Spaghetti *vs* blank control (p < 0.001);
Eth-Wakame	0.752 ± 0.157	0.020 ± 0.001	0.969; (0.985)	<0.001	Wakame *vs* Nori (p < 0.001);
Eth-Sea spaghetti	0.506 ± 0.077	0.007 ± 0.000	0.946; (0.972)	<0.001	Wakame *vs* Sea Spaghetti (p < 0.001) Nori *vs* Sea Spaghetti (p < 0.001)
Blank control	0.449 ± 0.190	0.022 ± 0.001	0.962; (0.981)	<0.001	Wakame *vs* blank control (p < 0.001);
W-Nori	1.430 ± 0.266	0.015 ± 0.001	0.864; (0.930)	<0.001	Nori *vs* blank control (p = 0.018);
W-Wakame	1.105 ± 0.221	0.006 ± 0.001	0.592; (0.769)	<0.001	Wakame *vs* Nori (p < 0.001);
W-Sea spaghetti	2.403 ± 0.498	0.027 ± 0.002	0.844; (0.919)	<0.001	Wakame *vs* Sea Spaghetti (p < 0.001); Nori *vs* Sea Spaghetti (p < 0.001)

p: differences between linear adjustments *Only significant differences shown. Ch, chloroform; Eth, ethanol; W, Water

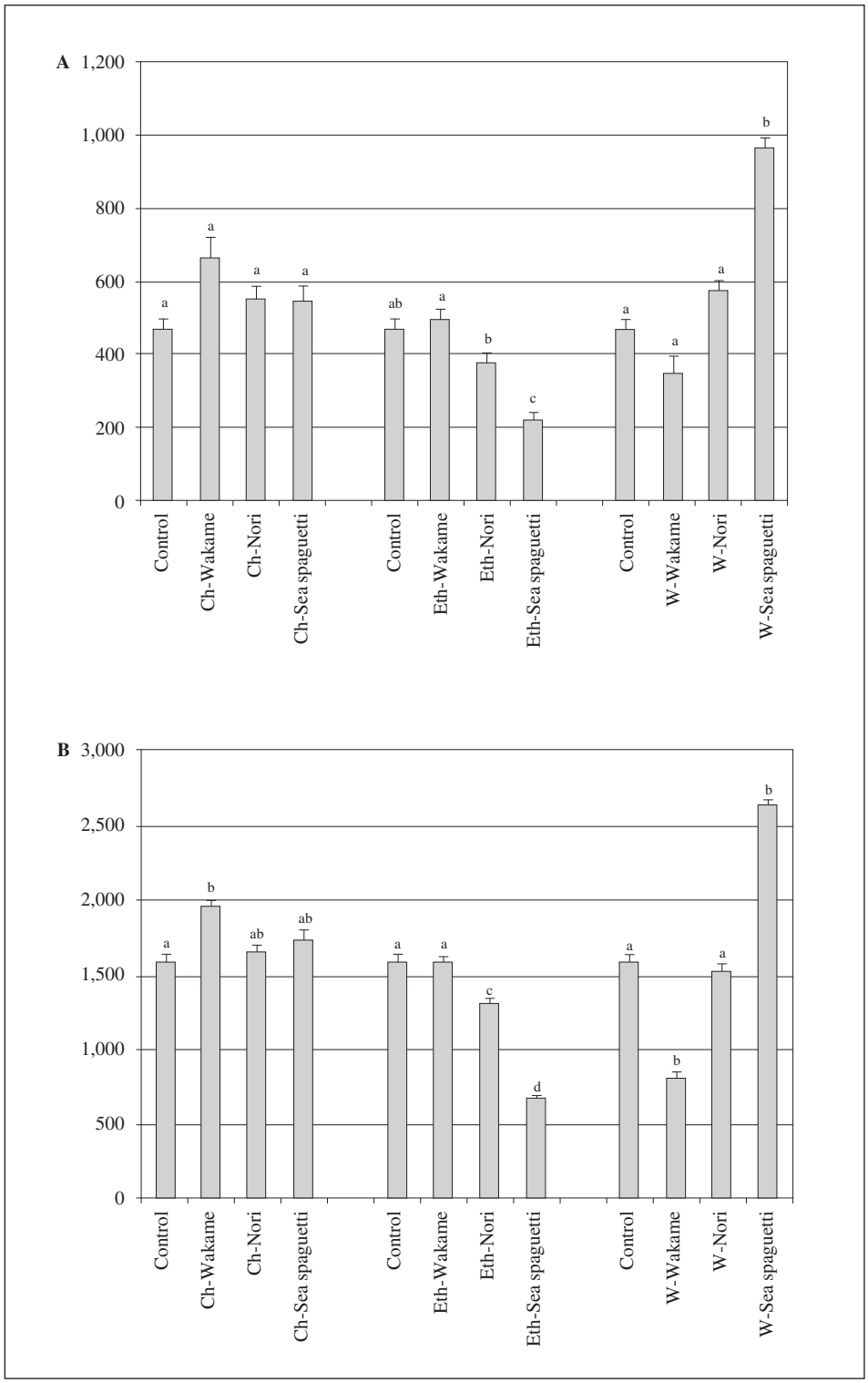

Fig. 2.—Effect of seaweeds extracts on the Area under the curve (AUC) values for glucose diffusion. A. AUC value at 180 min arbitrary square units. B. AUC values at 360 min. Values for the same type of extracts bearing different letter were significantly different ($p < 0.05$, Bonferroni test). All multiple comparison were significantly different (at least, $p = 0.021$) according to ANOVA test except for those of chloroform at 180 min (all, non significant). (Ch-, chloroform extract; Eth–, ethanol extract; W-, water extract).

time, and also correlated positively with the protein constituent. The third component (PC-3) accounted for 11.7% of the total variance, and correlated with the variance of SPP and SDF (both positively), and ash (negatively) components (table VI). Using the rotated component matrix, it could be inferred (fig. 3) that e.g. the water-Sea spaghetti, with the highest glucosidase inhibition percentage, was located further to the left along PC-1; while water-Nori, ethanol-Nori and ethanol-Wakame, showing the lowest inhibition enzyme activities, were located on the opposite side of the PC-1. Summarizing, the PCA scatter plot provides the following associations: For water-Sea spaghetti, the inhibition of α-glucosidase activity together with

Table VI

Rotated component matrix for the analysis of the relation between bioactive compounds in water and ethanol extracts from Porphyra umbilicalis *(Nori),* Undaria pinnatifida *(Wakame) and* Himanthalia elongata *(Sea spaghetti) on glucose diffusion and α-glucosidade assays*

Variable	*PC1*	*PC2*	*PC3*
Soluble Dietary Fibre	-0.036	0.149	0.983
Soluble Polyphenols	-0.535	-0.221	0.790
Ash	0.399	0.110	-0.903
Protein	0.756	0.505	-0.026
Diffusion Assay			
GDM	-0.612	0.749	0.230
GDF	0.612	-0.749	-0.230
% IGDM	-0.113	0.971	-0.109
% IGDF	0.124	-0.971	0.104
Enzymatic Assay			
AGM	0.905	-0.275	-0.239
AGF	-0.905	0.275	0.239
% IAGM	0.896	-0.311	-0.288
% IAGF	-0.896	0.311	0.288

Factors were extracted with the principal component analysis using the Varimax rotation. PC, principal component; AGF, α-glucosidase activity at final time (determination at 90 min); AGM, α-glucosidase activity at medium time (determination at 45 min); GDF, glucose concentration at final time (determination at 360 min); GDM glucose concentration at medium time (determination at 180 min); %IGDM, inhibition percentage of glucose diffusion at 180 min; %IAGM, inhibition of α-glucosidase activity at 45 min; %IAGF, inhibition of α-glucosidase activity at 90 min; %IGDF, inhibition percentage of glucose diffusion at 360 min.

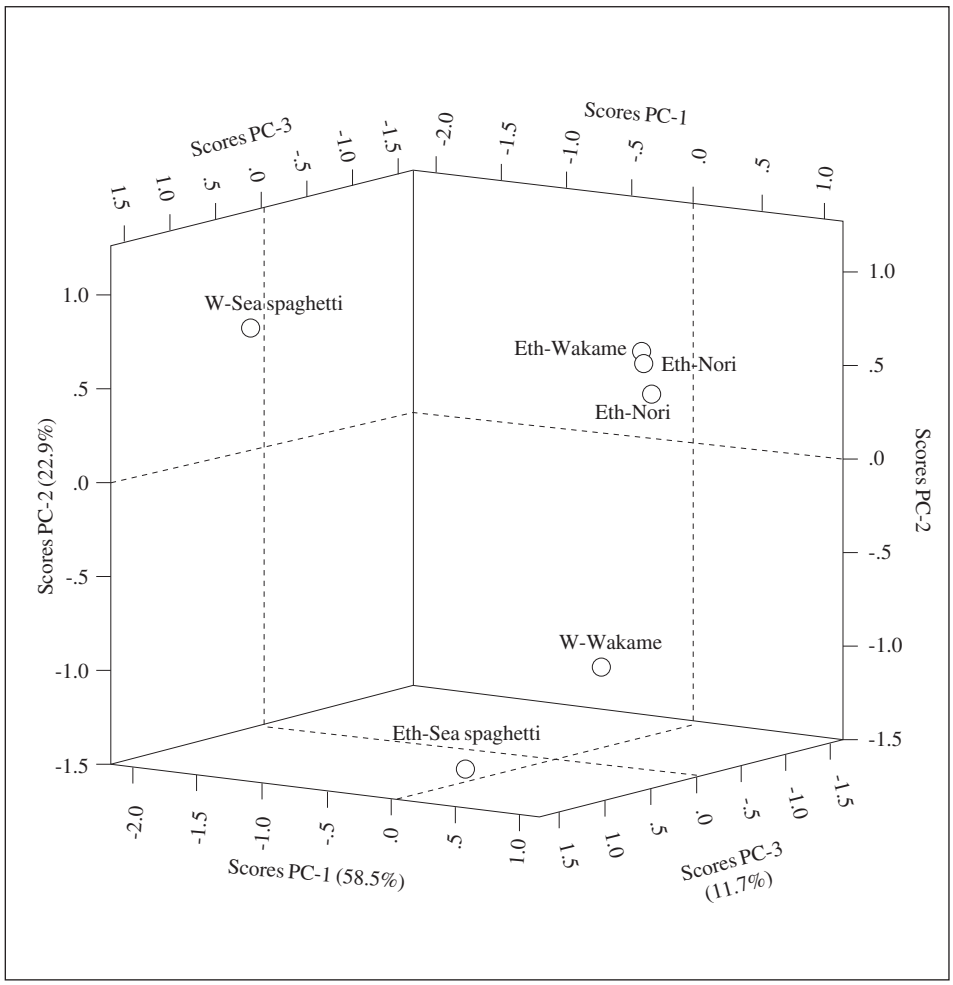

Fig. 3.—Principal Component analysis (PCA) scatter tri-plot using twelve variables in the study of the in vitro *glucose diffusion and α-glucosidase activity in ethanol- (Eth-) and water- (W-) extracts from Sea spaghetti (Ss), Wakame (Wk) and Nori (No) seaweeds. PC-1, PC-2, and PC-3 accounted for 58.5%, 22.9%, and 11.7% of the variance, respectively. Dot lines at 0 in the PC-1, PC-2 and PC-3 scales means no explanation by the specific PC to the variability found in each seaweed entity. Notice that chloroform extracts were not included due to their low effect on the* in vitro *glucose diffusion and α-glucosidase activity studies.*

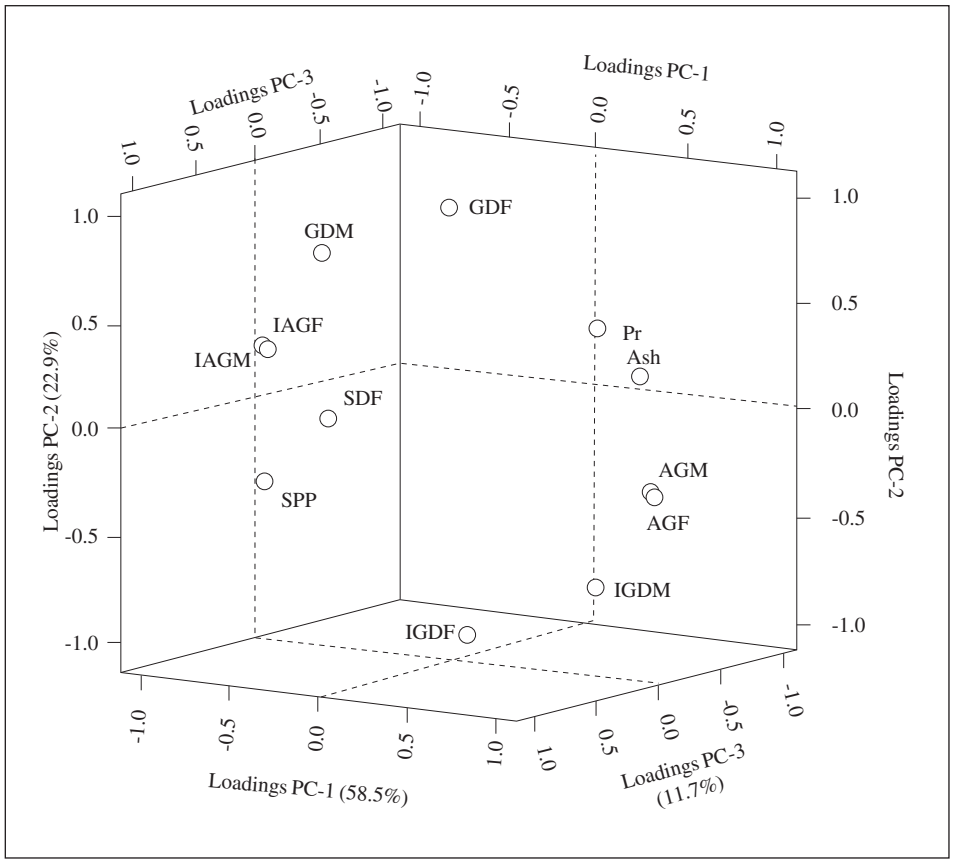

Fig. 4.—Loading PCA triplot for protein (Pr), ash (Ash), soluble polyphenols (SPP), and soluble dietary fibre (SDF) contents of the three seaweeds which were in vitro *tested for glucose concentration and inhibition percentage of glucose diffusion at medium (GDM and IGDM) and final times (GDF and IGDF) and for -glucosidase activity at medium (AGM and IAGM) and final times (AGF and IAGF) on water and ethanol extracts. Dot lines at 0 in the PC-1, PC-2 and PC-3 scales mean no contribution of each variable to the specific PC. This figure shows the different correlations between the different variables considered taking into account the three PCA components. Notice that chloroform extracts were not included due to their low effect on the* in vitro *glucose diffusion and α-glucosidase activity studies.*

the high glucose diffusion seemed related to its relative SDF and SPP high contents. For ethanol-Sea spaghetti and water-Wakame, the low glucose diffusion and low inhibition of α-glucosidase activity appeared related to a lower SDF and SPP contents. Finally, for ethanol-Wakame, ethanol-Nori and water-Nori, the high glucose diffusion and the low inhibition of α-glucosidase activity seemed related to low contents in soluble bioactive compounds. Figure 4 suggests a high correlation between the two soluble compounds, SDF and SPP, and the inhibition of the glucosidase activity both at medium and final incubation time. No significant correlations were found between glucose diffusion activity through the membrane and the compounds analysed.

Discussion

Although algal extracts are thought to exert potential glycemic control through inhibiting carbohydrate-digestive enzymes and/or delaying glucose absorption, the topic remains controversial and understudied. The present study indicated that algal extracts affected both on α-glucosidase activity and glucose diffusion but via different and independent mechanisms. Although complex mix of compounds could be responsible of results, data of this study emphasizes the importance per se of the extracts in the glycemic control.

As previously commented, the three tested algae differed qualitatively and quantitatively in composition (e.g. total fibre, SDF, polyphenols and mineral contents)[11]. Sea spaghetti is a brown seaweed rich in SDF and uronic acids but has a low-medium mineral content, presenting higher oil retention and swelling capacities than the other alga tested[24]. On the contrary, Wakame contains lower SDF but higher mineral content, and exhibits a higher water retention capacity[18]. Nori (red seaweed) displays medium values for polyphenols, ash, SDF and oil retention capacity but the highest protein content and the lowest swelling and water retention capacities[18]. Thus, differences in the effects of these algal extracts on carbohydrate digestion and glucose movement through dialysis membrane would be discussed in relation to algal composition.

Phenolic compounds are commonly found in plants and have been reported to have several biological activities. Polyphenols from edible seaweeds have been suggested to influence responses relevant to diabetes through modulation of glucose-induced oxidative stress[25], as well as through inhibition of starch-digestive enzymes[26]. Indeed, polyphenol-rich extracts from *Ascophyllum* inhibited glucosidase and showed promising anti-diabetic effects in mouse models[27] and polyphenols from *Ecklonia* have shown positive effects on genetically diabetic mice[3]. There is not sufficient information yet either on individual

phenols in algae or about their physiological function in the human organism.

Higher amount of polyphenols in brown algae than in red and green algae has been reported[28]. Phenolic compounds are generally more soluble in polar solvents. The major active compounds in different seaweed extracts have been reported to be phlorotannins and fucoxanthin[29]. Ethanol extracts from brown algae showed higher polyphenol contents than green algae[30]. Brown algae had a total phenol content of 5-45 mg GAE/g dm. In our study, Sea spaghetti extracts showed the highest polyphenol content. The differences between the phenolic compounds in water and ethanol extracts reflects, at least in part, the polarity of the same and the extent to which they can be esterified and/or glycosylated. The lower amount of phenolic compounds in chloroform extracts seems due to the lower polarity of this solvent in comparison to water and ethanol. These results are in line with those of Moller et al.[31] who unexpectedly found a higher amount of phenolic compounds in the water extract of dittany (*Origanum dictamnus*) as compared to those ones obtained with organic solvents. Ethanolic and water extracts are the most widely employed due to their more hygienic characteristics[32].

Intestinal α-glucosidase plays an important role in carbohydrate digestion and its inhibition is considered a suitable strategy for treating postprandial hyperglycemia, frequently associated with T2DM. Present results suggest that chloroform extracts displayed, among the extracts assayed, the lowest correlation among studied variables with the α-glucosidase activity inhibition (data not shown). Preliminary PCA results (data not shown) indicated that plots for chloroform extracts where centred, thus indicative of their low effect. Therefore, in the final PCA study only water and ethanol extracts were considered. Clearly, water-Sea spaghetti could be used as antidiabetic agent since these extracts induced the maximum α-glucosidase inhibitory activities. PCA analysis indicated that SDF and SPP are the most likely candidates for inhibiting α-glucosidase activity (fig. 4). Sea spaghetti is much richer in polyphenols than the other two algae (table I), explaining, at least in part, the present results. Several studies have determined that the antidiabetic properties of algae are mostly due to the effects of some polyphenols on carbohydrate digestion. Among brown algae, polyphenols (i.e. phlorotannins) present in alcoholic extracts, have been found to inhibit α-glucosidase activity. Phlorotannins in general[33] and particularly those isolated from *Ascophyllum nodosum* have shown potential antidiabetic effects through the inhibition of both α-amylase and α-glucosidase enzymes[34]. Methanolic extracts of *Ecklonia stolonifera* and *Eisenia bicyclis*, and their isolated phlorotannins are potent α-glucosidase inhibitors. Molecular size and number of hydroxyl groups were crucial for the grade/intensity of this inhibition.[35] Methanolic extracts of *Ecklonia stolonifera*, rich in phlorotannins, show stronger α-glucosidase inhibitory activity than water extracts, and contain more polyphenol.[3] Phloroglucinol from *Ecklonia cava* showed α-glucosidase and α-amylase inhibitory activities[26].

The influence of SDF on inhibition of starch digestive enzymes at a very low level and maintenance of the *in vivo* glycemic control has been suggested.[36] The hypoglycemic properties of fibre polysaccharides from Sea spaghetti were demonstrated in rabbits by Lamela et al.[37] Sea spaghetti is rich in SDF, and its fucoidans, rich in glucuronic acid, have a similar structure to fucoidans of *Ascophyllum*, which present a known α-glucosidase inhibitory activity[38]. *Fucus vesiculosus* showed inhibition of α-glucosidase activity that correlated with phenolic contents.[36] Furthermore, the interaction between SDF and SPP should not be discarded, as these types of compounds are often associated in seaweeds[9]. Wakame, although is a brown seaweed, presented lower amount of SDF, composed essentially by alginates[39], and its fucoidans structure is quite different from those of Sea Spaghetti[38]. The ineffectiveness of Wakame polysaccharides as α-glucosidase inhibitors was shown by Cho et al.[40] who observed that they only had inhibitory effect when sulphation degree increased. Moreover, aqueous extracts should contain both compounds (fucoidans and polyphenols), being much richer the aqueous extracts from Sea spaghetti than those from the other two algae, explaining our present results on α-glucosidase inhibition.

Among possible mechanisms related with the antihyperglycemic action of seaweeds, the decrease in glucose absorption should be emphasised. Seaweed extracts inhibit glucose movement dealing with viscosity of the plant extract and soluble polysaccharides, as glucose absorption is intimately related to gastric emptying and time inhibition[41]. Previous reports show the effect of seaweed extracts on glucose absorption models[15]. However, to the best of our knowledge this is the first report investigating extracts of Sea spaghetti. The extracts distinctly modified the movement of glucose across membrane. Comparing slope adjustments of all extracts assayed, ethanol-Sea spaghetti and water-Wakame demonstrated the highest inhibitory effects on glucose diffusion compared with control; while water-Sea spaghetti increased the trend diffusion. Interestingly, during the first hour all extracts, except for that of ethanol-Sea spaghetti, increased glucose diffusion through membrane. No hypothesis is available at present to explain this short-term effect. Coinciding with the lower glucose absorption trends, ethanol-Sea spaghetti and water-Wakame showed the lowest AUC for glucose. Thus, it can be suggested the potential utility to promote decreases of glucose diffusion in terms of trend and intensity of both previously cited extracts.

Present results suggest that chloroform extracts displayed, among the extracts assayed, the lowest effects in glucose diffusion activity. As in the case of the α-glucosidase inhibition analysis, preliminary PCA

results (data not shown) indicated that plots for chloroform extracts at final time where almost centred; thus, indicative of their lower effect on glucose diffusion. Therefore, in the final PCA study performed only water and ethanol extracts were considered. However, no clear association was found in the PCA between major compound composition and glucose diffusion (fig. 4). As Wakame was richer in alginate[39], and Sea spaghetti in polyphenols[11], alginates in the water-Wakame and polyphenols in the ethanol-Sea spaghetti seemed the potential candidates to exert inhibition on glucose diffusion. However, the type and total concentration of polyphenols and other compounds (e.g. alginates) or even their time-course effects did not overlap, explaining the absence of concurrence in the PCA. Several reports suggest the glucose absorption delay promoted by alginates. Thus, it has been demonstrated that alginates act on glucose absorption and insulin response in non-diabetic[42] and T2DM patients.[43] Moreover, methanolic extracts from *Ecklonia stolonifer,* rich i*n* phlorotannins[3], suppressed the increase of plasma glucose levels in diabetic kk-A mice, emphasizing the contribution of polyphenols and alginates to our hypothesis.

Conclusions

Present study suggests that the particular solvent used to extract the seaweed material will have a dramatic effect on the anti-diabetic activity[43]. Future studies should be conducted to ascertain which specific active compounds or group of compounds are mainly responsible for the effects observed and to address the best way to isolate them. According to present results, ethanol and water extracts of Sea spaghetti, and water extracts of Wakame could be useful for the development of functional foods with specific hypoglycemic properties. Since the effects of seaweed extracts on glucose diffusion and α-glucosidase activity do not coincide, the alga antidiabetic/hypoglycemic effects should be viewed as simplistic, and studies to discover the source of their activity need to be conducted.

Competing interests

The authors declare that they have no competing interests.

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