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Seaweed bioactivity

Effects on glucose liberation

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PREFACE

This dissertation is submitted for the degree of Doctor of Philosophy at the University of Copenhagen. The research was conducted under the supervision of Professor Lars Ove Dragsted and Professor Dan Stærk. The study was conducted at the Department of Nutrition, Exercise & Sports in collaboration with Department of Drug Design and Pharmacology as well as Department of Plant and Environmental Sciences, University of Copenhagen.

This thesis presents the results from *in vitro* studies on inhibition of α-amylase and α-glucosidase by some edible seaweeds and the effect of selected edible seaweeds on the postprandial blood glucose and insulin levels following a starch load in a human meal study.

This dissertation contains several parts including the introduction and background on hyperglycaemia and seaweeds, the aims of the research project, material and methods, results (included papers), discussion, conclusion, and perspectives.

The data from the thesis work has been gathered in 3 manuscripts included in the present thesis. Part of this study has been submitted in the following publications:

Paper 1

Zaharudin, N., Salmaen, A.A., Dragsted, L.O. (2017). **Inhibitory effects of edible seaweeds, polyphenolics and alginates on the activities of porcine pancreatic α-amylase**. *Food Chemistry*. (Accepted).

Paper 2

Zaharudin, N., Staerk, D., Dragsted, L.O. (2017). **Inhibition of α-glucosidase by selected edible seaweeds and fucoxanthin: Kinetic studies**. *Food Chemistry*. (Submitted).

Paper 3

Zaharudin, N., Tulin, M., Sloth, J.J., Rasmussen, R.R., Dragsted, L.O. (2017). **Effects of the edible seaweeds, *Laminaria digitata* and *Undaria pinnatifida* on postprandial glucose, insulin and appetite in humans**. (Ready to submit).

SUMMARY

Hyperglycaemia (high blood sugar levels) is one of the risk factor of type 2 diabetes (T2D) when it is sustained over a longer period of time. Various factors that can lead to high blood glucose levels include glucose absorption by the small intestine and the production of glucose by liver cells. Maintenance of normal plasma glucose concentration is essential for the human health. Diet and exercise play important role to control blood sugar level. Limiting intake of high Glycaemic Index (GI) foods as part of a balanced diet is known to be important. In addition, having the right food intake such ad functional foods that affect the blood sugar increase, e.g by containing inhibitors of α-amylase and/or α-glucosidase, may also help lowering the average blood sugar levels. Thus, such foods may in theory help to lower blood glucose postprandially and could potentially help delay the development of T2D in subjects with impaired glucose tolerance who regularly consume starchy foods.

The present study involved the investigation of crude extracts of dried edible seaweeds in inhibiting the carbohydrate digestive enzymes, α-amylase and α-glucosidase. Bioactive compounds from selected edible seaweeds that inhibit α-amylase and α-glucosidase were identified. The edible seaweeds that were showing high potential for inhibiting the enzymes were selected to investigate their effect on the postprandial blood glucose and insulin levels following a starch load in a human meal study.

In vitro studies and a human study were performed as part of this thesis. In **Paper 1** and **Paper 2**, the inhibition of α-amylase and α-glucosidase activity *in vitro* by edible red, green and brown seaweeds were investigated. Aqueous and alcoholic extracts of dried edible seaweeds were tested to investigate the inhibition kinetics on these enzyme activities. The most potent edible seaweed extracts were showing mixed-type inhibition (lowering both K_m and V_{max}) and were selected for bioactive compound identification. The brown seaweeds, *Laminaria digitata* and *Undaria pinnatifida,* were found to be the most potent inhibitors of α-amylase and α-glucosidase activities. Polyphenols, alginates and fucoxanthin found in the selected seaweeds are among the bioactive compounds that contributed to inhibition of the enzyme activities.

In **Paper 3**, the same two edible seaweeds were tested in a human study. The primary endpoint was the ability of the edible seaweeds to reduce human postprandial blood glucose and insulin concentrations following a starch load in a human meal study. There was no significant effect in plasma glucose but both brown seaweeds lowered postprandial insulin response following consumption of *Laminaria digitata* or *Undaria pinnatifida* compared to the control meal.

In conclusion, two brown seaweeds, *Laminaria digitata* and *Undaria pinnatifida,* inhibited α-amylase and α-glucosidase activities due to their content of several bioactive components with a potential use for future functional foods. Their effects on the postprandial insulin response and the *in vitro* findings regarding the phenolics, alginate and fucoxanthin in these seaweeds may further support that brown seaweeds, particularly *Undaria pinatifida*, might be used as a potential functional food to help control postprandial hyperinsulinaemia.

SAMMENDRAG (DANSK)

Hyperglykæmi (højt blodsukker) over en længere periode er en risikofaktor for udvikling af type 2 diabetes (T2D). Blandt faktorer, der kan bidrage direkte til koncentrationen af glukose i blodet er absorptionen fra tyndtarmen og glukoneogenese i leveren. Kost og motion er vigtige for blodsukkeret. Fastholdelse af et normalt blodsukker er vigtigt for helbredet og begrænsning i indtaget af fødevarer med højt glykæmisk indeks som led i en sund og balanceret kost er kendt som vigtigt i forebyggelsen af T2D. Dertil kan indtag af visse fødevarer bidrage til at sænke blodsukkeret, herunder funktionelle fødevarer, der for eksempel indeholder hæmmere af α-amylase og/eller α-glucosidase. Sådanne fødevarer kunne derfor i teorien hjælpe til at sænke blodsukkeret i perioden efter et måltid og kunne potentielt hjælpe med at forsinke udvikling af T2D hos personer som har en forstyrret glukosetolerance, men som har et hyppigt indtag af stivelsesholdige fødevarer.

Arbejdet bag denne afhandling indbefatter studiet af, om simple ekstrakter af forskellige typer af spiselig tang (makroalger) kan hæmme fordøjelsesenzymerne, α-amylase og α-glukosidase. Bioaktive komponenter i udvalgte spiselige makroalger med evnen til at hæmme disse enzymer blev identificeret. De arter af tang, der havde særligt stærk hæmmende effekt på enzymerne blev udvalgt til at studere effekten på blodsukker og -insulin, når de blev indtaget sammen med en belastning med stivelse i et måltidsstudie blandt frivillige forsøgspersoner.

Denne afhandling omfatter derfor såvel *in vitro* studier som et humanstudie. I **artikel 1 og 2** undersøges effekten af røde, grønne og brune tangarter på aktiviteten af α-amylase og α-glukosidase. Vandige og organiske ekstrakter af tørret tang blev anvendt i undersøgelsen af hæmningskinetikken. De mest aktive ekstrakter viste en blandet type af hæmningskinetik (sænkning af både K_m og V_{max}) og blev udvalgt til identifikation af de aktive komponenter. De brune makroalger, *Laminaria digitata* og *Undaria pinnatifida*, var de mest potente hæmmere af de to enzymaktiviteter. Polyphenoler, alginat og fucoxanthin fra de brune makroalger var blandt de komponenter, der bidrog til enzymhæmningen.

I **artikel 3** beskrives et måltidsstudie blandt frivillige med de samme to brune makroalger. Den primære hypotese var at de kunne sænke blodsukkeret efter en stivelsesbelastning og en af de

sekundære hypoteser var, at de kunne sænke blodets koncentration af insulin. Der var ingen effekt på blodglukose, mens der var en lavere stigning i insulin efter måltidet med brun tang, sammenlignet med kontrol (ærter). Der var tillige virkninger af tangen på følelsen af sult, appetit, mæthed og lysten til at spise noget.

Det konkluderes, at de to makroalger, *Laminaria digitata* og *Undaria pinnatifida,* kan hæmme α-amylase og α-glukosidase som følge af deres indhold af visse bioaktive komponenter, hvilket giver dem potentiale som fremtidige funktionelle fødevarer. Deres virkninger på det postprandielle insulinrespons og virkningerne *in vitro* af fenoliske komponenter, alginat og fucoxanthin fra disse makroalger underbygger at brun tang og især *Undaria pinnatifida* i fremtiden potentielt vil kunne bruges i funktionelle fødevarer til at hjælpe med kontrol af postprandiel hyperinsulinæmi.

LIST OF ABBREVIATIONS

ANOVA	Analysis of variance
CBM3a	Crystalline Cellulose-Binding Module
CoMPP	Comprehensive Microarray Polymer Profiling
ESI	Electrospray ionization
FAME	Fatty Acid Methylesters
FAO	Food and Agricultural Organisation
FDA	Food and Drugs Administration
F-value	Fisher test value
GAE	Gallic acid equivalent
GI	Glycaemic index
GLP-1	Glucagon-like peptide-1
HbA1c	Hemoglobin A1c
HPLC	High Performance Liquid Chromatography
HRMS	High Resolution Mass Spectrometry
IC_{50}	Half maximal inhibitory concentration
iAUC	Incremental Area Under the Curve
k	Rate constant
K_m	Michaelis-Menten constant
LM7	Monoclonal Antibody to Homogalacturonan
P	Probability
PHGG	Partially hydrolysed guar gum
pNPG	p-nitrophenyl-beta-D-glucoside
ppm	parts per million
PTP1B	protein-tyrosine phosphatase 1B
R^2	Co-efficient of determination
rpm	revolutions per minute
SCFA	Short-chain fatty acid
SD	Standard deviation
Spp.	Species
T	Temperature
t	Time
T2D	Type 2 diabetes
TDF	Total dietary fibre
TFC	Total flavonoid content
TPC	Total phenolic content
UV	Ultraviolet
V_{max}	Maximum velocity
wt/wt	weight over weight

TABLE OF CONTENTS

1. INTRODUCTION

Diabetes mellitus (DM) is recognized as a major health problem, as it is the fastest growing chronic condition globally. It is one of the metabolic diseases characterized by hyperglycaemia resulting from defects in insulin secretion, insulin action, or both (American Diabetes Association, 2009). The number of people with diabetes is growing worldwide and by 2040 it is estimated to rise to 642 million people (International Diabetes Federation, 2015). In Malaysia, 3.5 million (19.7 %) of the citizens, aged ≥ 30 years are diagnosed with diabetes, showing an increase in the number of patients (World Health Organization, 2016). In Denmark, 320,545 (5.7 %) of the citizens are diagnosed with diabetes (Danish Diabetes Association, 2017).

The upward trend of cases of diabetes has sparked awareness and worry in governments, that has prompted them to act to tackle this global health problem. The solution against this health problem requires researchers to do further studying on how to control, manage and treat this health problem.

Early management of the condition by controlling high blood glucose levels (hyperglycaemia) as well as by maintaining a healthy lifestyle and making better choices in diet can halt the development of Type 2 Diabetes (T2D). Balanced diet and increased physical activity may lead to improvement or normalization of blood glucose levels. A diet with non-starchy foods or food with low glycaemic index (GI) has been introduced for people to improve glycaemic control and insulin sensitivity (Brand-Miller; Hayne, Petocz, 2003; McGonigal & Kapustin, 2008). Reducing glucose uptake or inhibition of glucose liberation has also been explored as a means to reduce hyperglycaemia (Kabir et al., 2014; Kasner, Hunter, Ph, Kariko, & Ph, 2013). It is of high interest to study how consuming food from natural sources containing carbohydrate digestive inhibitors may affect glucose uptake and metabolism.

Marine-derived seaweeds may be one food candidate in terms of inhibitor content. The term "seaweed" refers to macroalgae and microalgae that belong to one of several groups of algae like red algae, green algae and brown algae (Baweja, Kumar, Sahoo, & Levine, 2016). Over the last decade, seaweeds have become ingredients in food and medicine (Fitton, Irhimeh, & Teas, 2007; Stengel & Walker, 2015). Seaweeds contain valuable nutrients and compounds such as fatty acids,

dietary fibres and polyphenols (Goñi, Valdivieso, & Garcia-Alonso, 2000; M. S. Kim, Kim, Choi, & Lee, 2008; B. Liu, Kongstad, Wiese, Jäger, & Staerk, 2016a; Lordan, Ross, & Stanton, 2011). Some of these bioactive compounds could be used for the management of hyperglycaemia. The identification of bioactive compounds from marine sources has seen increased interest among researchers of functional foods and in drug development. The application of seaweeds show an inhibiting effect on carbohydrate digestive enzyme activity *in vitro* (Gupta & Abu-Ghannam, 2011; Ikeda & Kusano, 1983; Liu, Kongstad, Wiese, Jäger, & Staerk, 2016; Lordan, Smyth, Soler-Vila, Stanton, & Ross, 2013).

Therefore, the investigations and exploitation of seaweeds in *in- vitro* and human studies have been suggested, as it may provide valuable evidence, which could potentially be used in to control blood glucose levels. This thesis focuses on the effects of seaweeds on carbohydrate digestive enzymes *in vitro* and possible effects on human postprandial blood responses.

2. AIM OF THE THESIS

The aims of this study were:

1) To investigate the potential of crude extracts of dried edible seaweeds in inhibiting carbohydrate digestive enzymes (α-amylase and α-glucosidase).

2) To identify the bioactive compounds from selected edible seaweeds that inhibit α-amylase and α-glucosidase.

3) To investigate the effect of selected edible seaweeds on the postprandial blood glucose and insulin levels following a starch load in a human meal study.

3. BACKGROUND

3.1 Hyperglycaemia

Prolonged high blood glucose levels (hyperglycaemia) is a characteristic sign of diabetes mellitus (DM) (American Diabetes Association, 2009). Therefore, blood glucose control is the most important way in which to manage blood glucose levels. In general, a person is diagnosed with diabetes when their fasting plasma glucose concentration reaches ≥7.0 mmol/L or 126 mg/dL or when their two-hour plasma glucose ≥11.1 mmol/L or 200 mg/dL (International Diabetes Federation, 2015). HbA1c is an alternative (or even preferred) diagnostic for diabetes in recent criteria, especially from ADA in the US. In healthy non-diabetic individuals, postprandial hyperglycaemia is defined when the blood glucose level rises to >7.8 mmol/L (>140 mg/dL) (Monnier & Colette, 2015).

Several factors may contribute to a higher blood glucose level. One of the factors is the individual's diet, including the quality and quantity of carbohydrates (glycaemic load) in the diet (Giugliano, Ceriello, & Esposito, 2008; McGonigal & Kapustin, 2008). Food with high glycaemic index for instance rice or white bread result in higher postprandial rises in blood glucose and insulin compared with low glycaemic index foods (Tavani et al., 2003). The amount of insulin available and the degree of insulin resistance are another factor to consider when investigating blood glucose levels (American Diabetes Association, 2008). Insulin is a hormone released from the pancreas that is important in keeping the blood glucose level from getting too high or too low. If the body does not produce enough insulin or the cells are resistant to the effect of the hormone, the result will be an elevated blood glucose level (Aronoff, Berkowitz, Shreiner, & Want, 2004). Over time, insulin resistance may lead to type 2 diabetes (T2D) because of insufficient insulin to maintain blood glucose level to a normal level (Kahn, 2001).

Studies have shown that postprandial hyperglycaemia may lead to complications of diabetes and to increased cardiovascular mortality (Giugliano et al., 2008; Monnier & Colette, 2015; De Vegt et al., 1999). An observational study done by Stratton (2000) revealed that the incidence of clinical complications in patients with T2D was significantly associated with previous hyperglycaemia. Another study carried out by Cavalot et al. (2011) found that postprandial

hyperglycaemia predicts cardiovascular events in T2D with a 14-year follow-up. Therefore, controlling postprandial hyperglycaemia is very important when it comes to the management of the T2D.

3.2 Carbohydrate digestion

Carbohydrates

Complex carbohydrates such as starch are polysaccharides made up of a mixture of long chains of glucose molecules and unbranched amylose (glucose residues linked by α-(1,4) bonds) and branched amylopectin (glucose residues linked by α-(1,4) and α-(1,6) branch points) (**Figure 1**) (Hames & Hooper, 2005). Special enzymes such as α-amylase break down starch into disaccharides such as maltose, which are in turn broken down by α-glucosidases into monosaccharides such as glucose that can be absorbed by the intestines (Heacock et al., 2005).
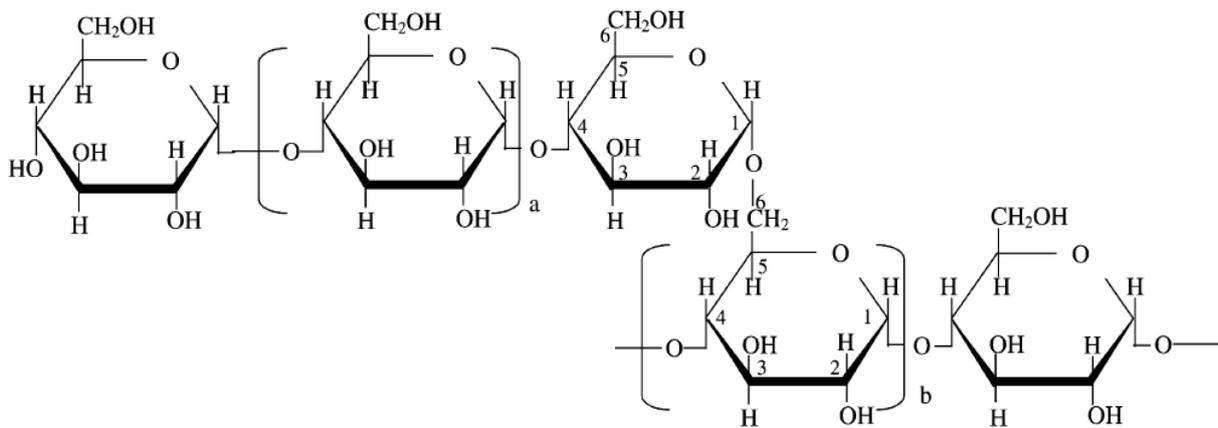

Figure 1. Structure of starch. Contains amylose and amylopectin with α-(1,4) bonds and α-(1,6) branching points. Adapted from Tester, Karkalas, & Qi (2004).

Digestion of carbohydrates in the mouth

Digestion of polysaccharides such as starch begins in the mouth. Here, salivary α-amylase hydrolyses internal α-(1,4) glycosidic bonds in linear glucose polymers. However, α-amylase is unable to hydrolyse α-(1,6) linkages, α-(1,4) linkages near branch points, or terminal α-(1,4) linkages. Hence the primary end products of amylase digestion are oligosaccharides and disaccharides (Sanders, 2016). Some undigested carbohydrates travel down to the stomach. As these smaller carbohydrates such as trisaccharides and disaccharides go into the stomach, salivary amylase is inactivated by the acidic gastric juices and the digestion of carbohydrates does not recommence until they reach the small intestine.

Digestion of carbohydrates in the small intestine

As the carbohydrates pass into the small intestine, the gastric juices are neutralised by bicarbonate (HCO_3^-). Pancreatic α-amylase is released and digests the remaining starch digestion products. Similar to salivary α-amylase, pancreatic α-amylase is only able to hydrolyse linear portions of glucose polymers. Therefore, brush border enzymes (glucoamylase, sucrose-isomaltase and glycosidase) are responsible for the next stage of the carbohydrate digestion (Sanders, 2016). Glucoamylase continues to hydrolyse starch with α-(1,4) linkages at the non-reducing end of the glucose polymers to release individual glucose moieties such as a disaccharide of glucose bound by an α-(1,6) linkage and isomaltose (Sanders, 2016). Then, the small intestinal brush border enzyme, sucrase–isomaltase, hydrolyses α-(1,6) glycosidic bonds in isomaltose and α-(1-4) glycosidic bond in maltose, breaking down maltoses to glucose. Other functions of the sucrase-isomaltase is to digest sucrose into glucose and fructose, while the glycosidase enzyme, ß-glycosidase (lactase-phlorizin hydrolase), hydrolyses the ß-(1,4) bond of lactose to release the monosaccharides, glucose and galactose.

The monosaccharide end products are quickly absorbed by the cells of the small intestine (enterocytes). The monosaccharides absorbed from the digestive tract enter into the bloodstream. The monosaccharides are then transported to the liver and the rest of the body (Sanders, 2016). Any carbohydrate that is not broken down by digestive enzymes and absorbed in the small intestine

such as resistant starch and dietary fibres will pass into the large intestine where they are fermented by microbes.

3.3   Glucose metabolism and insulin resistance

Blood glucose monitoring is essential for those who suffer from hyperglycaemia. A change of diet into one consisting of foods with a low glycaemic index (GI) is an important measure in maintaining stable blood glucose levels. Carbohydrates and their resulting glucose or other monosaccharide degradation products are an important part of the human diet. Therefore, understanding the process of maintaining a steady-state blood glucose level (homeostasis) is very important.

Plasma glucose concentration is a function of the rate of glucose entering the circulation balanced by the rate of glucose removal from the circulation. A steady-state blood glucose indicates equal rates of glucose production and utilization (Aronoff et al., 2004). This circulation of glucose is derived from the digestion of dietary carbohydrates, glycogenolysis and gluconeogenesis (Giugliano et al., 2008).

The glucose from the digestion of starch described above, is transported by glucose transporters (GLUT2) and released into the blood stream (Campos, 2012). As the glucose levels in the blood increase, ß-cells in the pancreas respond by releasing insulin (Hames & Hooper, 2005). Insulin controls the blood glucose level by activating the cell glucose absorption process and initiating storage of glucose in the muscles and liver as glycogen. It may be stored as glycogen, undergo glycolysis to yield energy and acetyl-coenzyme A, or be released into the circulation by the liver and kidneys (Poretsky, 2010). Excess glucose is also converted to fatty acids and stored as body fat.

When blood glucose levels rise after ingesting a meal, insulin initiates absorption of glucose from the bloodstream or storage in the muscle, fat and liver cells. A deficiency in insulin secretion such as insulin resistance (IR) gives rise to elevated levels of glucose in the blood (Aronoff et al., 2004). Over time, this abnormally high blood glucose level results in hyperglycaemia and can lead to prediabetes (Szablewski, 2001). Due to its reactivity, glucose binds to many proteins leading to their malfunction. This glycation can be used as a measure of continued hyperglycaemia and this

is why the relative abundance of glycated haemoglobin, HbA1c, is used as a diagnostic measure of hyperglycaemia and diabetes.

3.4 Control and management of hyperglycaemia

Untreated hyperglycaemia can cause long-term complications such as diabetes, kidney damage, nerve damage and eye problem (cataract) (Vieira-Potter, Karamichos, & Lee, 2016). The medical challenge posed by these complications is huge. Therefore, control and management plans are needed for maintaining blood glucose levels.

Primary management such as nutritional interventions, physical activity and weight control is important for the management of blood glucose levels (Giugliano et al., 2008). Lifestyle modification studies have shown that adopting a healthy diet and physical activity can prevent or at least delay the onset of diabetes with a 43% reduction over a 20-year period (Li et al., 2008). Findings from another study demonstrated that diets with a low glycaemic load are beneficial in controlling high plasma glucose levels (Ceriello, Colagiuri, Gerich, & Tuomilehto, 2008). By reducing the consumption of sugary as well as fatty foods and increasing the consumption of dietary fibre volunteers can improve blood glucose levels (Khalid Imam, 2013). Having foods with a lower glycaemic index (GI) such as legumes, fruits, vegetables and food products that contain starches and sugars that are more slowly digested and absorbed helps to reduce hyperglycaemia (Giugliano et al., 2008).

If needed, the use of oral glucose-lowering medication and food supplements is prescribed as another way to control high blood glucose levels. Monotherapy using α-amylase and/or α-glucosidase inhibitors are types of medication that lower blood glucose levels in subjects having a starchy diet. Acarbose (**Figure 2**), is one of the medications that are used to inhibit carbohydrate digestive enzyme such as α-amylase and/or α-glucosidase through competitive inhibition (Heacock, Hertzler, Williams, & Wolf, 2005). The inhibition of these enzymes delays starch degradation and slows down the absorption of sugars, consequently, reducing postprandial glycaemia (Hu, Li, Lv, Wu, & Tong, 2015). Thus, it may help to delay the development of T2D in patients with impaired glucose tolerance who consume starch.
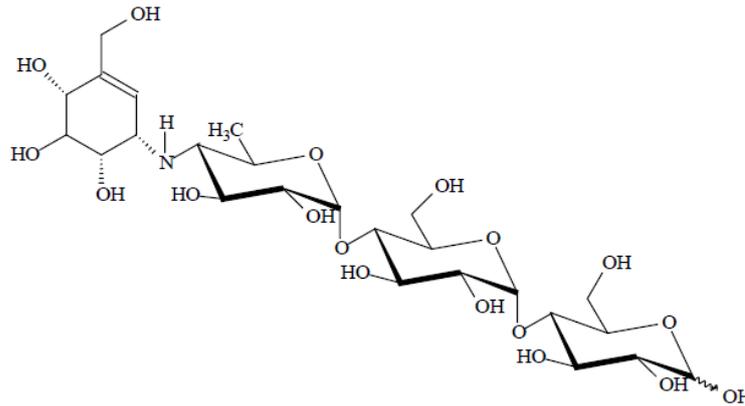
Figure 2. Acarbose. Adapted from Sales, Souza, Simeoni, & Magalhães (2012).

A meta-analysis shows positive effects of acarbose in preventing the progression of prediabetes to diabetes and in reversing prediabetes to normal glucose tolerance in Eastern and Western populations (Hu et al., 2015). In another study, 7-month treatment with acarbose also improved glycaemic control and homeostasis model assessment insulin resistance index (HOMA-IR), fasting plasma insulin, and post-prandial plasma insulin in type 2 diabetic patients (Derosa et al., 2011). Unfortunately, there are some side effects such as abdominal pain, flatulence, constipation and diarrhea from undigested starches because of the consumption of large doses of acarbose (Jones et al., 2011; Mertes, 2001). It is therefore advised to start with a low dose and gradually increase the dose to the desired amount.

Similarly, other plant compounds have demonstrated bioactivity against hyperglycaemia including polyphenols, polysaccharides, alkaloids and glycosides. Anthocyanins for instance, extracted from black currant berries inhibited α-glucosidase activity *in vitro* and was as effective as acarbose (Boath, Stewart, & McDougall, 2012). In a study conducted by Goh et al. (2015), it was shown that catechins derived from green tea also inhibited the activity of α-amylase and α-glucosidase. A study using male Wistar rats also showed that catechin solutions containing catechins from green tea (40-80 mg/mL) suppressed an increase in plasma glucose and increased plasma insulin and the activity of α-amylase (Matsumoto et al., 1993). Increasing the intake of dietary fibre might also be associated with the reduction of postprandial glucose levels and

increased insulin sensitivity (Weickert & Pfeiffer, 2008). The ability of dietary fibre to retard food digestion and nutrient absorption influences the carbohydrate metabolism. Evidence shows that by increasing the intake of fibre from sources such as cereal whole grain and oat that have a low in glycaemic index (GI) help to reduce the risk of metabolic syndrome, insulin resistance and obesity (Giacco, Costabile, & Riccardi, 2015; Lafiandra, Riccardi, & Shewry, 2014). An intervention study of 12 healthy subjects, given 6 g of partially hydrolysed guar gum (PHGG) for 12 months, showed significant reduction in postprandial plasma glucose and reduced the fasting and postprandial insulin levels (Kapoor, Ishihara, & Okubo, 2016). Barley that contains ß-glucans was fed to GK rats (male, type 2 diabetic) for 9 months and significantly improved the area under the plasma glucose concentration time curves and lowered the fasting plasma glucose level (Li et al., 2003).

High viscosity foods rich in dietary fibres may influence the glucose tolerance. For instance ß-glucan, which is highly viscous in solution increases the viscosity of contents in the stomach and small intestine (Gao et al., 2015; Lafiandra et al., 2014; J. Li et al., 2003). Increased viscosity of the intestinal bolus increases the resistance of the mucosal diffusion barrier and thereby slows gastric emptying rate and inhibits mixing and diffusion in the intestinal tract. This might prolong carbohydrate digestion and reduce the glucose absorption rate (Lafiandra et al., 2014). Dietary fibre can also significantly alter pro-glucagon gene expression and modulate glucagon-like peptide-1 (GLP-1) (Li et al., 2003). GLP-1 plays a significant role in the disposition of glucose absorbed from the gut and affects the basal islet output of glucagon and thereby also fasting glycaemia.

3.5   Seaweed

Seaweeds, also known as macroalgae, can be categorised into three different types Rhodophyta (red), Phaeophyta (brown) and Chlorophyta (green) algae (MacArtain, 2007), (**Figure 3**). There are more than 10,000 species of seaweeds found in the sea in areas from the warm tropics to the cold temperate seas (Mahadevan, 2015). Some of the species float on the water like *Sargassum* and *Gracillia*. Some of the seaweeds have adapted to live in tidal rock pools or around the shores.

Seaweeds are harvested and cultivated for various purposes. About 13 million tons of seaweeds are harvested every year around the world, including China, Korea, Japan, Philippines, Indonesia, Malaysia, India, Norway and Ireland (Mahadevan, 2015). Around 83% of seaweeds are produced for food consumption (Craigie, 2011). They are consumed as food for coastal cuisines, herbal use, and medicine (Mohamed, Hashim, & Rahman, 2012). In western countries such as Iceland, Scotland, Ireland, France, Canada and North America, seaweeds have been used as food ingredients in bread, butter and milk while in Japan, China and Korea, seaweeds are used in soups and sushi rap (Mahadevan, 2015).

3.5.1   Type of seaweeds used in the study

Eucheuma cottonii

Eucheuma cottonii, also known as *Kappaphycus alvarezii*, belongs to the red algae cultivated for the production of carrageenan (Baweja et al., 2016). *E. Cottonii* makes up 80% of the world's carrageenan production used in the phycocolloid industry in the Philippines, Indonesia and Malaysia (Lobban & Harrison, 2012). This species grows in open ocean waters with high levels of water motion, salinities greater than 30% with water temperatures between 25-30°C (Doty, Caddy, & Santelices, 1986). This seaweed is collected and dried under the sun prior to transportation. It can be eaten fresh or used as a food ingredient.

Sarcothalia crispata

Sarcothalia crispata, also known as *Iridaea ciliate,* also belongs to the red algae, found in the eulittoral to the sublittoral zone, down to a depth of 10 m (McHugh, 2003). This species grows naturally and is harvested in Chile for export to the US, Europe and Asia (Bixler, Johndro, &

Falshaw, 2001; Marambio, Mansilla, Ávila, & Rosenfeld, 2014). *S. crispata* contains kappa-carrageenan and lambda-carrageenan. They are used as a source of hydrocolloid for extracting carrageenan. The carrageenan extract of *S. crispata,* is used in dairy products as a stabilizing agent (Bixler et al., 2001).

Sargassum polycystum

Sargassum polycystum is one of the brown macroalgae. This seaweed is found in tropical and subtropical waters in China, Japan, Philippines, Malaysia and Indonesia in the midlittoral down to the sublittoral zone (Baweja et al., 2016; Stengel & Walker, 2015). It grows attached to rocks or shells and coral. *S. Polycystum* is characterised by having many branches and leaves that form large floating islands that also gives the Sargasso Sea its name (Baweja et al., 2016; Liu, Heinrich, Myers, & Dworjanyn, 2012). In earlier times, *sargassum* was collected for use in Traditional Chinese Medicine as a herbal remedy for the treatment of goitre (Liu et al., 2012). The high level of iodine in this and many other seaweeds obviously explains this empirical finding.

Laminaria digitata

Laminaria digitata, also known as one of the species used as kelp, is a type of brown seaweed that grows in cold temperate marine waters. It is the largest brown seaweed and thrives along the European littoral zone and in the northern Atlantic (Bartsch et al., 2008). Under normal conditions, it is to be found all year round with ideal habitats spread throughout Danish coastal zones and the entire coast of Norway (Raybaud et al., 2013). *L. digitata* can be described as variable in shape, with long stipe and a broad lamina (Lund, 2014).

Undaria pinnatifida

Undaria pinnatifida, also known as wakame, is a widely used seaweed in Japan (Gollasch, 2006). *U. pinnatifida* is a species of brown seaweed with a deep dark greenish brown colour and leaves being 1-3 m long and 30-40 cm wide. It grows in the Pacific Northwest and in cold temperate coastal areas along Japan, China, and Korea (Verlaque, 2007). It grows in the upper part of the infralittoral zone down to 10-20 m and it can grow on the surface of rocks, shells, artificial pillars and sea walls (Bloch, 2014). *U. pinatifida* has been cultivated for hundreds of years in Japan

and Korea, and in recent years also in China. Subsequently France and Spain have started to cultivate this popular brown seaweed (McHugh, 2003).
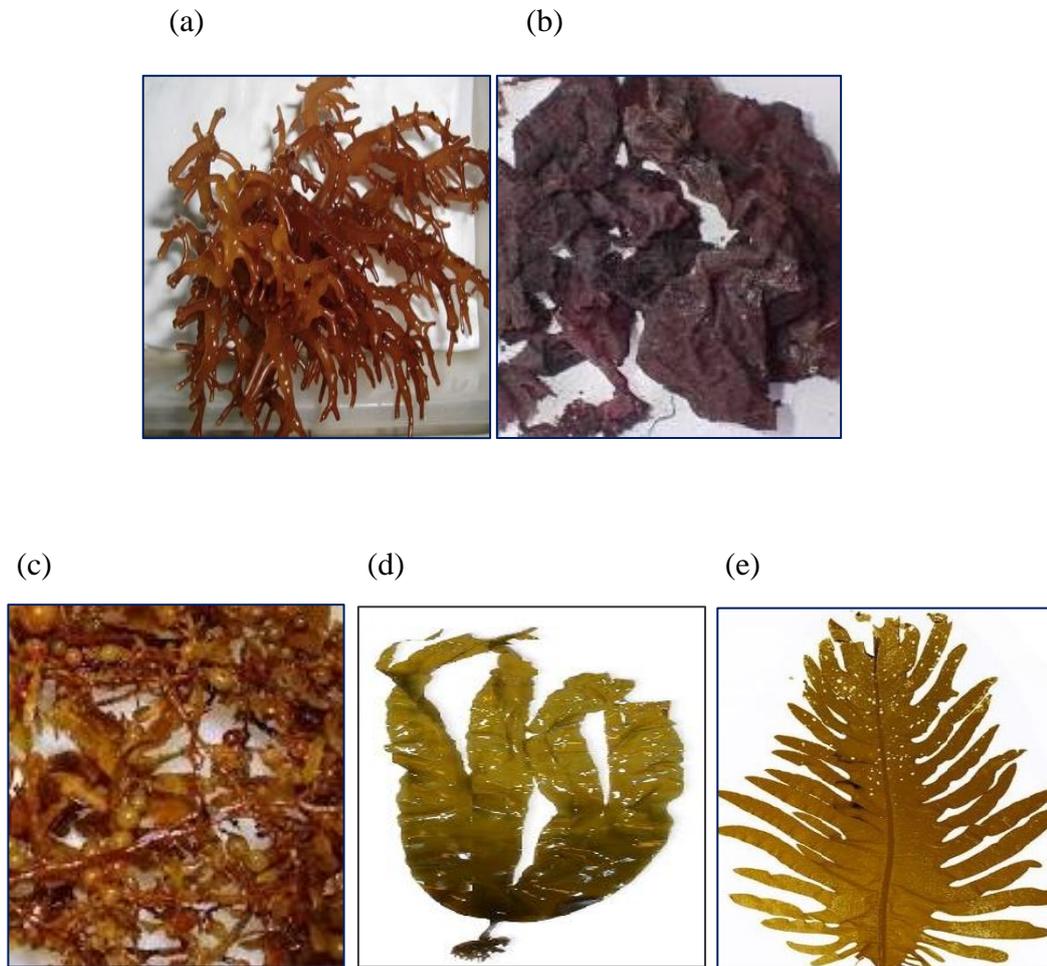
(a)   (b)

(c)   (d)   (e)

Figure 3. Various seaweeds used in the study. (a) *Eucheuma cottonii*; (b) *Sarcothalia crispata*; (c) *Sargassum polycystum*; (d) *Laminaria digitata*; and (e) *Undaria pinnatifida*. Adapted from Baweja et al. (2016); National Oceanic and Atmospheric Administration (2005); Wiktor & Tatarek (2007) .

3.5.2   Application of seaweeds as human food

In East Asian countries such as Japan, China, Korea, Taiwan and in South East Asian countries such as Thailand, Indonesia, Malaysia and Philippines, seaweeds are commonly used as food (Stengel & Walker, 2015). People who live in coastal areas in France, Scandinavia, South

West England, Ireland, and Scotland also consume seaweeds, albeit to a much more limited extent (McHugh, 2003; Stengel & Walker, 2015).

Traditionally, seaweeds are serve as salads, vegetables, sushi wrap, used in soups (**Table 1**) and as traditional medicines (Liu et al., 2012). Today, seaweeds are also used for gelatinous substances in the food or pharmaceutical industries (Mohamed et al., 2012). It was reported that approximately 1 million tons of wet seaweed is harvested annually and extracted to produce hydrocolloids (McHugh, 2003). For instance, carrageenan or complex polysaccharides from seaweeds are extracted for the production of food gelling agents and preservatives (Ole G, 2015). Carrageenan is used in powdered form as gelling agent in chocolate milk, jam and preservatives in bread, meat and fish products (Fleurence, 2016; Lobban & Harrison, 2012; Roohinejad et al., 2016). Other than carrageenan, the hydrocolloids such as alginate, agar and fucoidan found in seaweeds are extracted as gelatinous substances, used as gelling and emulsifying agents (Mabeau & Fleurence, 1993; Rajapakse & Kim, 2011). Traditional use of kelp species has been for burning to produce sodium carbonate for the European glass and chemical industries. However, this was discontinued when cheaper sources became available in the 19th century. Relatively minor uses have included various food products. Modern use includes pilot scale biofuel production.

Table 1. Seaweeds used as food ingredients

Application	Species	Country	References
Soup	*Laminaria japonica, Undaria pinnatifida*	Japan, China, France	(Bocanegra, Bastida, Benedí, Ródenas, & Sánchez-muniz, 2009; Mabeau & Fleurence, 1993)
Sushi wrappers	*Undaria pinnatifida*	Japan, Korea, China	(Lobban & Harrison, 2012; Teas, Vena, Cone, & Irhimeh, 2013)
Salad	*Laminaria japonica, Gracilaria chorda, Undaria pinnatifida, Porphyra umbilicalis*	France, Japan	(Fleurence, 2016; Mabeau & Fleurence, 1993)

Noodle	*Monostroma nitidum, Undaria pinnatifida*	Japan	(Bocanegra et al., 2009; Ole G, 2015)
Alginate powder (pork products, beef products)	*Himanthalia elongata, Undaria pinnatifida*	Spain	(Fernández-Martín, López-López, Cofrades, & Colmenero, 2009; López-López, Cofrades, Yakan, Solas, & Jiménez-Colmenero, 2010)
Carrageenan powder (bakery products, dairy products)	*Eucheuma cottonii, Chondrus crispus*	Philippine, Indonesia, Malaysia, Canada, France	(Fleurence, 2016; Mahadevan, 2015)

3.5.3 Nutritional composition of edible seaweeds

Seaweeds have a high fibre content and contain complex polysaccharides and mineral elements and a limited amount of lipids (Mabeau & Fleurence, 1993; MacArtain et al., 2007). The nutrient composition of seaweeds varies according to factors such as season, location, physiology, and species (Matanjun, Mohamed, Mustapha, & Muhammad, 2009). In Asian cuisines, the typical daily amount of seaweed consumed is 8 g on a dry matter basis (MacArtain et al., 2007). Previous studies have analysed the nutrient composition of various edible seaweeds, including their micronutrients and macronutrients (Fleury & Lahaye, 1991; Lahaye, 1991; Mabeau & Fleurence, 1993; Matanjun et al., 2009; Pereira, 2011). **Table 2** shows the reported nutrient composition of edible seaweeds. The composition of one of the red seaweed species, *S. crispata*, has apparently not been reported so far. However, other red seaweeds have reported to have high content of protein (21-47% of dry weigh) (Rajapakse & Kim, 2011).

Table 2. Nutrient composition of dried edible seaweeds.

Seaweeds	Nutrient composition				
	Carbohydrate (g/100 g dry weight)	Soluble fibre (% dry weight sample)	Insoluble fibre (% dry weight sample)	Protein (g/100 g dry weight)	The total fat content (% dry weight)
E. cottonii	26.5[a]	18.3[a]	6.8[a]	9.7[a]	1.10[a]
S. polycystum	33.5[a]	5.57[a]	34.1[a]	5.4[a]	0.29-2.19[a,h]
L. digitata	48.0[b]	32.6[c]	4.7[c]	8.0-15.0[e,g]	1.0[f]
U. pinnatifida	45.0-51.0[b,g]	30.0[d]	5.3[d]	11.0-24.0[e,g]	4.5[f]

[a]Values from Matanjun et al., 2009.
[b]Values from Pereira, 2011.
[c]Values from Fleury & Lahaye, 1991.
[d]Values from Lahaye, 1991.
[e]Values from Joel Fleurence, 1999.
[f]Values from Dawczynski, Schubert, & Jahreis, 2007.
[g]Values from Burtin, 2003.
[h]Values from Mwalugha, Wakibia, Kenji, & Mwasaru, 2015.

Fibres and carbohydrate contents

The average total dietary fibre content in edible seaweed is about 36-60% in dry condition (Rajapakse & Kim, 2011). Seaweeds are abundant in soluble fibres. Red seaweeds are rich in carrageenan and brown seaweeds are rich in alginates, fucoidan, and agar. These fibres cannot be digested completely in the human gut, thus help increase the feeling of satiety and aid digestive transit through their bulking capacity (MacArtain et al., 2007). Mac Artain and colleagues reported that *L. digitata and U. pinnatifida* contain more fibres than bananas and carrots. These seaweeds also do not contain a lot of carbohydrate, which might be causing them to have a negligible glycaemic load. High soluble fibre contents in seaweeds might be a potential functional food ingredient for lowering cholesterol and glycaemic index (Matanjun et al., 2009). The recommended average daily intake of dietary fibre in the United Kingdom is about 30 g and 25-30 g in the United States (Brirish Nutrition Foundation, 2017; Rajapakse & Kim, 2011; WHO, 2003).

Protein

Generally, protein contents in seaweeds are rich in the amino acids, aspartic acid, glycine, arginine, alanine and glutamic acid (MacArtain et al., 2007; Rajapakse & Kim, 2011). *L. digitata* contains glutamic acid that gives rise to the umami taste (Mouritsen, Williams, Bjerregaard, & Duelund, 2012). The protein content in brown seaweeds such as *L. digitata* and *U. pinnatifida* is relatively high ranging from (8-24) g per 100 g dry weight compared with *S. polycystum* which contains only 5 g protein per 100 g dry weight (Fleurence, 1999; Matanjun et al., 2009).

Lipids and fatty acids

Most seaweeds contain polyunsaturated fatty acids such as linoleic acid (C18:2ω6). In *U. pinnatifida*, however, the concentration of linoleic acid is relatively low, varying from (0.3-4.2)% of total fatty acids (Dawczynski et al., 2007). Other polyunsaturated fatty acids found in *U. pinnatifida* and *L. digitata* are arachidonic acids (C20:4ω6) with concentrations in the range of (11.3-14.3)% (Dawczynski et al., 2007). *L. digitata, E. cottonii and S. polycystum* also contained essential fatty acids such as α-linolenic acid (C18:3ω3) (Liu, Kongstad, Wiese, Jäger, & Staerk, 2016; Matanjun et al., 2009). According to Matanjun et al. (2009), *E. cottonii* also contained a high relative content of omega-3 fatty acids in the fat (45.72%) compared to *S. polycystum* (9.63%).

Minerals

Seaweeds are known for their high mineral contents. Most seaweeds are high in calcium, magnesium, potassium, sodium, iron and iodine contents as shown in **Table 3**. Pereira (2011) and Matanjun et al. (2009) reported that *U. pinnatifida* and *S. polycystum* are rich in calcium, even higher than in milk and *Laminaria* species have a sodium/potassium ration that is optimal for humans. Seaweed such as *L. digitata* is also rich in magnesium with contents that are 6 times higher than that of carrots and tomatoes (mg/100g of dry weight) (Bocanegra et al., 2009). Seaweeds also contain large quantities of iodine; however, EU recommends establishing a safe upper iodine limit for seaweed products.

Table 3. Mineral composition of seaweeds (mg/ 100 g dry weight)

Seaweeds	Mineral composition				
	Sodium	Calcium	Potassium	Iodine	Magnesium
E.cottonii	1771[a]	329[a]	13,155[a]	9.4[a]	271[a]
S. polycystum	1362[a]	3792[a]	8371[a]	7.6[a]	487[a]
L. digitata	3818[b,d]	1005[b,d]	11,579[b,d]	70[c]	659[b,d]
U.pinnatifida	1600-7000[b,d]	680-1380[b,d]	5500-6810[b,d]	3.9[c]	405[b,d]

[a]Values from Matanjun et al., 2009.
[b]Values from Pereira, 2011.
[c]Value from Institut de Phytonutrition, 2004.
[d]Value from Rupérez, 2002.

3.5.4 Bioactive compounds of seaweeds and their effect on glucose metabolism

Seaweeds are not only rich in minerals; they have also become sources of many different chemical compounds that includes a variety of biologically active compounds. **Table 4** shows the effect of seaweeds and their bioactive components on carbohydrate digestive enzymes and blood glucose levels.

Table 4. The effects of edible seaweeds on carbohydrate-digestive enzymes and glycaemic responses.

Bioactive compound	Seaweeds	Experimental model	Effect or mechanism of action	References
Fucoidan	*S. wightii*	α-D-glucosidase	Inhibit α-D-glucosidase (IC$_{50}$ 132.9 µg)	(Vinoth Kumar et al., 2015)
Fucoidan	*U. pinnatifida*	C57BL/KSJ*db/db* mice	Reduced blood glucose levels in C57BL/KSJ*db/db* mice	(Kim, Yoon, & Lee, 2012)
Alginate	*L.digitata*	Large white male pigs	Reduced blood glucose and insulin responses, 50% decrease in glucose absorption balance over 8 h	(Vaugelade et al., 2000)
Sodium alginate	Brown seaweed	Men with noninsulin-dependent diabetes	Reduced the postprandial glycaemic responses	(Torsdottir, Alpsten, Holm, Sandberg, & Tölli, 1991)
Polyphenols	*A. nodosum*	α-amylase and α-glucosidase	Inhibited α-amylase and α-glucosidase, IC$_{50}$ ~0.1 µg/mL GAE and IC$_{50}$ ~20 µg/mL GAE,respectively	(Nwosu et al., 2011; Pantidos, Boath, Lund, Conner, & McDougall, 2014)
Bromophenol	*G. elliptica*	α-glucosidase, rat-intestinal sucrose and rat intestinal maltase	Inhibited α-glucosidase (IC$_{50}$: 60.3-110.4 µM) and rat-intestinal	(Kim, Nam, Kurihara, & Kim, 2008)

				sucrose and rat intestinal maltase (IC$_{50}$: 3.6-5.0 mM)	
Fucoxanthin	*U. pinnatifida*	Female KK-A^y mice; Male C57BL/6J mice		Significantly lowered blood glucose and plasma insulin levels in a dose dependent manner	(Maeda, Hosokawa, Sashima, & Miyashita, 2007; Park, Lee, Park, Shin, & Choi, 2011)

Polysaccharides

There are two types of polysaccharides in seaweeds, those that are structural and those produced for storage. Seaweeds contain structural polysaccharides of the same type as surface plants, for examples cellulose and xylans while the storage polysaccharides consist of carrageenan, fucoidan and alginate, which exhibit textural and stabilizing properties for use in food application (MacArtain et al., 2007), **Figure 4**. Seaweeds contain an abundance of non-starch polysaccharides, potential sources of dietary fibre, prebiotics or other functional ingredients (Mabeau & Fleurence, 1993). Seaweed fibres have positive impact on regulating the blood glucose levels as they could slow down the action of carbohydrates in the gut or starch digestion in the diet (Rajapakse & Kim, 2011). According to Mabeau & Fleurence (1993) and Goñi, Valdivieso, & Garcia-Alonso (2000) the mode of action of soluble fibre may be to form viscous solutions. This could restrict the α-amylase access to complex and simple carbohydrates and thus limit enzymatic breakdown of the starch molecules. This would enable soluble seaweed fibres to slow down digestion and absorption of nutrients thereby reducing blood glucose levels.
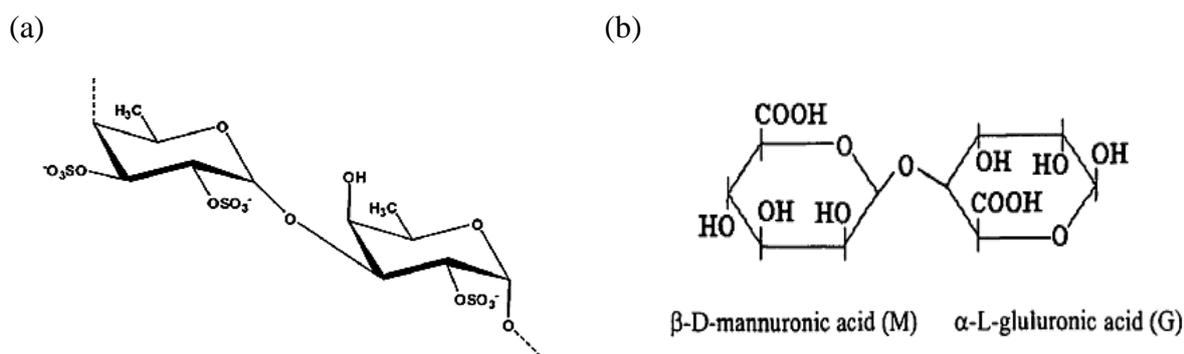
(a) (b)

Figure 4. Chemical structure of (a) Fucoidan; and (b) Alginate.

Adapted from Jani (2009) and Padua, Rocha, Gargiulo, & Ramos (2015).

Recently, seaweeds have been investigated for their potential as sources of enzyme inhibitors. Fucoidan (sulphated polysaccharides) found in brown seaweeds such as *Ascophyllum nodosum* have been identified as inhibitors of α-amylase and α-glucosidase (starch digestive enzymes), with IC_{50} 0.013-0.047 mg/mL and IC_{50} 0.12-4.64 mg/mL, respectively (Kim et al., 2014). According to Kim et al. (2014), fucoidan could inhibit enzymes through electrostatic interaction between the negatively charged sulphate groups of fucoidan with the enzyme. A study conducted by Vinoth Kumar et al. (2015), also reported that fucoidan isolated from *Sargassum wightii* inhibited α-D-glucosidase with an IC_{50} of 132.9 µg/mL.

Another seaweed fibre that is widely used in the food industry and tested on glycaemic regulation is sodium alginate. Sodium alginate consists of α-1-guluronic acid residues and ß-d-mannuronic acid residues (Yavorska, 2012). The presence of guluronic acid residues allow alginate fibre to form viscous fluids by binding Ca^{2+} ions and stomach H^+ ions (Milani & Maleki, 2012; Yavorska, 2012). Consumption of viscosity raising fibre might hinder macronutrient absorption, slow gastric emptying and reduce postprandial glucose responses (Weickert & Pfeiffer, 2008; Yavorska, 2012). An animal study done using different types of non-starch polysaccharides, showed that alginates (alginic acid) from *L. digitata* significantly reduced blood glucose absorption balance (50%) and insulin responses in pigs over 8 h (Vaugelade et al., 2000). Vaugelade and colleagues reported that this might be a product of the high viscosity polysaccharide in *L. digitata* compared with carrageenan from *E. cottonii* and xylans from

Palmaria palmate that could affect intestinal absorption of glucose and insulin response. Another study investigated crude polysaccharide obtained from *Himanthalia elongata* (Sea spaghetti), which significantly reduced blood glucose levels in alloxan-induced diabetic rabbits by approximately 50% and 18% in healthy rabbits after 8 h of 5 mg/kg intravenous administration (Lamela, Anca, Villar, Otero, & Calleja, 1989). In other work, fucoidan was isolated from the same species of seaweed (*Himanthalia elongata*) and shown to significantly lower blood glucose in healthy rabbits 8 h after intravenous administration of 2.5 mg/kg (27% reduction) (Lamela, Anca, Vazquez-Freire, Gato, & Calleja, 1993). Another study done by Kim, Yoon, & Lee (2012) reported that highly sulphated fucoidan from the *Sporophyll* of *U. pinnatifida* significantly reduced blood glucose levels in C57B/KSJm+/+*db* and C57BL/KSJ*db/db* mice.

In a human study, the consumption of 5 g of sodium alginate in T2D-patients significantly reduced the postprandial rise in blood glucose and serum insulin by 31% and 42%, respectively (Torsdottir et al., 1991). According to this group, this attenuation of glycaemic response correlated with high doses of viscous fibre. The reduction in postprandial glycaemia might be a result of highly viscous fibre causing an increased viscosity of meal or stomach contents, a slowdown of the gastric emptying rate and a lowered intestinal absorption per second. A crossover study conducted in 14 healthy male subjects demonstrated that treatment with 1.5 g of strong-gelling sodium alginate reduces glucose concentration (Paxman, Richardson, Dettmar, & Corfe, 2008).

Considering the above findings, seaweed fibre such as fucoidan and alginate found in brown seaweeds might be potent functional food ingredients for maintenance of blood glucose at near normal levels.

Polyphenol compounds

Previous research has suggested that functional groups of certain polyphenols from plants contribute to its biological importance and its efficacy in inhibition of starch digestive enzymes, as well as influence responses relevant to diabetes through modulation of glucose-induced oxidative stress (Lee & Jeon, 2013; Liu et al., 2016; Mojica et al., 2014; Nyambe-Silavwe et al., 2015; Wojdyło, Nowicka, Carbonell-Barrachina, & Hernández, 2016). Some polyphenols, specifically flavonoids, phlorotannins and bromophenols, show potential α-amylase and α-glucosidase inhibiting activities and have demonstrated anti-hyperglycaemic effects (Ibanez & Cifuentes, 2013; Lee & Jeon, 2013; Mojica et al., 2014; Pantidos et al., 2014). Seaweeds are potent sources of polyphenol compounds. Brown seaweed for instance contain phlorotannins (**Figure 5**), such as phlorofucofuroeckol, phloroeckols, eckol, dieckol and bieckol (Li, Wijesekara, Li, & Kim, 2011; Lordan et al., 2011). While red and green seaweeds contain low levels of polyphenol compounds such as catechins and epicatechins (Murphy & Hotchkiss, 2015).
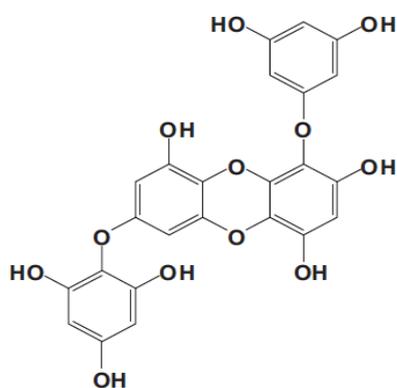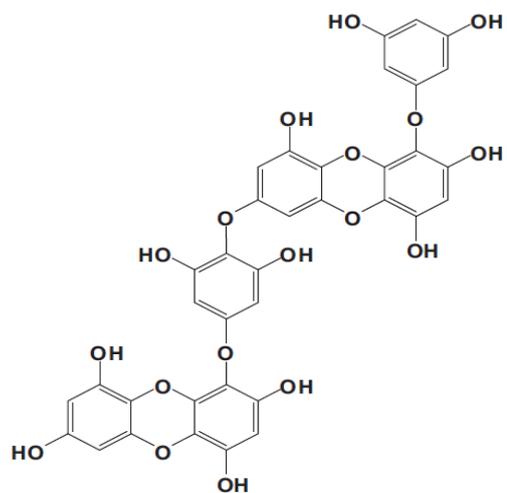
(a)
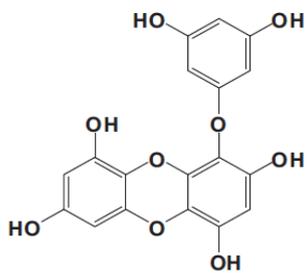
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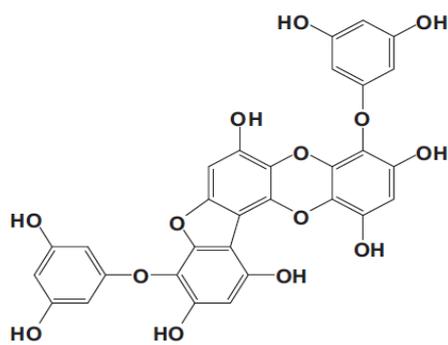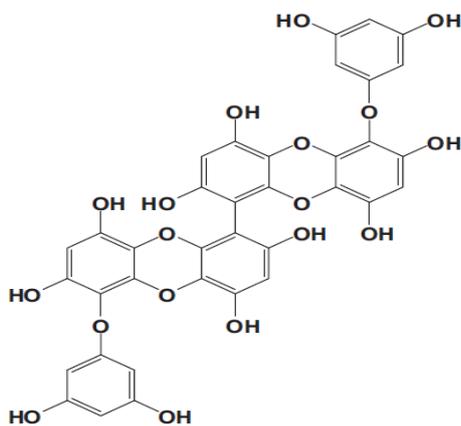
(c)

(d)

(e)

Figure 5. Chemical structure of (a) eckol; (b) phlorofucofuroeckol; (c) 7-phloroeckol; (d) dieckol; and (e) 6,6-bieckol. Adapted from Li et al. (2011).

Previous works reported that polyphenols from *Ascophyllum* inhibited amylase activity with $IC_{50}\sim$ 0.1 µg GAE/mL. It also inhibited α-glucosidase activity in vitro at low doses, $IC_{50}\sim$ 20µg GAE/mL and 10 µg/mL, respectively (Nwosu et al., 2011; Pantidos et al., 2014). Indeed, the phlorotannins from *Ascophyllum* extracts were more effective than acarbose, polyphenolic- rich extracts from teas (Yang & Kong, 2016), and berry fruits (Boath et al., 2012). In *Eisenia bicylis*, two phlorotannins, fucofuroeckol A and dioxinodehydroeckol, demonstrated significant inhibitory activities against α-glucosidase, with IC_{50} 131.34 nmol/L and 93.33 nmol/L, respectively (Eom et al., 2012). Both bioactive compounds inhibited α-glucosidase activities in non-competitive inhibitions, where they competed with the substrate to bind to the active site. While in red seaweed, bromophenol purified from *Grateloupia elliptica* actively inhibited intestinal α-glucosidase, rat-intestinal sucrase and rat-intestinal maltase (Kim, Nam, Kurihara, & Kim, 2008). In mouse models, polyphenol-rich extracts from *Ascophyllum* inhibited glucosidase and showed promising anti-diabetic effects (Zhang et al., 2007). In summary, polyphenols and in particular the phlorotannins from brown and red seaweeds, show potential anti-hyperglycaemic effects through the inhibition of α-amylase and α-glucosidase activities. The inhibitory activities of phenolic compounds against starch digestive enzymes may be associated to their hydroxyl groups and be dependent on differences in the positions or the number of hydroxyl groups (Eom et al., 2012).

Carotenoids

Carotenoids are organic pigments that are found in phototropic organisms. Seaweeds contain carotenoids such as ß-carotene, lutein, zeaxanthin, astaxanthin and fucoxanthin (Ibanez & Cifuentes, 2013). Fucoxanthin is the main carotenoid found in brown seaweed, specifically in *Undaria* and *Laminaria* species (Maeda, Tsukui, Sashima, Hosokawa, & Miyashita, 2008). Fucoxanthin has an allenic bond and a 5,6-monoepoxide in the molecule (Maeda, Tsukui, Sashima, Hosokawa, & Miyashita, 2008), **Figure 6**.
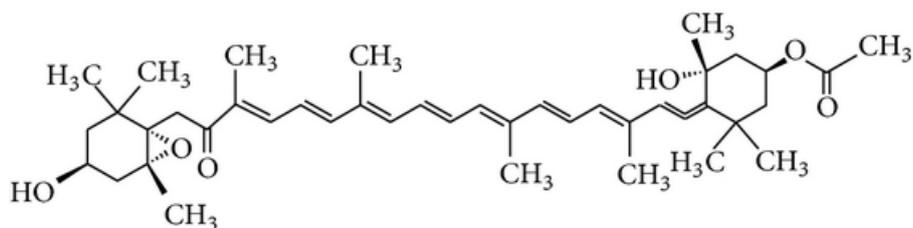
Figure 6. Chemical structure of fucoxanthin. Adapted from Padua et al. (2015).

Fucoxanthin has shown remarkable biological functions in animal model studies. It has been reported to have anti-obesity properties since it could help to attenuate the weight gain in white adipose tissue and to decrease the blood glucose level in obese and diabetic KK-A^y mice (Maeda et al., 2007; Mojica et al., 2014). A concentration of 0.02% fucoxanthin (wt/wt) significantly lowered plasma insulin, insulin resistance index and hepatic fat accumulation in (C57BL/6J) obese mice after 9 week regime (Park et al., 2011). These investigations showed that fucoxanthin has promising anti-hyperglycaemic effects, however more in-depth studies are needed to understand the mechanisms involved and the potential applications of this carotenoid in human health.

4. MATERIALS & METHODS

4.1 Sample materials

The seaweed species included in this study were selected based on a screening of different types of seaweeds that could be relevant for human health and consumption in Malaysia and Denmark. Most of these species are easily accessible and easy to use in the kitchen. The dried edible seaweeds *E. cottonii* and *S. polycystum* were purchased from Malaysia, *S. crispata* was collected from the Department of Plant and Environmental Sciences, University of Copenhagen, Denmark while *U. pinnatifida* and *L. digitata* was purchased as described in **Table 5**.

Table 5. Edible seaweed species used in this study, their class, origin and source.

Species	Class	Type	Country	Source
Eucheuma cottonii [a]	Rhodophyta	Red	Malaysia	Purchased from The Borneo Local Product, Semporna, Sabah, Malaysia.
Sargassum polycystum [b]	Phaeophyta	Brown	Malaysia	A generous gift from Uee Global Trade Sdn Bhd, Bkt Mertajam, Pulau Pinang, Malaysia.
Laminaria digitata [c]	Phaeophyta	Brown	Ireland	Purchased from AlgAran Teoranta, Kilcar Co. Donegal, Ireland.
Undaria pinnatifida [d]	Phaeophyta	Brown	Korea	Purchased from JFC Deutschland, Düsseldorf, Germany.
Sarcothalia crispata [e]	Rhodophyta	Red	Chile	A generous gift from Danisco, Copenhagen, Denmark to Department of Plant and Environmental Sciences, Section for Plant Glycobiology, University of Copenhagen, Copenhagen, Denmark.

[a]Purchased in June 2013; [b]Received in June 2013; [c]Purchased in May 2013; [d]Purchased in August 2013; [e]Collected in August 2013

The dried samples were washed with distilled water. They were freeze dried for 48 h using a freeze dryer (BFBT-101, Ontario, Canada). Next, the dried seaweeds were milled into powder at a frequency of 30 s-1 for 5 min by a TissueLyser II (Qiagen MM 200, Qiagen Nordic, West Sussex, UK). The powders were stored in the refrigerator at 2-4 ˚C for the next analysis. An overview of the experiments carried out in this study can be seen in **Figure 7**.
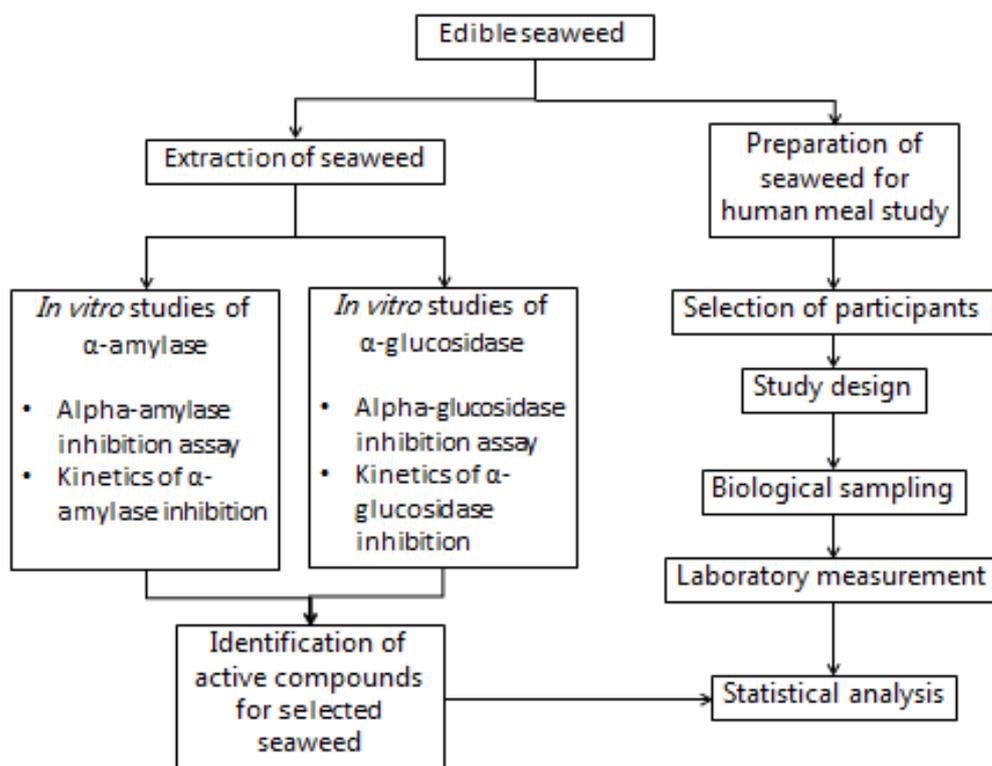
Figure 7. Experiments carried out in this study.

4.2 Preparation of seaweed extracts for α-amylase and α-glucosidase inhibition assay

The selection of solvents for extraction of seaweeds may depend on the type of the compounds targeted. In this study, water and alcohol were used to extract active ingredients from dried edible seaweeds, such as polyphenols and complex polysaccharides. As described in **Paper 1** and **Paper 2**, seaweed powders were extracted in three different solvents; water, 80% methanol and 70% acetone. The solvent-seaweed powder mixtures were extracted for 3 h and re-extracted for another 24 h. The mixtures were stirred using an orbital shaker at 140 rpm, at room temperature. After the extraction, the filtrates were evaporated using a rotary evaporator. The dried extracts were kept in the freezer for the analysis.

4.3 Identification of potent crude seaweed extracts on α-amylase and α-glucosidase inhibitory activities

In this study, α-amylase and α-glucosidase inhibition using methanol, acetone and water extracts of seaweeds were determined as their percentage of enzymes inhibition and their IC_{50} values. Stock solutions of all five seaweeds were prepared by dissolving the methanol and acetone extracts with dimethyl sulfoxide (DMSO). Water extracts were dissolved in 0.02 M sodium phosphate buffer solution. Acarbose was used as a positive control and as a negative control incubation mixture with no seaweed. The absorbances were measured for all samples, blank readings that contained no enzyme were subtracted from each well and results were compared to the control.

The percentage of enzyme inhibition was calculated using the following equation:

$$\% \text{ enzyme inhibition activity} = \frac{(\text{Absorbance of negative control} - \text{Absorbance of sample})}{\text{Absorbance of negative control}} \times 100\%$$

IC_{50} values (defined as the inhibitor concentration that inhibits 50% of the enzyme activity) were determined graphically by interpolation from the inhibitions determined with different concentrations of seaweed extracts, as a percentage of inhibition versus log inhibitor concentration. The α-amylase inhibitory effects of seaweeds were measured at 0.05-50 mg/mL while the concentrations of seaweed extracts for α-glucosidase inhibitory effects were 0.005-50 mg/mL.

4.4 Alpha-amylase inhibition assay

The α-amylase inhibition was determined by an assay modified from a method developed by Wu (Wu et al., 2011) and Balasubramaniam (Balasubramaniam et al., 2013). A method of detection using dinitro salicylic acid (DNS) colour reagent, sodium phosphate buffer, starch solution, and seaweed extract was used for measuring the inhibition of porcine pancreatic α-amylase, as described in detail in **Paper 1**. Each sample was placed in a 96-well microplate and

the α-amylase inhibitory activity was determined based on the colorimetric assay. The absorbance was recorded at 540 nm.

4.5 Alpha-glucosidase inhibition assay

The α-glucosidase inhibition assay was performed according to the method described in (Schmidt, Lauridsen, Dragsted, Nielsen, & Staerk, 2012). The inhibitory effects of 5 seaweed extracts were measured at lower concentrations. In each 96-well microplate, a solution of α-glucosidase enzyme was mixed with seaweed extract and pNPG. The solution was then incubated at 28°C and the ratio of pNPG to p-nitrophenol was determined at 405 nm every 30 s for 35 minutes as described detail in **Paper 2**.

4.6 Kinetics of enzyme inhibition

The reaction rate and modes of inhibition of α-amylase and α-glucosidase by the different seaweed preparations were determined by Michaelis-Menten kinetics using Lineweaver-Burk plots. Initial reaction rate experiments were performed to determine the Michaelis constant (K_m) and maximal velocity (V_{max}). Michaelis-Menten kinetic parameters were determined using different concentrations of starch as a substrate 5.84-29.21 mM for α-amylase inhibition, while 0.1-4.0 mM of pNPG for α-glucosidase inhibition. The substrates were each added into mixtures of seaweed extracts and enzymes. These experiments were performed as described in previous procedures for α-amylase and α-glucosidase inhibition assays (Balasubramaniam et al., 2013; Schmidt et al., 2012; Wu et al., 2011). The absorbance was measured immediately with a Multiscan FC microplate photometer to determine the initial reaction velocities, as described in **Paper 1** and **Paper 2**.

4.7 Identification of α-amylase inhibitors in seaweed extracts

Seaweed extracts that show the most potent inhibitors of α-amylase and α-glucosidase activities with low IC$_{50}$ and high percentage of inhibition were selected to identify their potent bioactive compounds. In this study, *L. digitata* and *U. pinnatifida* that showed strong inhibitory effects were selected for the identification of bioactive compounds.

Phenolic compounds

As referred in **Paper 1**, total phenolic content in seaweed extracts were determined by using a modified Folin-Ciocalteu method (Singleton, Orthofer, & Lamuela-Raventós, 1999). The absorbance was measured at 765 nm on UV/VIS spectrometer Lambda 25 (PerkinElmer, Waltham, MA, USA). Standard (gallic acid) was used to construct calibration curve. The total phenolic content was expressed as mg gallic acid equivalents per mg of seaweed extract (mg GAE/mg).

Polyphenolic compounds in seaweed extracts were then analysed on Reversed phase HPLC, Agilent 1200 series instrument (Santa Clara, CA). Polyphenolic compounds were identified by the retention time of chromatographic peaks compared with standards using the same HPLC operating conditions. Analytical grade standards of phenolic acids were used for further study of α-amylase inhibitory activity and kinetics.

Complex carbohydrates

Further investigation using comprehensive microarray polymer profiling (CoMPP), a method from Sørensen & Willats (2011) and Torode et al. (2015) was applied to identify potent complex carbohydrates in selected edible seaweeds. This study was conducted at the Department of Plant Glycobiology, Faculty of Science, University of Copenhagen. The heatmap used in this experiment indicated mean plot signals, showing the relative binding of the monoclonal antibodies (mAb LM7, BAM1 and CBM3a) to seaweed extracts. High values of mean spot signals indicate a strong binding of monoclonal antibodies to seaweed extracts, again being an indicator of complex polysaccharides. LM7, CBM3a and BAM indicate that seaweed extracts contain alginate, cellulose and fucoidan, respectively. Detailed procedures are described in **Paper 1**. Complex polysaccharides were then extracted using a modified method originally developed by Fertah et al. (2014) and Torres et al. (2007). The yield of the complex polysaccharides was measured based on their dried biomass obtained after the extraction as the percentage of the seaweed dry weight (% dry wt). The dried extracts were then used for further study of α-amylase inhibition and its kinetics.

4.8 Identification of α-glucosidase inhibitors in seaweed extracts

High-resolution α-glucosidase bio-chromatogram

This study was conducted at the Department of Drug Design and Pharmacology, University of Copenhagen. A chromatographic separation of seaweed extracts was performed using a High-Pressure Liquid Chromatograph (HPLC) (Agilent 1200 series) and fractionated at all time points during elution into 96-well microplates, leading to a resolution of data points per min, as described in **Paper 2**. The α-glucosidase inhibition for each well was determined as described above (α-glucosidase inhibition assay). High-resolution inhibition profiles (cleavage rates) were plotted at selected retention times as a α-glucosidase inhibition bio-chromatogram underneath the HPLC chromatogram.

Identification of active compounds using HPLC-HRMS

Separation of seaweed extracts were performed using the same procedure, solvent composition, gradient profile, column, and temperature as described in the high-resolution α-glucosidase biochromatogram's procedure. HPLC-HRMS analysis of analytes with α-glucosidase activity was performed on the above-described chromatograph using a T-piece splitter to direct 0.1% of the HPLC elute to a micrOTOF-Q II mass spectrometer (Bruker Daltonik, Bremen, Germany), equipped with an electrospray ionisation (ESI) interface, as described in detail in **Paper 2**. Mass spectra were acquired in positive-ion mode.

4.9 Human meal studies

Human meal study was conducted at the Department of Nutrition, Exercise & Sports, University of Copenhagen. The study was designed as a randomised, 3-way, blinded crossover trial. In total, 20 healthy participants completed the study. The test meal consisted of 150 mL of starchy drink, made up of 30 g of corn starch in water with 22 g of sugar free lemonade powder, with either of 3 different solid foods. The only difference between the meals was the addition of 5 g of the seaweeds, *Laminaria digitata* or *Undaria pinnatifida,* or the addition of 5 g pea protein. Together with the test meal, a glass of 500 mL of water was served.

The participants were instructed to eat only one type of meal each test day. The day before they received the meal, they had an overnight fasting period of 12 h. In the morning of each test day, the participants completed their first registration of subjective appetite sensations, the first blood sample was drawn, and the participants ate the test meal. Blood samples were drawn as follows: -20min (baseline) and then at 20, 40, 60, 90, 120 and 180 min. The blood was collected for plasma glucose and serum insulin analysis by standard clinical chemistry kits. Visual-analog scales (VAS) for a number of hunger- and satiety related feelings were also recorded intermittently for up to 3 h. After that an ad libitum meal consisting of pasta Bolognese was served and food intake was registered to calculate energy intake. Plasma glucose concentration was determined by a standard kit on an ABX Pentra 400 analyser (Horiba ABX SAS, Montpellier, Cedex, France). Insulin concentration was determined by a standard solid-phase, two-site chemiluminescent immunometric assay. GLP-1 was determined using ELISA; details about the human meal study are provided and discussed in **Paper 3**.

The primary hypothesis was that the incremental area under the curve (iAUC) for plasma glucose would be lower after seaweed meals than after the placebo meal. Secondary endpoints are the iAUC for insulin, mixed-model postprandial analyses of differences in glucose and insulin, and the post-meal energy intake which were also hypothesized to be lower.

4.10 Determination of mineral elements and nutrient composition in seaweed samples

The experiment to determine mineral elements in selected seaweeds was conducted at the National Food Institute, Technical University of Denmark. The concentration of selected mineral elements in the seaweed samples was determined following the principles in EN15762:2009 (European Commitee for Standardisation, 2009) and EN15111:2007 (European Committee for Standardisation, 2005). The total element concentration was determined using inductively coupled plasma mass spectrometry (ICP-QQQ-MS) (Agilent 8800, Agilent Technologies, Waldbronn, Germany). Quantification was done using external calibration with internal standardization. Analytical quality was assessed by running selected samples in duplicate (RSD values in the range 1-20% for all elements) and by the use of the certified reference material (CRM) ERM-CD200 Bladderwrack (IRMM, 2016).

The analysis to determine the nutrient composition of test meals were conducted by Eurofins A/S (Glostrup, Denmark) and the results were compared with the nutrient composition from Dankost. Three test meals (*L. digitata, U. pinnatifida and pea protein*) were analysed for its contents of energy, protein, fat, carbohydrate and dietary fibre.

5. RESULTS

5.1 Paper 1: Inhibitory effects of edible seaweeds, polyphenolics and alginates on the activities of porcine pancreatic α-amylase

Nazikussabah Zaharudin, Armando Asunción Salmeán, Lars Ove Dragsted

Inhibitory effects of edible seaweeds, polyphenolics and alginates on the activities of porcine pancreatic α-amylase

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Abstract

Edible seaweeds are valuable because of their organoleptic properties and complex polysaccharide content. A study was conducted to investigate the potential of dried edible seaweed extracts, its potential phenolic compounds and alginates for α-amylase inhibitory effects. The kinetics of inhibition was assessed in comparison with acarbose. The methanol extract of *Laminaria digitata* and the acetone extract of *Undaria pinnatifida* showed inhibitory activity against α-amylase, IC_{50} 0.74±0.02 mg/mL and 0.81±0.03 mg/mL, respectively; both showed mixed-type inhibition. Phenolic compound, 2,5-dihydroxybenzoic acid was found to be potent inhibitor of α-amylase with IC_{50} value of 0.046±0.004 mg/mL. Alginates found in brown seaweeds appeared to be potent inhibitors of α-amylase activity with IC_{50} of (0.075±0.010 - 0.103±0.017) mg/mL, also a mixed-type inhibition. Overall, the findings provide information that crude extracts of brown edible seaweeds, phenolic compounds and alginates are potent α-amylase inhibitors, thereby potentially retarding glucose liberation from starches and alleviation of postprandial hyperglycaemia.

KEYWORDS: Seaweed; Glucose liberation; α-Amylase; Phenolic compound; Alginate; Inhibitor; Hyperglycaemia

1. Introduction

Foods with a high glycaemic index (GI) such as simple carbohydrates, are rapidly digested and cause a rise in blood glucose levels (Opperman, Venter, Oosthuizen, Thompson, & Vorster, 2004). Enduring high blood glucose levels, known as hyperglycaemia, precedes type 2 diabetes mellitus (T2DM) (Oh, 2014). Dietary changes such as maintenance of a low dietary GI may aid in the prevention and management of hyperglycaemia. Another method to control blood glucose levels is through bioactive food components acting on the liberation of glucose during digestion. Inhibiting enzymes such as α-amylase is one such method for slowing down glucose liberation. It has previously been suggested that management of hyperglycaemia by inhibition of α-amylase may be used for the treatment or prevention of T2DM (Wu et al., 2011). Alpha-amylase found in pancreatic juice and saliva plays a significant role in the digestion of polysaccharides into maltose and glucose.

Seaweeds are marine algae that are commonly used as vegetables in Asian countries. Seaweeds are also used as medicines and other therapeutic applications, while in Western countries they are used as a functional ingredients in foods and beverages (Gupta & Abu-Ghannam, 2011; Mabeau & Fleurence, 1993). Numerous studies have shown that seaweeds have significant nutritional value as well as potential health benefits. Seaweeds contain minerals, nutrients and non-nutrient components such as phenolic compounds and terpenoids (Stengel, Connan, & Popper, 2011; Syad, Shunmugiah, & Kasi, 2013). In addition, seaweeds contain complex polysaccharides (alginate, carrageenan, fucoidan, agar, cellulose or xylan), which make up 30-71%, of their dry weight (Fleurence et al., 2012; O'Sullivan et al., 2010). A study carried out in stable diabetes patients revealed that seaweed fibre (alginate) decreased the postprandial rise of blood glucose and insulin levels (Torsdottir, Alpsten, Holm, Sandberg, & Tölli, 1991) . Other more recent studies reported that fucoidan and cellulose were showing α-amylase inhibitory activity *in vitro* (Kim, Rioux, & Turgeon, 2015; Dhital, Gidley, & Warren, 2015). Seaweeds are also known to have a low lipid content, only ~2.3% of their dry weight (Dawczynski, Schubert, & Jahreis, 2007). Since seaweeds contain complex

polysaccharides, which are resistant to enzymatic degradation by enzymes in the human body but undergo some degree of fermentation by the gut microbiota, consumption will slow down the release of absorbable monosaccharides such as glucose (Ramnani et al., 2012; Mohamed, Hashim, & Rahman, 2012).

As seaweeds contain chemical constituents with potential for inhibition of α-amylase, these foods and food constituents might be beneficial in promoting health or management of hyperglycaemia. Therefore, in this study, we investigated the α-amylase inhibitory effects of dried seaweed sources from red and brown seaweeds (*Sargassum polycystum, Laminaria digitata, Undaria pinnatifida, Eucheuma cottonii,* and *Sarcothalia crispata*), some of which have not been studied previously. These seaweeds were selected due to their abundance and prevalent use as foods in Malaysia and in European countries such as Spain, Ireland, Norway and Denmark. In addition, we investigated the potential of phenolic compounds and alginates found in brown seaweeds as α-amylase inhibitor. This is the first study to clarify the enzyme kinetics of α-amylase inhibition by several edible seaweeds and to compare their inhibition kinetics with alginate and phenolic acids.

2. Materials and methods

2.1 Chemicals

Monoclonal antibodies (LM7, CBM3a or BAM1) were purchased from Paul Knox Cell Wall Lab, Centre for Plant Sciences, Faculty of Biological Sciences, University of Leeds, UK. HPLC grade acetonitrile and methanol, Folin-Ciocalteu's reagent, standards of gallic acid, epicatechin, 2,5-dihydroxybenzoic acid and epigallocatechin were purchased from Merck (Darmstadt, Germany). Porcine pancreatic α-amylase (EC 3.2.1.1), soluble starch ($C_{12}H_{22}O_{11}$) (MW: 342.30 g/mol), 3, 5-dinitrosalicylic acid ($C_7H_4N_2O_7$), acarbose, bromo-4-chloro-3-indolyphosphate (BCIP), nitro-blue tetrazolium chloride (NBT), diethanolamine, commercially available alginate (A7003) and all buffers and salts were purchased from Sigma-Aldrich (Schnelldorf, Germany).

2.2   Materials

The dried edible seaweeds *Eucheuma cottonii* and *Sargassum polycystum* were imported from Malaysia, *Sarcothalia crispata* was collected from the Department of Plant and Environmental Sciences, University of Copenhagen, Denmark while *Undaria pinnatifida* and *Laminaria digitata* were purchased from Ireland and Germany, as described in the Supplementary data, **Table S1**. The collected samples were washed with distilled water and were frozen at -70 ^{o}C for 24 h and dried for 48 h using a freeze dryer (BFBT-101, Ontario, Canada). The dried seaweeds were milled into powder at a frequency of 30 s^{-1} for 5 min by a TissueLyser II (Qiagen MM 200, Qiagen Nordic, West Sussex, UK). Seaweed powders were then stored in the refrigerator at 2-4 $^{\circ}$C for up to 48 hours before analysis.

2.3   Preparation of seaweed extracts

Separate portions (5 g) of each seaweed powder were extracted in 50 mL of methanol (80%), 50 mL of acetone (70%) or 100 mL of water. During extraction, the solvent-seaweed powder mixtures were stirred using an orbital shaker at 140 rpm for 3 h at room temperature. Then, the methanol and acetone extracts were filtered using filter paper Whatman, Cat No 1001 125 (Frisenette, Knebel, Denmark) while the cold-water extract was filtered using a glass wool filter (18421 Glass wool, Sigma Aldrich, Schnelldorf, Germany). The residues were re-extracted for another 24 h under the same conditions and the filtrates from the first and second extractions were combined. The filtrates were evaporated using a rotary evaporator with a water bath temperature of 40 ^{o}C (Büchi Rotavapor R-114 and water bath B-480 from Büchi Labortechnik AG, Flavil, Switzerland). The dried extracts were kept in the freezer at -20 $^{\circ}$C within 48 hours for the analysis. In α-amylase inhibition assay, 200 mg dried matter/ mL of stock solutions were prepared by dissolving the methanol and acetone extracts in 10% DMSO. The stock solution for dried aqueous extract was prepared by dissolving the extract in 0.02 M sodium phosphate buffer solution.

2.4 α-Amylase inhibition assay

The α-amylase inhibitory activities of seaweeds were assayed using methods described by Wu et al. (2011) and (Balasubramaniam et al., 2013) with minor modifications. A volume of 100 μL of the diluted seaweed extract was mixed with 100 μL of porcine pancreatic α-amylase (0.5 U/mL of α-amylase in 0.02 M sodium phosphate buffer with 0.06 M NaCl, pH 6.9 at 20˚ C) in 2 mL Eppendorf tubes. The mixture was pre-incubated at 25 ˚C for 10 min. Acarbose (2 mg/mL) was used as a positive control. As a negative control (without seaweed extract), the sodium phosphate buffer was used for studies with water extracts and a mixture of 10% DMSO and sodium phosphate buffer was used for methanol and acetone extracts. After pre-incubation, 100 μL of 1% starch (dissolved in 0.02 M sodium phosphate buffer) was added into the solution and the mixture was incubated at 25 ˚C for 10 min. Next, 200 μL of 96 mM dinitro salicylic acid (DNS) colour reagent was added. The mixture in the tubes were heated in boiling water for 5 min to stop the reaction and then cooled to room temperature. Finally, 200 μL of each incubation mixture was transferred into a well in a 96-well microtiter plate and the absorbance of the mixture was measured at 540 nm. The absorbance was measured with a Tecan Spectra III Rainbow reader (Tecan, Grodig, Austria) using Magellan Standard 6.0 software.

The percentage of α-amylase inhibition was calculated using the following equation:

$$\% \ \alpha-\text{amylase inhibition activity} = \frac{(\text{Absorbance of negative control} - \text{Absorbance of sample})}{\text{Absorbance of negative control}} \times 100\%$$

IC_{50} values (defined as the inhibitor concentration that inhibits 50% of the enzyme activity) were determined graphically by interpolation from the inhibitions determined with different concentrations of seaweed extracts ranging from 0.05-50 mg/mL as percent of inhibition vs log inhibitor concentration (Gomathi, Kalaiselvi & Uma, 2012).

2.5 Kinetics of α-amylase inhibition

The reaction rate and modes of inhibition of α-amylase by the different seaweed preparations were determined by Michaelis-Menten kinetics using Lineweaver-Burk plots. Experiments were performed to determine the Michaelis constant (K_m) and maximal velocity (V_{max}). This experiment was also performed in the 96- well microplates: 50 µl of starch solutions (5.84, 11.69, 17.53, 23.73 and 29.21 mM) were added with 50 µL of 50 mg/mL seaweed extracts and 50 µl of 0.3 U/ml porcine pancreatic α-amylase. The absorbance was measured continuously with a Multiscan FC microplate photometer at 540 nm to determine the initial velocities.

2.6 Determination of total phenolic contents

The amount of total phenolic contents of selected seaweed extracts were determined according to the Folin-Ciocalteu method. Seaweed extract (1.0 mL) was mixed with 1.0 mL of Folin-Ciocalteu's reagent and 5.0 mL of distilled water. The mixture was incubated for 5 min at room temperature. Next, 1.0 mL of 20% Na_2CO_3 was added to the mixture. The mixture was made up to 10.0 mL, and incubated for 1 h at room temperature. The absorbance was measured at 765 nm on UV/VIS spectrometer Lambda 25 (PerkinElmer, Waltham, MA, USA). Standard (gallic acid) was used to construct calibration curve. The total phenolic content was expressed as mg gallic acid equivalents per mg of seaweed extract (mg GAE/mg).

2.7 Identification of phenolic acids by HPLC

Chromatographic separation of seaweed extract was performed on a C_{18} column (Phenomenex, 150 mm x 4.6 mm, i.d 3 µm) using HPLC, Agilent 1200 series instrument (Santa Clara, CA). The HPLC analysis was performed using eluent (A) consisted of water/acetic acid (99:1, v/v) and eluent (B) consisted water/acetonitrile/acetic acid (67:32:1, v/v/v). The injection volume of sample is 10 µL at 23 °C. The flow rate was 1 mL/min with the gradient profile: 0-10 min, 10% B; 10-16 min, 20%

B; 16-20 min, 40% B; 20-25 min, 50% B; 25-27 min, 40% B; 27-35 min, 10% B. UV trace was monitored at 275 nm. The identification of phenolic compounds in the chromatographic peaks were determined by the comparison of retention times with that of reference compounds.

2.8   *Identifying complex carbohydrates in selected seaweeds*

The composition of complex polysaccharides was analysed using comprehensive microarray polymer profiling (CoMPP), according to a published method (Moller et al., 2007; Salmeán et al., 2017). The analysis involved sample extraction, spotting using a microarray robot, probing using monoclonal antibodies (mAb) and quantification using microarray software (ImaGene 6.0, Biodiscovery Inc., El Segundo, CA, USA). The selected seaweeds were extracted 3 times by using the Alcohol insoluble residue (AIR) method. The collection of alcohol insoluble residue was performed by adding 5 mL of 70% ethanol to the powdered seaweeds and the mixtures were stirred for 1 h at 60 °C. The samples were centrifuged at 2500 x g for 10 min and the supernatants was discarded. This step was repeated until the supernatant was clear. Finally, the AIR samples were washed with acetone for 5 min and then air dried.

The dried samples (10 mg) were placed in 96-tube boxes and metal ball bearings were placed in each tube. The samples in the tubes were homogenized with the TissueLyser II. A volume of 300 µL 50 mM CDTA was added to each tube and the samples shaken at 30 Hz on the TissueLyser II for 2 min before a 2 h extraction at 6-10 Hz. The boxes were then centrifuged at 2500 x g for 10 min and the supernatants were collected.

Next, the samples were prepared for microarray printing. The extracted samples were printed onto nitrocellulose membranes using a microarray robot microarrayer robot (Piezoelectric Sprint Arrayjet, Roslin, UK). The membrane was cut into individual arrays and dissolved in 5 mL of blocking solution of 5% MP/PBS for 1 h. MP/PBS was discarded and 5 mL of primary mAb LM7,

BAM1 and carbohydrate binding protein (CBM3a) solution was added to extracted prints of each sample and left for 2 h. The mAb/CBM solution was discarded and the arrays were washed three times for 5 min in 10 mL PBS and 1 min in 10 mL deionised water on the orbit shaking table. Then, alkaline phosphatase (AP)-conjugated secondary mAb was added in 5 mL of MP/PBS and arrays were left at room temperature for 2 h. After 2 h with secondary mAb, the arrays were washed as described above to remove excess antibody. Finally, these arrays were developed using development solution, which contained alkaline phosphate buffer and stored in the darkness until they were ready for development. Once developed, the arrays were removed from the development solution and were put in a water bath to stop the reaction and rinsed with deionised water for 3 min before placement on a filter paper to dry.

For quantification of spot signals, the arrays were scanned on a standard commercial desktop flatbed scanner (CanoScan 9950F, Canon Denmark, Copenhagen, Denmark) at a resolution of 1200 dpi and images were saved as 16-bit tiff files. The tiff files were uploaded into the Imagene microarray analysis software. The spot signals were converted into heatmaps using an online heatmapper tool (www.bar.utoronto.ca/ntools/cgi-bin/ntools_heatmapper.cgi). The complete data set that provided the maximal mean spot signals was set to 100%.

2.9   Alginate extraction

The extraction of alginate was performed through a modified method originally developed by Fertah et al. (2014) and Torres et al. (2007). Seaweed powder was suspended in 100 mL of 2% $CaCl_2$. The solution was shaken for 1 h at 80°C using an orbital shaker at 240 rpm. After centrifugation at 16,000 x g for 10 min, the supernatant was collected. The supernatant was mixed well with 2 mL of 0.2 M HCl and the solution was shaken again using the same procedure as described above. The supernatant was then mixed with 5 mL of 3% NaC_2CO_3. The solution was re-extracted for another 1 h under the same conditions. The supernatants collected were labelled as crude extracts. The crude

extract was mixed with ethanol to obtain alginate. The solutions were then filtered, and the filtrates were dried using a rotary evaporator with a water bath temperature of 40°C (Büchi Rotavapor R-114 and water bath B-480 from Büchi Labortechnik AG, Flavil, Switzerland). The yield of the alginate was measured based on their dried biomass obtained after the extraction as the percentage of the seaweed dry weight (% dry wt). The dried extracts were kept in the refrigerator at -20 ˚C within 48 hours for the analysis.

2.10   Statistical analysis

The α-amylase inhibition assays were performed in triplicate. Data was expressed as mean ± standard deviation. Statistical analysis was performed by using GraphPad Prism software (version 6.0) (GraphPad Software Inc., San Diego, CA, USA) for IC_{50} and kinetic constants calculation. Values obtained were compared using Analysis of Variance (ANOVA). P-values were determined with corrections by Tukey's multiple comparison and $P < 0.05$ was considered statistically significant. Kinetic constants were calculated in Excel and used to plot the Michaelis-Menten and Lineweaver-Burk plots.

3. Results and discussion

3.1   Alpha-amylase inhibition

A comparative study was conducted to determine the ability of five species of seaweed extracts in three different solvents to inhibit α-amylase degradation of starch. As shown in **Fig. 1**, almost all seaweed extracts from, *Eucheuma cottonii, Sargassum polycystum, Laminaria digitata, Undaria pinnatifida and Sarcothalia crispata* showed the ability to inhibit porcine pancreatic α-amylase activity at a concentration of 2 mg/mL.
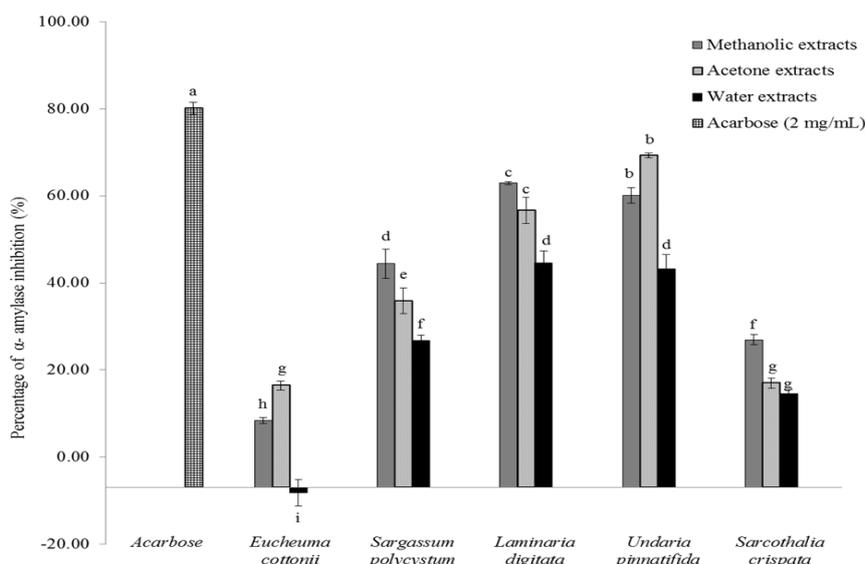
Fig. 1. α-Amylase inhibition using 2 mg/mL of seaweed extracts in methanol, acetone or water. Acarbose at 2 mg/mL was used as a positive control. Results were represented as mean ± standard deviation (n=3). Bars marked with different letters are significantly different at $P < 0.05$.

The α-amylase inhibitory activity was found to be stronger with the methanol and acetone extracts than with water. The acetone extracts of *Undaria pinnatifida* showed the highest percentage inhibition of 69.3 ± 0.5% amongst the tested seaweed extracts, followed by methanolic extracts of *Laminaria digitata* with an inhibition of 61.5 ± 0.7% at 2 mg/mL **(Fig. 1)**. Acetone extract of *Undaria pinnatifida* and methanol extract of *Laminaria digitata* had significant ($P < 0.05$) inhibitory effect on α-amylase activity but lower than the commercial inhibitor, 2 mg/mL of acarbose (positive control), which inhibited the enzyme by 80.8 ± 0.5%. In contrast, the extracts of *Eucheuma cottonii* had the least inhibitory effect against α-amylase, less than 20% of inhibition at a concentration of 2 mg extract/mL. The aqueous extract of *Eucheuma cottonii* showed no α-amylase inhibitory activity. Overall, it is noted that brown seaweeds such as *Undaria pinnatifida* and *Laminaria digitata* had appreciable effects on pancreatic α-amylase (> 40%) compared with red seaweeds like *Eucheuma cottonii* and *Sarcothalia crispata*.

Further data analysis was completed to determine the effectiveness of seaweed extracts in inhibiting α-amylase. The IC$_{50}$ was calculated from the concentration-by-inhibition plots and the IC$_{50}$

values are shown in **Table 1**. The standard inhibitor, acarbose was used as positive control (IC_{50}=1.12 ± 0.04 mg/mL).

Table 1
IC_{50} values of seaweed extracts against α-amylase activities.

Seaweed species	IC_{50} (mg/mL) [a, b]		
	Methanol	Acetone	Water
Eucheuma cottonii	6.5 ± 0.2	5.7 ± 0.7	>10
Sargassum polycystum	2.5 ± 0.0	2.8 ± 0.1	4.2 ± 0.5
Laminaria digitata	0.74 ± 0.02	0.96 ± 0.03	3.2 ± 0.2
Undaria pinnatifida	0.92 ± 0.02	0.81 ± 0.03	2.07 ± 0.1
Sarcothalia crispata	4.6 ± 0.5	5.0 ± 0.5	6.3 ± 0.2

[a]Data are presented as mean ± standard deviation (n=3)
[b]IC_{50} is the half maximal inhibitory concentration to produce 50% inhibition of the enzyme activity.

The highest inhibitory activity was found in methanol extract of *Laminaria digitata* and in acetone extracts of *Undaria pinnatifida* with IC_{50} less than 1 mg/mL. The lowest inhibitory activity was exhibited by the water extract of *Eucheuma cottonii* where IC_{50} was more than 10 mg/mL and with a non-recordable inhibition even at this maximal concentration. It was found that brown edible seaweeds like *Laminaria digitata* and *Undaria pinnatifda* have considerable potential for inhibition of α-amylase with efficiencies better than the red seaweeds (*Eucheuma cottonii* and *Sarcothalia crispata*). In previous studies, brown seaweeds such as *Ascophyllum nodosum* and *Fucus vesiculs* were reported to have high α-amylase inhibitory activity with IC_{50} 44.7 µg/ml and 59.1 µg/ml, respectively (Lordan, Smyth, Soler-Vila, Stanton, & Ross, 2013; Apostolidis et al. 2011; Apostolidis & Lee, 2010). According to Lordan et al. (2013), brown seaweeds (phaeophyceae) are an excellent source of α-amylase inhibitors and these seaweeds could tentatively slow down the activity of α-amylase and decrease postprandial hyperglycaemia. These brown seaweeds may therefore have the potential to reduce the rate of α-amylase digestion of complex carbohydrates in the digestion system.

The red seaweeds can also act as α-amylase inhibitors **(Fig. 1)**. Inhibition of α-amylase activity is observed to increase in the order *Eucheuma cottonii* < *Sarcothalia crispata*. However, IC_{50} values are high ($IC_{50} > 4$ mg/mL) compared with brown seaweeds and acarbose, indicating that high concentrations of *Eucheuma cottonii* and *Sarcothalia crispata* are required to inhibit porcine pancreatic α-amylase. The water extract of *Eucheuma cottonii* (2 mg/mL) slightly increased α-amylase activity with no significant α-amylase inhibition at any concentration **(Fig.1)**. As the inhibitor concentration was increased, the water extract of *Eucheuma cottonii* showed only negligible inhibitory effect ($IC_{50} > 10$ mg/mL) indicating lack of efficacy.

3.2   Alpha-amylase inhibition kinetics

A kinetic study was conducted to understand the type of inhibition exhibited by each seaweed extract. **Fig. 2** shows Lineweaver-Burk plots of a methanol extract of *Laminaria digitata* and an acetone extract of *Undaria pinnatifida*. Plots for other crude seaweed extracts are shown in the Supplementary data, **Fig. S1-S3.** The plots display 1/v versus 1/[S] where [S] is substrate concentration and v is the initial velocity of the reaction recorded according to Michaelis-Menten Kinetics. These plots provide values for the Michaelis-Menten constant (K_m) and maximal velocity (V_{max}).
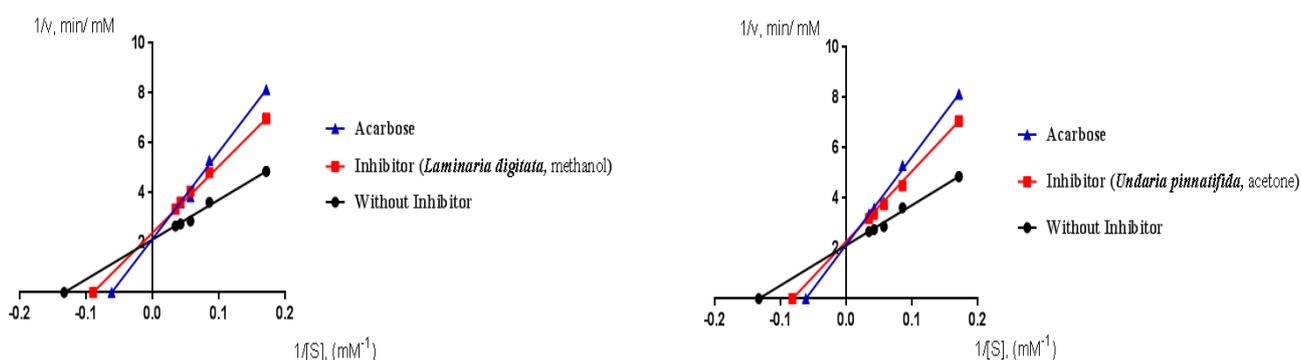

Fig.2. Lineweaver-Burk plots of inhibition kinetics of α-amylase inhibitory by (A) methanol extract of *Laminaria digitata* and (B) acetone extract of *Undaria pinnatifida* with variable starch concentrations (5.84, 11.69, 17.53, 23.73 and 29.21 mM).

These plots reveal mixed-type inhibition of α-amylase by the seaweed extracts, different from the competitive inhibition observed with acarbose. *Undaria pinnatifida* and *Laminaria digitata* display mixed-type inhibition since K_m values were higher and V_{max} values lower in comparison with control (inhibitor free) incubations. The addition of *Undaria pinnatifida* and *Laminaria digitata* extracts therefore most likely alter the K_m and V_{max} values by influencing the binding of substrates to the active sites. In mixed-type inhibition, a single inhibitor both hinders the binding of substrates and decreases the substrate turnover number for the enzyme (Berg, Tymoczko & Stryer, 2012), thereby providing a very robust inhibition. According to Narita & Inouye (2011), in a mixed-type inhibition, the apparent affinity of α-amylase for the substrate may either decrease or increase. In our case, since the K_m values are high, this α-amylase has a decreased affinity for starch.

3.3 Total phenolic content in selected seaweed extracts

As reported in the present study, at 1 mg/mL extracts of *Lamniaria digitata* and *Undaria pinnatifida* significantly reduced α-amylase activity more than 50% inhibition. Hence, these two brown seaweeds were analysed for total phenolic content. The amount of total phenolic content is presented as in **Table 2**.

Table 2
Total phenolic content and the extraction yield of selected seaweeds.

Seaweed	Total Phenolic Content (mg GAE/g)			Extraction yield (%)		
	Methanol	Acetone	Water	Methanol	Acetone	Water
Undaria pinnatifida	30.8 ± 1.2	12.5 ± 0.9	3.7 ± 0.6	17.5 ± 0.8	23.5 ± 1.0	28.6 ± 1.4
Laminaria digitata	23.0 ± 1.1	7.6 ± 0.2	0.3 ± 0.0	19.2 ± 01.2	9.5 ± 0.6	54.3 ± 2.2

Data are presented as mean ± standard deviation (n=3).

The highest phenolic content was found in *Undaria pinnatifida* extract. In general, methanol extracts of both brown seaweeds contained significantly (*P < 0.05*) higher levels of phenolic contents when compared with water extracts. In previous studies, the aqueous extracts of *Undaria* and

Laminaria species were reported to have small values of phenolic content which is 8 mg/g GAE and 0.1 mg/g GAE, respectively (Machu et al., 2015; Mojica et al., 2014). As reported by Machu et al. (2015), the amounts of total phenolic content are influenced by the origin and species of the sample, the type of solvents and the conditions of extraction.

3.4   Identification of phenolic compounds

Phenolic acids of *Undaria pinnatifida* and *Laminaria digitata* were identified by HPLC. As shown in **Table 3**, the major phenolic acids were found in *Undaria pinnatifida* extracts which contain higher amount of gallic acid, 2,5-dihydroxybenzoic acid, epicatechin and epigallocatechin compared to *Laminaria digitata* extracts. Both brown seaweed extracts contained higher amounts of 2,5-dihydoxybenzoic acid compared to other phenolic compounds. Gallic acid was absent in acetone extract of *Undaria pinnatifida*. Water extracts of *Undaria pinnatifida* and *Laminaria digitata* generally contained low concentrations of phenolic compounds.

Table 3
Phenolic acids (mg/g extract) in seaweed extracts.

Seaweed extracts		Gallic	2,5-dihydroxybenzoic	Epicatechin	Epigallocatechin
Undaria pinnatifida	Water	0.8 ± 0.0	1.8 ± 0.1	1.4 ± 0.1	1.2 ± 0.2
	Methanol	4.3 ± 0.1	17.4 ± 1.3	5.8 ± 0.4	7.1 ± 0.2
	Acetone	-	9.8 ± 0.2	2.5 ± 0.2	3.7 ± 0.1
Laminaria digitata	Water	0.3 ± 0.0	0.7 ± 0.0	-	-
	Methanol	2.8 ± 0.6	13.9 ± 1.4	3.7 ± 0.5	0.9 ± 0.0
	Acetone	0.7 ± 0.0	5.3 ± 0.0	1.1 ± 0.4	-

Result are expressed as mean ± standard deviation (n=3).

In other studies, some of the phenolic compounds such as gallic acid, hydroxybenzoic acid, and caffeic acid have been identified in brown seaweeds. Other researchers reported that *Laminaria* species contain lower gallic acid when compared to other brown seaweed such as *Ascophyllum nodosum* (Machu et al., 2015; Pantidos, Boath, Lund, Conner, & McDougall, 2014; Sabeena Farvin & Jacobsen, 2013).

3.5   Identification and quantification of selected seaweed polysaccharides

Since brown seaweeds showed potent α-amylase inhibitory activities compared to red seaweeds, they were selected for further analysis to identify and quantify their complex polysaccharide content. Quantification of the mean spot signals (n=3) indicated some correlation between complex polysaccharide concentrations and signal. A heatmap is shown in **Fig.3**, indicating mean spot signals, and showing the relative binding of the LM7, CBM3a and BAM1 to *Laminaria digitata*, *Undaria pinnatifida* and *Sargassum polycystum*.

Monoclonal antibodies/ Species	anti-alginate	anti-cellulose	anti-fucoidan
	LM7	CBM3a	BAM1
Laminaria digitata	66	0	20
Undaria pinnatifida	62	0	9
Sargassum polycystum	21	0	0

Scale						
0	10	20	30	40	>50	
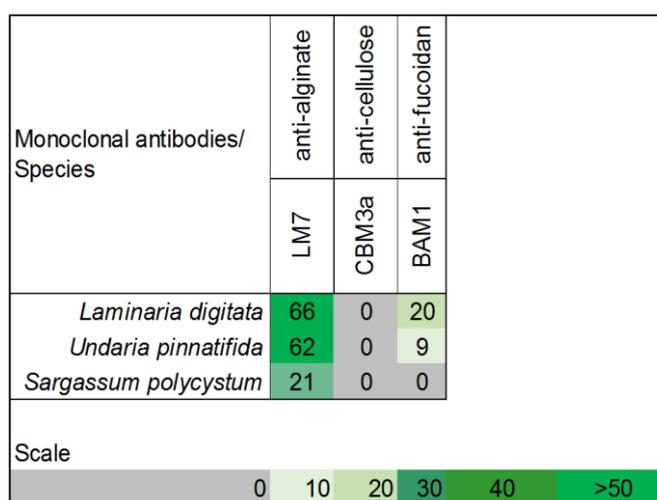
Fig. 3. Heatmap with the relative binding of the LM7, CBM3a and BAM1 to *Laminaria digitata* and *Undaria pinnatifida*. The values in the heatmap are mean spot signals (n=3) and in all cases standard deviations were *P* < 0.1. The maximal value was set to 100. The colour scale is in relation to absorbance values.

The mAb LM7 displays a strong binding to both *Laminaria digitata* and *Undaria pinnatifida*. This indicates a high concentration of alginates in *Laminaria digitata* and *Undaria pinnatifida*. The mAb BAM1 also binds to these brown seaweeds but with lower mean spot signals, indicating comparatively smaller concentration of fucoidan.

Findings in this study indicate that *Laminaria digitata* and *Undaria pinnatifida* contain high amounts of alginates and relatively smaller levels of fucoidans. As shown in another study

(Dawczynski et al., 2007), brown seaweeds contain alginate (1,4-linked polymer of β-D-mannuronic acid and α-L-guluronic acid). Therefore, alginates from selected brown seaweeds were extracted for further study of α-amylase inhibitory activity and kinetics.

3.6 *The inhibition of α-amylase activities by phenolic acids and alginates*

In this study, gallic acid, 2,5-dihydroxybenzoic acid, epicatechin and epigallocatechin (analytical-grade) that are potentially found in *Undaria pinnatifida* and *Laminaria digitata* **(Table 3)** were used to assess their ability to inhibit α-amylase activity. As in **Table 4**, 2,5-dihydroxybenzoic acid showed the most effective α-amylase inhibitor with IC_{50} 0.046 ± 0.004 mg/mL. In accordance with the present result, previous study has demonstrated that hydroxybenzoic acid (400 mg/L) (analytical grade) inhibited amylase activity (Wu, Shen, Han, Liu, & Lu, 2009). Contrary to this, epigallocatechin showed the lowest α-amylase inhibitory activity (IC_{50} 0.504 ± 0.003mg/mL). The finding is in agreement with Yilmazer-Musa et.al. (2015) findings which showed that catechin such as epigallocatechin is not a strong inhibitor for α-amylase. This may be due to the lack of specific A and B ring hydroxyl groups to effectively interact with the catalytic site of the enzyme (Goh et al., 2015; Piparo & Nestlé, 2008; Yilmazer-Musa et al., 2015).

Table 4 showed potential inhibition against α-amylase of alginates. Alginates were extracted from *Laminaria digitata* and *Undaria pinnatifida* with yields of 2.7 ± 0.33% and 2.3 ± 0.41%, respectively. A commercial alginate (A7003) (0.0001-20 mg/mL) was used as a reference. All alginates were assessed for their ability to inhibit α-amylase. A dose-response curve for the commercially available alginate is shown in **Fig.4**. The commercial alginate inhibited α-amylase with lower IC_{50} 0.096 ± 0.001 mg/mL compared to crude extracts of *Laminaria digitata*.
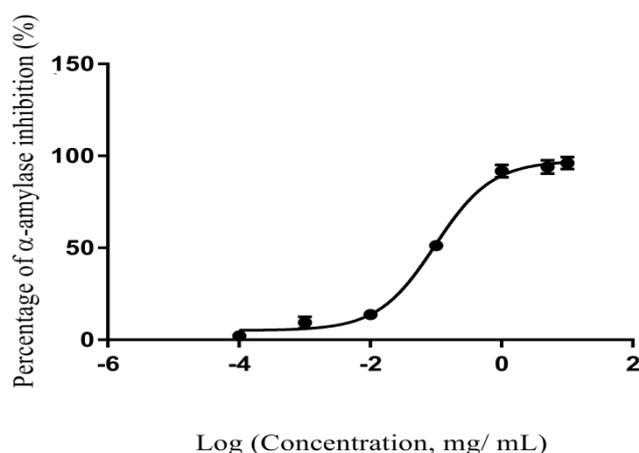
Fig.4. Dose-response curve of commercially available alginate (A7003). Each point represents the average of triplicate measurements.

Table 4
IC$_{50}$ values, K$_m$ and V$_{max}$ of samples.

Samples	IC$_{50}$ (mg/mL)	Type of inhibition	K$_m$ (mM)	V$_{max}$ (mM/ min)
Phenolic acids				
Control (Without inhibitor)	-		7.96 ± 0.52	0.4436 ± 0.0094
Gallic acid	0.212 ± 0.049	Mixed-type	13.72 ± 1.69	0.3326 ± 0.0174
2,5-dihydroxybenzoic acid	0.046 ± 0.004	Mixed-type	10.02 ± 1.20	0.2797 ± 0.0123
Epicatechin	0.387 ± 0.011	Non-competitive	7.96 ± 0.77	0.3067 ± 0.0087
Epigallocatechin	0.504 ± 0.013	Non-competitive	7.96 ± 0.46	0.3371 ± 0.0062
Alginates				
Control (Without inhibitor)	-		8.08 ±0.69	0.4864 ± 0.0133
Commercial alginate	0.096 ± 0.001	Mixed-type	12.89 ± 1.26	0.4042 ± 0.0163
Alginate *(Laminaria digitata)*	0.075 ± 0.010	Mixed-type	8.63 ± 0.79	0.3858 ± 0.0119
Alginate *(Undaria pinnatifida)*	0.103 ± 0.017	Mixed-type	11.14 ± 1.33	0.4107 ± 0.0189

Data are presented as mean ± standard deviation (n=3)

To investigate the type of inhibition, Lineweaver-Burk plots were constructed. Kinetic analyses revealed that phenolic compounds such as 2,5-dihydroxybenzoic acid and gallic acid display mixed-type inhibition while epicatechin and epigallocatechin are non-competitive inhibition **(Table 4)**. The commercial alginate and alginates extracted from *Laminaria digitata* and *Undaria pinnatifida* are mixed-type α-amylase inhibitors, similar to the kinetics observed for the brown seaweeds. The inhibition obtained with phenolic acids and alginates might affect how the α-amylase interacts with

starch. As shown in **Table 4**, epicatechin and epigallocatechin show non-competitive inhibition as the K_m values are unchanged and the V_{max} values are decreased when compare with control sample. In non-competitive inhibition, epicatechin and epigallocatechin may react to an enzyme with or without a substrate and changes the conformation of an enzyme and the active site, thus slow the rate of reaction to form the enzyme-product. As for 2,5-dihydroxybenzoic acid, gallic acid and alginates, K_m values increased with inhibitors since the inhibitors are competing with the substrates for a fixed number of active sites on α-amylase. V_{max} decrease with the addition of 2,5-dihydroxybenzoic acid, gallic acid and alginates compared with control sample. These show that the inhibitors do not bind to the active site of the enzyme. Instead they bind allosterically, to different site of α-amylase, thus affecting the enzyme-substrate complex and slowing the rate of reaction between starch and α-amylase.

This finding may also be explained by the fact that the alginates that contain a linear polymer of uronic acid, mannuronic (M) and guluronic acid (G) can form acidic and ionic gels. This may influence the ability of digestive enzymes and gastrointestinal enzymes to interact with substrates, as reported by Houghton et al. (2015) and Wilcox et al. (2014). Alginate from *Laminaria digitata* has relatively high viscosity compared with carrageenans from *Eucheuma cottonii*, significantly affecting carbohydrate digestion (Vaugelade et al., 2000). Previous work has suggested that alginate contained in seaweeds produce viscous solutions and may be a barrier to starch digestion and influence glucose uptake (Goñi, Valdivieso & Garcia-Alonso, 2000; Nwosu et al., 2011). Previous studies also revealed that other complex polysaccharides such as the sulphated polysaccharide, fucoidan, from a brown seaweed (*Ascophyllum nodosum*) delays carbohydrate digestion and glucose absorption by limiting α-amylase activity or slowing the diffusion of glucose from the enzyme's active site (Kim et al., 2014). These findings suggest that alginate, which is an indigestible (resistant) polysaccharide, could be a useful component to inhibit α-amylase activity.

The brown seaweeds, *Laminaria digitata* and *Undaria pinnatifida*, tested in this study and another brown seaweed, *Ascophyllum nodosum,* tested by others, exhibited strong inhibitory activity against α-amylase. Although this would fit with their potential bioactive compound such as phenolic compounds and alginate content, it could also be a product of other mechanisms, such as other bioactive compounds found in the seaweeds since the organic rather than the water extracts were the most effective inhibitors. Although previous studies in the literature have reported on α-amylase inhibition by seaweeds, the strength of our present study is that we compare the inhibitory effects of edible brown and red seaweeds that come from five distinct genera (*Laminaria, Undaria, Sargassum, Sarcothalia* and *Eucheuma*). Different solvent extractions of seaweeds were compared in the present study to evaluate the efficacy of crude extracts for inhibiting α-amylase. In addition, enzyme kinetic studies were also conducted for the first time to provide information about the kinetics of α-amylase inhibition in the presence or absence of edible seaweeds, phenolic compounds and their main dietary fibre, alginate. However, further studies are recommended to validate the current findings taking all seaweed constituents into account. *In vitro* studies with human recombinant enzymes as well as human meal studies are also needed to confirm the external validity of the outcome of this research.

4. Conclusion

Laminaria digitata and *Undaria pinnatifida* can be promising sources of dietary inhibitors of α-amylase. The present study demonstrates that selected seaweeds inhibited porcine pancreatic α-amylase through a mixed-type inhibition mechanism. The three brown seaweeds (*Sargassum Polycystum*, *Laminaria digitata* and *Undaria pinnatifida*) showed more potent α-amylase inhibition compared to two red seaweeds. This study also indicates that brown seaweeds containing 2,5-dihydroxybenzoic acids, gallic acids and alginates may in general be potent inhibitors of α-amylase. The investigation provides *in vitro* evidence for α-amylase inhibitors from marine algae with details of the kinetic pattern of inhibition for the first time.

5. Acknowledgement

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Supplementary data for

Inhibitory effects of edible seaweeds, polyphenolics, and alginates on the activities of porcine pancreati α-amylase

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Table S1.

Edible seaweeds used in this study, their class, origin and source.

Species	Class	Type	Country	Source
Eucheuma cottonii [a]	Rhodophyta	Red	Malaysia	Purchased from The Borneo Local Product, Semporna, Sabah, Malaysia.
Sargassum polycystum [b]	Phaeophyta	Brown	Malaysia	A generous gift from Uee Global Trade Sdn Bhd, Bkt Mertajam, Pulau Pinang, Malaysia.
Laminaria digitata [c]	Phaeophyta	Brown	Ireland	Purchased from AlgAran Teoranta, Kilcar Co. Donegal, Ireland.
Undaria pinnatifida [d]	Phaeophyta	Brown	Korea	Purchased from JFC Deutschland, Düsseldorf, Germany.
Sarcothalia crispata [e]	Rhodophyta	Red	Chile	A generous gift from Danisco, Copenhagen, Denmark to Department of Plant and Environmental Sciences, Section for Plant Glycobiology, University of Copenhagen, Copenhagen, Denmark.

[a] Purchased in June 2013; [b] Received in June 2013; [c] Purchased in May 2013; [d] Purchased in August 2013; [e] Collected in April 2013

Figure S1. Lineweaver-Burk plots of inhibition kinetics of α-amylase inhibitory by methanolic extracts of seaweeds, (A) *Sargassum polycystum*, (B) *Undaria pinnatifida* and (C) *Sarcothalia crispata* with variable starch concentrations (5.84, 11.69, 17.53, 23.73 and 29.21) mM. The values represent the mean ± standard deviation (n=3).
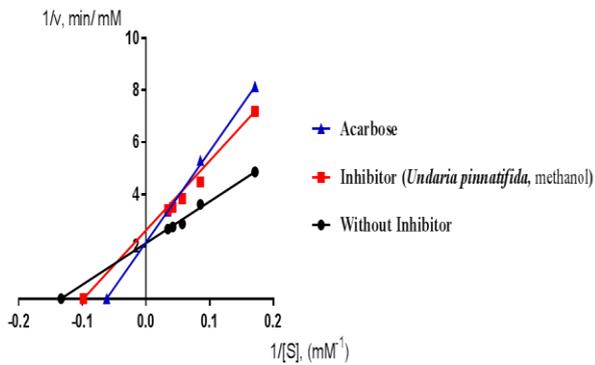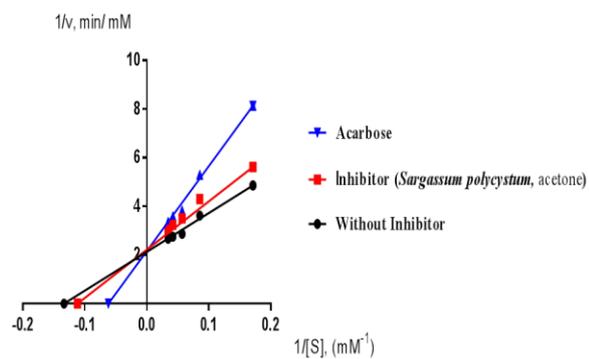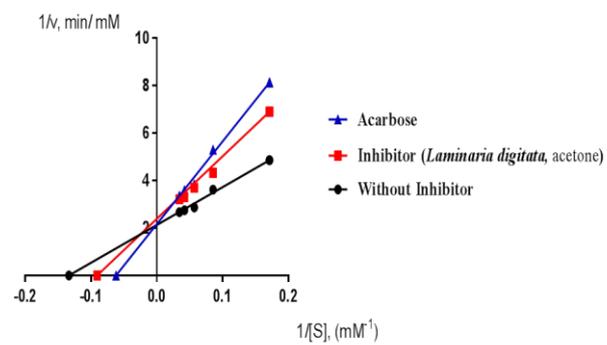
(a)
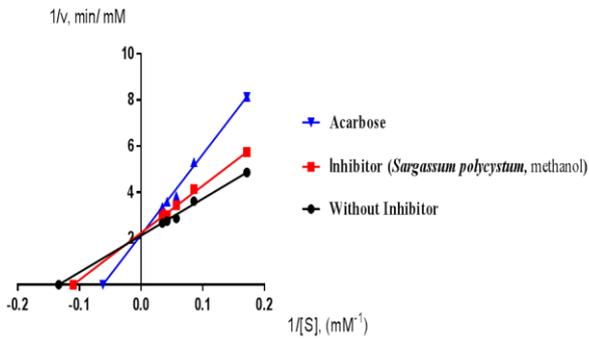
(c)
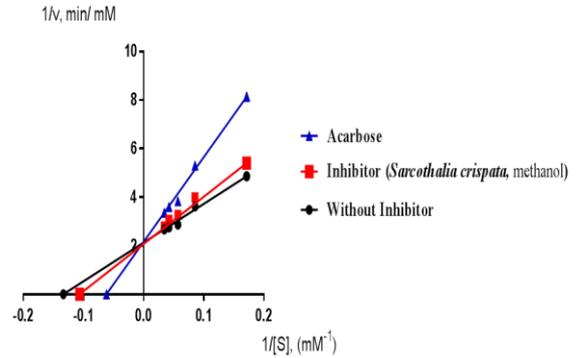
(b)

Figure S2. Lineweaver-Burk plots of inhibition kinetics of α-amylase inhibitory by acetone extracts of seaweeds, (A) *Sargassum polycystum* and (B) *Laminaria digitata* with variable starch concentrations (5.84, 11.69, 17.53, 23.73 and 29.21) mM. The values represent the mean ± standard deviation (n=3).

(a)

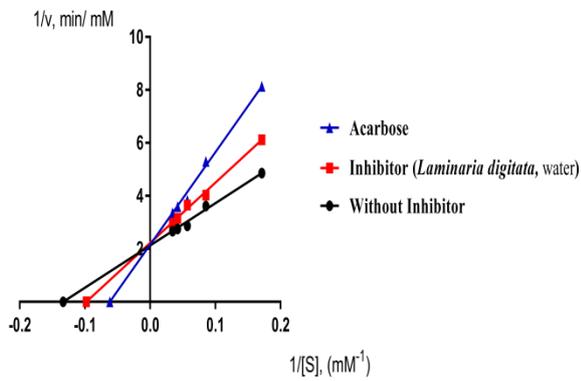(b)

Figure S3. Lineweaver-Burk plots of inhibition kinetics of α-amylase inhibitory by aqueous extracts of seaweeds, (A) *Sargassum polycystum*, (B) *Laminaria digitata* and (C) *Undaria pinnatifida* with variable starch concentrations (5.84, 11.69, 17.53, 23.73 and 29.21) mM. The values represent the mean ± standard deviation (n=3).

(a)
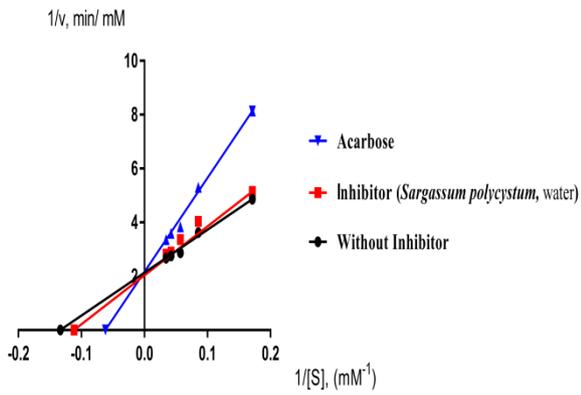
(c)
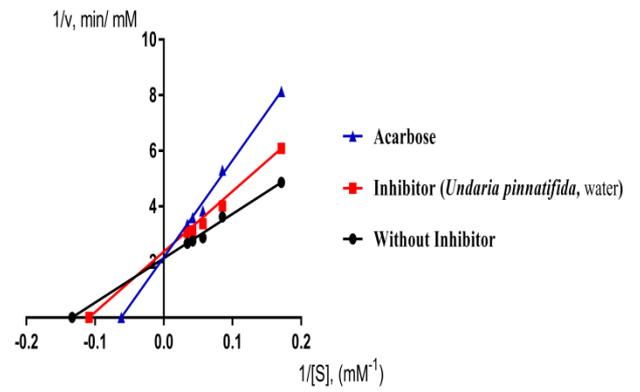
(b)

5.2   Paper 2: Inhibition of α-glucosidase by selected edible seaweeds and fucoxanthin: Kinetic studies

Nazikussabah Zaharudin, Dan Stærk, Lars Ove Dragsted

(*Submitted*)

Inhibition of α-glucosidase activity by selected edible seaweeds and fucoxanthin: Kinetic studies

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Abstract

Alpha-glucosidase inhibitors are used to control blood glucose levels for people suffering from hyperglycaemia. The inhibitors delay the breakdown of carbohydrate into simple sugars thus helping to lower blood glucose levels in people who have high blood glucose after eating. In this study, five species of dried edible seaweeds were tested for α-glucosidase inhibitory effect. A 5 mg/mL of methanol, acetone and water extracts of seaweeds were used for a high-resolution α-glucosidase inhibition assay hyphenated with high performance liquid chromatography-mass spectrometry (HPLC-ESI-MS). Our results show that acetone extracts of *Undaria pinnatifida* has the strongest inhibitory effect against α-glucosidase activity with IC_{50} 0.08 ± 0.02 mg/mL. The active compound was identified as associated with the peak from fucoxanthin (IC_{50} 0.0467 ± 0.0013 mg/mL). An inhibition kinetics study indicates that fucoxanthin is showing mixed-type inhibition. These results suggest that *Undaria pinnatifida* has a potential to inhibit α-glucosidase and may be used as a bioactive food ingredient for glycaemic control.

KEYWORDS: Seaweed, glycaemic control, hyperglycaemia, α- glucosidase, fucoxanthin

1. Introduction

Prolonged high blood glucose levels (hyperglycaemia) is a characteristic sign of diabetes mellitus (DM) (American Diabetes Association, 2009). According to American Diabetes Association, (2009), the disorder is characterized by improper functioning or secretion of insulin hormone from the pancreas or abnormal glucose homeostasis. Excessive and frequent intake of quickly digestible carbohydrates may also lead to prolonged elevation in the blood glucose level (O'Keefe & Bell, 2007). Over time, the continued postprandial state where the blood glucose is high (postprandial hyperglycaemia) may increase glycation and increase the risk of metabolic dysfunctions. In combination with other factors this may in turn increase the risk of type 2 diabetes where uncontrolled high blood glucose levels can contribute to a number of complications such as blindness, cardiovascular complications, renal failure, foot ulcers and need for limb amputation (Ceriello et al., 2006; O'Keefe & Bell, 2007; Szablewski, 2001).

It is important to control hyperglycaemia because it can contribute to serious complications. Hyperglycaemia can be managed by maintaining stable blood glucose levels inside the normal range. This can be achieved by various strategies such as diet, medications and exercise (O'Keefe & Bell, 2007). Several life-style changes and medications have been introduced for managing hyperglycaemia. One of the strategies to manage the disorder is through inhibition of carbolytic enzymes such as α-amylase and α-glucosidase. Inhibiting these enzymes will result in slower absorption of sugars during digestion (Mojica, Meyer, Berhow, & de Mejía, 2015). Clinical studies using acarbose and miglitol as α-glucosidase inhibitors showed a reduction in postprandial blood glucose and an increase in insulin sensitivity (Su, Wang, Chen, Wu, & Jin, 2011; Meneilly et al., 2000). These inhibitors act by blocking the α-glucosidase enzyme in the small intestine where breakdown of complex carbohydrates occur. This enzyme reaction reduces carbohydrate hydrolysis and glucose absorption into the bloodstream and thus lowering postprandial blood glucose levels

(Khalid Imam, 2013; Ahmad, 2013). However, acarbose has side effects such as flatulence and abdominal pain (Rosenstock et al., 1998). Such side effects are caused by the fermentation of undigested carbohydrate by the microbiota in the large intestine (Khalid Imam, 2013; Samulitis, Goda, Lee, & Koldovsky, 1987).

Besides acarbose many studies have been carried out to evaluate foods as a means to lower and control high blood glucose levels. The use of low glycaemic index foods in mixed meals (Grant, Wolever, O'Connor, Nisenbaum, & Josse, 2011), food intake with dietary fibre (Kapoor, Ishihara, & Okubo, 2016; Lattimer & Haub, 2010), intake of supplements and herbal medicines (Akilen, Tsiami, Devendra, & Robinson, 2012; Najm & Lie, 2010) have been shown to result in reduced blood glucose levels and an improvement in prevention of type 2 diabetes mellitus (T2DM). Natural sources that contain complex polysaccharides and potential bioactive compounds are also some of the food sources that can help regulate blood sugar and improve insulin sensitivity (Babio, Balanza, Basulto, Bullo, & Salas-Salvado, 2010; Valls et al., 2010; Wang, Zhao, Yang, Wang, & Kuang, 2016). It is reported that *Salacia* species inhibit α-glucosidase and slows the breakdown of carbohydrates into monosaccharides, thus lowering the postprandial blood glucose levels (Heacock, Hertzler, Williams, & Wolf, 2005; Matsuda H; Murakami T; Yashiro K; Yamahara J, 1999). A recent study also reported that plant extracts from *Phyllantus* species such as *P. amarus* and *P. urinaria* which are widely used in Vietnam as traditional medicines for diabetes showed the most promising inhibition of α-glucosidase activity Trinh, Staerk, & Jäger (2016). Their findings provide additional evidences that inhibition of carbohydrate digestive enzymes such as α-glucosidase can be one of the means to control blood glucose levels by delaying the degradation of polysaccharides and starch to glucose (Lebovitz, 1997).

Marine algae such as seaweeds used as food sources in Asia, especially in Japan, Korea and China, are also providing similar potential health benefits (Pomin, 2012). The present use of various species

of seaweeds as human foods from all parts of the world has seen a growing interest from researchers to study seaweeds as potential functional foods. Seaweeds contain nutrients such as dietary fibre, protein and biologically as well as potentially bioactive compounds like algal polysaccharides, phenolic compounds, carotenoids, and marine fatty acids (Ibanez & Cifuentes, 2013; Miyashita et al., 2011; Sharifuddin, Chin, Lim, & Phang, 2015; Lordan, Ross, & Stanton, 2011). With this information, we undertook a study and applied selected edible seaweeds in order to evaluate their potential in inhibiting α-glucosidase activity.

In this study, edible seaweeds were selected based on their availability. We selected five of the most commonly consumed brown and red seaweeds in Asia and Europe. Active compounds were extracted using three different polar solvents, methanol, acetone and water. The main aim was to investigate the potential of crude extracts from edible seaweeds in inhibiting α-glucosidase activity. Secondly, we wanted to identify the most potent α-glucosidase inhibitors and finally to evaluate the kinetics of inhibition. In addition, efficient techniques were applied to identify targeted α-glucosidase inhibitors using a high resolution α-glucosidase inhibition assay hyphenated with high performance liquid chromatography-mass spectrometry (HPLC-ESI-MS).

2. Experimental

2.1 Chemicals

Alpha-glucosidase from *Saccharomyces cerevisiae* (EC 3.2.1.20), *p*-nitrophenol-α-D-Glucopyranoside (*p*NPG), sodium azide, sodium phosphate monobasic dihydrate, dibasic sodium phosphate, dimethyl sulfoxide, acarbose and fucoxanthin were purchased from Sigma-Aldrich (St. Louis, MO). HPLC-grade acetonitrile and methanol were obtained from VWR International (Fontenay-sous-Bois, France). Water was prepared by deionization and 0.22 µm membrane filtration using a Millipore system (Billerica, MA). Methanol-d_4 was purchased from Eurisotop (Gif-Sur-Yvette, Cedex, France) and formic acid was purchased from Merck (Darmstadt, Germany).

2.2 Sample material

The dried edible seaweeds were purchased from companies in Malaysia, Ireland, Germany and Denmark or received from the Department of Plant and Environmental Sciences, University of Copenhagen, Denmark as described in **Table 1**. The dried samples were washed with distilled water. They were freeze dried for 48 h using a freeze dryer (BFBT-101, Ontario, Canada). Next, the dried seaweeds were milled into powder by a TissueLyser II (Qiagen MM 200, Qiagen Nordic, West Sussex, UK) at a frequency of 30 s^{-1} for 5 min. The powders were then stored in the refrigerator at 2-4 ˚C for further analysis.

Table 1

Seaweed species used in this study.

Species	Class	Type	Country	Source
Eucheuma cottonii [a]	Rhodophyta	Red	Malaysia	Borneo Local Product, Semporna, Sabah, Malaysia.
Sargassum polycystum [b]	Phaeophyta	Brown	Malaysia	Uee Global Trade Sdn Bhd, Bkt Mertajam, Pulau Pinang, Malaysia.
Laminaria digitata [c]	Phaeophyta	Brown	Ireland	AlgAran Teoranta, Kilcar Co., Donegal, Ireland.
Undaria pinnatifida [d]	Phaeophyta	Brown	Korea	JFC Deutschland, Düsseldorf, Germany.
Sarcothalia crispata [e]	Rhodophyta	Red	Chile	Danisco, Copenhagen, Denmark and Department of Plant and Environmental Sciences, Section for Plant Glycobiology, University of Copenhagen, Copenhagen, Denmark.

[a] Purchased in June 2013; [b] Received in June 2013; [c] Purchased in May 2013; [d] Purchased in August 2013; [e] Received in August 2013.

2.3   Sample extraction

A sample of 5 g of each seaweed powder, was extracted in 50 mL of methanol (80%), 50 mL of acetone (70 %) or 100 mL of water. The extraction mixtures were stirred using an orbital shaker at 140 rpm for 3 h. Then the methanol and acetone extracts were filtered using filter paper (Whatman, Cat No 1001 125) while the water extracts were filtered using glass wool filters. The residues were re-extracted for 24 h under the same condition. All the filtrates were combined. The filtrates were dried using a rotary evaporator (90 mbar, 40°C) (Büchi Rotavapor R-114 and water bath B-480 from Büchi Labortechnik AG, Flavil, Switzerland). The dried extracts were kept at -20°C for until analysis. Stock solutions of all five seaweeds were prepared by dissolving the methanol and acetone extracts with dimethyl sulfoxide (DMSO). Water extracts were dissolved in distilled water. Stock solutions of seaweed extracts (50 mg/mL) were prepared for α-glucosidase assays and HPLC analyses.

2.4   Alpha-glucosidase inhibition assay

The alpha-glucosidase inhibition assay was performed using a method from Schmidt et.al (2012). In a 96-well microplate, each well was added with 10 µl of extract dissolved in DMSO and 90 µl of 0.1 M sodium phosphate buffer (SPB), pH 7.5 containing 0.02% sodium azide. A solution 80 µl of α-glucosidase (2.0 U/mL) in SPB was added in each well and the mixture was pre-incubated at 28 °C for 10 min. Acarbose was used as a positive control. As a negative control (without seaweed extract), sodium phosphate buffer was used for water extracts and a mixture of 10% DMSO and sodium phosphate buffer was used for methanol and acetone extracts. After the incubation, 20 µl of *p*NPG (dissolved in SPB) was mixed into the solution to initiate the reaction. The rate of *p*NPG conversion to *p*-nitrophenol was determined by the measurement of absorbance of p-nitrophenol at 405 nm every 30 s for 35 minutes using a Multiskan FC microplate photometer (Thermo Fisher Scientific, Waltham, MA, USA) controlled by SkanIt software version 2.5.1.

The percentage of α-glucosidase inhibition was calculated by the following equation:

$$\% \: \alpha-\text{glucosidase inhibition} = \frac{(\text{Slope}_{\text{blank}} - \text{Slope}_{\text{sample}})}{\text{Slope}_{\text{blank}}} \times 100\%$$

IC_{50} is the concentration at which the α-glucosidase enzyme activity is inhibited to 50% of maximum activity. The IC_{50} concentration was determined using different concentrations of seaweeds extracts, ranging from 0.005 - 50 mg/mL. These IC_{50} values were determined from the mean inhibitory values plotted in a graph of the percentage inhibition vs log inhibitor concentration (Gomathi et al., 2012).

2.5   High-resolution α-glucosidase inhibition assay of seaweed extracts

Experiments were performed according to a procedure developed previously with minor modifications (Schmidt, Lauridsen, Dragsted, Nielsen, & Staerk, 2012b). HPLC separation for high-resolution α-glucosidase was performed using an Agilent 1200 series instrument (Santa Clara, CA) comprising a G1311A quaternary pump, a G1322A degasser, a G1316A thermostated column compartment, a G1315C photodiode-array detector, a G1367C high–performance auto sampler, and a G1364C fraction collector, controlled by Agilent Chemstation ver. B.03.02 software.

A C_{18}(2) Luna column (Phenomenex, 150 mm x 4.6 mm, 3 µm, 100 Å) was used for separation and maintained at 40 °C with a flow of 0.5 mL/ min. The flow was maintained using a mixture of solvent A, acetonitrile: water (5%: 95%, v/v) and solvent B, acetonitrile: water, (95 %: 5%, v/v); both acidified with 0.1 % formic acid. A volume of 50 µl of (10 mg/mL) of acetone seaweed extract was injected and separated using the following gradient profile: 0 min, 0 % B; 40 min, 100 %; 50 min, 100 %; 52 min, 0 % B; 55 min, 0 % B. UV traces were monitored at 210 nm, 254 nm, 280 nm, 330 nm and 450 nm.

Micro-fractionations were performed using 96-well microplates. A total of 180 fractions were collected with HPLC eluate from 10 to 50 min (100 µl aliquot in each well). The fractionated solutions in the microplate were evaporated to dryness using a SPD121P Savant Speed-Vac concentrator (Thermo Scientific, Waltham, MA) equipped with an OFP400 Oil Free Pump and a RVT400 Refrigerated Vapor Trap. The α-glucosidase assay was performed using contents in each well. Inhibition of α-glucosidase activity was plotted against chromatographic retention time to produce high-resolution biochromatogram.

2.6   HPLC-HRMS analysis

Separations were performed using the same solvents, gradient profile, column, and temperature as described above. HPLC-HRMS analysis of analytes with α-glucosidase activity was performed on the above-described chromatograph using a T-piece splitter to direct 0.1% of the HPLC elute to a micrOTOF-Q II mass spectrometer (Bruker Daltonik, Bremen, Germany), equipped with an electrospray ionisation (ESI) interface. Mass spectra were acquired in positive-ion mode using drying temperature of $200^{\circ}C$, capillary voltage of 4000V, nebulizer pressure of 2.0 bar, and drying gas flow of 7 L/min.

2.7   Kinetics of α-glucosidase inhibition

The reaction rate and modes of inhibition of α-glucosidase by the different seaweed extracts were determined by Michaelis-Menten kinetics using Lineweaver-Burk plots. Initial reaction rate experiments were performed to determine the Michaelis constant (K_m) and maximal velocity (V_{max}). This experiment was performed in 96-well microplates. The substrate, pNPG at concentrations of 0.1-4.0 mM, was added into mixtures of seaweed extracts and α-glucosidase, as describe in the procedure for the α-glucosidase inhibition assay. The absorbance of p-nitrophenol was measured at 405 nm for every 30 s for 35 minutes as described in the previous method.

2.8   *Statistical analysis*

The α-glucosidase inhibition assays were performed in triplicate. All data was expressed as mean ± standard deviation. Statistical analysis to calculate IC_{50} and kinetic constants was performed by using GraphPad Prism software (version 6.0) (GraphPad Software, Inc, San Diego, CA, USA). The Km and Vmax values obtained were compared using Analysis of Variance (ANOVA). P values were determined by Tukey's multiple comparison test, where $P < 0.05$ was considered statistically significant. Linear Michaelis-Menten and Lineweaver-Burk plots were plotted in Excel (Microsoft Denmark, Kgs. Lyngby, Denmark) using the experimental kinetic values.

3. Results and discussion

3.1. Alpha-glucosidase inhibitory activity

The inhibition of α-glucosidase was found to be more effective with the acetone and methanol extracts. As seen in **Fig. 1**, the acetone extracts of *Undaria pinnatifida* showed the highest percentage inhibition of $92.04 \pm 1.60\%$ amongst the seaweeds extracts, followed by *Laminaria digitata* with inhibition of $64.15 \pm 0.81\%$ of the α-glucosidase activity. In comparison with that, 1 mg/mL of acarbose (positive control) inhibited the enzyme by $94.67 \pm 1.65\%$. In contrast, the crude extracts of *Eucheuma cottonii* extracted in any of the three different solvents showed no inhibitory effect against α-glucosidase at a concentration of 5 mg extract/mL. In addition, it is noted that *Undaria pinnatifida* and *Laminaria digitata* had appreciable inhibition of α-glucosidase (> 50 %) compared with *Sargassum polycystum* and *Sarcothalia crispata*.
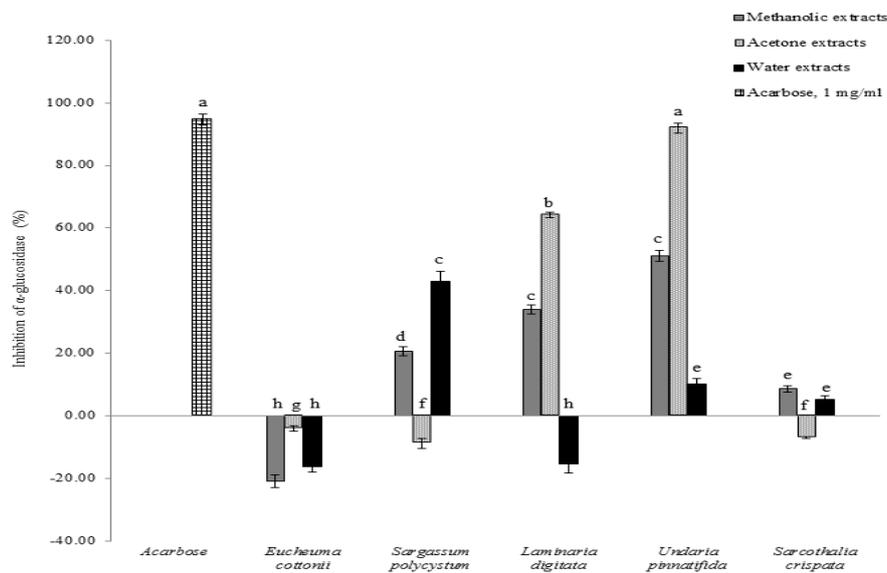
Fig. 1. Bars show α-glucosidase inhibition by 5 mg/mL crude extract of seaweeds in 80% methanol, 70% acetone, and water. Acarbose (1 mg/mL) was used as a positive control. Bars represent mean $\pm$ standard deviation of three experiments. Bars marked with different letters are significantly different ($P < 0.05$).

Further data analysis was done to assess the effectiveness of seaweed extracts in inhibiting α-glucosidase activity. The IC_{50} was calculated from the concentration-by-inhibition plots. As shown in **Table 2**, the IC_{50} of some of the seaweed extracts were comparable to those for the standard inhibitor, acarbose ($IC_{50}= 0.6 \pm 0.01$ mg/mL).

Table 2. IC_{50} values of crude seaweeds extracts.

Seaweed species	α-glucosidase assay, IC_{50} (mg/mL)[a,b,c]		
	80% methanol	70% acetone	Water
Eucheuma cottonii	n.d.	n.d.	n.d.
Sargassum polycystum	3.8 ± 0.3	n.d.	1.5 ± 0.2
Laminaria digitata	1.6 ± 0.1	0.8 ± 1.5	>10
Undaria pinnatifida	1.2 ± 0.2	0.08 ± 0.02	>10
Sarcothalia crispata	>10	n.d.	>10

[a] Data is presented as mean ± standard deviation values of triplicate measurements.
[b] IC_{50} is the concentration to produce 50% inhibition of the enzyme activity.
[c] n.d.: not determined since there is no α-glucosidase inhibition at the maximal concentration, 5 mg/mL.

The highest inhibitory activity was found in the acetone extract of *Undaria pinnatifida* with IC_{50} less than 0.1 mg/mL. The acetone extract of *Laminaria digitata* also showed a potent α-glucosidase inhibitory activity with IC_{50} value of less than 1 mg/mL. The findings from this study showed that crude extracts of brown seaweeds, like *Undaria pinnatifida* and *Laminaria digitata*, have inhibitory effect on α-glucosidase activity. The results of this study also indicate that brown seaweeds are better inhibitors of α-glucosidase than red seaweeds like *Sarcothalia crispata* and *Eucheuma cottonii*. The inhibition of α-glucosidase activity is observed to increase in the order of *Sarcothalia crispata < Sargassum polycystum <Laminaria digitata <Undaria pinnatifida*. Suprisingly, *Eucheuma cottonii* was found to give rise to an increase in α-glucosidase activity compared to its respective negative control. The present findings seem to be consistent with other research, which found *Undaria pinnatifida* and *Laminaria digitata* showing more than 50% of α-glucosidase inhibitory activity (Liu, Kongstad, Wiese, Jäger, & Staerk, 2016). In contrast others found that water extract of *Laminaria digitata* was effective at inhibiting α-glucosidase (Lordan and colleagues 2013).

3.2.   High-resolution α-glucosidase inhibition profiling

Our results demonstrate that a crude acetone extract of *Undaria pinnatifida* has the most potent inhibitory effect of α-glucosidase with high percentage inhibition ($> 90\%$) and low IC_{50} value (< 0.1 mg/mL) compared to other crude seaweed extracts. Therefore, we performed high-resolution α-glucosidase profiling of the crude acetone extract of *Undaria pinnatifida*. High-resolution α-glucosidase inhibition profile of this 70% acetone extract of *Undaria pinnatifida* overlaid with the HPLC chromatogram at 450 nm is shown in **Fig. 2**.
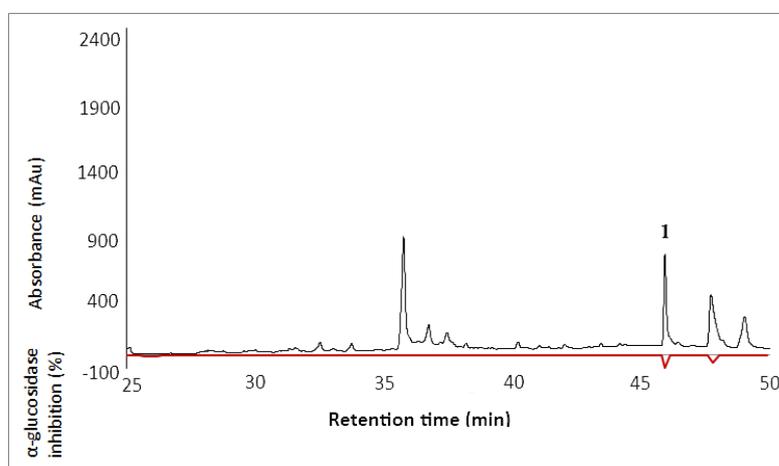
Fig.2. High-resolution α-glucosidase inhibition profile of a 70% acetone extract of *Undaria pinnatifida* overlaid with the HPLC chromatogram at 450 nm.

The potent α-glucosidase inhibitor (peak 1) found in *Undaria pinnatifida* was confirmed by HPLC-HRMS analysis. A mass spectrum in positive mode ESI-MS was recorded from the peak at approximately 47 min in the UV-Vis chromatogram at 450 nm as shown in **Fig. 3**.
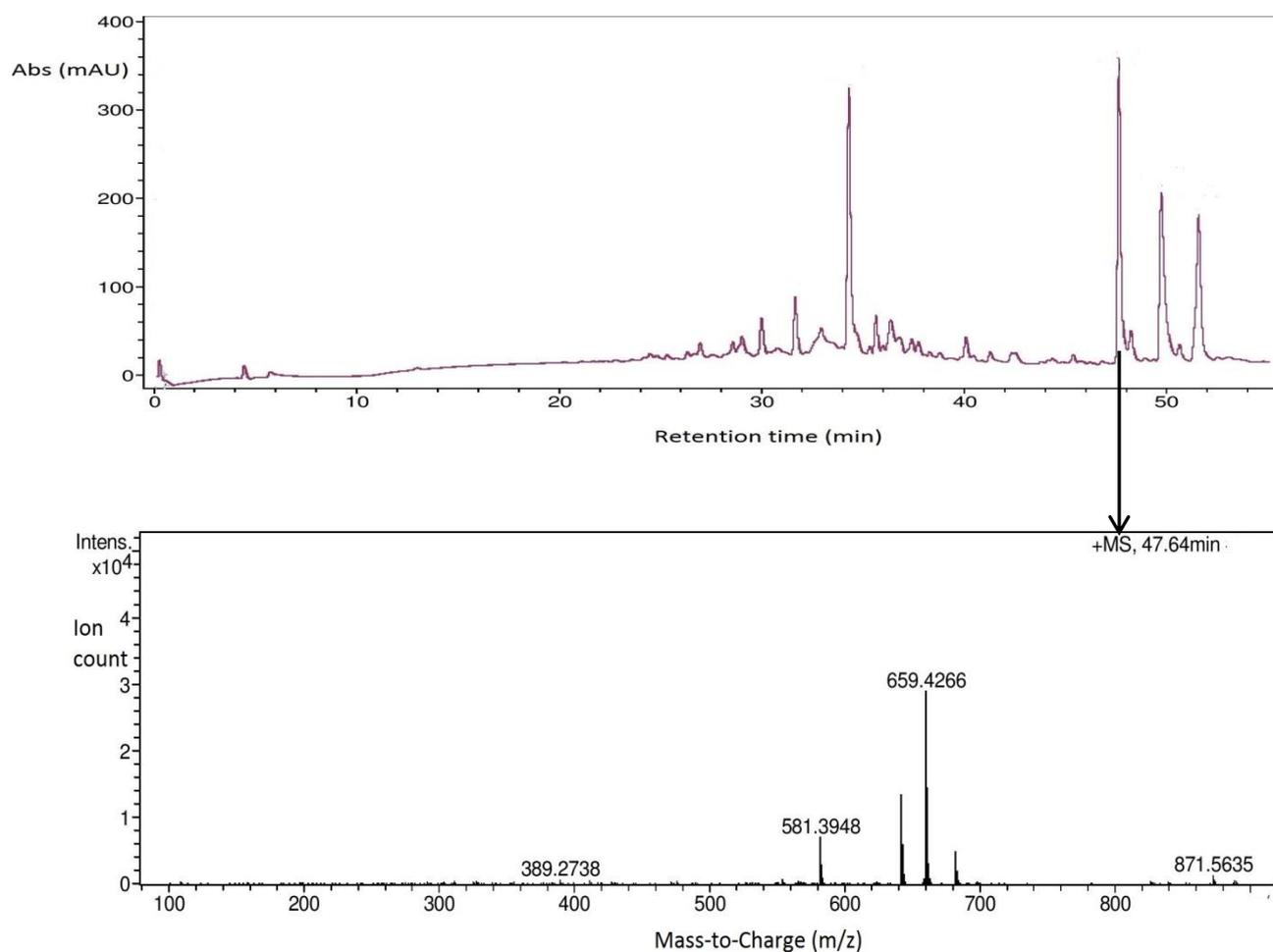
Fig.3. Chemical ionization mass spectrum (bottom) of the broad peak observed at approx. 47.6 min (top) from the chromatogram of the most inhibitory acetone extract fraction from *Undaria pinnatifida*.

The compound eluting as peak 1 with α-glucosidase inhibition of approximately 92% was identified from the mass spectrum as being fucoxanthin. It was found that fucoxanthin, which is a carotenoid from brown seaweed, had a high α-glucosidase inhibitory activity. Fucoxanthin ($C_{42}H_{58}O_6$) extract was found at retention time, RT 47.6 min with m/z 659.43 $(M+H)^+$. The compound was confirmed by comparing the MS-data with spectral information. The finding of this active compound was in accordance with the literature (Palermo, Seldes, & Areschoug, 1991; Xiao, Si, Yuan, Xu, & Li, 2012). Fucoxanthin has previously been isolated from *Eisenia bicyclis* and *Undaria pinnatifida* and it showed potent inhibitory activity against PTP1B, which is another key therapeutic target in type 2 diabetes (Jung et al., 2012). However, this is the first report of the potent α-glucosidase

inhibitor of fucoxanthin from *Undaria pinnatifida* on its α-glucosidase inhibitory activity and type of inhibition.

3.3. Alpha-glucosidase inhibitory activity and inhibition kinetics of fucoxanthin

Further analysis of the α-glucosidase inhibitory activity and the type of inhibition by a fucoxanthin standard was assessed. **Fig.4** shows the dose-response curves with the IC_{50} value of 0.0710 ± 0.0021 mM (0.0467 ± 0.0013 mg/mL). Fucoxanthin turns out to be a strong inhibitor for α-glucosidase activity with a lower IC_{50} value than that of the specific inhibitor acarbose (0.0592 ± 0.0015 mg/mL).
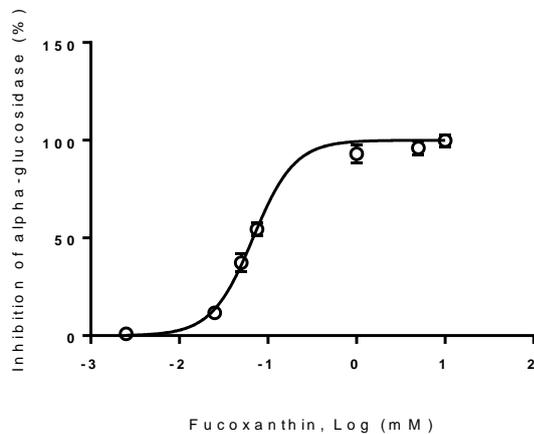
Fig.4. Dose-response (IC_{50}) curve of fucoxanthin. Each point represents the average of triplicate measurements.
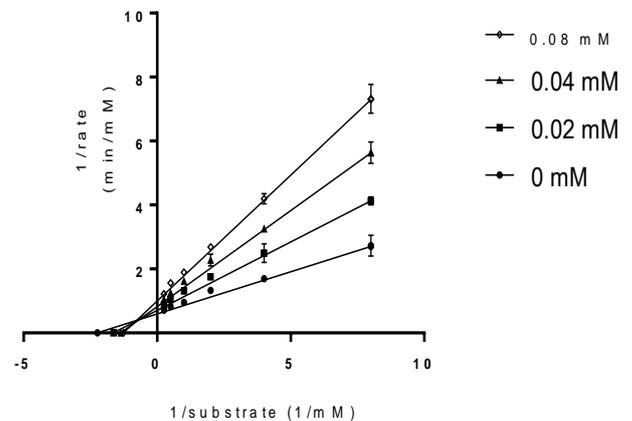
Fig.5. Lineweaver-Burk plots showing inhibition kinetics of α-glucosidase by fucoxanthin.

The mode of inhibition was determined by Lineweaver-Burk plots (1/v versus 1/[S] where [S] analysis of data according to Michaelis-Menten Kinetics. As seen in **Fig.5,** the plots reveal mixed type of inhibition of α-glucosidase. This is based on Michaelis-Menten constant (K_m) and maximal velocity (V_{max}) values from the control incubation (free from inhibitor) and from incubations with fucoxanthin. The addition of a mixed inhibitor alters the K_m and V_{max} values by influencing the binding of substrates to the active site. In a mixed type inhibition, a single inhibitor both hinders the binding of substrates and decreases the turnover number of the enzyme (Berg, Tymoczko, & Stryer, 2012). Fucoxanthin may therefore have the potential to reduce the rate at which α-glucosidase digests

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complex carbohydrates in the small intestine. It has been reported that fucoxanthin lowered the fasting blood glucose concentration and plasma insulin concentration in C57BL/6J mice (Park, Lee, Park, Shin, & Choi, 2011). Another study also reported that small amounts of purified fucoxanthin (0.2%) resulted in significantly lower blood glucose concentrations in KK-A^y mice (Maeda, Hosokawa, Sashima, & Miyashita, 2007); (Maeda, Tsukui, Sashima, Hosokawa, & Miyashita, 2008).

4. Conclusion

The crude extract of brown seaweeds could be a promising source of α-glucosidase activity inhibitor. The present study demonstrated that fucoxanthin from *Undaria pinnatifida* is the specific compound responsible for the inhibition of α-glucosidase activity through mixed type inhibition. In addition, other brown seaweeds like *Laminaria digitata* and *Sargassum polycystum* showed potent inhibitory effect compared to red seaweeds. The investigation provides *in vitro* evidence for α-glucosidase inhibitors from marine algae and its kinetic pattern that could be used for future animal and human studies. Nevertheless, more studies are needed to discover the ability of other carotenoids to inhibit alpha-glucosidase. Further investigation is also needed to understand if extracts from brown seaweeds could inhibit other carbolytic enzymes.

5. Acknowledgement

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5.3 Paper 3: Effects of the seaweeds, *Laminaria digitata* and *Undaria pinnatifida*, on postprandial glucose, insulin and appetite in humans

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(Ready to submit)

Effects of the seaweeds, *Laminaria digitata* and *Undaria pinnatifida* on postprandial glucose, insulin and appetite in humans

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Abstract

Background: High habitual consumption of carbohydrate with a high glycaemic index (GI) may cause unintended hyperglycaemia and be a factor off-setting glucose homeostasis. Starch has a high GI liberating glucose by the fast action of the digestive enzymes, alpha-amylase and beta-glucosidase. Low-GI carbohydrate sources from seaweed with high nutrient contents may be efficacious for a glycaemic management strategy. Seaweeds also contain factors that may affect glycaemia as well as appetite.

Objective: We investigated the effect of the two brown edible seaweeds, *Laminaria digitata* and *Undaria pinnatifida*, on postprandial blood glucose, insulin concentrations, GLP-1 secretion and appetite following a starch load in a human meal study.

Method: We selected the seaweed species based on their culinary popularity, concentrated nutritional content, and potential for reducing glycaemia. Twenty healthy subjects were enrolled in a randomized, 3-way, blinded crossover trial. After an overnight fasting period of 12 h, the subjects received at each session one of three meals comprising 30 g of linear corn starch with 5 g of *L. digitata* or *U. pinnatifida* or an energy-adjusted control meal. Fasting and postprandial blood glucose, insulin and glucagon like peptide 1 (GLP-1) concentrations were measured over a period of 180 min and 120 min, respectively. Aspects of subjective appetite sensation were scored 9 times from baseline to 180 min using visual analogue scales (VAS). An ad libitum meal was served after this time in order to assess hunger more objectively. The change over time by mixed model analyses and the incremental area under the curve (iAUC) for plasma glucose, insulin, GLP-1 and VAS scores were compared between the seaweed test meals and the control meal.

Results: There was no significant effect observed in plasma glucose and serum log-insulin iAUC response following consumption of *L. digitata* or *U. pinnatifida* compared to the control meal. However, linear mixed model (LME) analysis showed that log-insulin was reduced after intake of

U. pinnatifida overall and at time 20 min and 40 min after the test meal. LME and iAUC was increased for serum GLP-1 after intake of *L. digitata* compared to control and LME analysis specifically showed an increase at 120 min. Increase was observed also for iAUC and LME for satiety and fullness; LME analysis further showed increases at time 20 min for satiety and at time 20 and 50 min and for fullness after intake of *U. pinnatifida* compared to control. Also the perception of fullness was amplified at time 20 min after intake of *L. digitata* compared to control. Intake of *U. pinnatifida* showed reduction in iAUC and from the the LME analysis at time 20 and 70 min for hunger. iAUC was reduced for prospective food consumption; the LME analysis showed reduction between 20 to100 min after intake of *U. pinnatifida* compared to control.

Conclusions: The brown seaweeds, *L. digitata* and *U. pinnatifida* did not lower plasma glucose after a single meal but lowered plasma insulin and GLP-1 while increasing satiety and fullness as well as reducing feelings of hunger and prospective food intake compared to control. Thus, these brown seaweeds may play an important role in improving glycaemic and appetite control in healthy adults.

1. Introduction

Postprandial hyperglycaemia is characterised by a plasma glucose level > 7.8 mmol/L (140 mg/dL) 2 h after ingestion of food (Ceriello, Colagiuri, Gerich, & Tuomilehto, 2008). Normal fasting blood glucose levels are typically < 6.1 mmol/L with 2-h postprandial plasma glucose < 7.8 mmol/L (postprandial) (WHO, 2006). Continued fasting and/or postprandial hyperglycaemia is characterised by a progressive decline in hepatic and peripheral insulin sensitivity, deterioration of β-cells, and deficiencies in the incretin hormones, glucagon-like peptide-1 (GLP-1) and glucose-dependent gastric inhibitory peptide (GIP), secreted by the gut (Ceriello et al., 2008). Restoring a normal blood glucose level within a short interval after a meal is important for health, as hyperglycaemia is associated with increased risk of type 2 diabetes and the condition may therefore be a predecessor for diabetes development (American Diabetes Association, 2009). Therefore, minimising postprandial exposures to high blood glucose levels through dietary modification is one of the remedies for glycaemic management.

In recent years, attention has increased to the potential health benefits and therapeutic properties of seaweed. For centuries, seaweed has been consumed in Asian countries such as China, Malaysia and Japan where they are a frequent ingredient in the daily diet. Remnants of edible seaweed species such as Arame (*Eisenia bicyclis*), Hiziki (*Hizika fusiforme*) and several Hondawara species such as *Sargassum* species have been found at archaeological excavation sites being ten thousand years old (Bocanegra et al., 2009). Elsewhere in the world, other seaboard countries such as Ireland, Scotland, Wales, Iceland, Norway, Canada and Spain have traditionally eaten seaweed but to a lesser extent (Mouritsen, 2009; Murphy, 2015). Seaweeds were previously more commonly eaten in the Northern European areas. Seaweed use has now been revived with the New Nordic kitchen (Mithril et al., 2012) in the Nordic countries and due to the influx of Asian food in Europe in general. Since ancient times, seaweed has been used in Asian countries as a functional food and medical herb, and it is therefore considered as an important resource for exploring new therapeutic compounds for humans (Moussavou et al., 2014). Multiple potential health-related actions of seaweed have been described (Motshakeri et al., 2014) and potential bioactive compounds from seaweed have been studied in a number of *in vitro* and animal studies. These include specific proteins and peptides, complex polysaccharides including sulphated polysaccharides and alginate, as well as polyphenols (e.g. phlorotannin), carotenoids and diterpenes (Gupta & Abu-Ghannam, 2011).

Edible seaweeds are a particularly rich source of a variety of resistant dietary fibres, including xylans, carrageenan, fucoidan, laminaran and alginate. These different dietary fibres have different health-related properties (MacArtain et al., 2007). Some of the fibres are known to reduce glycaemia and insulin levels and some of them are known to improve satiety (Sharifuddin et al., 2015) (MacArtain et al., 2007) (Lange et al., 2015). From other studies, it has been shown that some of the edible seaweed species have a positive effect on glucose metabolism in animal models (Motshakeri et al., 2014; Vaugelade et al., 2000). However, there is a scarcity of human studies on the short- and long-term effects of seaweed on these endpoints. Therefore, the main aim of this study was to investigate whether the two brown seaweeds, *Laminaria digitata* and *Undaria pinnatifida*, affect postprandial glucose, insulin and GLP-1 concentrations in healthy adults. Moreover, we sought to evaluate the effect of these two seaweed species on subjective appetite sensation using visual analogue scales (VAS) and an *ad libitum* meal.

2. Methods

2.1　Ethics and protocol registration

The study was carried out at the Department of Nutrition, Exercise, and Sports (NEXS), in the section for Preventive and Clinical Nutrition, University of Copenhagen, Denmark. The study protocol was approved by the municipal Ethical Committee of Copenhagen (journal no.: H 15004500) in accordance with the Helsinki-II declaration. The study was registered on Clinicaltrials.gov (ID# NCT02608372). Before entering the study, all participants gave their written consent after having received written and oral information about the study.

2.2　Participants

Twenty healthy participants aged 28.8 ± 5.4 y with body mass index (BMI) 21.4 ± 2.1 kg/m^2 were recruited through posters at the University of Copenhagen and via website advertisement on http://www.forsogsperson.dk and www.sundhed.dk. Participants were excluded, if they were suffering from systemic infections, had acute or chronic metabolic disorders, were smokers, were breastfeeding, pregnant or planning a pregnancy, were or had been drug addicts, or if they had an iodine related intolerance or allergy. Participants were also excluded if they had a history of surgical intervention for treatment of obesity, had been enrolled in any human dietary or medical intervention study less than 4 weeks before the study, or if they had habitual alcohol consumption above the maximal limit as recommended by the Danish Health Authorities (14 drinks per week for men or 7 drinks per week for women).

2.3　Study design

The study had a randomized, 3-way, blinded crossover design consisting of three test meals given in a random order to each participant on the test days, which were separated by at least 7 days for washout. Participants were instructed to refrain from all kinds of seaweed and paracetamol 48 h prior to and throughout each test day, except for what was provided. In addition, the participants were instructed to refrain from any caffeinated beverages including coffee, black, green, or white tea, cola, energy drinks and chocolate as well as alcohol during this same period. Furthermore, they should avoid intense physical activity 24 h proceeding each test day and until the following morning.

In the evening before each test day, the participants were fasting from 20:00, but drinking 0.5 L of water was required between 20:00 and 08:00 the next day and again 0.5 L during the test day (08:00-12:00). For each test day, the participants had to meet at the Department, in a 12-hour fasting state at 08:00 with minimal use of energy by either walking or cycling at a slow pace, by public transportation, or by car.

Upon arrival, participants were asked to urinate and collect this baseline sample. Participants were then weighed, their height and waist circumference were measured, and they were instructed to lie down and rest for 10 min before measurement of baseline blood pressure (BP). A venflon catheter was afterwards inserted into the antecubital vein, preferably of the right arm, allowing repeated blood sampling throughout the day.

2.4   Biological sampling

The test subjects had three test visits, each with seven separate blood draws by trained phlebotomists. Blood samples were drawn as follows: baseline samples at timed intervals -20 min and then at 20, 40, 60, 90, 120 and 180 min. The blood was collected for plasma glucose analysis in 3 mL FC-mixture tubes (VF-053SFC36, TERUMO Corporation, Tokyo, Japan). Blood collection for serum insulin and GLP-1 was collected in 4 and 6ml additive-free tubes (369032 and 366815 from Becton Dickinson, Plymouth, UK). Samples for plasma collection were centrifuged immediately after sampling while serum tubes were allowed to stand at room temperature for 20 min; serum and plasma were dispensed into cryotubes and subsequently frozen at -80°C. Samples were thawed and assayed after they had all been collected.

2.5   Laboratory measures

Plasma glucose was determined by a standard kit on an ABX Pentra 400 analyzer (Horiba ABX SAS, Montpellier, Cedex, France). Insulin was determined by solid-phase, two-site chemiluminescent immunometric assay, using Immulite 2000 XPi (Siemens Healthcare Diagnostic Ltd, Llaneris Gwynedd, United Kingdom). Serum concentrations of GLP-1 were determined using an enzyme-linked immunosorbent assay (ELISA) based kit (Multi Species GLP-1 total ELISA, EZGLP1T-36K) obtained from EMD Millipore, USA. The kit measures both the inactive and active form of GLP-1 (7-36- and 9-36amides) and was chosen based on recent findings (Bak et al. 2014).

2.6 Determination of mineral elements in seaweed samples

The concentration of selected mineral elements in the seaweed samples was determined following the principles in EN15762:2009 (European Commitee for Standardisation, 2009). Briefly, subsamples of seaweed (approx. 0.3 g) were digested using 5 mL of concentrated nitric acid (SCP Science, France) in a microwave oven (Multiwave 3000, Anton Paar, Graz, Austria). Prior to analysis, the digests were diluted with milli-Q water and subsequently the total element concentration was determined using inductively coupled plasma mass spectrometry (ICP-QQQ-MS) (Agilent 8800, Agilent Technologies, Waldbronn, Germany). Quantification was done using external calibration with internal standardization. Analytical quality was assessed by running selected samples in duplicate (RSD values in the range 1-20% for all elements) and by including the certified reference material (CRM) ERM-CD200 Bladderwrack (IRMM, 2016) in the analytical run (obtained results were in good agreement with certified target values).

2.7 Determination of iodine in seaweed samples

The content of iodine in the seaweed samples was determined following the principles in EN15111:2007 (European Committee for Standardisation, 2005). Briefly, subsamples of seaweed (approx. 0.3 g) were extracted using 4% TMAH in an oven at 90°C for 3 h. Prior to analysis the extracts were diluted with water and filtered and subsequently the iodine concentration determination using inductively coupled plasma mass spectrometry (Agilent 7500ce, Agilent Technologies, Japan). Quantification was done at m/z 127 using external calibration with internal standardization with tellurium at m/z 125. Analytical quality was assessed by running selected samples in duplicate ($RSD_{pooled} = 2,7\%$ (N=3) and the use of the reference material CD200 Bladderwrack, where the obtained results was in good agreement with the target value for iodine recently established in a collaborative trial (DTU Food, 2016).

2.8 Measurements of subjective appetite sensations

Appetite registration was measured at all three test days by repeated visual analogue scales (VAS). VAS was used as a replacement for a categorical questionnaire to register scores for satiety, hunger, prospective food intake, fullness, comfort, and *ad libitum* energy intake as continuous variables. The first appetite assessment was carried out before consumption of the test meal (after 10-12 hours fasting). Subjects were hereafter instructed to register VAS every approximately 20

min, following a guideline on a tablet screen, until the last registration at 180 min postprandially after the *ad libitum* meal.

The VAS registration was done using a digital tablet (Lenovo ThinkPad 10) running a VAS-assessment program, Acqui (Laugesen, J. L. at XYZT, Denmark, www.sensory.dk). VAS was constructed as a digital horizontal line, equal to a 100 mm analogue line on a paper, with the question of interest set above the line. The extremes of the response options were indicated as vertical marks at each end of the line. The VAS equal to 0 and 100 mm is equivalent as follows: satiety ("I am completely empty" and "I cannot eat another bite"), hunger ("I am not hungry at all" and "I have never been more hungry"), prospective food intake ("How much do you think you can eat?" "Nothing at all" and "A lot"), fullness ("How full are you?" "I am totally full" and "Not full at all") and comfort ("How comfortable do you feel?" "Not comfortable at all" and "Very comfortable"). The participants were instructed to assess each question and mark with a vertical line presented on the tablet screen. All data was downloaded from the tablets and quantified using Excel (Microsoft Denmark, Kgs. Lyngby, Denmark).

2.9   Test meals

The test meal was served in the morning at 08:45. A volume of 150 mL starchy drink consisting of 30 g of corn starch in water with 22 g sugar free lemonade powder (Fun One, Stevia lemonade with guava/lime, Kavli A/S Hvidovre, Denmark) was served with either of three different meals. They consisted of 5 g of *Laminaria digitata* (obtained from AlgAran Teoranta, Kilcar Co. Donegal, Ireland) or 5 g of *Undaria pinnatifida* (obtained from JFC Deutschland, Dusseldorf, Germany) or 5 g of pea protein (Pea protein Mega 83%, Natur Drogeriet, Hørning, Denmark). The test meals were prepared by the kitchen staff at the Department of Nutrition, Exercise & Sports, University of Copenhagen. The dried seaweed were soaked in 200 mL of water for 10 min, then rinsed and drained to remove excess water. Finally, they were cut into small pieces and added with 0.5 g iodine enriched salt (6.5 µg iodine), 0.2 g black pepper and 4 g of fresh lemon juice. Together with the test meal, a glass of 500 mL of drinking water was additionally served. The amount of 5 g of seaweed corresponds with the currently recommended maximal daily intake of this food in Denmark. The minor differences in contents of carbohydrate, fat, and protein in the three meals were adjusted with pea protein (83%), rapeseed oil and cornstarch.

Three hours after the test meal and immediately after the last VAS score participants were offered an *ad libitum* test meal to assess their hunger. The *ad libitum* meal consisted of 7987.6 kJ pasta with meat sauce, served with 250 mL water (energy: 554.5 kJ/100 g with macronutrient content (protein: 15.5 E %, carbohydrate: 54.5 E % and fat: 30.1 E %). The volunteers were given 30 min to complete this meal. **Table 1** and **Table 2** show an overview of the various test meals**.**

Table 1
Nutrient composition of test meals (g/serving)

Nutrient composition	*Laminaria digitata*	*Undaria pinnatifida*	Pea protein, control
Energy (kJ)	502.7	536.6	518.9
Protein (g/serving)	0.8	1.1	1.0
Fat (g/serving)	0.3	0.2	0.3
Carbohydrate (g/serving)	27.3	29.2	28.8
Dietary fibre (g/serving)	1.8	1.7	0.2
Water (g)	206.7	206.7	206.7

(Dankost, 2015; Eurofins, 2016)**.**

Table 2
Content of selected minerals in soaked, blotted *Laminaria digitata* and *Undaria pinnatifida*

Mineral composition	*Laminaria digitata*	*Undaria pinnatifida*
Arsenic (As) (mg/100g)	4.00	5.81
Cadmium (Cd) (mg/100g)	0.018	0.137
Chromium (Cr) (mg/100g)	0.048	0.096
Iodine (I) (mg/100g)	164.6	32.0
Zinc (Zn) (mg/100g)	5.53	4.00
Calcium (Ca) (g/100g)	2.01	1.27
Magnesium (Mg) (g/100g)	0.683	0.406
Potassium (K) (g/100g)	1.46	0.275
Sodium (Na) (g/100g)	2.77	5.46

The mineral contents were determined according to (Sloth, 2016)

2.10 Statistical analysis

Statistical analysis was performed using RStudio software (version 1.0.153, ©2009-2016 Rstudio, Inc.) and R (version 3.4.1, R Core Team 2017). Graphs were prepared in GraphPad Prism version 7.0 (GraphPad Software Inc., 2017). The descriptive data is presented as mean ± standard deviation (SD), ± standard error (SE) or ± 95% Confidence Interval (CI). All variables were

checked for outliers and missing data. Dependent variables were inspected for homogeneity of variance and normal distribution using residuals plots and normal probability plots/histograms. Non-normally distributed data were logarithmically transformed and reassessed for normal distribution before further analysis. Glucose, insulin and GLP-1 responses and VAS scores were calculated as the incremental area under the curve, iAUC from baseline values. Blood concentrations of glucose, insulin and GLP-1 are presented as mmol/L, pmol/L and pmol/L respectively. Data for VAS questions are shown as mm within the range of 0-100mm. We used R lme4 (Bates et al., 2012) to additionally perform a linear mixed effects (LME) analysis (ANOVA analysis) for repeated measures on all outcomes, with time and treatment as fixed effects and subject, sex and visit (randomization order) added as random effects. Post hoc pairwise comparisons were made with R multcomp (Hothorn, Bretz, &Westfall), at each time point with time 0 as a co-variate if the model showed statistical significance. The same was applied for all VAS questions. All *P*-values < 0.05 were considered statistically significant.

3. Results

3.1   Participant characteristics

Recruitment of subjects started May 2015; forty subjects were screened and 20 healthy subjects (9 men and 11 women) were enrolled in the study. **Table 3** shows the baseline characteristics of the subjects. All 20 volunteers completed the study, so the drop-out rate turned out to be 0%.

Table 3
Subjects characteristics at baseline

Subject characteristics	All ($n = 20$)	Men ($n = 9$)	Women ($n = 11$)
Age (y)	28.8 ± 5.4	30.3 ± 7.1	27.5 ± 3.4
Height (cm)	171.3 ± 14.5	183.6 ± 10.0	160.7 ± 7.7
Weight (kg)	63.6 ± 11.5	73.2 ± 9.7	55.9 ± 5.4
BMI (kg/m^2)	21.4 ± 2.2	21.6 ± 1.2	21.7 ± 1.9
Waist circumference (cm)	76.4 ± 8.0	81.0 ± 6.7	71.8 ± 6.9
Blood pressure, systolic (mm Hg)	110.3 ± 0.9	112.5 ± 1.5	108.3 ± 0.4
Blood pressure, diastolic (mm Hg)	67.8 ± 0.2	63.6 ± 0.6	70.6 ± 0.6
Fasting blood glucose mmol/L	5.3 ± 0.4	5.3 ± 0.4	5.3 ± 0.5
Fasting blood insulin pmol/L	45.5 ± 26,1	39.3 ± 17.3	50.6 ± 30.9
Fasting blood GLP-1 pmol/L	17.9 ± 7.0	17.5 ± 4.9	18.3 ± 8.3

Numbers represent mean ± standard deviation.

3.2 Nutrient content and mineral elements

The nutrient and mineral contents varied between the two species, *L. digitata* and *U. pinnatifida*. In general, both of them had approximately 1.5 g higher dietary fibre content (**Table 1**) compared to the control meal. Furthermore *L. digitata* also has 5 times higher iodine content and almost 20 times higher K/Na ratio than *U. pinnatifida* (**Table 2**).

3.3 Glucose, insulin and GLP-1 response

There were no differences in iAUC for glucose or log-trans-formed insulin after intake of any of the two seaweed meals. Changes in glucose determined by LME analysis showed no effect of the two meals compared with control. However, postprandial changes in insulin by LME analysis were different between the meals ($P < 0.05$). From the LME analysis for insulin women had a lower insulin response at time 20 minutes ($P = 0.004$) after eating *U. pinnatifida*. Furthermore after eating *L. digitata* men had a lower insulin response at time 20 and 40 minutes ($P = 0.026$, $P = 0.003$, respectively), compared to control (**Figure 1**).
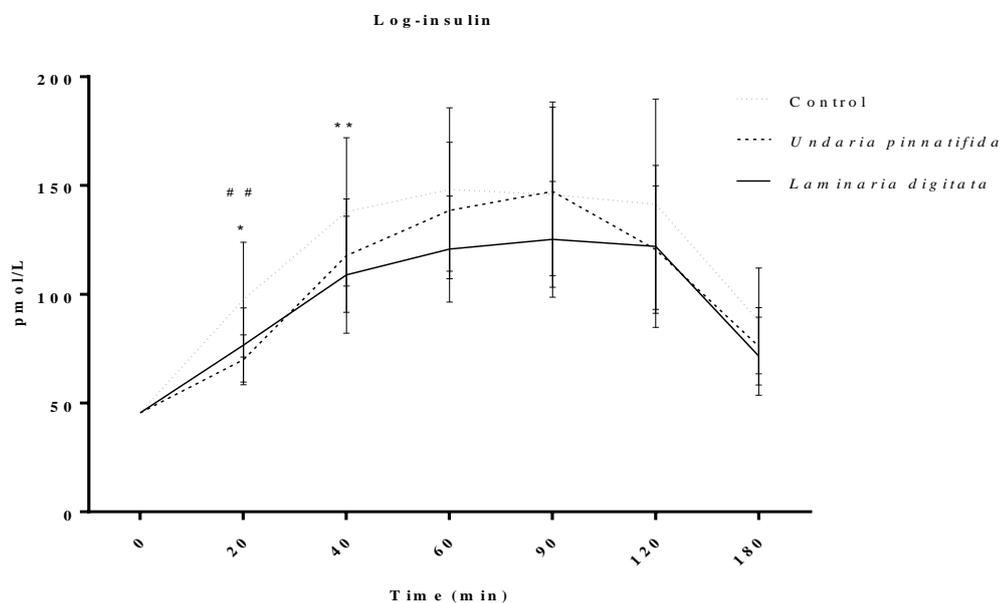
Figure 1. Effects of *Undaria pinnatifida* and *Laminaria digitata* on postprandial plasma insulin concentrations (pmol/L). Postprandial plasma insulin levels at 0, 20, 40, 60, 90, 120 and 180 min after the intake of control and seaweed meals. Values are represented as mean ± 95% CI (n=20).

The iAUC for GLP-1 was higher after intake of *L. digitata* when compared to control ($P=$ 0.017). The LME analysis for postprandial GLP-1 concentrations demonstrated an overall increase for *L. digitata* compared with control. Thus, the GLP-1 secretion was increased at time 120 minutes ($P=$ 2.2e-05) after intake of the *L. digitata* meal compared to the control meal (see **Figure 2**).
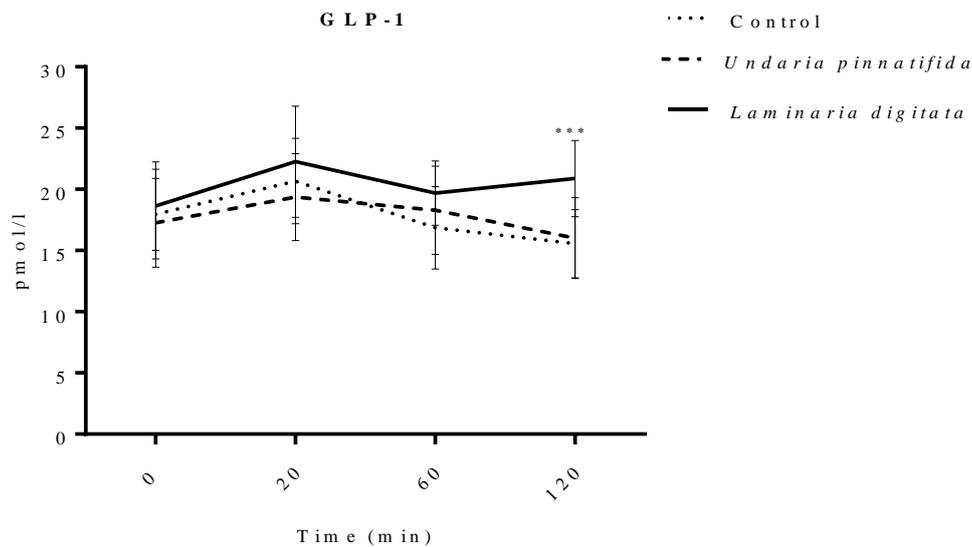

Figure 2. Effects of *Undaria pinnatifida* and *Laminaria digitata* on postprandial plasma GLP-1 concentrations (pmol/L). Postprandial blood GLP-1 levels at 0, 20, 60 and 120 min after the intake of control and seaweed meals. Values are represented as mean ± 95% CI (n=20).

3.4 Appetite and comfort scores

The postprandial changes in ratings of satiety, hunger, fullness, anticipated prospective food consumption, comfort and *ad libitum* energy are tabulated in **Table 4**. There were marked acute effects for all scores related to appetite, however there was no remaining effects after 200 min, no change in comfort at any time, and no effect on ad libitum energy intake at 200 min.

Satiety. There was a difference in satiety iAUC after intake of the test meals ($P= 0.003$), increasing it about 1314.1 ± 363.21 (SE) after intake of *U. pinnatifida* ($P= 0.0002$) and increasing it about 784.2 ± 367.02 (SE) after intake *L. digitata* ($P= 0.032$) compared to control. Correspondingly, from the LME analysis an acute time dependent effect on the feelings of satiety ($P= 0.009$) were found. Thus, the subjects felt more satiated postprandially after ingesting *U. pinnatifida,* in particular at time 20 min (15.6 mm ± 3.7 (mean±SE), $P= 302e-05$) in comparison to the control meal. The response curves for satiety are presented in **Figure 3**.
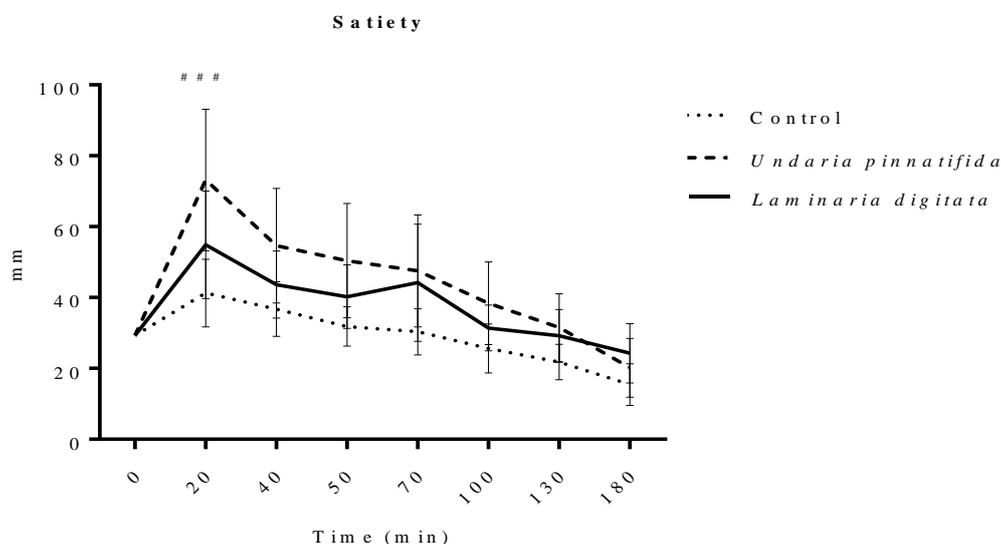
Figure 3. Satiety scores postprandially after meals containing *Laminaria digitata*, *Undaria pinnatifida* or control (pea protein) in 20 healthy normal-weight subjects. VAS equal to 0 and 100 mm is equivalent to "I am completely empty" and "I cannot eat another bite, respectively. $^{\#\#\#}P< 0.001$ for *Undaria pinnatifida* versus control. Values are represented as mean $\pm 95\%$ CI (n=20).

Hunger. Comparison of hunger iAUC showed no effects on the two test meals. However, from the LME model the feeling of hunger was affected after intake of the test meals ($P= 0.016$). Hunger was reduced due to the intake of the *U. pinnatifida* meal at time 20 min (-14.6 mm ± 4.1 (mean±SE), $P= 0.0004$) and at time 70 min (-8.1 mm ± 4.1 (mean±SE), $P= 0.048$) compared to control (**Figure 4**). No specific time points were affected after intake of *L. digitata*.
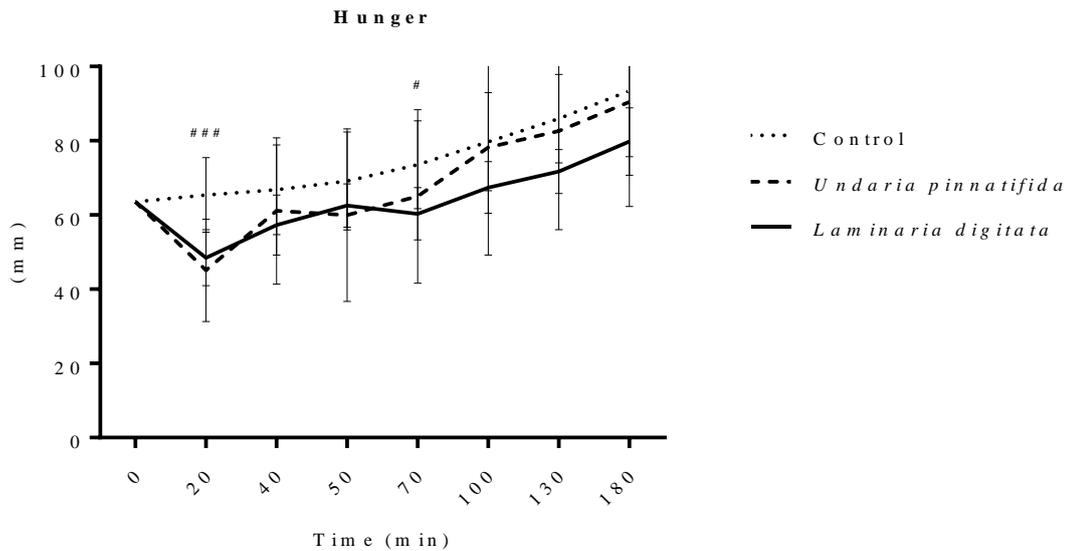
Figure 4. Hunger scores postprandial after meals containing *Laminaria digitata*, *Undaria pinnatifida* or control (pea protein) in 20 healthy normal-weight subjects. VAS equal to 0 and 100 mm is equivalent to "I have never been more hungry" and "I am not hungry at all", respectively [###]P< 0.001 for *Undaria pinnatifida* versus control. Values are represented as mean ± 95% CI (n=20).

Fullness. Comparison of iAUC for the three meals demonstrated an effect on fullness after ingesting the test meals (P= 0.008), increasing it about 1247.1 ± 379.7 mm (mean±SE) after intake of *U. pinnatifida* (P= 0.001) compared to control. Changes in fullness were observed for both meals when analysing the data using LME (P= 0.019). From the following *post hoc* analysis, it appeared that the participants had an increased feeling of fullness after intake of the *U. pinnatifida* meal at time 20 min (14.5 mm ± 3.9 (SE), P= 0.0002) and time 50 min (10.0 mm ± 3.4 (SE), P = 0.01). The subjects also felt more full after intake of the *L. digitata* meal at time 20 min (7.1 mm ± 3.9 (SE), P= 0.041) (**Figure 5**).

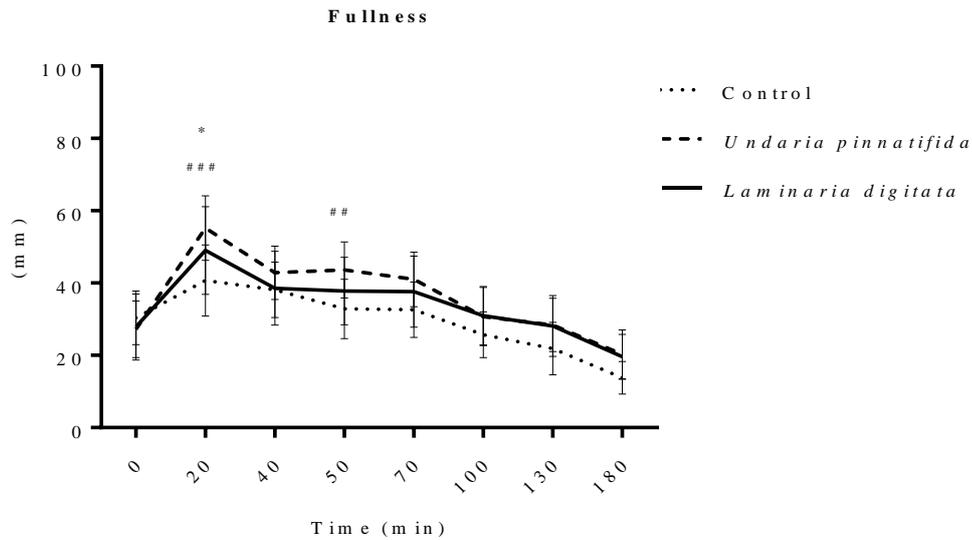**Fullness**

Figure 5. Fullness scores postprandially after meals containing *Laminaria digitata*, *Undaria pinnatifida* or control (pea protein) in 20 healthy normal-weight subjects. VAS equal to 0 and 100 mm is equivalent to "I am totally full" (fullness), and "Not at all", respectively. [###]$P < 0.0001$ and, [##]$P < 0.001$ for *Undaria pinnatifida* and [*]$P < 0.05$ for *Laminaria digitata* versus control. Values are represented as mean ± 95% CI (n=20).

Anticipated prospective food consumption. There were no differences for iAUC between the three groups. The following LME analysis showed an acute time-dependent effect of the test meals ($P = 0.034$). The subjects had a reduced desire to eat after the *U. pinnatifida* test-meal compared to the control meal at the following time points: time 20 (-11.7 mm ± 3.4 (SE), $P = 0.001$), time 40 (-7.7 mm ± 3.4 (SE), $P = 0.02$), time 50 (-8.8 mm ± 3.4 (SE), $P = 0.01$), time 70 min (-7.1 mm ± 3.4 (SE), $P = 0.04$) and at time 100 min (-6.8 mm ± 3.4 (SE), $P = 0.05$) (see **Figure 6**).
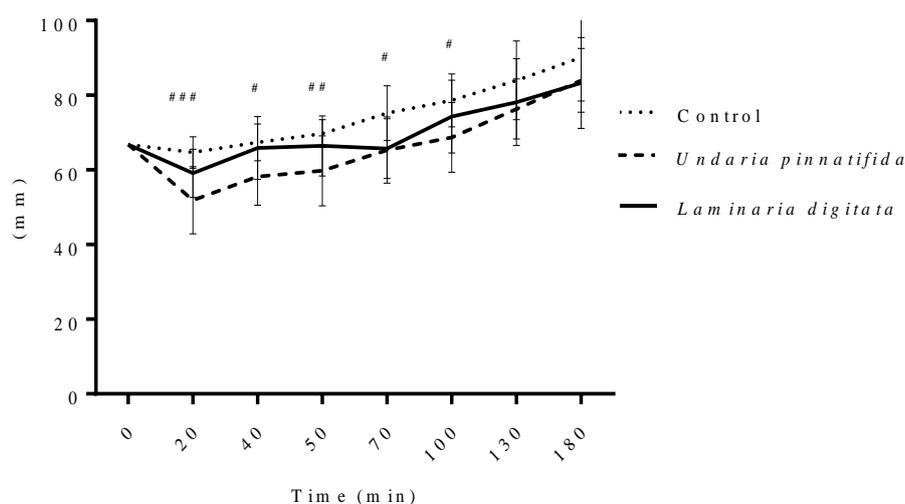
Figure 6. Anticipated prospective food consumption scores postprandially after meals containing *Laminaria digitata*, *Undaria pinnatifida* or control (pea protein) in 20 healthy normal-weight subjects. VAS equal to 0 and 100 mm is equivalent to "How much do you think you can eat?" "Nothing at all" and "A lot", respectively. $^{\#\#\#}P< 0.001, ^{\#\#}P< 0.01 \ ^{\#}P< 0.05$ for *Undaria pinnatifida* versus control. Values are represented as mean ± standard error (n=20).

Table 4
Result for iAUC of all measured endpoints after ingestion of the three test meals

Outcome	Control	*Laminaria digitata*	*Undaria pinnatifida*	*P*-value iAUC	*P*-value LME_{rm}
Biochemistry					
Glucose (mmol/L)	155.4 ± 19.8	-23.9 ± 20.2	29.5 ± 19.9	0.30	0.07
Log-insulin (pmol/L)	76.1 ± 8.66	-8.30 ± 6.34	-5.19 ± 6.25	0.42	0.04*
Glp-1 (pmol/L)	126.2 ± 80.4	176.6 ± 74.5	47.5 ± 73.4	0.02*	0.05*
Appetite scores					
Satiety (mm)	1459.8 ± 394.1	719.3 ± 369.6	1282.1 ± 361.4	0.004 **	0.009**
Hunger (mm)	2481.7 ± 552.9	-1040.1 ± 557.2	641.6 ± 544.9	0.18	0.02*
Fullness (mm)	1296.4 ± 386.7	568.2 ± 388.3	1247.2 ± 379.7	0.008 **	0.02*
Prospective food-consumption (mm)	1806.8 ± 429.3	-591.6 ± 443.2	-574.6 ± 33.5	0.32	0.03*
Comfort (mm)	1182.9 ± 427.0	-279.0 ± 292.3	-5.82 ± 285.8	0.55	0.81
Ad libitum (g)	512.7 ± 79.2	-99.8 ± 54.0	-5.23 ± 53.6	NA	0.13[1]

Comparison of iAUC for all outcomes using linear mixed model (LME) with iAUC$_{outcome}$ ~ meal + visit, as the dependent variable. The fixed effect "meal" is presented as mean ± SE. The fixed effect "visit" is not shown as there was no effect observed for visit. *P* – values are obtained from the full model.

The significant codes are: *** for 0.001; ** for 0.01 and * for 0.05. NA; not applicable; the [1]*ad libitum* could be compared using a linear mixed-effect model only.

4. Discussion

In this randomized controlled trial, we investigated the postprandial effects in healthy adults after meals containing whole brown seaweeds in order to study their potential for management of glycaemic and insulinemic responses, incretin effect, and appetite sensation. We used whole seaweeds in the meals as consumed by people in Asia, where seaweed salads with whole or chopped leafs are common. This provides minimal processing, thereby preserving the seaweed constituents. Consumption of whole seaweed may provide a combination of bioactive components which may be more effective than the sum of the individual compounds in seaweed when tested individually (Teas et al., 2013). The main findings from this study suggest that the brown seaweeds, *Laminaria digitata* and *Undaria pinnatifida* have effects on postprandial insulin and GLP-1 concentrations in healthy adults after starchy meals. *U. pinnatifida* also significantly reduced postprandial insulin concentrations in women at 20 min.

In previous studies, brown seaweeds were reported to contain potentially bioactive compounds that inhibited α-amylase and α-glucosidase enzymes *in vitro* and reduced blood glucose and plasma insulin concentrations in mice (Liu et al., 2016); (Kang et al., 2016); (Lordan, Smyth, Soler-Vila, Stanton, & Ross, 2013); (Lordan et al., 2011); (Zhang et al., 2007); (Aeda et al., 2007); (Hosokawa et al., 2010); (Nwosu et al., 2011). Polyphenols, fucoxanthin and fatty acids found in *L. digitata* and *U. pinnatifida* have been reported to inhibit α-glucosidase activities resulting in a reduced rate of glucose liberation thereby reducing the postprandial rise in blood glucose. A crude polyphenol extract from a brown seaweed, *Ascophyllum nodosum*, reduced the fasting serum glucose level in diabetic mice by inhibiting the intestinal α-glucosidase (Zhang et al., 2007). Other findings suggest that carotenoids such as fucoxanthin have potential to improve glycaemic control in mice (Hosokawa et al., 2010; Jung et al., 2012). *U. pinnatifida* contains from 1.77-2.89 mg of fucoxanthin per g of dry weight (Fung, Hamid, & Lu, 2013). In diabetic KK-A^y mice intake of 0.2% fucoxanthin in the feed reduced fasting blood glucose and plasma insulin concentrations compared with the control mice (Maeda et al., 2007). Fucoxanthin fed to diabetic and obese KK-A^y mice was also found to attenuate hyperglycaemia (Hosokawa et al., 2010). Other nonpolar components including oleic acid and linoleic acid found in *L. digitata* also seem to inhibit α-glucosidase *in vitro*, however their importance *in vivo* still needs to be investigated (Liu et al., 2016). Different Irish seaweed species have been shown to possess inhibitory properties on alpha glucosidase and to increase GLP-1 secretion. Subsequently, cold-water extracts of *L. digitata* were shown to have

potent inhibitory effects on DPP-4 similar to the effect of the well known DPP-4 inhibitor, berberine (Calderwood et al. 2013). Chin and colleagues (2015) report anti-diabetic potential of different seaweeds from Malaysia. Crude water extracts from the brown seaweeds, *Paralia sulcata*, *Sargassum binderi* and *Turbinaria conoides* strongly inhibited DPP-4 and stimulated secretion of the incretins, GLP-1 and GIP, from pGIP neo STC-1 cells *in vitro*. Various organic extracts from other *Sargassum* species (*S. polycystum* and *S. wightii)* have shown similar properties in vitro. Thus, different extracts from several brown seaweed species have been shown to inhibit DPP-4 at several concentrations (Unnikrishnan et al., 2015). The results from our study provide additional evidence that relatively small dietary intakes of whole seaweed may affect postprandial serum insulin and GLP-1 concentrations in healthy adults.

These effects might be due to several factors including the specific nutrient content in seaweed. High contents of dietary fibre in the seaweed species used in our study may lead to reductions in blood glucose, insulin concentrations concentrations, however such effects are usually seen only at higher fibre intake levels than the 1.8 g provided in our study (Wolfram & Ismail-Beigi 2011). Brown seaweeds contain between 19.6-64.9% of soluble fibre depending on the species (Ruperez & Saura-Calixto, 2001) and the contents of around 40% observed for our batches is therefore not extraordinary. The soluble fibre from seaweed dissolves in water to form a viscous gel (Blackburn, Jarjjs, & Hanning, 1984), which might lead to a reduced rate of gastric emptying (Blackburn et al., 1984) or simply to reduced substrate diffusion resulting in a reduction of the intestinal glucose absorption rate. Alternatively, glucose liberation from degradable polysaccharides such as starch may be delayed after entering into the duodenum thereby retarding the rate of glucose reaching the intestinal contents when seaweeds are consumed together with a meal rich in starch (here provided as a starchy drink). Seaweeds rich in dietary fibre have been reported to play an important role in glycaemic management (Sharifuddin et al., 2015). In a placebo-controlled crossover study involving 30 healthy subjects, Wolf and colleagues (2002) found that an acid-induced viscosity complex containing 3.75 g alginate supplied together with a glucose beverage attenuated the postprandial glycaemic response, indicating that inhibition of enzymatic glucose release is not necessary for this response. However, the lack of reduction in postprandial blood glucose concentrations after intake of *L. digitata* and *U. pinnatifida* observed in our study could be explained to some extent by the low dose provided despite their high content of this dietary gel-forming fibre. In comparison, the control meal contained only a limited amount of pea fibre. Pea protein (10 g) combined with pea hull fibre (7 g) is well known to have effects on postprandial blood glucose and this effect was not reduced

by adding insoluble hull fibre (Mollard et al., 2014). Therefore, our use of 5 g pea protein as control represents a low dose of another bioactive antiglycaemic meal component and may therefore have partially masked an effect of the seaweeds in this study.

L. digitata and *U. pinnatifida* also increased satiety sensation and reduced any feelings of hunger. As opposed to the lack of effects of pea protein on postprandial measures of satiety (Mollard et al., 2014) our findings show that consumption of brown seaweed affects satiety and hunger. From the corresponding VAS questions, it appears that intake of whole *U. pinnatifida* and *L. digitata* affect several appetite related feelings for more than 1 h postprandially and satiety for more than two hours. This implies that these seaweed species are potential candidates to reduce energy intake for several hours after intake of just 5 g of dried *U. pinnatifida* and *L. digitata*, possibly because of its content of the polysaccharide, alginate, and perhaps other soluble dietary fibres having a satiating effect that may be due to bulking or to reduced gastric emptying rate. The data shows that average fasting levels of GLP-1 are lower than the postprandial GLP-1 concentrations at time 20 min after all test meals with *Laminaria*, *Undaria,* or control. The increment correlates with previous findings showing that after ingestion of a liquid glucose load, nutrients reach the proximal duodenum about 6-8 min later (Deacon & Ahrén, 2011). The maximum serum concentrations (C_{max}) of GLP-1 was reached at time 20 min for all test meals in accordance with a commonly observed peak-response approximately 30 min postprandially (Baggio & Drucker, 2007; Pala et al., 2010). However, as no data is available from baseline to 20 min and at 60 min, it cannot be ruled out that the C_{max} of GLP-1 was higher or occurred before or after time 20 min. Significant differences in GLP-1 levels were seen at 120 min after intake of *Laminaria* compared to control. Similar findings were observed in an *in vitro* study, where laminarin, from Irish brown seaweed, was found to increase GLP-1 secretion and to inhibit DPP-4 (Calderwood et al., 2013). From our results, it can be speculated that the time course for laminarin to inhibit DPP-4 is delayed because of the dietary fibre load from *Laminaria* causing a delayed absorption of the meal and as a result, there is an increase in GLP-1 levels again at time 120 min.

This discovery could potentially benefit many people, both in terms of possibly helping in maintaining a given weight, as well as to potentially prevent and/or control weight gain; however, this needs to be carefully tested in longer-term trials. In support of this interpretation alginate from brown seaweed has been hypothesized to increase satiety by delaying gastric clearance, stimulating gastric stretch receptors, and attenuating nutrient absorption (Lange et al., 2015).

The analysis of nutrient and mineral contents showed that *L. digitata* and *U. pinnatifida* contain some protein but only small amounts of fat. Both of them, and especially *L. digitate*, are rich in minerals such as potassium (K), calcium (Ca) and magnesium (Mg), which have been hypothesized to improve glycaemic control (Song et al., 2006). *L. digitata* and *U. pinnatifida* also contain zinc (Zn) and chromium (Cr) in relatively high amounts. Both minerals have been reported to improve blood sugar levels and improve the ability of insulin to bind to membrane receptors and to increase the transportation of glucose into muscle cells (Suliburska et al., 2014; Jou et al., 2010). However, care should be taken with some trace elements like iodine, where an upper tolerable intake level of iodine has been recommended at 600 µg/day for adults (European Commission, 2002). Despite the high iodine content in the test meals, the acute exposure was judged as safe for subjects who are not hypersensitive to iodine since single acute doses from foods are not known to give adverse effects (European Commission, 2002). However, caution may be needed in case of frequent consumption, as 0.36 g *L. digitata* and 1.9 g *U. pinnatifida* would exceed the recommended maximal average daily intake level of 600 µg/day. However, seaweed is a good natural resource of iodine that can help to increase thyroxine and reduce iodine deficiency disorder (Reinhardt et al., 1998).

Further studies on isolated bioactive compounds from seaweed and a longer-term study with different groups of volunteers such as healthy subjects or subjects with insulin resistance are suggested in order to see the individual contributions of seaweed components to the satiating effects and to glucose/insulin maintenance over time. Cooking and processing of seaweed may also be of importance due to changes in nutrient and mineral levels and the resulting concentration of bioactive compounds in the meal. For example, iodine levels were decreased by cooking of *L. digitata* (the iodine is 99% water soluble) (Houa et al., 1997) and 99% of the iodine from *U. pinnatifida* was also found in the cooking water after 15 min of boiling (Ishizuki, 1989). Cooking may thus provide a means of reducing the iodine intake while increasing the exposure to other functional components. In addition, it would be interesting to investigate the effects of cooked vs. raw seaweed on glucose, insulin, incretins and appetite response and on food acceptability in a mixed-meal study. Further work is therefore needed in order to identify the bioactive compounds, longer-term effects, and mineral bioavailability in humans after *L. digitata* and *U. pinnatifida* intake.

5. Conclusions

This study did not show an effect of low amounts (5 g) of selected brown seaweeds on postprandial glycaemia but provides human trial evidence for an effect of the brown seaweeds on the insulinaemic response, GLP-1 secretion and appetite. Brown seaweed lowered the postprandial insulin response as well as hunger in humans exposed to a highly degradable linear starch and increased the postprandial feeling of satiety and fullness in healthy subjects of both sexes. Consumption of brown seaweed may be recommended for people with hyperglycaemic disorders provided issues with excessive iodine intake can be avoided by water extraction or cooking.

6. Acknowledgements

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6. DISCUSSION

The influence of seaweeds on α-amylase and α-glucosidase activities

In the *in vitro* studies, crude extracts of dried edible seaweed, collected and purchased from different western and Asian countries, were investigated for their effects on carbohydrate-digestive-enzymes. *L. digitata* and *U. pinnatifida* showed potent inhibiting effects on α-amylase and α-glucosidase, in mixed-type inhibition. These two brown seaweeds have a considerable potential for inhibition of α-amylase and α-glucosidase with efficiencies better than the red seaweeds tested here. Acetone and methanol extracts of *U. pinnatifida* and *L. digitata* had relatively higher α-amylase and α-glucosidase inhibitory activities.

This result is in concordance with other studies where the brown seaweed, *Ascophyllum nodosum,* which is an Irish seaweed, was found to be a strong inhibitor of both α-amylase and α-glucosidase activities at low concentrations (Lordan, Smyth, Soler-Vila, Stanton, & Ross, 2013; Nwosu et al., 2011). Another brown seaweed, *Himanthalia elongata,* also known as Sea spaghetti was also capable of inhibiting α-glucosidase activity (Mojica et al., 2014). Additionally, *A. nodosum* and *H. elongata* are capable of inhibiting α-glucosidase using water extracts (Lordan et al., 2013; Mojica et al., 2014). These findings further support the idea that brown seaweeds are highly potent in inhibiting carbohydrate digestion enzymes but also underline that several different bioactive components may be involved.

Our results differ from another published study (Nagappan et al., 2017), in which the consumption of fresh seaweed of *S. polycystum* from Malaysia was shown to have higher inhibitory effect against α-amylase (IC$_{50}$ 0.58 ± 0.01 mg/mL) and α-glucosidase (IC$_{50}$ 0.69 ± 0.02 mg/mL) compared to our dried *S. polycystum*. This was also observed by Kim et. al. (2008), who discovered a potent inhibitory activity of α-glucosidase by the fresh seaweed of *Grateloupia elliptica* from Korea. There are several possible explanations for these results such as the seaweed source, consumption of seaweeds being fresh or dry, climate and water conditions, that may cause differences in the potential of each seaweed preparation for inhibitory effects against these enzyme activities. However, a possible explanation for our results might be the degradation or removal of relevant bioactive compounds during the drying of the seaweeds. Drying also affects some differences in the seaweed nutrient composition (Matanjun et al., 2009). For

instance, a study conducted by Jenny et al. (1997) showed that the sun-dried seaweed, *S. hemiphyllum,* contains lower concentrations of total amino acids, total polyunsaturated fatty acids, minerals and total vitamin C compared with freeze-dried seaweed.

Overall, these findings show that, the crude extracts of *L. digitata* and *U. pinnatifida* inhibited α-amylase and α-glucosidase activities. This implicates that these seaweeds may contribute to anti-hyperglycaemic effect. Further studies on the inhibitory effect of polyphenolics, alginates and fucoxanthin from seaweeds and the effect of selected dried edible seaweeds on human postprandial glycaemia were subsequently conducted to investigate its effects on anti-hyperglycaemic effect.

Potential α-amylase and α-glucosidase inhibitors

Polyphenolics, alginate and fucoxanthin present in brown seaweeds were bioactive compounds shown in this study to be α-amylase and α-glucosidase inhibitors. Several mechanisms may be involved in the inhibition of the enzymes activities. It was found that, *L. digitata* and *U. pinnatifida* both showed mixed-type inhibition. These results might be related to the polyphenolic compounds, alginate and fucoxanthin, as discussed below.

Polyphenolic compounds

Both preparations of the brown seaweeds, *L. digitata* and *U. pinnatifida,* contained high amounts of 2,5-dihydrobenzoic acids and small amount of gallic acids. Using 2,5-dihydroxybenzoic acid and gallic acid (analytical-grade), it was found that 2,5-dihydroxybenzoic acid showed as the most effective α-amylase inhibitor with IC_{50} 0.046 ± 0.004 mg/mL. In accordance with the present result, a previous study has demonstrated that 4-hydroxybenzoic acid (400 mg/L) (analytical grade) inhibited amylase activity (Wu, Shen, Han, Liu, & Lu, 2009) and other phenolic acid such as 2,4-dihydrobenzoic acid showed strong inhibitory effect against α-glucosidase enzyme compared to the standard of acarbose (Abdullah, Salim, & Ahmad, 2016). In this study, the kinetic analyses showed that both phenolic acids display mixed-type inhibition. In mixed-type inhibition, K_m values increase with inhibitors since the inhibitors are competing with the substrates for a fixed number of active sites on α-amylase. V_{max} decreases with the addition of 2,5-dihydroxybenzoic acid and gallic acid compared with control (no inhibitor).

These results indicate that the inhibitors do not bind to the active site of the enzyme. Instead they bind allosterically, to a different site on the α-amylase, thus affecting the enzyme-substrate complex and slowing the rate of reaction between starch and α-amylase. Contrary to this, epigallocatechin showed the lowest α-amylase inhibitory activity (IC_{50} 0.504 ± 0.003mg/mL). The finding is in agreement with Yilmazer-Musa et.al. (2015) who found that catechins such as epigallocatechin are not strong inhibitors of α-amylase. This may be due to the lack of specific A- and B-ring hydroxyl groups to effectively interact with the catalytic site of the enzyme (Goh et al., 2015; Piparo & Nestlé, 2008; Yilmazer-Musa et al., 2015).

Alginate

Alginate is a complex polysaccharide found in brown seaweeds. The results of our study showed that *L. digitata* and *U. pinnatifida* display strong binding affinity to mAb LM7 antibodies, which indicates high concentration of alginates in both. Alginate used in this study possessed the strongest inhibitory effect on α-amylase activity. Surprisingly, this alginate was a potent mixed type inhibitor against α-amylase, even stronger than crude extracts and acarbose. This result has not previously been reported in an *in vitro* study. Some authors have only reported that dietary fibres such as alginates promote a delayed glucose absorption and act on insulin response in T2D patients (Thorsdottir, Alpsten, Holm, Sandberg, & Tölli, 1991). Their results may be explained by the fact that alginate, which is a viscous fibre, slows gastric emptying rate and the intestinal absorption of glucose. Some other complex polysaccharide such as fucoidan found in brown seaweeds (*A. nodusum*) was reported to have the same findings where fucoidan inhibited α-amylase and α-glucosidase, IC_{50} 0.12-4.64 mg/mL and IC_{50} 0.0013-0.047 mg/mL, respectively (Kim, Rioux, & Turgeon, 2014).

Fucoxanthin

It is interesting to note that among all five-seaweed species, *U. pinnatifida* had the strongest inhibitory effect against α-glucosidase. This effect was associated with the bioactive compound, fucoxanthin. The inhibition kinetic study indicates that fucoxanthin elicits mixed inhibition. In this study, fucoxanthin was found to be a strong inhibitor of α-glucosidase activity with a lower IC_{50} value than the acarbose. Fucoxanthin may therefore have the potential to reduce the rate at which α-glucosidase digests complex carbohydrates in the small intestine.

These findings further support the idea of Hwang et al. (2015), where fucoxanthin isolated from acetone extract of *S. hemiphyllum* inhibited carbohydrate digestive enzymes such as α-amylase and α-glucosidase (sucrose and maltase). Jung et al. (2012) also reported that fucoxanthin showed potent inhibitory activity against protein tyrosine phosphatase 1B (PTP1B) and α-glucosidase. Interestingly, Park et al. (2011) and Maeda et al. (2007) reported that fucoxanthin significantly reduced the fasting blood glucose concentration and plasma insulin concentration in C57BL/6J mice and KK-A[y] mice, respectively.

However, in accordance with previous studies, the mechanism by which fucoxanthin inhibits carbohydrate digestive enzymes and reduce the blood glucose has yet to be clarified. There are, however, some possible explanations based on our findings. The addition fucoxanthin showed it to be a mixed-type inhibitor, altering the K_m and V_{max} values. This usually happens by influencing the binding of substrates to the enzyme's active site. Fucoxanthin probably binds to an allosteric site, a site different from the substrate binding in site. Fucoxanthin may therefore have the potential to reduce the rate at which α-glucosidase digests complex carbohydrates in the small intestine.

Influence of seaweeds' bioactive compounds on carbohydrate digestion

The results of this study indicate that polyphenolics, alginate and fucoxanthin found in *L. digitata* and *U. pinnatifida* are potent inhibitors of α-amylase and α-glucosidase activities. There are growing findings that polyphenols and dietary fibre may influence carbohydrate metabolism. In animal studies polyphenols found in food have attenuated postprandial glycaemic responses and improved acute insulin secretion and insulin sensitivity (Hanhineva et al., 2010) .

There are various possible mechanisms involved to attenuate postprandial glycaemic responses. Based on our findings, the most plausible mechanism might be by inhibition of carbohydrate digestion enzymes. Alpha-amylase and α-glucosidase are the enzymes used to digest carbohydrates to glucose. Carbohydrate such as starch contain amylose (linear α-1,4-linked glucose polymer) and amylopectin (linear α-1,4-linked glucose chains and α-1,6-linked branch chains). The inhibition of salivary and pancreatic α-amylase activity may reduce the hydrolysis of α-1,4-glucosidic linkages and its product, maltose. The inhibition of α-glucosidase activity in the small intestinal brush border will slow down the hydrolysis of the terminal α-1,4-linked glucose, and the production of glucose. In addition, studies conducted in *in vitro* and animal models indicate that several flavonoids and phenolic acids such as ferulic, caffeic, and tannic acids, quercetin and catechin have been shown to inhibit glucose transport (Hanhineva et al., 2010; Nyambe-Silavwe et al., 2015; Sun et al., 2016).

In this study, we also identified alginate in *L. digitata* and *U. pinnatifida*. Alginate is a source of dietary fibre. As mentioned before, alginate is a viscous dietary fibre in solution and was a potent inhibitor of α-amylase. Possible effect of this alginate may be interference with the digestion of carbohydrate and the absorption of glucose. Ikeda & Kusano (1983) have indicated that several dietary fibre sources can inhibit digestive enzymes. According to Kapoor et al. (2016), the consumption of soluble fibre increases the viscosity of gut environments, reducing diffusion through the stationary water stratum and the accessibility of α-amylase to its substrates and thus decrease the enzyme activity. Soluble, viscous fibre like alginate may therefore slow the absorption of glucose by the small intestine. This soluble fibre is passing through into digestive system intact; since it is not broken down by the human digestive enzymes, this alginate does not raise blood glucose levels. When this soluble fibre reaches the large intestine, it will be fermented by the colonic microflora with the production of short fatty acids (SCFA). Some fibres are degraded in the large intestine and the rest being excreted in the stool.

Seaweeds influence the blood glucose and insulin levels

The present study was designed to investigate the effect of selected edible seaweeds on the postprandial blood glucose and insulin concentrations following a starch load in humans. *L. digitata* and *U. pinnatifida* were tested for their effects on plasma glucose and serum insulin levels.

The results show that there were no significant changes in iAUC of plasma glucose and serum insulin concentrations using these seaweeds when compared with the control meal. Although the changes were not significant, where $P > 0.05$ but both seaweed meals suggested a small decrease in plasma glucose and serum insulin concentrations compared with control meal. This was also statistically corroborated for serum insulin by using a mixed model showing that especially at the early time points after seaweed ingestion there was reduced responses.

The results of this study will now be compared to the findings of previous works. In previous works, fucoxanthin from *U. pinnatifida* significantly lowered the fasting blood glucose concentration and the plasma insulin concentration in diet-induced obese (C57BL/6J) mice (Park et al. 2011). After 9 weeks of feeding with fucoxanthin the insulin resistance index improved. However, in our study, we used the whole dried *U. pinnatifida*, and the bioaccessibility of the carotenoid may be affected by drying and also by the food matrix, which was quite low in fats. This may have resulted in a weaker inhibition of starch degradation and hence a weaker inhibition of the glycaemic response in the volunteers.

The findings of our study suggested that alginate from brown seaweeds inhibited α-amylase. In a study by Vaugelade et al. (2000), it was found that *L. digitata,* which contains alginate, strongly reduced blood glucose and insulin responses in Large White male pigs. In a human study, Paxman et al. (2008) used a crossover design to monitor the uptake of glucose. Overweight male subjects given 1.5 g of alginate from seaweed, revealed a significant decrease in glycaemia AUC. From these findings it seems quite clear that brown seaweeds that contain alginate influence blood glucose levels and have α-amylase inhibitory activities that may explain these actions.

In another randomised study, a seaweed supplement that contained *L. japonica* and *U. pinnatifida* consumed by 10 subjects with T2D, resulted in significantly decreased fasting blood glucose levels (Kim, Kim, Choi, & Lee, 2008). This shows that the consumption of a seaweed supplement containing 30.1 g/day of total dietary fibre in T2D patients can result in lower levels

of fasting blood glucose. The authors state that the seaweed supplement may delay glucose absorption because it is rich in indigestible polysaccharide fibre. Although their preparation contains *U. pinnatifida* the authors do not discuss the potential influence of fucoxanthin on their findings. Similarly, to our human study results regarding insulin response and the *in vitro* findings on alginate and on fucoxanthin these findings may further support that brown seaweeds, particularly *U. pinatifida*, might be used as a potential functional food.

Limitations and challenges

This study had some limitations and challenges, which influenced the findings. One limitation was the focus on dried edible seaweeds to inhibit α-amylase and α-glucosidase activities and their potential effect on lowering the postprandial blood glucose and insulin concentrations following a starch load in human meal study. We used dried edible seaweeds compared with fresh edible seaweeds since it is much easier to buy dried edible seaweeds on the market compared with fresh seaweeds.

As in **Paper 1** and **Paper 2**, the investigation was limited by selected number of edible seaweed samples. All samples were collected at different locations such as Malaysia, Korea, Ireland, and Chile. It was therefore difficult to have fresh seaweeds at hand. Thus, dried seaweeds were used to study their effects and compare their enzyme inhibiting capabilities and their potential for lowering plasma glucose and serum insulin levels.

Furthermore, the current study has only examined some of the potent bioactive compounds that inhibit enzyme activities. Only some of the compounds such as polyphenolics, alginate (**Paper 1**) and fucoxanthin (**Paper 2**) may have shown potent bioactive compounds due to the extraction methods and solvents used to extract the compounds from seaweeds. Other compounds may be liberated during digestion, but this process was not simulated in our setup but could be targeted in a follow-up study.

Thirdly, in **Paper 3**, to explain some of the *in vitro* results, a human meal study was conducted in a randomized, 3-way, blinded crossover design. Twenty healthy volunteers were recruited to participate in three meals containing either of two brown seaweeds or pea protein as placebo in random order and with at least one-week washout between meals. Before and up until 3 h after the meal blood was collected for measurement of plasma glucose and serum insulin. The study was powered to observe an effect on the postprandial glucose iAUC provided dropout

was below 10%. The selection of pea protein may not be optimal since it has been found to affect postprandial glycaemia as well. This may have reduced any potential contrast with the seaweeds. In this study, we focused on the selected raw edible seaweeds (whole food). The study was not designed to investigate the effects of any specific bioactive compounds on the postprandial blood glucose and insulin levels. All meals also contained corn starch to provide an accessible substrate for the amylase and glucosidases in the upper digestive tract. We selected a linear starch needing only the amylase and glucosidase for degradation rather than using any branched starches which may use additional enzymes and/or be degraded more slowly. Incremental area under the curve (iAUC) for glucose was the primary end point and iAUC for insulin was secondary along with other statistical models. Repeated measures analyses were completed using linear mixed models. The mixed model gives some advantages by treating time as continuous variable rather than collapsing time as in the iAUC.

We also investigated spontaneous energy intake after the last blood sample had been collected. The test meal may have contained too little energy and/or the time for the subsequent meal may have been too remote so that all subjects were quite hungry and ready to consume the energy expected for their body size. Also, the visual-analog scores for appetite, hunger and satiety indicate that effects of the seaweeds exist but only for the first hour or so. Only the smallest subjects consumed less than expected at the last test meal (data not shown), possibly because they had an adequate amount of energy from the test meal to feel some satiation still after 3 h. The test meals should therefore have been scaled to the energy needs of the subjects in order to get an optimal test of the seaweeds for effects on subsequent energy intake 3 h after intake.

We carried out the investigation on healthy subjects rather than in pre-diabetics. This was done because the immediate effect on starch degradation in the upper gastrointestinal tract was expected to be independent of the presence of glycaemia *per se*. In a longer-term study the effect on glycaemia in pre-diabetic and diabetic patients would be much more relevant.

The overall strengths of the study were the randomisation reducing confounding and the crossover design reducing bias because each subject is their own control. It was also a strength that the meals were well accepted by the volunteers and that the drop-out rate was low to help keep a reasonable power. The major weaknesses are the still limited size of the study, which could cause a few individuals to strongly affect the results. Moreover, the amount of seaweed provided and the selection of pea protein for the control meal and the use of the same meal size

for all volunteers, independent of their energy needs may have reduced the ability to observe effects of the seaweeds in this study.

7. CONCLUSIONS

The main aim of this thesis work was to investigate the potential of crude extracts of dried edible seaweeds to inhibit α-amylase and α-glucosidase activities and to examine the effect of selected edible seaweeds on the postprandial blood glucose and insulin levels following a starch load in a human meal study. Based on the three papers, the findings can be concluded as follows:

In **Paper 1** and **Paper 2**, the dried edible seaweeds, *U. pinnatifida* and *L. digitata,* showed effective inhibition of α-amylase and α-glucosidase activities, with both showing mixed-type inhibition. Alpha-amylase and α-glucosidase inhibitors play important roles in achieving better glycaemic control independent of insulin, potentially retarding glucose liberation from starches. *U. pinnatifida* and *L. digitata* containing phenolic acids and alginates inhibited α-amylase activities while fucoxanthin from *U. pinnatifida* inhibited α-glucosidase activities, showing chemical diversity with a possibly use for future functional foods.

In **Paper 3**, we investigated the effect of raw *U. pinnatifida* and *L. digitata* on the postprandial blood glucose and insulin concentrations following a starch load in a human meal study. In a randomized, 3-way blinded crossover trial; there was no significant effect in mean difference of plasma glucose and serum insulin incremental areas under the curve with the consumption of 5 g of seaweeds compared with the control meal ($P > 0.05$). However, insulin was affected in a mixed model and at several time points. Also increased satiety and fullness iAUC and decreased hunger and prospective food consumption iAUC were observed after intake of *U. pinnatifida*, while no effects were observed on energy intake 3 hours after the seaweed test meal.

Overall, relatively small amounts, only 5 g, of brown seaweeds have no effect on the incremental area under the curve for plasma glucose but affect several secondary endpoints within the first 1-2 hours after ingestion. It is therefore not possible to conclude that the inhibition of α-amylase and α-glucosidase observed *in vitro* can affect postprandial blood glucose while it may indirectly affect insulin, GLP-1 and feeling of satiety, fullness, prospective food intake and hunger up to 1½ hours after a meal with 5 g of brown seaweed.

8. PERSPECTIVES

The future perspectives are to further investigate seaweeds for effects on postprandial glycaemia and possibly to apply the potential bioactive compounds from seaweeds in populations with hyperglycaemia. If possible, different cooking methods or preparation of seaweed meals can be done to compare the findings and factors that affect the plasma glucose and serum insulin levels. Especially due to the high levels of iodine and certain other elements it may be of particular value to find methods for preparing seaweeds that reduce these components while keeping the alginate and fucoxanthin. The consumption of fresh edible seaweeds would also provide for an interesting future study about how freshness influences the bioactive and macronutrient compositions of the diet, which could be beneficial in reducing plasma glucose and serum insulin levels.

Regarding the study design for both meal studies and longer-term studies, larger numbers of participants should be recruited as they would represent better the population at large and may reduce the impact of outliers. In a longer-term study the effect on glycaemia in pre-diabetic and diabetic patients would be much more relevant.

The concluding perspective is that bioactive compounds from *U. pinnatifida* and *L. digitata* that has the potential to inhibit carbohydrate digestive enzymes may be potential applications in the formulation of functional food. The bioactive compound can be added as food ingredient in food or beverages products to produce functional food that provide health benefits to human.

9.   ACKNOWLEDGEMENTS

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