Release of polyphenols from brown seaweeds following an *in vitro* enzymatic digestion predicts antioxidant capacity and potential to inhibit the digestive enzymes α-amylase, α-glucosidase and lipase

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Abstract

Brown seaweeds are known to contain phlorotannins, polyphenol compounds unique to brown seaweeds. Concentrated phlorotannin extracts of brown seaweeds have demonstrated high antioxidant activity *in vitro* . Seagreens® harvests three brown seaweeds of the Fucaceae family from the Outer Hebrides for human consumption. An *in vitro* digestion methods was used to extract the brown seaweeds in order to determine the release of polyphenols during digestion and provide a physiologically relevant evaluation of the antioxidant capacity of the brown seaweeds. The release of polyphenols was highest from *F. vesiculosus* which also demonstrated the highest antioxidant capacity determined by ferric reducing antioxidant assay (FRAP), and Scavenging of the 2,2-Diphenyl-1-picrylhydrazyl radical (DPPH).

Other biological activity of the brown seaweeds attributed to their phlorotannin content is the inhibition of enzymes important in the gastro-intestinal digestion of carbohydrate. These enzymes are currently targeted by Acarbose® used to reduce the increase in postprandial blood glucose to help in the management of type II diabetes. Polyphenols from green tea have also demonstrated inhibition of lipase in previous studies. Lipase is targeted by the drug Orlistat® used in the treatment of obesity by reducing fat uptake and therefore reducing calorie intake. A comparison of the three brown seaweeds and a commercially available blend of the seaweeds was made in their potency to inhibit the enzymes α-amylase, α-glucosidase and lipase. *P. canaliculata* demonstrated the most potent inhibition of α-amylase while the blend of the three seaweeds demonstrated the most potent inhibition of α-glucosidase and lipase. This highlights the brown seaweeds as potential candidates in the management of diabetes and obesity.

1. Introduction

Ascophyllum nodosum, *Pelvetia canaliculata* and *Fucus vesiculosus* are brown seaweeds which are currently being harvested for human consumption in the Outer Hebrides by Seagreens ® UK . Seagreens ® work closely with those harvesting the seaweed from the source in the Outer Hebride to preserve the quality of the seaweed by completing the drying process at a low temperature and within 12-24hrs. of being cut. They ensure the seaweed is sustainably harvested by cutting the youngest part of the seaweed leaving enough to allow it to regrow. This also improves the consistency of the product as the same part of the plant is used in all batches.

The consumption of seaweed in the UK has a long history but has dwindled in more recent times as coastal populations have declined. Consumers in the UK are now more likely to encounter seaweed in Asian cuisine. Consumption of seaweed is high in countries such as China, Japan and Korea. Seagreens® has introduced brown seaweed to the market in the form of a fine powder encapsulated as a daily supplement and as a medium grain for inclusion in food. The daily supplement contains a mixture of all three brown seaweed species.

Research into the biological activity of brown seaweeds is increasing. The complex mixture of bioactive components within the seaweed has led many to investigate concentrated extracts of specific types of compounds such as alginates, fucoidans and polyphenols. The literature on whole seaweed extracts is limited. As the seaweed is being provided as a whole food it is important to understand whether the biological activity of isolated extracts occurs when the whole food is ingested.

Brown seaweeds have demonstrated high antioxidant activity including the species *A. nodosum, P. canaliculata* and *F. vesiculosus*. The antioxidant activity is attributed to their high polyphenol content and carotenoid pigments. Polyphenols are an important class of plant secondary metabolites found in high concentrations in land plants and seaweed. The role of polyphenols in the diet however is under intense scrutiny as peak plasma concentrations of polyphenols are low. The biological activity

of polyphenols beyond antioxidant activity is becoming increasingly important to determine their contribution to the maintenance of human health.

1.1 Aims

The purpose of the study is to demonstrate the biological activity of whole seaweed extracts of the brown seaweed species *A. nodosum*, *P. canaliculata* and *F. vesiculosus*. The antioxidant capacity of the individual species will be compared . The ability of the brown seaweeds to inhibit enzymes important in the gastro-intestinal digestion of carbohydrates and lipids will be determined. The antioxidant capacity and enzyme inhibition of a commercially available blend of the three brown seaweed species will also be determined.

1.2 Objectives

- To conduct an *in vitro* enzymatic digestion in order to provide a physiologically relevant estimation of the biological activity of extracts of whole brown seaweed .
- To measure the total polyphenol release during the *in vitro* enzymatic digestion .
- To measure the antioxidant release from the brown seaweeds during the *in vitro* enzymatic digestion by 2 different assays.
- To measure the inhibition of the digestive enzymes α-amylase, α-glucosidase and lipase by selected extracts of the brown seaweeds from the *in vitro* enzymatic digestion using individual assays specific to each enzyme.

1.3 Hypothesis

The total polyphenol contents and antioxidant capacities of the selected seaweeds, as well as their abilities to inhibit the activities of enzymes important in the gastro-intestinal digestion of carbohydrates and lipids will vary and be species specific. The antioxidant capacities and the ability of the selected seaweeds to inhibit specific gastro-intestinal enzymes are likely to be correlated with their total polyphenol content.

1.4 Literature Review

1.4.1 Seaweeds – Classification, Location

The Algal kingdom is hugely diverse ranging from single celled microscopic organisms to giant seaweeds which can reach over 50 meters long. Seaweeds are macro algae of which there are over 10,000 species which grow within the intertidal zone of oceans across the world[2]. Seaweeds have been classified into red, green and brown based on the pigments they contain.

Brown seaweeds belong to the class Phaeophyceae. The brown seaweeds under investigation *A. nodosum, F. vesiculosus, P. canaliculata* belong to the Fucaceae family of the order Fucales. Brown seaweeds are the principle seaweeds found around the coastlines of the Northern Atlantic. *A. nodosum*, *F. vesiculosus*, and *P. canaliculata* are predominantly found on the coastlines of Ireland, Scotland, Norway, Iceland and Canada. The characteristics used to identify the species are described in Figure 1.

Seaweeds have to deal with a number of adverse environmental conditions because of their location within the intertidal zone. They must be able to adapt to desiccation, salinity fluctuations, UV radiation, and temperature changes[3, 4]. Seaweeds in the inter tidal zone spend long periods exposed when they are not submerged. Depending on the time of year this can lead to high UV exposure or freezing conditions. Seasonal changes require that the seaweeds are able to withstand temperatures between - 40°C to +20 °C. The tidal changes also lead to dramatic changes in salinity as water evaporates from the surface of the seaweed salinity increases [3].

A. nodosum, *F. vesiculosus* and *P. canaliculata* are found at different positions within the intertidal zone. *P. canaliculata* is found at the high water mark[4]. *F. vesiculosus* and *A. nodosum* are found further down the shore around the midline. *P. canaliculata* being highest up the shoreline can spend several days uncovered and can completely dry out. The position of the seaweeds on the shore is believed to influence the composition of the seaweeds and their biological activity.

1.4.2. Identification

Pelvetia canaliculata
Common name Channelled Wrack

Colour fresh, olive-brown or olive yellow; when dry, black

Identification The fronds are rolled lengthwise to form a channel. Fronds are repeatedly branched in a fork. There are bumpy, v-shaped swellings at the ends of the fronds, which are its reproductive structures. It has no bladders.

Length : Fronds can grow up to 15cm

Fucus vesiculosus
Common name Bladder wrack

Colour fresh, olive-brown when dry, black

Identification The fronds are flat, midribbed, its margins are smooth. Bladders are round and found in pairs either side of the midrib.

Length : The fronds can grow up to 1m in length.

Ascophyllum nodosum
Common Name :Egg wrack

Colour fresh, olive green, glossy; when dry black

Identification The fronds are narrow strips with single, large (1-5cm long) oblong air bladders growing along its length. Pinnate branches along the frond. The margins are serrated at remote intervals

Length : Fronds can grow up to *1.5m long*

Figure 1. Profile and identification characteristics of the brown seaweeds *A nodosum*, *F. vesiculosus*, *P. canaliculata* [1]

1.4.3. History of seaweeds as food and medicine

Seaweeds are renowned as components of the Japanese diet. Japan and China have consumed seaweeds since ancient times. Seaweed has also been consumed by cultures in many other parts of the world including Indonesia, Polynesia, South America and Hawaii.

Only a few references to seaweed consumption by humans in Europe are known of. An early recording of its use in Europe is in a Gaelic poem for St Columba written in 600AD. The poem describes the collection of *Palmaria palmata* (Dulse) by the monks of Iona for distribution amongst the poor[5]. In the UK, the most familiar seaweed for human consumption is laver (*Porphya umbilicalis*). The laver is boiled and then fried and served with a breakfast dish known as laverbread. This dish is local to South Wales, Devon and Cornwall[6]. *A. nodosum*, *F. vesiculosus* and *P. canaliculata* are more commonly known for their use in agriculture. The use of seaweed as a fertiliser became common from around the 12th Century in Ireland, France, Scotland and Norway. Animals that graze by the coast were often moved to the shoreline to graze on seaweed. In Scotland *P. canaliculata* is known as cow-tang as it is preferred by the cows while *F. vesiculosus* is known as paddy tang because it is favoured by pigs [7]. The use of the brown seaweeds in agriculture continues to this day . As the land is becoming less nutrient dense they are an important source of minerals for these livestock.

While some seaweed species are mentioned in ancient Chinese Materia medica's [6], there are few references in European texts to medicinal uses of seaweed. The most notable use of *P. canaliculata*, *F. vesiculosus* and *A. nodosum* in European history is for their high iodine concentration. Infusions of *F. vesiculosus* were used to treat goitre[7]. In Victorian times, the brown seaweeds became popular as teas to promote weight loss[8]. In Scotland there are records of the application of *F. vesiculosus* to the knee for rheumatism [8]. *F. vesiculosus* is included in the British Herbal Compendium (volume 1) [9] for use in obesity, rheumatism, demulcent activity and thyroid metabolism .

1.4.4. Bioactive components within Brown seaweeds

A number of potentially bioactive components have been identified in brown seaweeds including polysaccharides, carotenoids and polyphenols. Alginates, fucoidans and laminarans are high molecular weight polysaccharides unique to the brown seaweeds [10]. A diverse range of biological activities have been described for each type of polysaccharide including antioxidant, anti-proliferative, anticoagulant and anti-inflammatory effects [10, 11]. The predominant carotenoid in brown seaweeds is fucoxanthin which has demonstrated anticancer and antioxidant effects *in vitro* [10, 12]. Brown seaweeds have been found to contain the highest levels of polyphenols compared with red or green seaweeds [12]. The major polyphenols within brown seaweeds are phlorotannins. Phlorotannins consist of polymers of phloroglucinol subunits. Phlorotannins range in size and contain up to 8 subunits of phloroglucinol[11] . The phlorotannins found within the seaweeds of the Fucaceae family can be grouped into one of three types[13]; fucols, fucophlorethols and phlorethols as shown in Figure 2. They each differ in the connection between the phloroglucinol subunits.

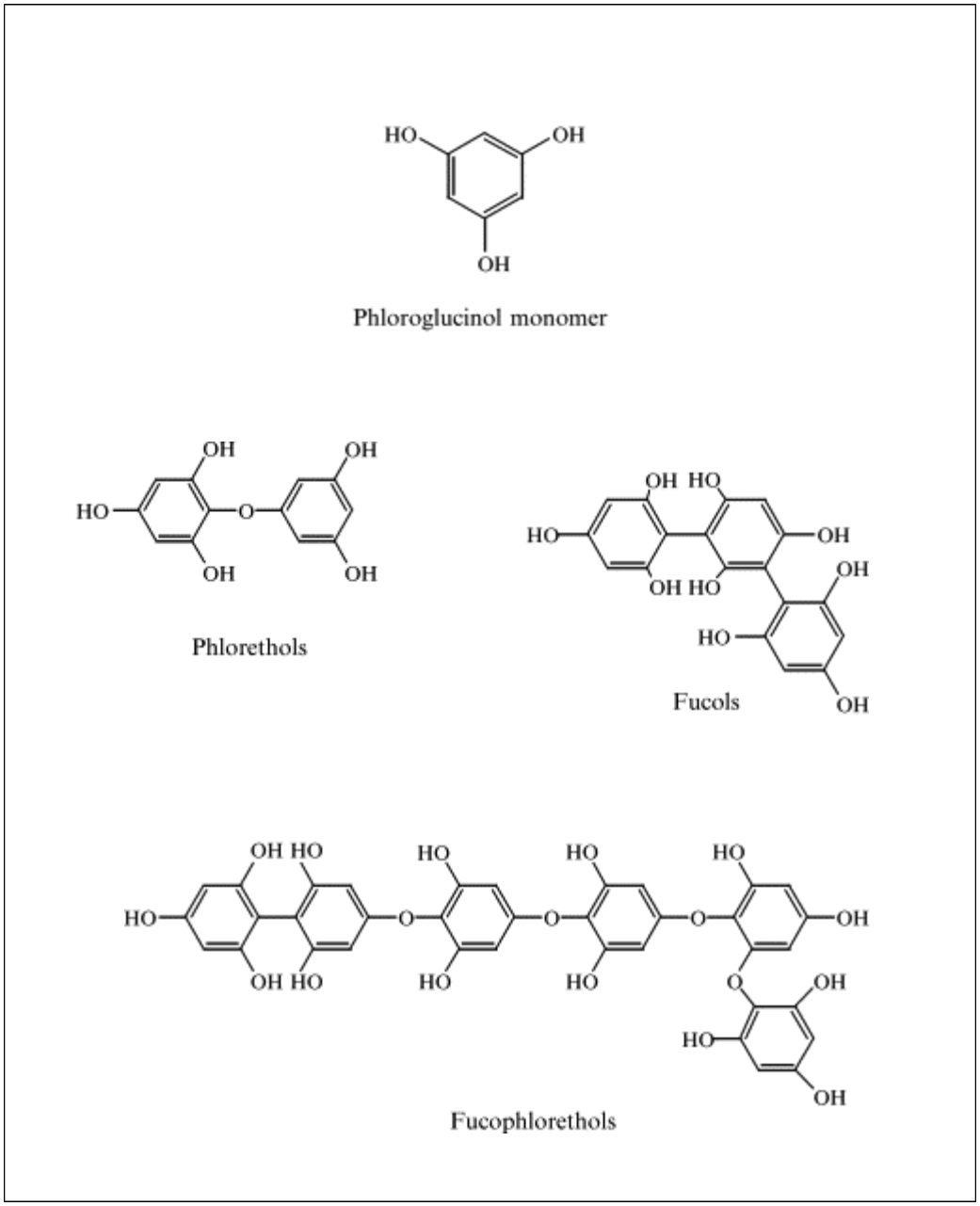

Figure 2. Phlorotannins of the Fucaceae family of seaweeds.[14]

1.4.5. Seaweeds as a source of Antioxidants

Brown seaweeds are considered the group of seaweeds with the highest polyphenol content. Within the brown seaweeds evidence is emerging that *A. nodosum, P. canaliculata* and *F. vesiculosus* have the highest content. *F. vesiculosus* and *A. nodosum* had the highest total polyphenol content in a comparison of 10 Icelandic red, green and brown seaweeds [15]. These three species were also found to have the highest total polyphenol content of five brown seaweeds found along the coast of Ireland [16]. Apostolidis and Lee [17] found *A. nodosum* to have the highest polyphenol content of four brown seaweeds found on the coastline of Canada. Nwosu et al.,[18] found *A. nodosum* to have the highest polyphenol content of four seaweeds found in the UK. The high polyphenol content of these seaweeds is proposed to distinguish these species amongst the brown seaweeds and confer unique biological activity.

A number of studies have demonstrated high antioxidant capacity of *A. nodosum, P canaliculata* and *F. vesiculosus* [15-18]. The polyphenols are believed to be responsible for the high antioxidant activity. The phenolic groups within polyphenols can accept an electron allowing polyphenols to scavenge for pro-oxidant species forming stable radicals. Seaweeds are believed to generate a number of antioxidant molecules in response to the extreme environmental conditions they must sustain. It is postulated that the antioxidant capacity of the brown seaweeds will correspond with their position on the shoreline and therefore the amount of UV exposure they must sustain [16].

A large number of epidemiological studies suggest that intake of fruit and vegetables is inversely associated with the development of chronic disease [19]. It is proposed that polyphenols play a role in the protective effects of fruit and vegetable intake because of their antioxidant properties.

Oxidative damage is believed to play a causal role in many chronic diseases including cancer, cardiovascular disease, diabetes and neurodegenerative diseases. Oxidative damage results from pro-oxidant species reacting with biological targets including nucleic acids, lipids and proteins. Pro-

oxidants include O_2^{-+}, H_2O_2 ROO•, and OH• . They are endogenously produced by the cell. Pro-oxidants are essential to the functioning of many cells and organelles including the mitochondria, peroxisomes and immune cells (REF). Antioxidants are molecules which prevent or slow down the oxidation of another molecule [20]. Redox regulation is an important control mechanism for a number of signalling pathways[20]. Antioxidants must maintain a balance within the cell that allows proxidants to carry out redox functions but limit damage caused to the cell. If intracellular antioxidant defence mechanisms are overwhelmed by reactive species then oxidative stress occurs [20].

1.4.6. Assessing the antioxidant potential of Seaweeds

The Folin-Ciocalteu method of Singleton et al., (1999) [21] for measuring total phenolic content (TPC) has been universally applied to land plants and seaweeds [22]. The Folin-Ciocalteu (FC) method is a colorimetric assay which measures the oxidation of phenolic components by a mixture of strong inorganic oxidants phosphotungstic and phosphomolybdic acids[23]. The FC assay can overestimate TPC because it does not have a high specificity for phenols. The FC reagent will react with a wide variety of hydroxyl containing species including amino acids, ascorbic acid, and reducing sugars [24]. Therefore the FC assay more than likely overestimates TPC and should be seen as an estimation of TPC content. The total polyphenol content can be seen as a measure of potential antioxidant activity. A number of studies have demonstrated an association between polyphenol content of plant extracts and *in vitro* assessments of antioxidant activity [25, 26].

The most common methods for screening of antioxidant activity of plant extracts are ferric reducing antioxidant potential (FRAP), 2,2-Diphenyl-1-picrylhydrazyl radical scavenging potential (DPPH), and Trolox equivalent antioxidant capacity (TEAC) [25]. These methods are relatively straight forward

colorimetric assays. The FRAP assay measures the reduction of the ferric ion(Fe^{3+}) to the ferrous ion(Fe^{2+}) by the formation of a blue-coloured compound ferrous-tripyridyltriazine (TPTZ)which occurs at low pH[27]. Reduction of Fe^{3+} to Fe^{2+} is favoured in the presence of antioxidants. Excess Fe^{3+} is used and so the rate-limiting factor in Fe^{2+} formation is the reducing ability of the plant extract. The increase in absorbance at 593nm is measured and related to the a standard solution containing known concentrations of ferrous ions to calculate FRAP values. The TEAC assay is based on the generation of ABTS (2,2-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) radical cation [28]. A stable $ABTS^{\cdot+}$ is formed from the oxidation of ABTS with potassium persulfate and then reacted with the plant extract. Any antioxidants present in the plant extract will reduce the radical. $ABTS^{\bullet+}$ is a blue/green chromophore. The reduction of $ABTS^{\bullet+}$ can be measured through the reduction in absorbance at 734nm. Trolox a vitamin E derivative is used as the standard which the antioxidant activity of the plant extract can be compared with. The DPPH assay is similar to the TEAC assay. It involves the formation of a stable radical ($DPPH^{\bullet}$) and measures the reduction of the radical by the plant extract in an organic solvent. $DPPH^{\bullet}$ has an absorption maxima at 517nm the decrease in absorbance as $DPPH^{\bullet}$ is reduced is measured.

Antioxidant compounds within a cell protect oxidizable substrates from pro-oxidants . The *in vitro* assays above do not involve oxidizable substrates. The TEAC and DPPH assay do not involve radicals which are found in biological systems and the FRAP assay measures the reduction of Fe^{3+} which is not a proxidant. Therefore it is concluded that these are indirect measures of antioxidant activity. In a review of the analytical methods to assess antioxidant capacity *in vitro* Prior and Cao et al., [29]recommend that a number of assays is recommend to get an overall measure of antioxidant capacity. This view is supported by others [30] and consideration for selecting the appropriate standardised methods for which there is an appropriate amount of comparable data is paramount.

1.4.7. Physiologically relevant assessment of antioxidant capacity

The antioxidant capacity of brown seaweeds from the Fucaceae family has been demonstrated by a number of studies [15, 16, 18]. The polyphenols within the brown seaweeds are believed to be responsible for the antioxidant potential of the brown seaweeds[15, 16, 18]. Polyphenols are generally more soluble in polar organic solvents than water [15]. Therefore organic solvent extraction procedures have been used to concentrate the polyphenols from the brown seaweeds. However these studies do not indicate the antioxidant capacity of the seaweeds following human consumption of the whole seaweed. *In vitro* enzymatic digestion methods have been developed to measure the biological activity of the compounds which are released from the food matrix during passage through the gastrointestinal tract. The *in vitro* enzymatic digestion involves a number of sequential steps to mimic the different stages of the digestive process [31] . The conditions within the stomach are replicated by adding hydrochloric acid (HCL) and pepsin. This is followed by the addition of sodium hydroxide (NaOH) and pancreatin to simulate conditions in the small intestine. The *in vitro* digestive methods vary in the length of incubation for each sequential step, enzyme concentration and concentration of HCL and NaOH can vary between methods [31].

1.4.8. Polyphenols in the diet

The *in vitro* enzymatic digestion is not a measure of bioavailability. It is known that polyphenols can be absorbed from the GI tract but polyphenols are extensively metabolised by phase 1 metabolism [32]. The impact of polyphenols in the diet has been measured by measuring the antioxidant capacity of the plasma following the consumption of polyphenol rich foods. Increases in plasma antioxidant capacity have been observed following the ingestion of tea, cocoa, wine and some fruit and vegetables [33]. However, these studies presume that the increases in plasma total antioxidant by polyphenol rich foods and beverages decreases oxidative damage without measuring it[20].

Studies using biomarkers of oxidative damage have produced less reproducible evidence for *in vivo* antioxidant activity of polyphenols [32]. Biomarkers of oxidative stress include F_2 –isoprostane which is a measure of lipid peroxidation and 8-hydroxy-2'-deoxyguanosine (8OHdG) the DNA adduct most abundantly formed by ROS. 8OHdG is removed from DNA by base excision and is excreted in the plasma. Thompson et al., [34]found decreased levels of 8OHdG and isoprostanes in the urine of subjects who consume more fruit and vegetables. No research as yet has been published out on the effect of consuming brown seaweed on markers of oxidative stress. However the University of Ulster is currently running a randomised controlled trial with a brown seaweed polyphenol extract is [35]. The study involves 80 subjects in an 8 week cross-over design. The participants were asked to consume 400mg of the seaweed polyphenol extract or placebo per day. Urinary levels of isoprostane are being taken as biomarkers of oxidative stress.

1.4.8.1. Polyphenols in the gut

The potent antioxidant activity of polyphenols may be utilised within the human gut before absorption occurs [32]. The gastro-intestinal(GI) tract is exposed to a large number of reactive species. The GI tract releases reactive species from epithelial cells, reactive species may be consumed in the diet, or dietary compounds may lead to the generation of reactive species in the gut [32, 36]. Bacteria and toxins in the diet may activate immune cells which increases generation of reactive species [32, 36]. Therefore radical scavenging activity by polyphenols could be an important protective mechanism within the GI tract.

1.4.9. Biological activity of polyphenols in addition to their antioxidant effects

The benefit of consuming polyphenols within the diet has moved beyond just assessing their antioxidant potential. Polyphenols are a large class of secondary metabolites and it is likely that they posses as a wider range of biological activity.

A number of studies have demonstrated *in vitro* inhibition of α-amylase and α-glucosidase by extracts of the brown seaweeds *A nodosum* and *F. vesiculosus* [17, 18, 37, 38]. The phlorotannins present in the brown seaweed are believed to be responsible for the inhibition. A number of the studies have measured the inhibition by polyphenol rich extract of the brown seaweeds which may explain some of the variation in the potency of inhibition of the enzymes (see Table 1).

The inhibition of the enzymes important in the gastro-intestinal digestion of carbohydrates and lipids by polyphenols may have application to help in the prevention and treatment of diabetes and obesity. Acarbose is a drug currently used in the treatment of type 2 diabetes which targets α-amylase and α-glucosidase. Acarbose reduces the post-prandial increase in blood glucose by preventing the hydrolysis of carbohydrates and reducing the amount of glucose available for uptake from the gastro-intestinal tract

Obesity is another global health epidemic affecting both the developed and developing countries. Currently there are only two drugs licensed for term treatment of obesity. One of these is Orlistat® which is a potent inhibitor of pancreatic lipase an enzyme key in the digestion of fats. It works on the principle of inhibiting the majority of lipid digestion thereby reducing fat absorption and energy uptake. Excess fat intake is believed to be an important factor in the increasing prevalence of obesity.

Table 1. Comparison of potency of inhibition of enzymes which hydrolyse carbohydrate by brown seaweed extracts

Study	Species	α-glucosidase IC_{50}	α-amylase IC_{50}	Extraction method
Nwosu et al .,2011 [18]	*A. nodosum*	~19µg/ml	< 25µg/ml	100% methanol 50% acetonitrile/50% UPW
Zhang et al., 2007 [38]	*A. nodosum*	77µg/ml		50% aqueous ethanol extract
Apostolidis et al.,2010 [17]	*A. nodosum*	208µg/ml 55µg/ml	2088 µg/ml 311 µg/ml	20°C Water extraction 80°C Water extraction
Roy et al ., 2011 [37]	*Extract containing A. nodosum and F. vesiculosus*	5 µg/ml	2.8µg/ml	Commercially available extract InSea$^{2®}$

Polyphenol rich plant extracts from tea and soft fruits (e.g blueberries and raspberries) have demonstrated inhibition of pancreatic lipase [39]. There is also yet unpublished work which has demonstrated that alginates extracted from brown seaweeds can inhibit pancreatic lipase with a similar potency to Orlistat®[40]. Only one of the studies featured in table 1 also measured the inhibition of pancreatic lipase brown seaweed extracts [18]. However they did not publish the data as they found no inhibition in extracts with the phenolic concentration of 100µg/ml. An unpublished study by Lordan and Ross[41] identified *A nodosum ,F. vesiculosus* and *P. canaliculata* as the most potent inhibitors of α-amylase and α-glucosidase after screening 15 Irish seaweeds in a high-throughput assay.

A common assay for measuring inhibition of α-amylase by plant extracts utilises 3,5 dinitrosalicylic acid. The enzyme is preincubated with the plant extract and then starch is added. 3,5-Dinitrosalicylic acid reacts with reducing sugars produced when starch is digested by α-amylase . 3-amino-5-nitrosalicylic acid is formed by this reaction which absorbs strongly at 540nm.

All the assays measuring inhibition of α-glucosidase in Table 1 use an artificial substrate p-nitrophenyl-α-D-glucopyranoside[42]. The enzyme and the plant extract are pre-incubated and then p-nitrophenyl-α-D-glucopyranoside is added. When it is hydrolysed it releases p- nitrophenol which can be measured by an increase in absorbance at 400nm.

A team at Newcastle University has developed an assay for measuring lipase activity which uses olive oil to provide a natural substrate for lipase. The olive oil is passed over aluminium oxide to remove any free fatty acids. The purified olive oil is then emulsified in an acetone solution producing a cloudy liquid. The alginate and lipase are added to the assay. The activity of the lipase is measured by the change in turbidity of the solution over time. As the triglyceride is broken down the solution becomes less cloudy.

The inhibition of digestive enzymes by the brown seaweeds could have applications in the prevention and treatment of diabetes and obesity. The enzyme assays discussed are useful in helping to demonstrate potential activity and provide the stimulus for further *in vivo* studies in animals and humans.

The biological activity of the brown seaweeds A. nodosum ,F. vesiculosus and P. canaliculata has been reviewed. This investigation will build on the research and enable comparisons to be made of the antioxidant activity and enzyme inhibition of the three species and the commercially available blend. This will provide the first insight into the biological activity of these seaweed species when harvested from the Outer Hebrides.

2. Materials and Methods

2.1. Chemicals and Reagents

All reagents were purchased from Sigma-Aldrich Ltd, Gillingham, Dorset, UK unless stated otherwise.

Pancreatin from porcine pancreas

Pepsin from porcine gastric mucosa

Folin-Ciocalteu reagent

DPPH· (2,2-Diphenyl-1-picrylhydrazyl)

2,4,6-Tris(2-pyridyl)-s-triazine (TPTZ)

3,5-dinitrosalicylic acid

α-Amylase from porcine pancreas

Porcine pancreatic lipase

Acarbose

Orlistat®

α-glucosidase (provided by Jeff Pearson laboratory, Institute for Cell and Molecular Biosciences, Newcastle University)

2.2. *In vitro* enzymatic digestion procedure

Fine milled *P. canaliculata*, *F. vesiculosus*, and *A. nodosum* were supplied by Seagreens® . .

The *in vitro* digestion procedure was based on the method used by Nagah and Seal [43] to assess the antioxidant activity of bioavailable extracts from wholegrain foods. The method was adapted from the procedure used by Aura et al., [44]. One gram of each seaweed species provided and a commercially available blend of the three species were added to individual 50ml plastic centrifuge tubes. Four marbles were added to each tube. Twenty-millilitres of deionised water was added to the samples before placing the tubes in a shaking water bath at 37°C set to 140 strokes min^{-1}. A control reaction was run containing 21ml of water. After 10 minutes, 10ml of 0.05M hydrochloric acid (HCl) was added to each tube. After 30 minutes 0.5ml of pepsin (10mg pepsin dissolved in 5ml of HCl) was added to each tuber. After 50minutes 1ml of 0.5M sodium hydroxide (NaOH) was added followed by the addition of 5ml of pancreatin (6mg dissolved in 40ml deionised water, centrifuged for 10 minutes at 1500 x g and then 25ml of the supernatant was removed) after 90 minutes.

Aliquots of 1ml were taken in triplicate from each tube (including control) immediately before each step; these were centrifuged and decanted into fresh eppendorf tubes. The extracts were stored at -20°C before analysis.

The successive steps involved in the *in vitro* enzymatic digestion are shown in the appendix 1. As the *in vitro* digestion involved dilution of the initial concentration of seaweed (0.05g/ml) at each successive step the calculations to determine the concentration of seaweed in the extracts taken during the digestion are shown,

2.3. Folin-Ciocalteu method for determination of total polyphenol content

The total polyphenol content (TPC)of the extracts was determined using Folin-Ciocalteu reagent following the method developed by Zhang et al.,[22] and carried out on a 96-well microplates. Gallic acid was used as the standard. A stock solution of 500µl/ml^{-1} gallic acid was prepared in deionised water. The stock solution was used to make a number of serial dilutions (250, 100, 50, 25, 12.5µl/ml^{-1}). Folin-Ciocalteu reagent was diluted with deionised water (1:10 (v/v)). The blank was taken from the 1ml aliquots of the control reaction during the *in vitro* enzymatic digestion. The 96-well microplate was loaded with 10 µl of seaweed extract, standard or blank (see Fig. 3 for plate template), followed by 130 µl of the diluted Folin-Ciocalteu reagent to each well. After 5 minutes, 100 µl of 7.5% anhydrous sodium carbonate was added to each well and the plate was incubated at 40°C for 30mins. The absorbance was read at 750 nm .

A 1:10 dilution of the seaweed extracts was made to ensure that the absorbance measurements were within the linear range of the standard curve. All extracts, blanks and standards were run in triplicate. Deionised water was used as a blank for the standards.

The TPC of the extracts was expressed in terms of mg gallic acid equivalents/g dry weight of sample (mg/gdw GAE).

	1	2	3	4	5	6	7	8	9	10	11	12
A	As1	As1	As1	Pe1	Pe1	Pe1	Fu1	Fu1	Fu1	Blend1	Blend1	Blend1
B	As2	As2	As2	Pe2	Pe2	Pe2	Fu2	Fu2	Fu2	Blend2	Blend2	Blend2
C	As3	As3	As3	Pe3	Pe3	Pe3	Fu3	Fu3	Fu3	Blend3	Blend3	Blend3
D	As4	As4	As4	Pe4	Pe4	Pe4	Fu4	Fu4	Fu4	Blend4	Blend4	Blend4
E	As5	As5	As5	Pe5	Pe5	Pe5	Fu5	Fu5	Fu5	Blend5	Blend5	Blend5
F	B1	B2	B3	B4	B5	St01	St02	St03	St04	St05	St06	B-w
G	B1	B2	B3	B4	B5	St01	St02	St03	St04	St05	St06	B-w
H	B1	B2	B3	B4	B5	St01	St02	St03	St04	St05	St06	B-w

Figure. 3 Map of 96-well microplate for TPC,FRAP and DPPH assays. Standards: St01- St06, Extracts from in vitro digestion: As1-As5 (Ascophyllum nodosum), Pe1-Pe5 (Pelvetia canaliculata), Fu1-Fu5 – (Fucus vesiculosus), Blend1-5 (Blend). Blanks from control reaction in vitro digestion(B1-B5), Blank water (B-w)

2.4. Ferric reducing antioxidant assay (FRAP)

The ferric reducing ability of the seaweed extracts was determined following the method of Benzie and Strain [27]adapted for use on a 96-well microplate. A FRAP working solution was prepared from 2.5ml of 10mM TPTZ solution (TPTZ dissolved in 40mM HCl), 2.5ml of 20mM Ferric Chloride and 25ml 300mM Sodium acetate buffer. Ferrous sulphate was used as the standard. A stock solution of 2000µM ferrous sulphate was prepared (55.6mg in 100ml deionised water). Serial dilutions at the concentrations of 1500µM, 1000µM, 800 µM,600µM,400µM,200µM were made. The 96-well microplate was loaded with 10µl of seaweed extract, standard or blank (see fig 3 for plate template), followed by 300µl of the FRAP working solution and the plate incubated at 37°C for four minutes. The absorbance was read at 593nm.

The brown seaweed extracts were diluted with deionised water by 1:10 (v/v) so the absorbance readings were within the range of the ferrous iron standards. All extracts, blanks and standards were run in triplicate. Deionised water was used as a blank for the standards.

The total antioxidant capacity of the extracts as determined by FRAP was expressed as µM ferrous sulphate equivalent/ g dry weight of sample (µM/g).

2.5. **Scavenging of the 2,2-Diphenyl-1-picrylhydrazyl radical (DPPH·)**

The method was adapted from Brand-Williams et al., [45] to be carried out in a 96-well microplate. A stock solution 2,2-Diphenyl-1-picrylhydrazyl of (DPPH•) was prepared to a concentration of 0.6mM in methanol and stored at -20° prior to analysis. The DPPH • working solution was prepared by mixing 30ml of the stock solution with 80ml methanol to obtain a final concentration of 0.16mM. A stock solution of 2.5mM trolox was prepared (0.1564g in 250ml ethanol). Serial dilutions of the stock solution were made to the concentrations 1.5mM, 1mM, 0.5mM, 0.25mM, 0.125mM, 0.0625mM. The 96-well microplate was loaded with 10µl of seaweed extract, standard or blank (see Fig 3 for plate template),followed by 300µl of the working solution and the microplate incubated at 30°C for 30mins. The change in absorbance was measured at 517nm.

The brown seaweed extracts were diluted with deionised water by 1:10 (V/V) so the absorbance readings were within the range of the trolox standards. All extracts, blanks and standards were run in triplicate. Methanol was used as a blank for the standards. The control reaction from the *in vitro* enzymatic digestion was used as the blank for the samples. The total antioxidant capacity of the extracts was expressed as mM trolox equivalent (TE)_/g dry weight of sample.

2.6. α-amylase inhibition assay

A 96-well microplate was loaded with 30µl of seaweed extract or acarbose (1mg/ml) or 30mM phosphate buffer (see figure 4 for plate map) and 30µl of α-amylase solution (2.5mg α-amylase in 10mls 30 mM phosphate buffer pH 7)and incubated at 37^{o} C. A 0.5% soluble starch solution was prepared (0.5g potato starch dissolved in 30 mM phosphate buffer pH 7 heated to 80^{o} C). After 30 mins preincubation 60µl of 0.5% soluble starch solution was added to each well and incubated for a further 30mins at 37^{o} C. This was followed by the addition of 120µl of 3,5-dinitrosalicylic acid to each well . The 96-well micro plates were heated to 100^{o}C for 5 mins and the absorbance was read at 540nm.

Not all seaweed extracts were chosen for their inhibitory activity of α-amylase. The extracts at 10 mins and 70 mins were assayed. All assays were run in triplicate. Serial Dilutions of the seaweed extracts were made to calculate the IC_{50}. The inhibition of α-amylase was calculated using the equation below:

$$\%\ \text{inhibition} = \left(\frac{\Delta Abs\ control - \Delta Abs\ sample}{\Delta Abs\ control}\right) \times 100$$

The IC_{50} (concentration of extract at which 50% inhibition of the enzyme occurs) was calculated with a minimum of 6 serial dilutions of the seaweed extracts using Sigma Plot.

	1	2	3	4	5	6	7	8	9	10	11	12
A			acarbose + buffer	extract + buffer	extract + buffer	extract + buffer	extract + buffer	extract + buffer	extract + buffer	extract + buffer		
B			acarbose + buffer	extract + buffer	extract + buffer	extract + buffer	extract + buffer	extract + buffer	extract + buffer	extract + buffer		
C			acarbose + α-amylase	extract + α-amylase	extract + α-amylase	extract + α-amylase	extract + α-amylase	extract + α-amylase	extract + α-amylase	extract + α-amylase		
D			acarbose + α-amylase	extract + α-amylase	extract + α-amylase	extract + α-amylase	extract + α-amylase	extract + α-amylase	extract + α-amylase	extract + α-amylase		
E			α-amylase + buffer	α-amylase + buffer	α-amylase + buffer	α-amylase + buffer	α-amylase + buffer	α-amylase + buffer	α-amylase + buffer	α-amylase + buffer		
F			α-amylase + buffer	α-amylase + buffer	α-amylase + buffer	α-amylase + buffer	α-amylase + buffer	α-amylase + buffer	α-amylase + buffer	α-amylase + buffer		
G												
H												

Figure 4. Plate map for α-amylase inhibition assay. Rows E3-10, F3-F10 control reactions.

2.7. α-glucosidase inhibition assay

The method used by Apostolidis et al.,[46] was used without modification. A 96-well micro plate was loaded with 50µl of seaweed extract / acarbose (1mg/ml)/ 0.1M phosphate buffer pH 7 and 100µl of α-glucosidase solution (1.0 U/ml) and incubated at room temperature for 10 minutes (see Figure 5 for plate template). Following the preincubation, the absorbance was read at 405nm and 50µl of 5mM p-nitropheny-α-D-glucopyranoside (0.0375g in 25ml 0.1M phosphate buffer pH 7) was added to each well and incubated at 25^{o}C for 5 minutes. The absorbance was read again at 405nm.

All assays were run in triplate. The inhibition of α-glucosidase was calculated using the equation below.

$$\%\ \text{inhibition} = \left(\frac{\Delta\text{Abs control} - \Delta\text{Abs sample}}{\Delta\text{Abs control}}\right) \times 100$$

The IC_{50} (concentration of extract at which 50% inhibition of the enzyme occurs) was calculated with a minimum of 6 serial dilutions of the seaweed extracts using Sigma Plot.

	1	2	3	4	5	6	7	8	9	10	11	12
A		enzyme + buffer	acarbose + buffer	extract + buffer	extract + buffer	extract + buffer	extract + buffer	extract + buffer	extract + buffer	extract + buffer		
B		enzyme + buffer	acarbose + buffer	extract + buffer	extract + buffer	extract + buffer	extract + buffer	extract + buffer	extract + buffer	extract + buffer		
C		enzyme + buffer	acarbose + enzyme	extract + enzyme	extract + enzyme	extract + enzyme	extract + enzyme	extract + enzyme	extract + enzyme	extract + enzyme		
D		enzyme + buffer	acarbose + enzyme	extract + enzyme	extract + enzyme	extract + enzyme	extract + enzyme	extract + enzyme	extract + enzyme	extract + enzyme		
E												
F												
G												
H												

Figure 5. The plate map for α-glucosidase inhibition assay. Control reaction A2-D2.

2.8. <u>Lipase Inhibition assay</u>

The turbidimetric lipase activity analysis assay was modified from Vogel and Zieve [47]using olive oil as the substrate for the lipase.

The olive substrate requires preparation before the assay can be performed. The substrate was provided by Geoff Pearsons Laboratory, Newcastle University Medical School. In brief, olive oil was passed through aluminium oxide in a glass chromatography column. A 10% olive oil solution was made by diluting the purified olive oil in acetone.

The 10% olive solution was further diluted to produce a 1% olive oil solution using acetone as a final preparation for use in the assay. A working substrate solution for the assay was prepared by heating 100 ml of 0.0539M Tris buffer adjusted to pH 7.7 with 0.37%Taurodeoxycholate to 70^{o}C. After 1 hour, 4 mls of the 1% olive oil solution was added to the heated buffer and blended using a homogeniser for 5 minutes maintaining the solution at 70^{o}C. The working substrate solution was used within 3 hours of preparation.

The seaweed extracts were added to 5 mls of the working substrate solution in a 50ml centrifuge tube and homogenised for 10 seconds. The concentration of seaweed extract achieved was 3.6mg/ml. A 96 well microplate was loaded with 10 µl of lipase (1mg/ml plus colipase at a ratio of 3:200 colipase to lipase)/ Tris buffer as control (see Figure 6 for plate template). A second 96-well microplate was loaded with 240µl of the working substrate solution containing the seaweed extract, buffer or Orlistat® was added (see Figure 5 for plate template). A multichannel pipette was used to pipette 200µl of the samples from plate 2 to plate 1 containing the lipase enzyme or control to start the assay. The plate was incubated at 37^{o}C while the turbidity was measured at 405nm every 5 minutes for 90 minutes. The control assay was calculated by subtracting the change in turbidity of the working substrate solution with lipase from the working solution with buffer in which no change in turbidity occurred.

The extracts and control reactions were run in triplicate. The inhibition of lipase was determined using the equation below.

$$\%\ \text{inhibition} = \left(\frac{\Delta\text{turbidity control rxn} - \Delta\text{turbidity sample rxn}}{\Delta\text{turbidity control rxn}}\right) \times 100$$

2.9. <u>Statistical analysis</u>

ANOVA and Tukey's multiple comparison test (Minitab, version 16) were used to measure the effects of the *in vitro* digestion on the brown seaweeds. P≤0.05 was treated as significant. They were also used to make comparisons between the species at each stage of the digestion. To determine the IC_{50} values for inhibition of α-amylase and α-glucosidase dose –response curves were plotted using Sigma plot (version 11.0).

	1	2	3	4	5	6	7	8	9	10	11	12
A	lipase	lipase	lipase	lipase	lipase	lipase	lipase	lipase	lipase	lipase	lipase	
B	lipase	lipase	lipase	lipase	lipase	lipase	lipase	lipase	lipase	lipase	lipase	
C	lipase	lipase	lipase	lipase	lipase	lipase	lipase	lipase	lipase	lipase	lipase	
D	lipase	lipase	lipase	lipase	lipase	lipase	lipase	lipase	lipase	lipase	lipase	
E	control	control	control	control	control	control	control	control	control	control	control	
F	lipase	lipase	lipase	lipase	control	control	control	control		control	control	
G	lipase	lipase	lipase	lipase	control	control	control	control		control	control	
H	lipase	lipase	lipase	lipase	control	control	control	control		control	control	

Figure 4. Plate map 1 for lipase assay (control =buffer sol)

	1	2	3	4	5	6	7	8	9	10	11	12
A	substrate +extract	substrate +extract	substrate +extract	substrate +extract	substrate +extract	substrate +extract	substrate +extract	substrate +extract	substrate +extract	substrate + buffer	substrate +Orlistat	
B	substrate +extract	substrate +extract	substrate +extract	substrate +extract	substrate +extract	substrate +extract	substrate +extract	substrate +extract	substrate +extract	substrate + buffer	substrate +Orlistat	
C	substrate +extract	substrate +extract	substrate +extract	substrate +extract	substrate +extract	substrate +extract	substrate +extract	substrate +extract	substrate +extract	substrate + buffer	substrate +Orlistat	
D	substrate +extract	substrate +extract	substrate +extract	substrate +extract	substrate +extract	substrate +extract	substrate +extract	substrate +extract	substrate +extract	substrate + buffer	substrate +Orlistat	
E	substrate +extract	substrate +extract	substrate +extract	substrate +extract	substrate +extract	substrate +extract	substrate +extract	substrate +extract	substrate +extract	substrate + buffer	substrate +Orlistat	
F	substrate	substrate	substrate	substrate	substrate	substrate	substrate	substrate		substrate + buffer	substrate +Orlistat	
G	substrate	substrate	substrate	substrate	substrate	substrate	substrate	substrate		substrate + buffer	substrate +Orlistat	
H	substrate	substrate	substrate	substrate	substrate	substrate	substrate	substrate		substrate + buffer	substrate +Orlistat	

Figure 6. Plate template 2 for lipase assay

3. Results

3.1. Total Polyphenol Content

Total polyphenol content was determined using the Folin-Ciocalteu assay and Gallic acid was used as the standard. The standard curve is shown in Figure 7.

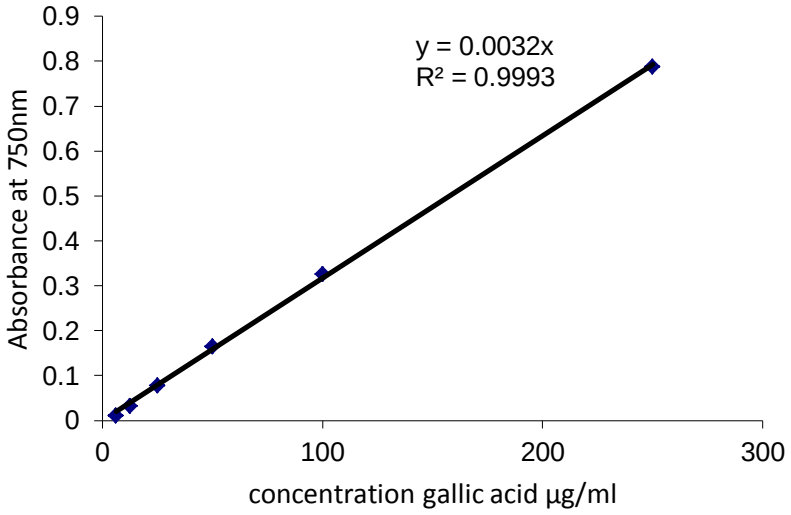

Figure 7. Standard curve for the Folin- Ciocalteu Method for determining total polyphenol content

The total polyphenol content (TPC) of all extracts released from the brown seaweeds increased across the in vitro digestion procedure. The TPC of the *A. nodosum* extract at 70 mins was significantly higher (three fold) than the TPC of the extract at 10 mins. While for the two other seaweed species and the blend the TPC of the extract at 70 mins was two fold higher than the TPC at 10 mins. For all species and the blend the TPC of the extract at 70 mins was significantly different ($p<0.001$) from the TPC at 10mins.

From Figure 8, it can be clearly seen that the increase in TPC occurred at two different stages. For all species and the blend there was a significant and substantial increase in TPC from the 10 min stage to the 30 min stage during which the pH was lowered by the addition of 0.05 M hydrochloric acid. The addition of pepsin after 30 mins did not significantly affect the TPC of *F. vesiculosus* extracts or the blend. While for *P. canaliculata* there was a significant increase ($p<0.001$) and for *A. nodosum* a

significant decrease occurred which is seen as an anomaly. Another significant and substantial increase in TPC of extracts occurred for all species and the blend between 50 mins and 70 mins during which the pH was raised by the addition of 0.5 M sodium hydroxide. A significant change in colour was noticed on raising the pH as brown pigments increased.

The TPC of extracts of *F. vesiculosus* were the highest across the *in vitro* enzymatic digestion. All values differed significantly from the other species at each stage of the digestion as shown in Table 2.

The TPC of extracts of *A. nodosum* were the lowest across the *in vitro* enzymatic digestion. The TPC of the extracts of the blend reflects the composition of the species within the blend with all values being in between the TPC of *F. vesiculosus* and *A.nodosum*.

The TPC of extracts after 90 mins could not be determined. The pancreatin used was a crude extract from porcine pancreas which contained a mixture of enzymes including amylases, lipases and proteases. There was significant interference from the pancreatin in the blank. Therefore TPC of extracts after the addition of pancreatin could not be determined.

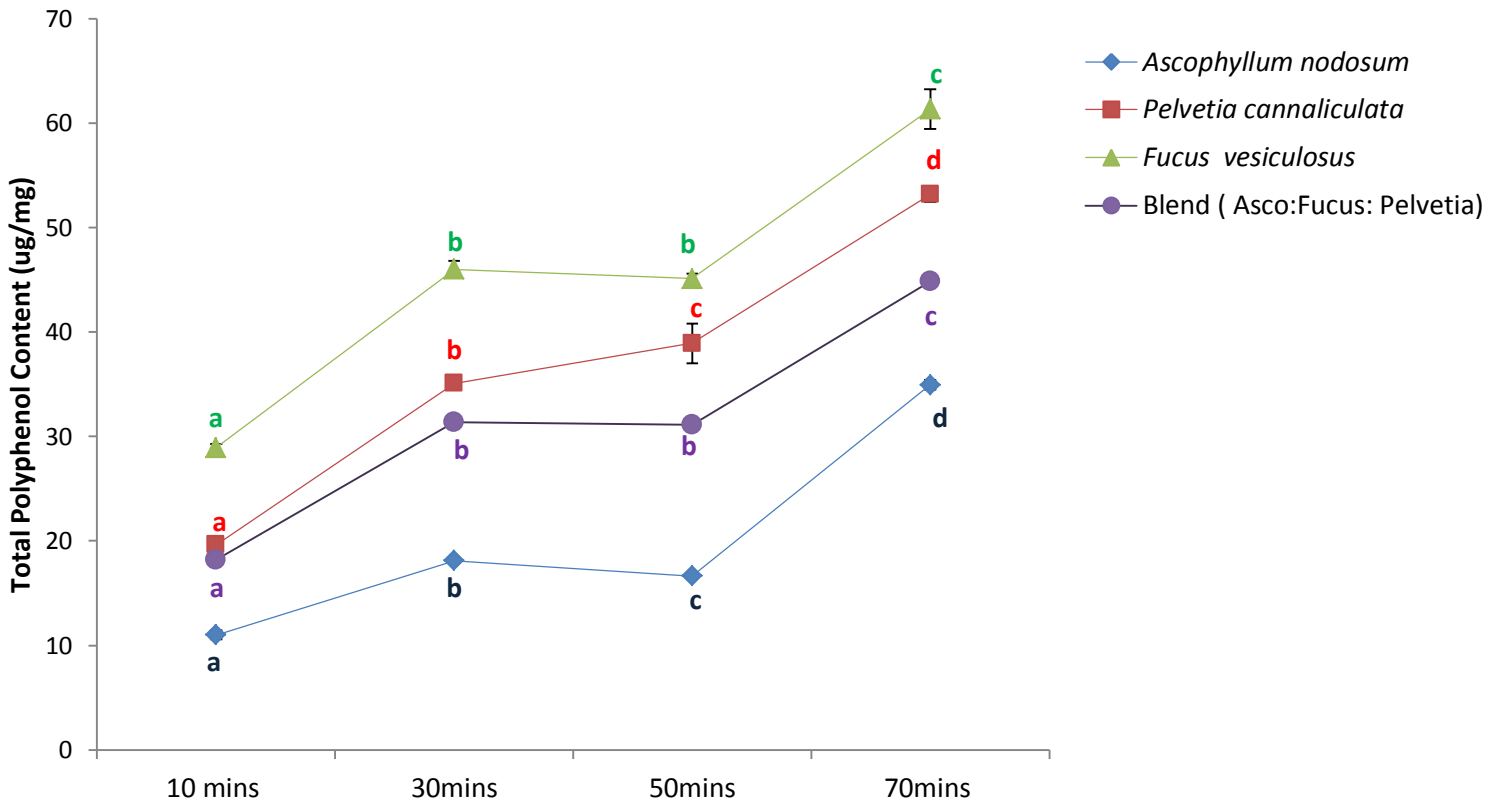

Figure 8. Total polyphenol content of extracts released from brown seaweeds during the *in vitro* enzymatic digestion. All values are means of triplicate assays ±SE. (a-d) denotes a significant difference of the TPC of extract of brown seaweed species between stages of in vitro enzymatic digestion. e.g. for *A. nodosum* (a) denotes a significant difference of TPC of extract taken at 10mins between (b) at 30 mins, (c) at 50 mins and (d) at 70 mins.

Table 2. Total Polyphenol Content of brown seaweeds during enzymatic digestion at 10mins, 30mins 50mins and 70mins (mg GAE/gdw)

	In Vitro Digestion stage							
	10mins		30mins		50mins		70mins	
Ascophyllum nodosum (a)	11.0 (b,c,d)	± 0.5	18.0 (b,c,d)	± 0.2	16.6 (b,c,d)	± 0.2	34.9 (b,c,d)	± 0.5
Pelvetia canaliculata (b)	19.6 (a,c,d)	± 0.3	35.1 (b,c,d)	± 0.6	38.9 (b,c,d)	± 1.9	53.2 (a,c,d)	± 0.7
Fucus vesiculosus (c)	28.9 (a,b,d)	± 0.4	45.0 (a,b,d)	± 0.8	45.09 (a,b,d)	± 0.5	61.3 (a,b,d)	± 1.9
Blend *(d)	18.2 (a,b,c)	± 0.0	31.3 (a,b,c)	± 0.5	31.1 (a,b,c)	± 0.3	44.8 (a,b,c)	± 0.4

All Values are means of triplicate assays ± SE. Outliers were removed. (a-d) denotes a significant difference between mean values, at each stage of *in vitro* enzymatic digestion, where (a) denotes *A. nodosum* (b) denotes *P. canaliculata.*

3.2. Antioxidant capacity measured by FRAP

The total antioxidant capacity of the extracts released from the brown seaweeds during the *in vitro* enzymatic digestion as measured by FRAP are shown in Figure 10. The standard curve used for determining antioxidant capacity by FRAP is shown in Figure 9.

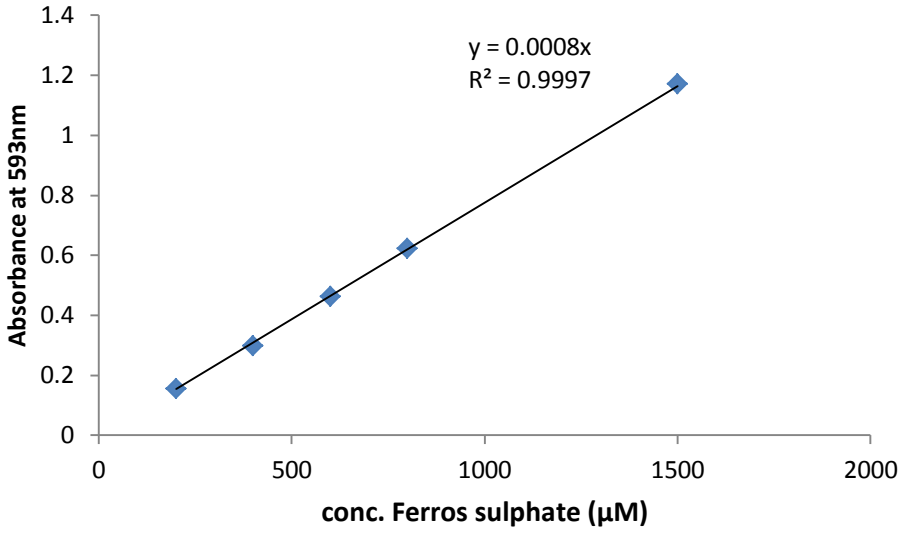

Figure 9. Standard curve for FRAP assay

There was no significant affects on the total antioxidant capacity of the extracts through stages 10 mins to 50 mins for any of the species or the blend. However there was a substantial and significant ($p<0.001$) increase after 70 mins for all species and blend. This was followed by a significant decrease ($p<0.001$)in total antioxidant capacity in all species and the blend at 90 mins. This decrease in antioxidant capacity after the addition of pancreatin can be seen as an anomaly as Nagah and Seal [43] did not find a decrease at this stage during *in vitro* enzymatic digestion of wholegrain products. However the AO capacities of the extracts after 90mins are significantly higher than the extracts after 10 mins for all species and the blend.

The total antioxidant release from *F. vesiculosus* was highest during each stage of the enzymatic digestion (see Table 3). The extracts of *A. nodosum* and *P. canaliculata* had the lowest total antioxidant capacity with no significant difference between the species at 10 mins, 30 mins and 90 mins. The total antioxidant capacity of the extracts of the blend appears to reflected its composition.

As all values during the *in vitro* enzymatic digestion fell between the lower values of (*A. nodosum* and *P. canaliculata*) and the higher values of *F. vesiculosus*.

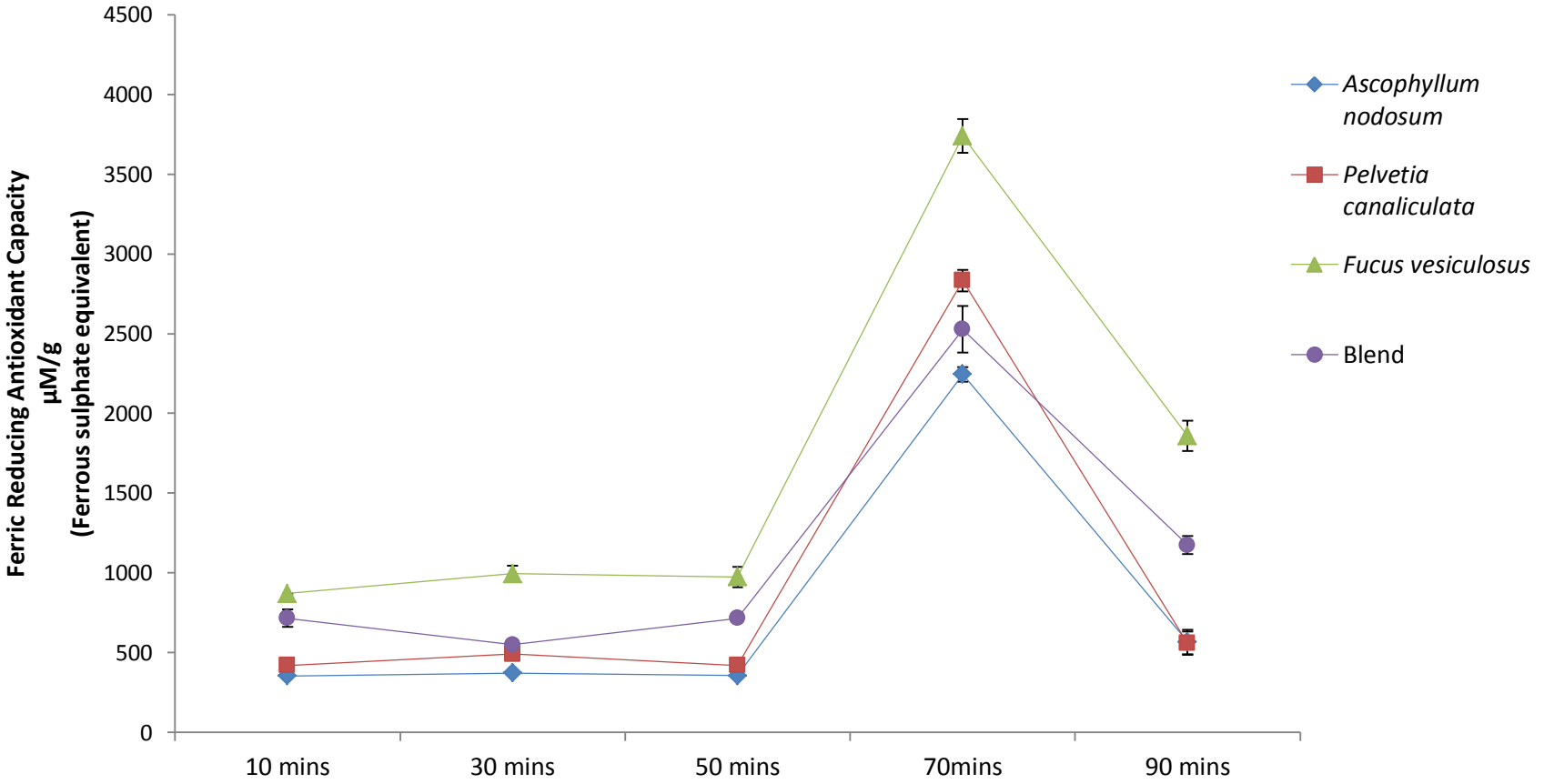

Figure 10. Total antioxidant capacity of extracts released from brown seaweeds during in vitro enzymatic digestion measured by FRAP. All values are means of triplicates ± SE.

Table 3. Total antioxidant capacity of extracts released from brown seaweeds during enzymatic digestion as measured by FRAP (µM/g Ferrous Sulphate equivalent) All values are means of triplicates ±SE. (a-d) denotes a significant difference between species at individual stage of *in vitro* enzymatic digestion, where (a) denotes significant difference from *A. nodosum* (b) denotes significant difference from *P. canaliculata*.

	In Vitro Digestion stage									
	10 mins		30 mins		50 mins		70 mins		90 mins	
Ascophyllum nodosum (a)	354.1 (c,d)	± 6.3	372.7 (b,c,d)	± 2.5	356.3 (c,d)	±4.5	2245.2 (b,c,d)	± 26.7	568.2 (c,d)	± 44.3
Pelvetia canaliculata (b)	420.4 (c,d)	± 8.5	490.6 (a,c)	± 15.2	419.4 (c,d)	±10.6	2833.7 (a,c,d)	± 39.0	558.4 (c,d)	± 42.1
Fucus vesiculosus (c)	869.8 (a,b,d)	± 14.7	994.2 (a,b,d)	± 25.8	974.8 (a,b,d)	± 36.5	3739.6 (a,b,d)	± 52.5	1858.6 (a,b,d)	± 55.1
Blend * (d)	716.1 (a,b,c)	± 31.8	549.3 (a,c)	± 15.0	715.3 (a,b,c)	± 3.6	2527.7 (a,b,c)	± 83.8	1174.6 (a,b,c)	± 29.2

3.3. Antioxidant capacity measured by DPPH radical scavenging assay

The total antioxidant capacity of the extracts released from the brown seaweeds during the in vitro enzymatic digestion as measured by DPPH are shown in Figure 12. The standard curve used for determining antioxidant capacity by DPPH is shown in Figure 11.

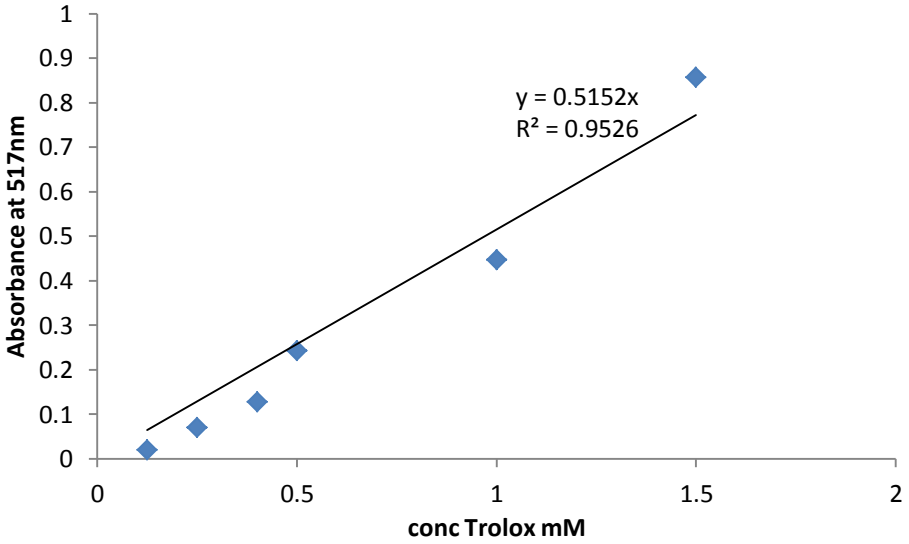

Figure 11. Standard curve for the DPPH assay

There were no significant affects on the antioxidant capacity of the extracts after lowering the pH with HCl between 1 0mins and 30 mins of the *in vitro* digestion on any of the species or the blend. Although there was a trend for a slight decrease in antioxidant capacity *for F. vesiculosus*, *A. nodosum* and the blend.

For *F. vesiculosus* and the blend a gradual and significant increase in AO capacity was observed from following the addition of pepsin. Which was followed by a further increase in AO capacity after the pH was raised with the addition of NaOH. For *A .nodosum* and *P. canaliculata* there was no significant affects of pepsin on the AO capacity but raising the pH significantly increase the AO capacity. For all species and the blend there was a significant decrease in AO capacity following the addition of pancreatin between 70 mins and 90 mins. There was no significant difference in the AO

capacity of the extracts after 10 mins of the *in vitro* digestion and the 90 mins the final stage. This is different from what was observed in the FRAP assay in which there was a significant increase.

A. nodosum had the lowest AO activity of all the species and the blend throughout the digestion apart from at the 70 mins stage when there was no difference in AO capacity between any of the species or the blend. *F. vesiculosus* had the highest activity after 10 mins of the digestion. After 90 mins the AO capacity of *F. vesiculosus* was significantly higher ($p<0.001$) than *P. canaliculata* and *A. nodosum* but not the blend. The blend had a similar AO capacity to *P. canaliculata* and *F. vesiculosus* lying between the values of the two species. Like the FRAP assay the AO capacity of the blend reflected its composition.

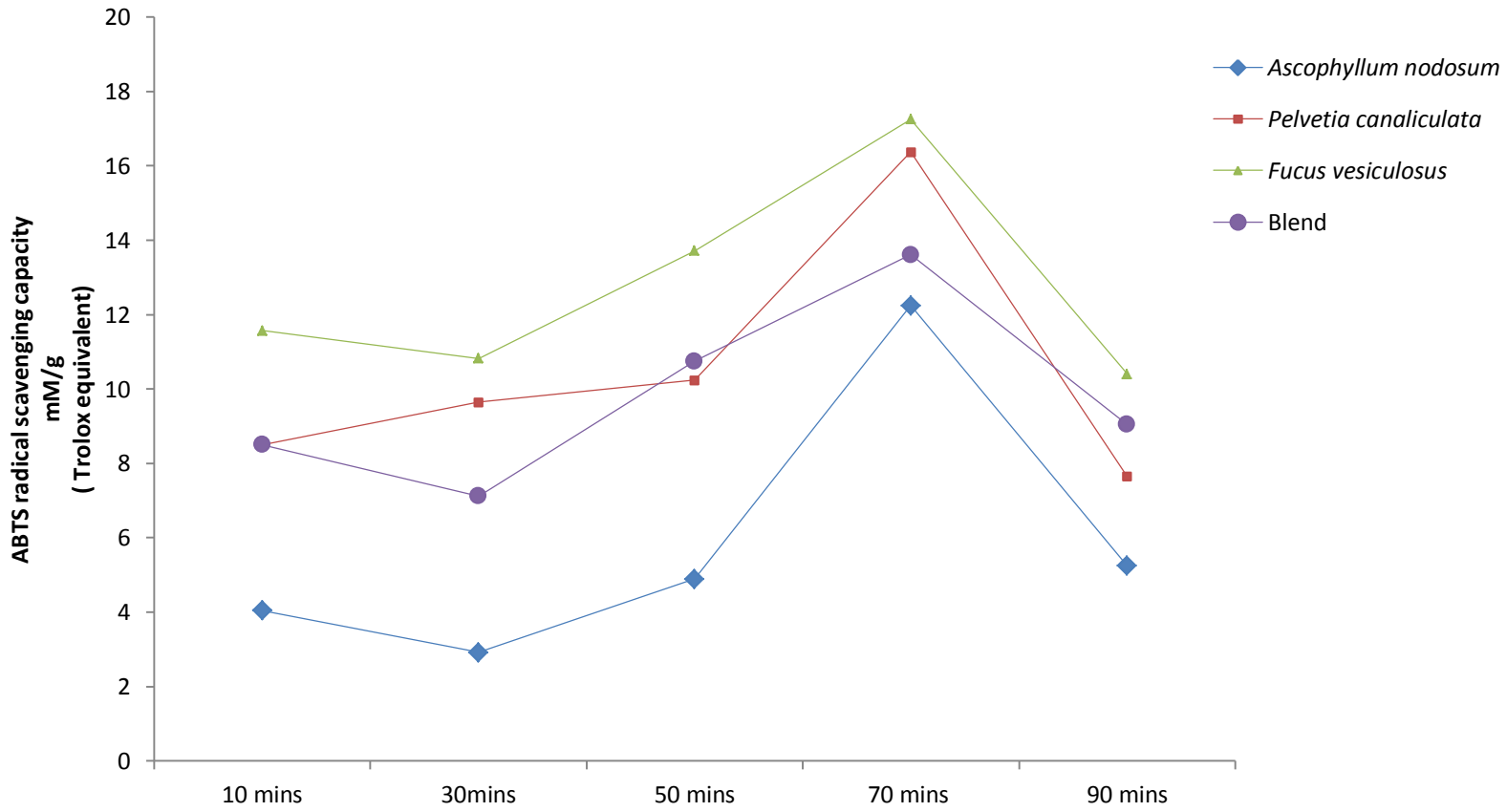

Figure 12. Total antioxidant capacity of extracts released from brown seaweeds during in vitro enzymatic digestion measured by DPPH radical scavenging ability (mM Trolox equivalent/g). All values are means of triplicates ±SE.

Table 4. Total antioxidant capacity of extracts released from brown seaweeds during enzymatic digestion as measured by DPPH radical scavenging ability (mM Trolox equivalent/g) All values are means of triplicates ± SE. (a-d) denotes a significant difference at 10,30,50,7 or 90 mins of *in vitro* enzymatic digestion between species, where (a) denotes significant difference from *A. nodosum* (b) denotes significant difference from *P. canaliculata*.

	In Vitro Digestion stage									
	10 mins		30 mins		50 mins		70 mins		90 mins	
Ascophyllum nodosum (a)	4.1 (b,c,d)	±0.5	2.9 (b,c,d)	±0.6	4.9 (b,c,d)	±0.8	12.3	±0.6	5.3 (b,c,d)	±0.4
Pelvetia canaliculata (b)	8.5 (a,c)	±0.6	9.7 (a)	±0.5	10.2 (a)	±1.2	16.4	±0.4	7.7 (c,d)	±0.4
Fucus vesiculosus (c)	11.6 (a,b,d)	±0.4	10.8 (a)	±0.9	13.7 (a)	±0.5	17.3	±2.1	10.4 (a,b)	±0.4
Blend * (d)	8.5 (a,c)	±0.4	7.1 (a)	±1.0	10.7 (a)	±0.4	13.6	±0.8	9.1 (a)	±0.3

3.4. α-amylase inhibition assay

All of the brown seaweed species caused inhibition of α-amylase activity. The IC_{50} values ranged from 411µg/ml to 2018µg/ml. *P. canaliculata* had the lowest IC_{50} so therefore demonstrated the most potent inhibition of α-amylase of all the species. The extract of *A. nodosum* demonstrated the least inhibition of α-amylase. The IC_{50} value of the blend for α-amylase appears to be a reflection the mixture of the three species it contains lying between *A. nodosum* and *P. canaliculata*.

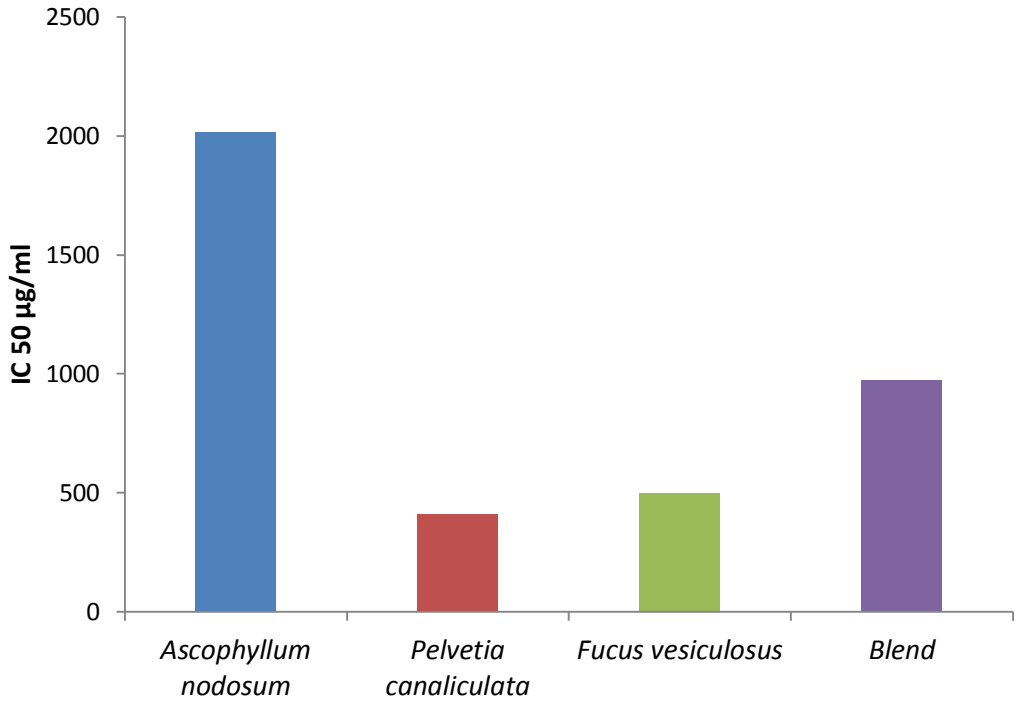

Figure 13. Comparison of α-amylase IC_{50} values of brown seaweeds extracts from in *vitro enzymatic* procedure after 10 mins.

The dose response curves used to determine the IC_{50} values can be found in Appendix 3.

3.5. α-glucosidase inhibition assay

All of the brown seaweed species caused inhibition of α-glucosidase activity. *F. vesiculosus* demonstrated the most potent inhibition of α-glucosidase of the three seaweed species (IC_{50} 44mg/ml) while *A. nodosum* demonstrated the least inhibition (IC_{50} 92mg/ml). The blend of seaweeds inhibited α-glucosidase more potently than any of the three species individually.

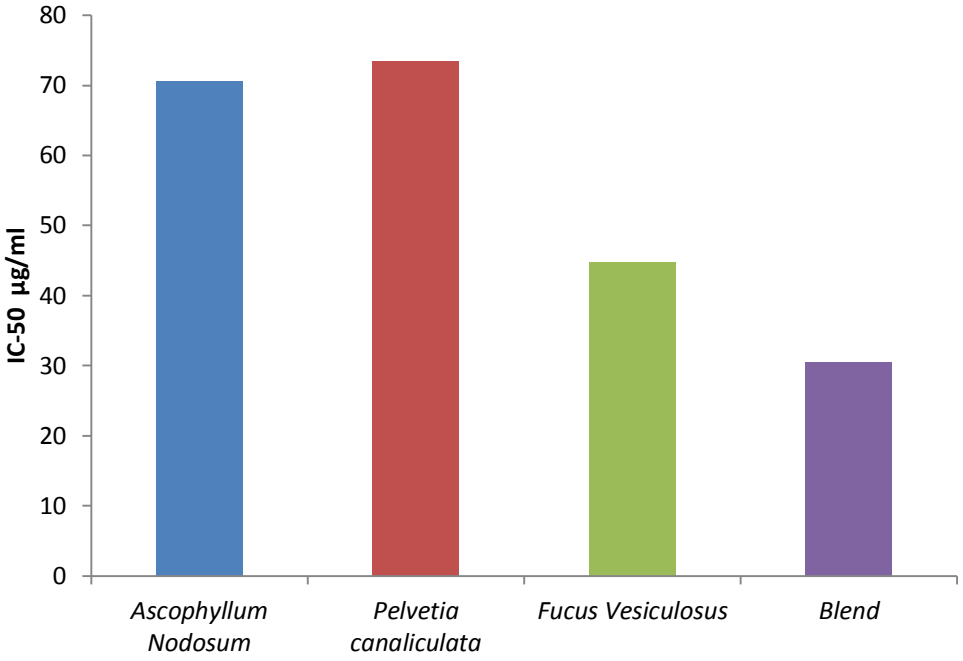

Figure 14. Comparison of α-glucosidase IC_{50} values of brown seaweed extracts from the *in vitro* enzymatic procedure after 10 mins

The dose response curves used to determine the IC_{50} values can be found in Appendix 2.

3.6. Lipase activity analysis

All of the brown seaweed species caused inhibition of lipase activity. Extracts of the brown seaweeds after 10 mins of the *in vitro* enzymatic digestion were tested at the concentration 3.6mg/ml.

A comparison of the rate of reaction for the control conditions and with extracts of the brown seaweed present is shown in figure 6 . The reaction is limited by the amount of substrate which is present. The rate of reaction is constant for the control up to ~ 15mins. The inhibition of lipase by the seaweed extracts was calculated within the time interval when reaction was not limited by the substrate concentration. This was taken as the linear portion of the control reaction in Figure 15 between 5-15mins.

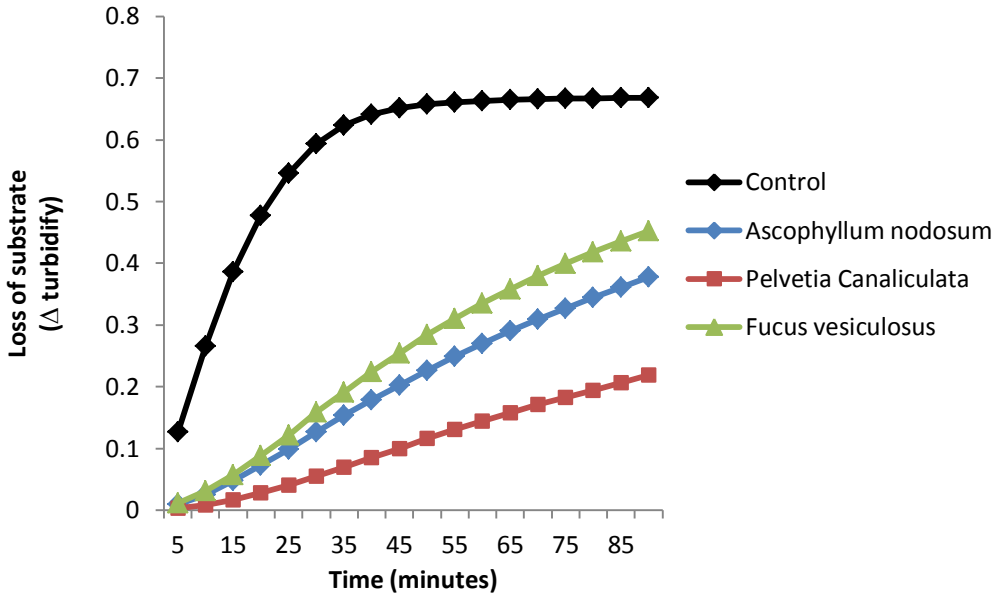

Figure 15. Kinetic of triglyceride breakdown by porcine pancreatic lipase with olive oil as the substrate in the presence of brown seaweed extracts at the concentration 3.6mg/ml

All of the brown seaweed extracts caused <80% inhibition of pancreatic lipase at the concentration 36mg/ml. *P. canaliculata* and the blend caused the most inhibition of lipase activity ~96% and 97% respectively . Due to time restrictions the seaweed extracts were not assayed for lipase inhibition at different concentrations and so the IC_{50} values were not calculated.

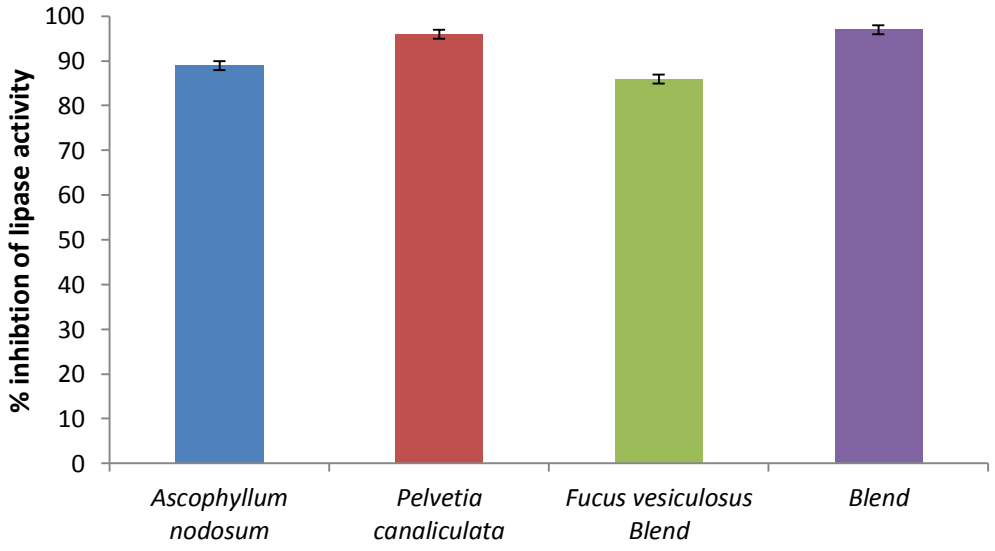

Figure 16 . Effect of brown seaweed extracts on pancreatic lipase activity. The lipase activity was determined using olive oil as the substrate and is expressed as a percentage of the control reaction. All values are means of triplicates ± SE

4. Discussion

4.1. Total polyphenol content

4.1.1. Variation between the species

The total polyphenol content (TPC) of the brown seaweeds differed significantly between species. *F. vesiculosus* had the highest polyphenol content ranging from 29-61mg/g through the *in vitro* digestion while *A. nodosum* had the lowest polyphenol content ranging from 11-35mg/g. O'Sullivan et al., [16, 48] is the only other study to have compared the TPC of the brown seaweeds *A. nodosum, P. canaliculata* and *F. vesiculosus.* They found no significant differences between the species and reported ~ 10 fold lower range in the total polyphenol content of the species 2.5-4.5mg/g. This is surprising because they used methanol to extract the seaweed an extraction method that would be expected to be more efficient than the *in vitro* enzymatic digestion. However Apostolidis et al., [17] found the TPC of *A. nodosum* to be within the range 22mg/g- 35mg/g when looking at the seasonal variation in TPC of *A. nodosum* harvested from Canada. The samples were extracted with water at 80 ° C for 30mins. This is similar to the range found in *A. nodosum* in this study. Wang et al.,[15] also found the TPC of *F. vesiculosus* to be higher than that of *A. nodosum .*

4.1.2. Total polyphenol release during *in vitro* enzymatic digestion

The TPC of the extracts increased during the *in vitro* enzymatic digestion for all brown seaweeds and the blend. A significant increase occurred after the gastric phase of the digestion (after 70 mins), an increase of ~ 40% was observed for all species. The blend of seaweeds did not impact the polyphenol release from the seaweeds during the *in vitro* enzymatic digestion.

4.1.3. Important findings

This is the first study to try and estimate the total polyphenol release from brown seaweeds during an *in vitro* enzymatic digestion. The TPC is difficult to compare with other data on plant materials which have undergone *in vitro* enzymatic digestion e.g vegetable juices due to differences in units. However a comparison can be made with another plant species *Camellia sinensis* (tea) whose biological activity is attributed to its polyphenol content [49]. Gondoin et al., [50]demonstrated that green, black and white teas have polyphenols within the range 60mg/g-100 mg/g following extraction of the teas with boiling water. The concentration of polyphenols within the brown seaweeds ranged between 11-61mg/g . Therefore the TPC of the brown seaweeds is comparable to another polyphenol rich source.

4.1.4. Limitations

The stability of the polyphenols after the gastric phase of the digestion is unknown. The total polyphenol release of the final stage of the digestion could not be determined. There was interference with the assay in extracts after 90mins following the addition of pancreatin to the *in vitro* enzymatic digestion. The crude source of pancreatrin is presumed to be the cause of the interference. Wootton-Beard et al., [26] measured the TPC of 23 vegetable juices through an *in vitro* digestion model. Similar to this study they found a significant increase in total polyphenol content occurred after gastric digestion. They were able ascertain the TPC of the vegetable juices after replication of conditions in the small intestine which included the addition of pancreatin. The majority of the juices had a further increase in total polyphenol release . The concentration of pancreatin used in the *in vitro* digestion by Wootton-Beard et al.,[26] study was higher and so the interference that occurred at the final stage of this experiment warrants further investigation.

A repeat assessment of TPC of the three brown seaweed species is necessary to confirm the findings here especially to confirm *F. vesiculosus* as the species with the highest TPC. As discussed previously the Folin –Ciocalteu method most likely overestimates TPC because it also reacts with other hydroxyl containing species. An evaluation[51] of the methods available for determining the phlorotannin content of brown seaweeds found FC method to be more precise than assays using 2,4-dimethoxybenzaldehyde (DMBA) or ability to bind polyvinylpyrrolidone (PVPP). The other method which matched the precision of FC method was H-NMR. However H-NMR is more complicated and time consuming especially when whole extracts containing complex mixtures of compounds are being measured. Brown seaweed extracts contain complex mixtures of phlorotannins which are polymers of phloroglucinol. Their chemical characteristics are therefore very similar which makes separation of the compounds difficult. Attempts so far to characterise the phlorotannins from brown seaweeds has involved separation by a sequential extraction with organic solvents followed by separation into lower and higher molecular weight fractions [52, 53].

4.2. Antioxidant assays

4.2.1. Variation between the species

F. vesiculosus had the highest antioxidant capacity measured by FRAP. A significant difference in antioxidant capacity between *A. nodosum* and *P. canaliculata* was not measured by the FRAP assay. O'Sullivan et al.,[16] found no significant differences in antioxidant capacity as measured by FRAP when comparing the three species.

A. nodosum had the lowest antioxidant capacity and significantly differed from the other species at most stages throughout the *in vitro* digestion when measured by DPPH. For the DPPH assay the antioxidant capacity of *F. vesiculosus* was not significantly different from *P. canaliculata* at all stages of the digestion. This is in contrast again to O'Sullivan et al., [16] who found *P. canaliculata* to have significantly lower DPPH radical scavenging ability than the other species. In support of the findings Wang et al.,[15] also found the DPPH radical scavenging ability to be higher in *F. vesiculosus* than *A. nodosum.*

4.2.2. *In vitro* release of antioxidants from brown seaweeds

A similar pattern of antioxidant release from the seaweeds during the digestion was found by measurement of the antioxidant capacity of the extracts by FRAP and DPPH. A spike in antioxidant capacity occurred after 70mins of the *in vitro* digestion followed by a decrease after 90mins in both assays (see figures 8 and 9, chapter 4)

However the antioxidant release from the brown seaweeds increased overall from the initial to the final stage as measured by FRAP. An increase in antioxidant release of more than 40% occurred for all species and the blend. While for DPPH there was no significant differences in the antioxidant capacity of the extracts at the start and the end of the digestion.

This did not occur in the *in vitro* digestion of wholegrain foods by Nagar and Seal[43] whose method for the *in vitro* enzymatic digestion was followed. For all their products tested there was a significant increase in antioxidant release after 90mins of the *in vitro* enzymatic digestion measured by FRAP and TEAC. However a more suitable comparison can be made with Wootton-Beard et al.,[26] which compared the antioxidant capacity of 23 vegetable juices during a similar *in vitro* enzymatic digestion . They also observed similar effects on the antioxidant capacity of the vegetable juices at the same stages in the digestion for both DPPH and FRAP. There was an increase in antioxidant capacity of the vegetable juices following the increase in pH after the gastric stage and a subsequent decrease after the addition of pancreatin.

4.2.3. Antioxidant activity and Total Polyphenol content

The antioxidant activity of the brown seaweeds appears to relate to their total polyphenol content. The spike in antioxidant activity for all species after 70mins of the *in vitro* digestion was also the point where the total polyphenol content was highest for all species (see figures 7,8,9 chapter 3). The trend for *F. vesiculosus* to have the highest antioxidant activity appears to relate to it containing the highest total polyphenol content. This supports the findings of Wang et al., [15] which also found a correlation of TPC and the antioxidant assays DPPH and ORAC for *A. nodosum* and *F. vesiculosus.* Wootton- Beard et al., [26] did a comparative study of antioxidant capacity of vegetable juices through an *in vitro* digestion. They found a significant correlation between FRAP and the TPC but a poor correlation between DPPH and TPC. However a comparative study of the antioxidant and total polyphenol content of 30 plant extracts found a strong correlation between both TPC and FRAP and DPPH[54].

4.2.4. Important findings

This is the first study to compare the antioxidant capacity of *A. nodosum, P. canaliculata* and *F. vesiculosus* sourced by Seagreens® from the Outer Hebrides . The brown seaweeds demonstrated high antioxidant activity in comparison to other commonly consumed fruit and vegetables. Nagah and Seal [43]measured the antioxidant release from wholegrain foods following the same *in vitro* enzymatic digestion as well as broccoli, sundried tomatoes and dried mushrooms. The peak of antioxidant capacity of the fruit and vegetables occurred at the end of the enzymatic digestion (after 90mins). Comparing the same step in this study, the antioxidant capacity of *F. vesiculosus* measured by FRAP was ~ 50-fold higher than sundried tomatoes and more than 5 fold higher than broccoli. While the antioxidant capacity of *A. nodosum* and *P. canaliculata* was greater than 10 fold higher than sundried tomatoes and almost 2 fold higher than broccoli. This suggests brown seaweeds are a concentrated source of antioxidants.

This is the first study to imitate and quantify the release of antioxidants from brown seaweeds which occurs during digestion in the stomach and small intestine. The aim of the study was to quantify the antioxidants which are released from the food matrix and their activity during passage through the initial stages in gastro-intestinal digestion. Two methods (DPPH and FRAP) were used to measure the antioxidant capacities of the extracts of brown seaweed. Overall they support *F. vesiculosus* to be the species with the highest antioxidant capacity. They demonstrate that the antioxidant capacity of the brown seaweeds remains high through digestion in the initial stages of gastro-intestinal digestion as no significant decreases in antioxidant capacity was observed by either assay. This suggests that a high concentration of antioxidants will be released from the brown seaweeds within the gut lumen where it can provide protection from oxidative damage.

4.2.5. Limitations

A comparative study is recommended when investigating the antioxidant capacity of plant extracts[25, 30, 43]. The two methods used in this study were chosen because they are straight forward, require relativity standard equipment, and are routinely used. The DPPH assay measures the ability of compounds in the plant extract to react with the stable radical DPPH•. A drawback to this assay for measuring brown seaweed extracts is the steric accessibility of the DPPH • radical. Smaller molecules can access the DPPH radical more easily[30] . Brown seaweeds contain a number of large phlorotannin structures and so the DPPH assay may underestimate the antioxidant activity of brown seaweed extracts. Also this is a colorimetric assay of which the absorbance wavelength used is 517nm. Carotenoids are known to absorb light at this wavelength. Fucoxanthin is a carotenoid found in brown seaweed and so could interfere with the assay results. The FRAP assay measures the ability of compounds to reduce Fe^{3+}. The reaction time generally used for this method is 4 minutes. However this may not have been long enough for all the antioxidant compounds to reduce Fe^{3+}. Polyphenols such as quercitin and caffeic acid have been shown to react much slower in this assay. It is unknown how long the phlorotannins take to reduce Fe^{3+}. The FRAP assay cannot measure the antioxidant capacity of lipophilic compounds in plant extracts. However the ABTS • radical used in the TEAC assay is able to measure both the antioxidant capacity of lipophilic and hydrophilic antioxidants[30, 43] . Unfortunately due to time constraints a TEAC analysis was not carried out.

A relationship between the phenolic content of the seaweed extracts and their antioxidant capacity was found which is in agreement with other studies. However the components of the extracts from the *in vitro* digestion are unknown. The *in vitro* digestion is likely to extract a complex mixture of bioactive compounds. Other compounds likely to have been present in the brown seaweed which have also demonstrated antioxidant activity include fucoxanthin and fucoidans [4, 10, 12].

4.3. Inhibition of digestive enzymes

4.3.1. Variation between the species

All brown seaweeds demonstrated inhibition of the digestive enzymes α-amylase, α-glucosidase and lipase. *F. vesiculosus* inhibited α-glucosidase most potently (IC_{50} 45 μg/ml). Interestingly the blend of seaweeds inhibited α-glucosidase more potently than *F. vesiculosus* alone (IC_{50} 31 μg/ml). In contrast *P. canaliculata* inhibited α-amylase most potently (IC_{50} 411 μg/ml) and the potency of the blend reflected its composition. All the brown seaweed extracts inhibited α-glucosidase more potently than α-amylase.

Although a number of studies have looked at the inhibition of α-amylase and α-glucosidase by *A. nodosum [17, 18, 37, 38]* the majority are difficult to compare because of different methods of extraction of the seaweeds. Organic solvents and complex extraction have been used to concentrate the polyphenols from the seaweed. However Apostolidis et al.,[17] measured the inhibition of α-amylase and α-glucosidase by extracts of *A. nodosum* in water at two different temperatures. As can be seen by table 5 the potency of *A. nodosum* for inhibition of α-amylase and α-glucosidase are comparable to the potencies of *A. nodosum* extracts extracted at 80°C by Apostolidis et al.,[17]. They also found *A. nodosum* to inhibit α-glucosidase more potently than α-amylase.

Lipase inhibition was detected at the concentration 3.6mg/ml for all seaweeds. This is in contrast to the findings of Nwosu et al., [18] which did not demonstrate any inhibition of lipase by *A. nodosum* by extracts with a polyphenol concentration 100μg/ml. The concentration of polyphenols in the brown seaweed extracts used in this study is ~ five fold higher as determined by the FC method.

Table 5. Comparison of potency of inhibition of enzymes which hydrolyse carbohydrate by brown seaweed extracts

Study	Species	α-glucosidase IC_{50} (μg/ml)	α-amylase IC_{50}(μg/ml)	Extraction method
Results	*A. nodosum*	70	2018	(see chapter 2)
	P. canaliculata	73	411	
	F. vesiculosus	45	499	
	Blend	31	973	
Nwosu et al .,2011 [18]	*A. nodosum*	~19	< 25	100% methanol 50% acetonitrile/50% UPW
Zhang et al., 2007 [38]	*A. nodosum*	77	N/A	50% aqueous ethanol extract
Apostolidis et al.,2010 [17]	*A. nodosum*	208 55	2088 311	20°C Water extraction 80°C Water extraction
Roy et al ., 2011 [37]	*Extract containing A. nodosum and F. vesiculosus*	5	2.8	Commercially available extract InSea$^{2®}$

4.3.2. Correlation between TPC and inhibition of enzymes important in the gastrointestinal digestion of carbohydrates and lipids

The relationship between the total polyphenol content of the extracts and the potency of inhibition of the digestive enzymes is not clear. *F. vesiculosus* has the highest total polyphenol content. However it did not display the most potent inhibition of all the enzyme assays as can be seen from table 6. The results are not in line with the hypothesis.

Table 6. Relative potencies of the brown seaweeds *A. nodosum, P. canaliculata and F. vesiculosus* in inhibiting enzymes important in the gastro intestinal digestion of carbohydrates and lipids.

α-amylase	α-glucosidase	Lipase
P. canaliculata	Blend	Blend
F. vesiculosus	*F. vesiculosus*	*P. canaliculata*
Blend	*A. nodosum*	*A. nodosum*
A. nodosum	*P. Canaliculata*	*F. vesiculosus*

As the extracts contain a mixture of bioactive components released from the brown seaweeds it is likely that a number of components are responsible for the inhibition. A relationship between the polyphenol content and enzyme inhibition has not been demonstrated.

The enzyme inhibition was only determined for the inhibition by the brown seaweed components released into water in the first 10mins of the digestion. Therefore the effects of the *in vitro digestion* on the ability of the brown seaweeds to inhibit digestive enzymes remains unknown.

4.3.3. Important findings

The study supports current literature that the brown seaweeds *A. nodosum, P. canaliculata and F. vesiculosus* have potential antidiabetic effects through inhibition of the carbohydrate digesting enzymes α-amylase and α-glucosidase. All of the seaweed species demonstrated more potent inhibition of α-glucosidase than α-amylase. This could be an advantage over the drug Acarbose which targets these enzymes. A number of side effects have been reported by diabetic patients taking acarbose including flatulence, abdominal distension and diarrhoea. This is believed to be caused by the fermentation of undigested carbohydrate in the colon which results from excessive inhibition of α-amylase [17]. The potency of the brown seaweeds in inhibiting pancreatic lipase remains to be determined *in vitro* before potential anti-obesity effects can be investigated further.

4.3.4. Limitations

The potent inhibition of α-glucosidase by the blend of seaweeds appears was unexpected. The blend was 30% more potent than *F. vesiculosus* the most potent of the three species and more than 50% more potent than the other two species. A repeat assessment of the ability of the blend to inhibit α-glucosidase is required. The assays used to determine the enzyme activity bear no relevance to the effect of consuming whole seaweed in animals or humans. Both the α-amylase and lipase assays used biological substrates for the enzymes in the assays to measure inhibition. Biological substrates are considered to be superior to artificial substrates because the potency of the inhibition is determined for the same reaction as which occurs under normal conditions. The extracts of the brown seaweeds from later stages in the *in vitro* digestion could not be determined because the enzymes are affected by pH and the pH of the extracts varies through the digestion . The pH in the small intestine is within the range pH 6-7.4 where α-amylase and α-glucosidase are active. The pH of the extracts after 70mins and 90mins meant to replicate the conditions in the small intestine

were ~pH 5. The inhibition of α-amylase and α-glucosidase by the extracts after 70mins was assayed but the results are not presented as the pH of the extracts is believed to have caused interference.

This study provides more evidence to demonstrate that the brown seaweeds *A. nodosum, P. canaliculata and F. vesiculosus* inhibit carbohydrate digesting enzymes α-amylase and α-glucosidase *in vitro*. However there is not sufficient evidence to demonstrate that the inhibition of these enzymes occurs after consumption of the brown seaweeds. Zhang et al.,[38] was unable to detect significant effects of *A. nodosum* extracts on blood glucose levels in mice in a four week trial. However Paradis et al., [55] demonstrated that a commercially available extract of *A. nodosum and F. vesiculosus* was able to reduce the insulin response to the consumption of 50g of carbohydrate in bread. The study involved 23 men and women in a cross-over design, 500mg of the extract or placebo was given 30mins prior to the bread. Blood glucose and insulin measurements were taken for three hours afterwards. Another small human trial has investigated the effects of *A. nodosum* on postprandial blood glucose levels [56]. Participants consumed 100g of a loaf containing 4% *A. nodosum.* There were no detectable effects on blood glucose.

5. Conclusions

Three brown seaweeds of the Fucaceae family were digested by an *in vitro* enzymatic digestion procedure designed to mimic the initial stages of digestion in the gastro-intestinal tract. There was variation in the release of polyphenols between the species following the *in vitro* digestion. *F. vesiculosus* released the highest amount of polyphenols. The total polyphenol release from the brown seaweeds is comparable to green tea extracts following extraction with boiling water. Digestion of the brown seaweeds with sequential acid/enzymatic steps led to an increase in polyphenol release through the digestion. The antioxidant capacity of extracts of brown seaweeds following *in vitro* digestion were measured by FRAP and DPPH. There were fluctuations in the antioxidant capacity of the brown seaweed extracts during the *in vitro* digestion. However the antioxidant capacity of the extracts at the start of the digestion was comparable to the antioxidant capacity of the extracts at the end of the *in vitro* digestion. The brown seaweeds are consistent source of antioxidants to the GI tract through digestion to the small intestine. *A. nodosum*, *F. vesiculosus* and *P. canaliculata* are a more concentrated source of antioxidants compared with broccoli, tomatoes, and mushrooms. The antioxidant capacity of the brown seaweed demonstrated correlation with the total polyphenol content of the extracts.

P. canaliculata inhibited α-amylase most potently while the blend of seaweeds inhibited α-glucosidase and lipase the most potently. There was no correlation between the inhibition of the enzymes and total polyphenol content of the brown seaweeds. The whole brown seaweeds have demonstrated significant evidence *in vitro* as potential agents in the prevention and treatment of diabetes and obesity . Further research is required to demonstrate the inhibition of enzymes important in the gastro-intestinal digestion of carbohydrates and lipids *in vivo*.

6. Recommendations for future research

- The *in vitro* enzymatic digestion procedure should be adapted to enable analysis of the total polyphenol content of the brown seaweed extracts at each successive stage of the digestion. This could be achieved by changing the source and concentration of the pancreatin used in the procedure.
- The *in vitro* digestion procedure should be adapted to bring the pH of the samples after gastric digestion with the range pH 6-7.4. This would allow ability to inhibit digestion enzymes to be measured.
- The antioxidant capacity of the brown seaweeds from the same source following an *in vitro* digestion should be repeated and measured by at least 3 different in vitro methods preferably DPPH, FRAP and TEAC to enable comparisons with currently available literature and a comprehensive measure of the antioxidant capacity of the seaweeds.
- A repeat of the *in vitro* digestion procedure should also include another plant material suitable for comparison of total polyphenol and antioxidant release. Green tea would be suitable as previous studies have shown it to have a similar total polyphenol content by weight. This would provide further insight into the relationship between total polyphenol content of the brown seaweeds and their antioxidant capacity. It is proposed that phlorotannins within brown seaweeds are more effective radical scavengers than polyphenols such as epigallocatechin gallate found within green tea because they have greater polymerisation and so will produce more stable radicals. However characterisation of the phlorotannins found within the brown seaweeds extracts from the *in vitro* enzymatic digestion is needed. The chemical structures involved in the biological activity of the brown seaweeds need to be determined.
- I would like to take the study forward into a human feeding study, initially to measure postprandial glucose response after consuming the seaweed before a carbohydrate rich

meal. This would investigate whether the in vitro inhibition had any effect in vivo. This would be relatively simple to carry out and include a sufficient number of participants to enable significant differences between postprandial glucose area under the curve.

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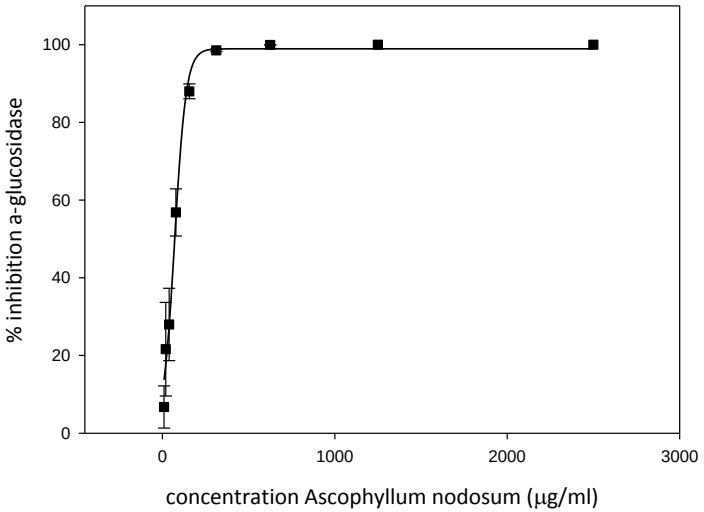
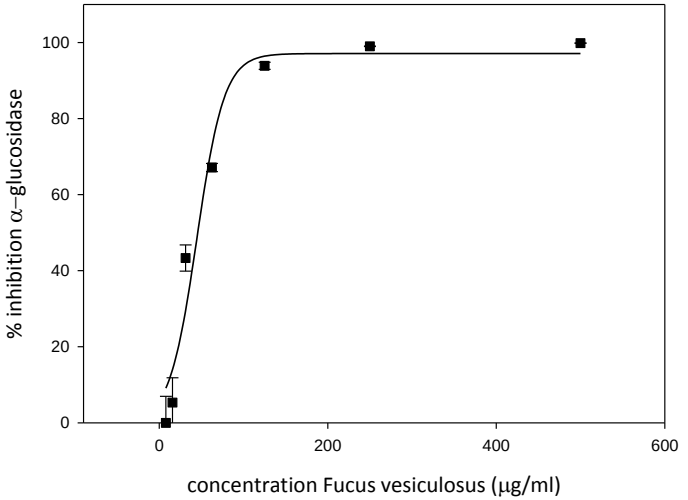
Appendix.

Appendix 1.*In vitro enzymatic digestion*

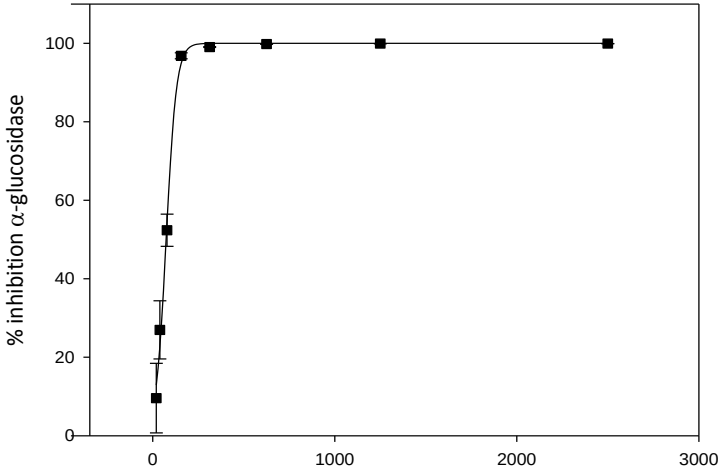
1g seaweed in 20mls	Total volume	Step	Dilution Factor
Add sample and blank to water bath	**=20mls**		
After 10mins ↓ **- 3ml sample**	**=17mls**	**10 mins**	Concentration = 0.05g/ml
Add 10 ml of HCl (0.05M)	**=27mls**		
After 30mins ↓ **- 3ml sample**	**=24mls**	**30 mins**	$= \frac{20}{17} \times \frac{27}{17} = 1.87$
Add 0.05 ml of pepsin solution	**=24.5mls**		
After 50mins ↓ **- 3ml sample**	**=21.5mls**	**50 mins**	$= 1.87 \times \frac{24.5}{24} = 1.91$
Add 1 ml of NaOH (0.5M)	**=22.5mls**		
After 70mins ↓ **- 3ml sample**	**=19.5mls**	**70 mins**	$= 1.91 \times \frac{22.5}{21.5} = 2.00$
Add 5 ml of pancreatin solution	**=24.5mls**		
After 90mins ↓ **- 3ml sample**		**90 mins**	$= 2.00 \times \frac{24.5}{19.5} = 2.50$
End of *in vitro* enzymatic digestion			



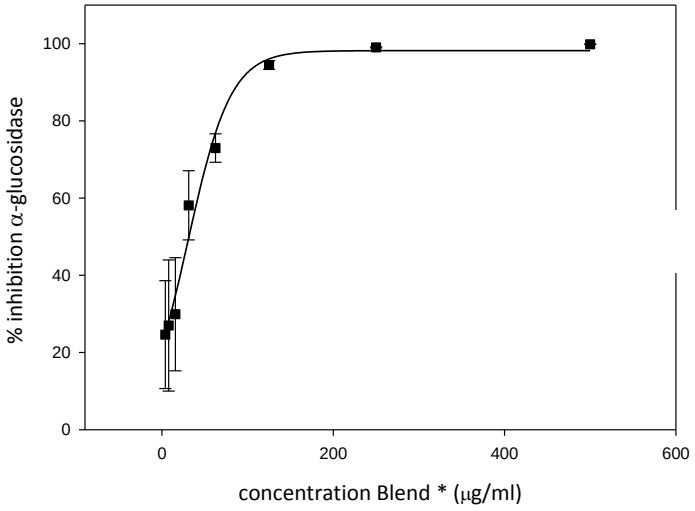
Ascophyllym nodosum inhibition of α-glucosidase (10 mins)



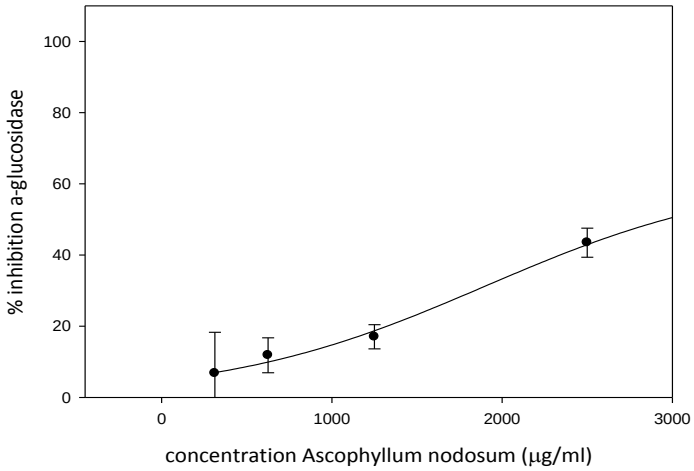
Fucus vesiculosus inhibition of α- glucosidase (10 mins)



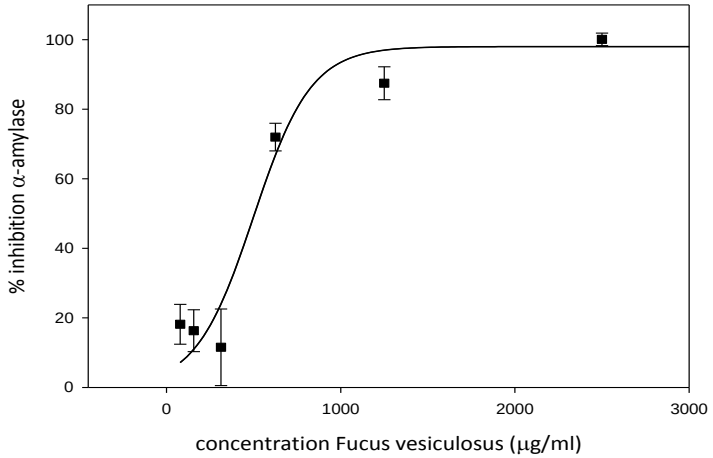
Pelvetia canaliculata inhibition of α-glucosidase (10 mins)



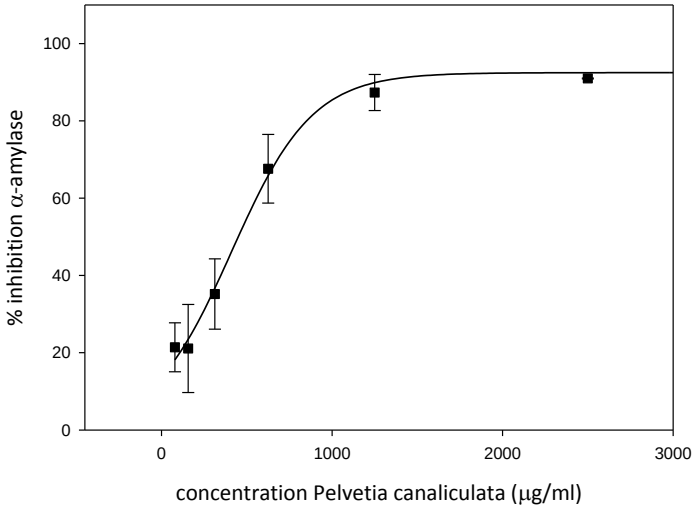
Inhibition of α- glucosidase by Blend* (10 mins)



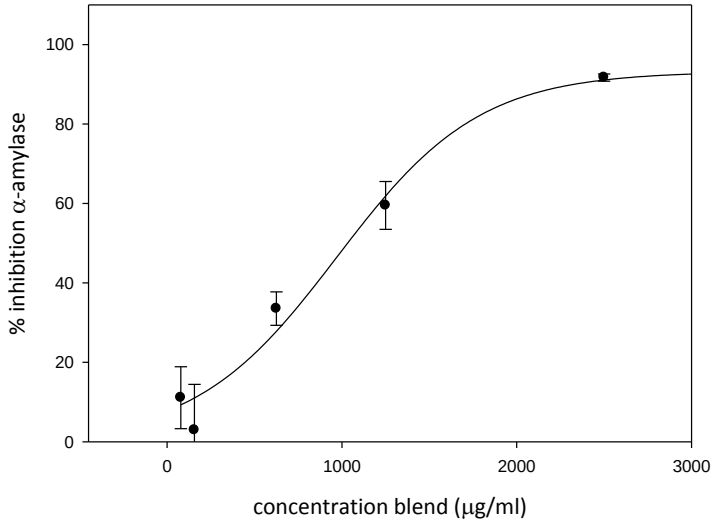
Ascophyllym nodosum inhibition of α-amylase (10 mins)



Fucus vesiculosus inhibition of α- amylase (10 mins)



Pelvetia canaliculata inhibition of α-amylase(10 mins)



Inhibition of α- amylase by Blend* (10 mins)