# Microwave assisted extraction of phenolic compounds from four economic brown macroalgae species and evaluation of antioxidant activity and inhibitory effects on α-amylase, α-glucosidase, pancreatic lipase and tyrosinase

Yuan YUANa, Jian ZHANGb, Jiajun FANc, James CLARKc, Peili SHENb\*, Yiqiang LIa\*, Chengsheng ZHANGa\*[[1]](#footnote-1)

a Marine Agriculture Research Center, Tobacco Research Institute of Chinese Academy of Agricultural Sciences, Qingdao, 266101, China

b State Key Laboratory of Bioactive Seaweed Substances, Qingdao Brightmoon Seaweed Group Co Ltd, Qingdao, 266400, China

c Green Chemistry Centre of Excellence, University of York, Heslington, York YO10 5DD, United Kingdom

# Abstract

Four economically important brown algae species (*Ascophyllum nodosum, Laminaria japonica, Lessonia trabeculate* and *Lessonia nigrecens*) were investigated for phenolic compounds extraction and evaluated for their antioxidant, anti-hyperglycemic, pancreatic lipase and tyrosinase inhibition activities. Microwave assisted extraction (MAE) at 110 oC for 15 min resulted in both higher crude yield and total phenolic content (TPC) for all algae species compared with conventional extraction at room temperature for 4 hours, among which *Ascophyllum nodosum* yielded highest TPC. Antioxidant test indicated that extracts from MAE of four species all exhibited higher DPPH, ABTS free radical scavenging ability and reducing power than conventional method. The extract of *Lessonia trabeculate* exhibited good α-amylase, α-glucosidase, pancreatic lipase and tyrosinase inhibition activities, especially the MAE extract showed even better α-glucosidase inhibitory activity than acarbose.

**Keywords:** Brown macroalgae; Phenolic compounds; Microwave assisted extraction (MAE); Anti-hyperglycaemic activity; Pancreatic lipase inhibition activity; Tyrosinase inhibition activity; Antioxidant

# Introduction

Macroalgae has been consumed by humans for centuries due to their high contents of carbohydrate, protein, minerals and vitamins ([Chakraborty, Joseph, & Praveen, 2015](#_ENREF_6); [Lorenzo et al., 2017](#_ENREF_22)). Brown algae are second most abundant group of marine algae, consisting about 2000 species([Yuan & Macquarrie, 2015a](#_ENREF_38)). In recent years, focus on brown algae has significantly increased due to the numerous bioactive compounds it contains, among which phenolic compounds have attracted particularly attention. Polyphenols derived from brown algae comprise a series of compounds such as catechins, phlorotannins, flavonoids and flavonol glycosides, which have been associated with effective biological activities, including antioxidant, antimicrobial and anti-inflammatory activities([Heffernan, Smyth, Soler-Villa, Fitzgerald, & Brunton, 2015](#_ENREF_15); [Pantidos, Boath, Lund, Conner, & McDougall, 2014](#_ENREF_26)). Considering their great taxonomic and environmental diversity, investigation on different macroalgae species for exploration of new biological active compounds can be regarded as an almost unlimited field ([Rajauria, Foley, & Abu-Ghannam, 2016](#_ENREF_29)).

Phenolic compounds from macroalgae were typically extracted by an aqueous mixture of methanol, ethanol and acetone at room temperature for several hours or days([Farasat, Khavari-Nejad, Nabavi, & Namjooyan, 2014](#_ENREF_10); [van Hees, Olsen, Wernberg, Van Alstyne, & Kendrick, 2017](#_ENREF_33)), and a few reported the hot extraction at about 60 oC ([Chakraborty et al., 2015](#_ENREF_6); [Heffernan et al., 2015](#_ENREF_15)). Recently, microwave assisted extraction (MAE) has been developed as an alternative to conventional extraction technologies due to its advantage of environment friendly, short extraction time and high efficiency([Peng, Cheng, Xie, & Yang, 2015](#_ENREF_28)). Microwave heating is generated by dipole rotation of polar solvent and ionic conduction of dissolved ions, and this rapid volumetric heating leads to the effective cell rupture, releasing the compounds into the solvent([Yuan & Macquarrie, 2015b](#_ENREF_39)). Variety of natural resources have been extracted by MAE for various active ingredients ([Chen, Zhang, Huang, Fu, & Liu, 2017](#_ENREF_8); [Peng et al., 2015](#_ENREF_28); [Yuan et al., 2018](#_ENREF_40)), however, little information is available on MAE of phenolic compounds from macroalgae.

*Ascophyllum nodosum, Laminaria japonica, Lessonia trabeculate* and *Lessonia nigrecens* are four economically important brown macroalgae species around the world. *Ascophyllum nodosum* can be found all coasts of Britain and Ireland, and around 32,000 t of *Ascophyllum nodosum* is harvested per year([Yuan & Macquarrie, 2015a](#_ENREF_38)). *Laminaria japonica* is extensively cultivated in East Asia. *Lessonia spp*. is a genus of large kelp native to the southern Pacific Ocean and is distributed along the coasts of South America, New Zealand, Tasmania, and the Antarctic islands([Cho, Klochkova, Krupnova, & Sung, 2006](#_ENREF_9)). Presently, major utilisation of the four brown algae is for alginate production and food consumption. The investigation of high-value products for nutraceuticals and pharmaceuticals is still on the way.

Therefore, the objective of this study is to extract phenolic compounds by MAE from the four species, which were further scanned for biological activities including antioxidant, anti-hyperglycemic, pancreatic lipase and tyrosinase inhibition activities. Phenolic profile of the extracts were analysed by liquid chromatography-diode array detection coupled to negative electrospray ionization-tandem mass spectrometry (LC-PDA-ESI-MS/MS). To the best of our knowledge, this is the first report on phenolic compounds extraction from *Lessonia trabeculate* and *Lessonia nigrecens,* and also the first time to report tyrosinase lipase inhibition activities of phenolic extracts from brown algae.

# 2. Materials and methods

## 2.1 Raw materials and chemicals

The four seaweed species *Ascophyllum nodosum* (AN), *Laminaria japonica* (LJ), *Lessonia trabeculate* (LT), *Lessonia nigrecens* (LN) were kindly supplied by Bright Moon Seaweed Group, Qingdao, China. Pancreatic lipase (type Ⅱ, from porcine pancreas), α-amylase (type Ⅳ-B，from porcine pancreas ), α-glucosidase (type Ⅰ, from Saccharomyces cerevisiae), tyrosinase from mushroom were purchased from Sigma-Aldrich LLC. Other chemicals and reagents were of analytical grade.

## 2.2. Microwave assisted extraction of phenolic compounds

Microwave assisted extraction of polyphenols was carried out using Uwave-2000, Sineo Microwave Chemistry Technology Co, LTD, Shanghai, China. 30 g seaweed sample was suspended in 300 mL 70% methanol and thoroughly mixed. The slurry was then placed into a 500 mL Teflon vessel which was subjected into the UWave-2000 reactor for microwave irradiation (2.45 GHz) for 15 min (5 min climbing and 10 min holding) at 110 oC. Temperature control was monitored by a platinum resistance thermometer and a magnetic stirring bar was used for agitation, 300 rpm. After extraction, the suspension was filtered with gauze cloth and centrifuged to separate residual alga. Supernatant was firstly rotary evaporated to remove methanol, and then freeze dried. All extracts were subsequently ground to fine powder and kept at -20 oC for further analysis.

To make a comparison, macroalgae sample was also extracted using conventional solid-liquid extraction at room temperature with agitation for 4 hours. The following separation was same as MAE process.

## 2.3 Determination of total phenolic content (TPC)

Total phenolic content of each seaweed extract was determined according to the method of ([Kaewnarin, Suwannarach, Kumla, & Lumyong, 2016](#_ENREF_17)). 0.1 mL extract was added to 7.9 mL of deionized water, followed by 0.5 mL of Folin-Ciocalteu reagent. 3 minutes later, 1.5 mL 20% NaCO3 was added into the mixture and it was then shaken with a vortex and incubated at room temperature for 1 h. The absorbance of the mixture was measured at 760 nm against a reagent blank. TPC was calculated by the standard curve of gallic acid and expressed as gallic acid equivalent. Absorbance measurement was recorded using a Shimadzu UV-2700 UV-VIS spectrophotometer.

## 2.4 Antioxidant activities

**2.4.1 DPPH free radical scavenging activity**

The DPPH radical scavenging activity of phenolic extract was determined according to the method of ([Xu et al., 2016](#_ENREF_37)) with some modifications. 0.5 mL extract was added to 4.5 mL 0.1 mM methanol solution of DPPH. After 30 min incubation in the dark at ambient temperature, absorbance was measured at 517 nm. The scavenging activity of DPPH radicals was calculated using the following equation:

DPPH scavenging activity (%) = (1 − Asample/Acontrol) ×100 (1)

where Acontrol is absorbance of the methanol solution of DPPH without sample (which was replaced by 70% methanol) and Asample represents absorbance of the methanol solution of DPPH with tested samples.

The DPPH radical scavenging activity was expressed as the Trolox equivalent antioxidant capacity (TEAC) per 100 gram of dry weight (DW).

**2.4.2 ABTS radical scavenging activity**

The ABTS radical scavenging activity of phenolic extract was determined according to the method of ([Giao et al., 2007](#_ENREF_12)). The stock solution of ABTS cation chromophore was prepared by a reaction between a 7mM ABTS solution (100 mL) and 2.45 mM potassium persulphate (final concentration) (100 mL) and was kept in a dark place at an ambient temperature for 16 h. The ABTS radical solution was diluted with phosphate buffer (100 mM, pH 7.4) to an absorbance of 0.70 ± 002 at 734 nm. 0.5 mL of extract was added to 1.5 mL ABTS solution. After 30 min incubation in the dark at ambient temperature, absorbance was measured at 734 nm. The scavenging activity of ABTS radicals was calculated using the following equation:

ABTS scavenging activity (%) = (1 − Asample/Acontrol) ×100 (2)

where A control is absorbance of the ABTS solution without sample (which was replaced by 70% methanol) and A sample represents absorbance of the ABTS solution with tested samples.

Trolox was used as a reference compound, and the ABTS radical scavenging activity was expressed as the Trolox equivalent antioxidant capacity (TEAC) per 100 gram of dry weight (DW).

**2.4.3 Reducing power**

The reducing power was determined according to our previous method ([Yuan & Macquarrie, 2015a](#_ENREF_38)), with slight modifications. 0.5 mL extract was mixed with 0.5 mL of phosphate butter (pH 6.6) and 0.5 mL of 1% potassium ferricyanide. The mixture was incubated at 50◦C for 20 min. The reaction was terminated by the addition of 0.5 mL of 10% trichloroacetic acid (TCA) to the reaction mixture. The solution was then mixed with 1 mL distilled water and 0.4 mL of 0.1% (w/v) ferric chloride, and the absorbance was measured at 700 nm: higher absorbance indicates higher reducing power. Trolox was used as a reference compound, and the reducing power of sample was expressed as the Trolox equivalent antioxidant capacity (TEAC) per 100 gram of dry weight (DW).

## 2.5 Hypoglycaemic assay

**2.5.1 α-Amylase inhibitory activity assay**

The α-amylase inhibitory activity assay was performed using the method of ([Li, Li, You, Fu, & Liu, 2017](#_ENREF_20)). Potato starch solution (1%, w/w) and NaCl solution (6.7 mM) were prepared using 100 mM phosphate-buffered saline (pH 6.9). Porcine pancreatic α-amylase (1 U/mL) was prepared by 6.7 mM NaCl solution. Briefly, 500 uL sample solution (10 mg/mL extract in 3% DMSO) was mixed with 500 uL α-amylase and incubated at 37 oC for 10 min. Then, 500 uL starch solution was added and incubated for another 10 min. The reaction was stopped with 1 mL DNS reagent and kept in a boiling water bath for 5 min. Finally, the resulting mixtures were cooled to room temperature, diluted with 10 mL of deionized water and the absorbance was measured at 520 nm. The solution without a sample (which was replaced by 3% DMSO) was used as the control and acarbose was used as the positive control. The percentage inhibition of α-amylase was calculated as follows:

α-Amylase inhibition rate (%) =[1- (Asample- Abackground/Acontro - Abackground)] × 100 (3)

where A control is absorbance of the control solution without sample (which was replaced by 3% DMSO), Abackground is absorbance of background solution without α-amylase (which was replaced by NaCl solution), and A sample represents absorbance of the tested samples.

**2.5.2 α-Glucosidase inhibitory activity assay**

The α-glucosidase inhibitory activity was measured by the method of ([Li et al., 2017](#_ENREF_20)) with some modifications. α-glucosidase solution (3.92 U/mL) and p-nitrophenyl-α-D-glucopyranoside solution (PNPG: 6 mM) were prepared by 100 mM phosphate buffer saline (pH 6.9). Briefly, 100 μL sample solution (10 mg/mL extract in 3% DMSO) was mixed with 10 μL α-glucosidase and incubated at 37 oC for 10 min. Then 200 μL PNPG was added and incubated for another 20 min. The reaction was terminated by adding 1 mL Na2CO3 and further dilute with 4 mL distilled water. The absorbance was measured at 400 nm. The solution without a sample (which was replaced by 3% DMSO) was used as the control and acarbose was used as the positive control. The percentage inhibition of α-glucosidase was calculated as follows:

α-glucosidase inhibition rate (%) =[1- (Asample- Abackground/Acontrol - Abackground)] × 100 (4)

where A control is absorbance of the control solution without sample (which was replaced by 3% DMSO), Abackground is absorbance of background solution without α-glucosidase (which was replaced by buffer solution), and A sample represents absorbance of the tested samples.

## 2.6 Determination of pancreatic lipase inhibitory activity

The pancreatic lipase activity was measured using 4-methylumbelliferyl oleate (4-MU oleate) as substrate([Kurihara, Asami, Shibata, Fukami, & Tanaka, 2003](#_ENREF_18)). Briefly, 0.25 mL extract (10 mg/mL extract in 3% DMSO) was mixed with 0.5 mL of 0.1 mM 4-MU solution dissolved in a buffer consisting of 13 mM Tris-HCl, 150 mM NaCl, and 1.3 m M CaCl2 (pH 8.0), and then 0.25 mL of lipase solution (1 mg/mL) in the above buffer was added to start the enzyme reaction. After incubation at 25 oC for 30 min, 1 mL of 0.1 M sodium citrate (pH 4.2) was added to stop the reaction. The amount of 4-methylumbelliferone released by the lipase was measured with a F-4600 fluorescence spectrophotometer (Hitachi, Ltd., Japan) at an excitation wavelength of 320 nm and an emission wavelength of 460 nm. The inhibitory activity was expressed as follows:

Pancreatic lipase inhibition rate (%) =[1-(Asample-Abackground/Acontrol-Abackground)]×100 (5)

where A control is absorbance of the control solution without sample (which was replaced by 3% DMSO), Abackground is absorbance of background solution without pancreatic lipase (which was replaced by buffer solution), and A sample represents absorbance of the tested samples.

**2.7 Determination of tyrosinase inhibitory activity**

The monophenolase inhibitory activity of the extract from seaweed was determined by the method of ([Chang, Ding, Tai, & Wu, 2007](#_ENREF_7)) with some modifications. 1 mL extracts (10 mg/mL extract in 3% DMSO) were pre-incubated with 500 uL tyrosinase (268.7 U/mL) at 25 oC for 10 min, then 1 mL 0.5 mM substrate (L-DOPA dissolved in 50 mM phosphate buffer, pH 6.8) were added to initiate the reaction. The assay mixture was incubated at 25 oC for 10 min. Absorbance of the dopachrome product was measured at 475 nm. Kojic acid was used as positive control. Percentage of tyrosinase inhibitory activity was calculated using the following equation:

Tyrosinase inhibitory activity (%) = [1-(Asample-Abackground/Acontrol-Abackground)]×100 (6)

where A control is absorbance of the control solution without sample (which was replaced by 3% DMSO), Abackground is absorbance of background solution without tyrosinase (which was replaced by buffer solution), and A sample represents absorbance of the tested samples.

## 2.8 Phenolic profile by LC-PDA-ESI-MS/MS

The analysis of phenolic compounds was performed with an Alliance 2695 HPLC system (Waters Corp., Milford, MA), equipped with a Hypersil GOLD column (C18, 150 ×2.1 mm, Thermo Scientific). The mobile phase was (A) 0.25% aqueous acetic acid and (B) acetonitrile/water (80/20) containing 0.25% acetic acid with 0.2 mL/min flowrate, 25 °C column temperature and 10 μL injection volume. The following elution gradient was used: 0–20min, 40% B; 20–35 min, 75% B; 35–46 min, 90% B and finally 46–50 min, 40% B.

Mass spectral data were recorded using an amaZon SL quadrupole equipped with an ESI (electro spray ionization) source (Bruker Daltonics, Bremen, Germany). The operating conditions were: temperature 350 °C, nebulizer pressure 25 psi, N2 drying gas flow rate 6.0 L/min, fragmentor voltage 135V, and capillary voltage 4000 V. Full mass scan spectra were recorded in negative ionization mode over the range of m/z 100-1500 Da. The Bruker Daltonics Data Analysis Version 4.0 SP 4 software (Bruker Daltonics, Bremen, Germany) was used for data analysis.

## 2.9 Statistical analysis

Results were presented as means ± standard deviation of three independent determinations. Statistical analyses were determined at p<0.05 by one-way ANOVA followed by a Duncan’s significant test using SPSS 19.0. Correlation among variables were conducted using Pearson’s correlation method with P < 0.01 for significance.

# 3. Results and discussion

## 3.1 Extraction yield and total phenolic content (TPC)

The extraction yield of crude extracts from four algae species is shown inTable 1. The yield of MAE fraction was higher than conventional extraction in all cases, indicating the efficiency of MAE with much shorter extraction time (15 min) than conventional extraction (4 hours). *Laminaria japonica* yielded highest crude extract that was 20.93±0.58%, followed by *Ascophyllum nodosum* (12.46±0.76%), *Lessonia nigrecens* (9.28±0.50%) and *Lessonia trabeculate* (5.22±0.58%), respectively. Agregán et al reported a phenolic yield of 25.86% for *Ascophyllum nodosum* by ultrasound-assisted extraction for 30 mins, which was higher than our work([Agregán et al., 2018](#_ENREF_2)). This might be due to the more polar solvent system they used (ethanol: water 50；50，v:v), as extraction yield was reported to be positively correlated to solvent polarity when extracting bioactive compounds from seaweed species([Agregán, Lorenzo, et al., 2017](#_ENREF_1)). The high yield of *Laminaria japonica* is attributed to simultaneous extraction of mannitol along with phenolic compounds, as *Laminaria japonica* is a well-known resource for extracting mannitol, which is also water/alcohol mixture soluble([Wen-Jie & Fan, 2006](#_ENREF_35)).

The amount of phenolic compounds from biological substances are dependent on macroalgae species and extraction conditions. The total phenolic content (TPC) was determined by the Folin-Ciocalteu method and expressed as milligram gallic acid equivalent (GAE) per 100 gram of dry weight. As shown inTable 1, MAE fractions had higher TPC than conventional fractions for all species, and significant differences were found among different macroalgae species, ranging from 73.13±1.67 to 139.80±10.82 mg GAE/ 100g dry seaweed. The highest amount of TPC was observed in extract of *Ascophyllum nodosum*, which is in agreement with previous study that higher levels of TPC were found in Fucales macroalgae species([Wang, Jonsdottir, & Olafsdottir, 2009](#_ENREF_34)). There is no previous data on phenolic compounds of *Lessonia nigrecens* and *Lessonia trabeculate*, therefore, this work could provide a rough idea of TPC levels in above two macroalgae species. It is worth mentioning that MAE condition in this work was 110 oC, which was actually at elevated temperature and pressure of liquid solvent. Although it was pointed out that phenolic compounds in algae seem to be particularly sensible to heating exposure([Agregán, Munekata, et al., 2017](#_ENREF_3)), the results in our work demonstrated that MAE in short time at elevated temperature could not only enhance extraction yield, but also effectively avoid degradation of phenolic compounds.

## 3.2 Antioxidant capacities

In human bodies, oxidation is an essential process to produce energy, however, reactive oxygen species (ROS) formed under oxidative stress conditions in human cells results in oxidative damage which may contribute to the development of a variety of chronic disease including coronary heart disease, rheumatoid arthritis, chronic inflammatory disease of the gastrointestinal tract, Alzheimer disease and other neurological disorders associated with the ageing processes ([Heffernan et al., 2015](#_ENREF_15)). Marine algae has been considered as a potential resource of natural antioxidant as a consequence of dynamic environmental conditions of their habitat. Many research has been carried out on the antioxidant of phenolic compounds from brown algae, which generally contained higher amounts of polyphenols than red and green algae([Farvin & Jacobsen, 2013](#_ENREF_11); [Heffernan, Smyth, FitzGerald, Soler-Vila, & Brunton, 2014](#_ENREF_14); [Heffernan et al., 2015](#_ENREF_15); [Wang et al., 2009](#_ENREF_34)). In this work, antioxidant activities of phenolic extracts were measured by DPPH, ABTS free radical scavenging ability and reducing power assay. Antioxidant capacities of the extracts was expressed as milligram Trolox equivalent antioxidant capacity (TEAC)/ 100 g dry seaweed.

**3.2.1 DPPH free radical scavenging ability**

**Fig. 1**A shows the effect of different extracts on the DPPH free radical scavenging ability of four algae species. The highest DPPH scavenging ability was achieved with the MAE extract of *Ascophyllum nodosum*, which was about 2.7 times higher than its conventional extracts. In comparison, the MAE extracts of other three algae species were slightly higher than conventional extracts. The higher DPPH radical scavenging activity of fractions from MAE implies that MAE technology could effectively avoid the decomposition of bioactive compounds. Pearson correlation analysis was conducted to analyse the correlation between antioxidant activity and phenolic substances. A moderate coefficient was obtained (r=0.702, P ＜0.01, n=24) between the phenolic content and DPPH free radical scavenging activity, indicating some specific active compounds in the extracts that could have impact on DPPH free radical scavenging capacity. For instance, small molecular weight polysaccharides present in the extracts may influence the activity. In addition, the number and location of the hydroxyl groups of phenolic compounds also have impact of the free radical scavenging capacity. It has reported that compounds with the second hydroxyl group in the ortho or para position have higher activity than when it is in meta position([Farvin & Jacobsen, 2013](#_ENREF_11)).

**3.2.2 ABTS free radical scavenging ability**

. **Fig. 1**B presents the effect of different extracts on the ABTS free radical scavenging ability of four algae species. MAE fractions of all four species had higher ABTS free radical scavenging ability than the conventional fractions, with the highest from *Lessonia nigrecens* (95.13±1.42 mg TEAC/100 g dry seaweed). Interestingly, the TPC of *Lessonia nigrecens* was less than *Ascophyllum nodosum* , whereas the ABTS free radical scavenging ability of *Lessonia nigrecens* was better than that of *Ascophyllum nodosum*. This indicates that this species contain some very efficient compounds which are responsible for its high ABTS scavenging activity. Similarly, MAE fraction of *Ascophyllum nodosum* exhibited significantly higher ABTS free radical scavenging ability than conventional fraction, indicating that MAE could effectively enhance the extraction of phenolic compounds responsible for free radical scavenging in *Ascophyllum nodosum.* Correlation analysis indicated that correlation between phenolic content and ABTS free radical scavenging activity was strong ( r=0.815, P ＜0.01, n=24), suggesting that the phenolic compound contents of seaweed extracts are associated with their ABTS scavenging activity.

**3.2.3 Reducing power assay**

In this assay, the yellow color of test solution would change into green and blue colors when the reductant in the test sample reduces Fe3+/ferricyanide complex to the ferrous form (Fe2+)([Yuan & Macquarrie, 2015a](#_ENREF_38)). The reductive capabilities of extracts of four algae species are shown in **Fig. 1**C. Among the various extracts, MAE extracts of *Ascophyllum nodosum* had the highest reducing power (75.23±5.41 mg TEAC/100 g dry seaweed), followed by MAE fractions of *Lessonia nigrecens* (63.78±7.11), *Laminaria japonica* (58.52±9.49) and *Lessonia trabeculate* (56.54±7.35), respectively. In agreement with previous study ([Heffernan et al., 2014](#_ENREF_14)), the *Fucus* species exhibited the highest ferric reducing power activity. The correlationbetweenthe phenolic content and reducing power was 0.782 (P ＜0.01, n=24).

In general, brown algae have better antioxidant activities than red and green seaweed, and *Fucus* species are better antioxidants. Our results also showed that phenolic compounds from *Ascophyllum nodosum* exhibited effective antioxidant activities with regard to the scavenging of free radicals and reducing power, suggesting *Ascophyllum nodosum* could potentially be a resource for natural antioxidants. Interestingly, Heffernan *et al*’s research demonstrated that the high temperatures and pressures in pressurized liquid extraction (PLE) did not enhance the antioxidant activities relative to conventional solid-liquid extraction (SLE) ([Heffernan et al., 2014](#_ENREF_14)), however, microwave assisted extraction with high temperature and pressure in this work efficiently enhanced the antioxidant activities compared to conventional methods, indicating MAE a prior method to extract phenolic compounds from seaweed.

**Fig. 1.** Antioxidant activities of polyphenol extracts of different brow algae measured by (A) DPPH, (B) ABTS, (C) Reducing power assay. The results were expressed as mean valueSD (n = 3). Different letters within the same figure mean statistical difference (p<0.05).

## 3.3 Inhibitory effects on α-amylase and α-glucosidase activities

Diabetes is a group of metabolic diseases in which there are high blood sugar levels over a prolonged period. Prolonged hyperglycaemia in diabetic patients contributes to diabetic complications, such as atherosclerosis and cardiovascular disease ([Kaewnarin et al., 2016](#_ENREF_17)). Type 2 diabetes is generally caused by a number of lifestyle-related risk factors including obesity, smoking, poor diet and physical inactivity ([Boath, Stewart, & McDougall, 2012](#_ENREF_4)), and can be managed by using drugs to delay or prevent the absorption of glucose from meals. The digestive enzymes, α-amylase and α-glucosidase, are the key enzymes in the breakdown of carbohydrate into glucose before its subsequent uptake into the bloodstream. The common used drugs for providing inhibition of enzymes includes miglitol, voglibose and acarbose, however, these drugs can cause side-effects such as abdominal discomfit, flatulence, and diarrhea which reduce patient compliance and treatment effectiveness([Pantidos et al., 2014](#_ENREF_26)). Thus, it is necessary to explore the natural inhibitors that could replace these drugs.

The inhibitory effects of phenolic extracts from four algae on α-amylase activity are shown in **Fig. 2**A. MAE fractions of all species exhibited better α-amylase activity than conventional fraction. The highest inhibition performance was extracts from *Lessonia trabeculate*, with 69.75±3.49% of MAE fraction and 34.69±2.31% of conventional fraction, and this was much higher than the rest three species. In comparison with positive control acarbose which showed IC50 value of 0.42 mg/mL, the inhibition of extracts from *Lessonia trabeculate* was relatively less effective, suggesting the necessity of further separation and purification of crude extracts.

**Fig. 2**B displayed the inhibitory effects of phenolic extracts from four algae species on α-glucosidase activity. It can be seen that extracts (both MAE fraction and conventional fraction) from *Lessonia trabeculate* showed 100% α-glucosidase activity at 10 mg/mL, followed by extracts from *Ascophyllum nodosum* (MAE fraction 84.12±0.19%, convention fraction 77.24±0.26%) and *Laminaria japonica* (MAE fraction 55.28±1.75%, convention fraction 14.77±3.62%), while extracts from *Lessonia nigrecens* showed no α-glucosidase activity at all. To accurately compare with acarbose, extracts of *Lessonia trabeculate* were further diluted for α-glucosidase activity test, and the results were shown in **Fig. 2**C. Out of expectation, MAE fraction of *Lessonia trabeculate* exhibited extremely good α-glucosidase activity, with IC50 value of 0.36 mg /mL, which was more effective than pharmaceutical inhibitor, acarbose (IC50 1.40 mg/mL). The inhibition effect of conventional fraction was slightly lower than acarbose, with IC50 value of 1.66 mg/ml.

There has been many research on the anti-hyperglycemic effects of phenolic extracts from variety of natural resources, including berry fruits, mushrooms, tea leaves as well as marine algae([Boath et al., 2012](#_ENREF_4); [Kaewnarin et al., 2016](#_ENREF_17); [McDougall et al., 2005](#_ENREF_23)). Among algae resources, extensive study has focused on *Ecklonia* species and *Ascophyllum nodosum,* in which the phlorotannins components have been demonstrated to show promising anti-hyperglycemic effects ([Lee & Jeon, 2013](#_ENREF_19); [Pantidos et al., 2014](#_ENREF_26)). However, no research has been done on *Lessonia* species about their bioactivities despite the traditional utilization for alginate production. Therefore, the results of this work enrich the scientific data of *Lessonia* species and suggest the potential pharmaceutical value of *Lessonia trabeculate* to be explored as anti-hyperglycemic reagent.

**Fig. 2.** (A)α-amylase inhibitory activities of different extracts of four brown algae species, (B) α-glucosidase inhibitory activities of extracts from different brown algae species, (C) α-glucosidase inhibitory activities of extract from LT with different concentrations. The results were expressed as mean valueSD (n = 3). Different letters within the same figure mean statistical difference (p<0.05).

## 3.4 Pancreatic lipase inhibition activity

Obesity is a key risk factor for some metabolic syndromes, such as cardiovascular disease, hypertension, and diabetes([C. Zhang et al., 2018](#_ENREF_42)). Pancreatic lipase is a critical enzyme responsible for digestion and absorption of 50-70% dietary triacylglycerol (TG) in the intestinal lumen([Patil, Patil, Bhadane, Mohammad, & Maheshwari, 2017](#_ENREF_27)). Inhibition of lipase has been considered as an effective way to treat obesity, and investigation of natural products for anti-obesity has recently become a research hotspot.

The inhibitory effects of phenolic extracts from four algae on pancreatic lipase activity are shown in **Fig. 3**. Among the four species, extracts from *Lessonia trabeculate* has the highest inhibition performance, with 70.41±4.80% of MAE fraction and 36.80±4.42% of conventional fraction. In comparison with positive control orlistat, a commercial pancreatic lipase inhibitor which showed IC50 value of 0.08 mg/mL, the inhibition effect of extracts from *Lessonia trabeculate* was relatively less effective. Previous research has indicated that polyphenol-rich extracts from tea, legumes and fruit can inhibit the lipase activity ([Glisan, Grove, Yennawar, & Lambert, 2017](#_ENREF_13); [B. Zhang et al., 2015](#_ENREF_41); [C. Zhang et al., 2018](#_ENREF_42)). The increasing phenolic hydroxyl group in polyphenols could increase their binding affinities and inhibition for lipase([Wu et al., 2017](#_ENREF_36)). However, few research has been done about lipase inhibition effect of phenolic extracts from algae resources. Only extracts from *Ascophyllum nodosum* has been reported to inhibited pancreatic lipase activity and the phlorotannin-enriched fraction was more potent([Ceri Austin, 2018](#_ENREF_5)).

**Fig. 3.** Pancreatic lipase inhibitory activities of different extracts of four brown algae species. The results were expressed as mean valueSD (n = 3). Different letters within the same figure mean statistical difference (p<0.05).

## 3.5 Tyrosinase inhibition activity

Tyrosinase is a type-3 copper protein with a dinuclear copper active site and is involved in melanin formation. The tyrosinase inhibitors have been used for the suppression of undesirable enzymatic browning in food products such as fruits and vegetables, to keep their color and sensory properties, extend shelf life, increase market value, and reduce the loss of nutritional value during postharvest peocess ([L. Zhang, Zhao, Tao, Chen, & Zheng, 2017](#_ENREF_43)). In this study, phenolic extracts of four macroalgae were evaluated for the tyrosinase inhibition activity. The results showed that only the extract of *Lessonia trabeculate* inhibited tyrosinase activity, with MAE fraction of 33.73% and RT fraction of 12.36% at concentration of 10 mg/mL(data not shown). Compared with kojic acid, a well-known tyrosinase inhibitor which showed 100% inhibition at 1 mg/mL, extracts from *Lessonia trabeculate* was relatively low. Many flavonoids from fruit, vegetables, spices, tea and traditional herbal medicine have been identified as tyrosinase inhibitors([L. Zhang et al., 2017](#_ENREF_43)), however, no information is available about tyrosinase inhibition activity of extracts from algae resources. The results of this work may open up a new sight for exploitation of natural tyrosinase inhibitor from algae resources.

## 3.6 Tentative identification of phenolic compounds of brown algae extracts

Compared with terrestrial phenolic compounds (e.g. flavanols, flavones and phenolic acid), less knowledge exists about characterization of complex mixture of algae polyphenols and their potential health benefits. The phenolic profile of extracts from four algae species were analysed by HPLC-PAD-ESI-MS and chromatogram are shown in Fig.4. 17 peaks were observed and some of the compounds were tentatively identified by related references (**Table 2**). The UV profile showed the occurrence of compounds with absorption bands at 210-270 nm, corresponding to typical phenolics ([Rajauria et al., 2016](#_ENREF_29)). As can be seen, the major components of extracts include phenolic acid derivatives (peak 1, 2, 14 and 15), phlorotannin derivatives (peak 6 and 7) and catechins derivatives (peak 10, 11, 13 and 16). Peak 1 exhibited a molecular ion [M-H]- at m/z 343, and a fragment ion [F-H]- at m/z 137 corresponding to hydroxybenzoic acid, which was also reported by Agregan et al. in *Ascophyllum nodosum*([Agregán, Munekata, et al., 2017](#_ENREF_3))*,* therefore, this compound was tentatively identified as hydroxybenzoic acid derivative. A fragment ion [F-H]- at m/z 163 corresponding to *p*-coumaric acid was observed for peak 2 and 15, suggesting *p*-coumaric acid derivatives, which was detected in *Fucus vesiculosus* previously ([Agregán, Munekata, et al., 2017](#_ENREF_3)). Peak 14 exhibited a fragment ion [F-H]- at m/z 153 corresponding to dihydroxybenzoic acid. Peak 6 showed a negative molecular ion [M-H]- at m/z 755, and fragment ions [M-18]- at m/z 737, [M-144]- at m/z 611, [M-286]- at m/z 469, while peak 7 showed a negative molecular ion [M-H]- at m/z 297, and fragment ions [M-18]- at m/z 279, [M-126]- at m/z 171, the same as those of phlorotannin oligomers reported in *Fucus spp* ([Lopes et al., 2018](#_ENREF_21)). Therefore, peak 6 and 7 were tentatively identified as phlorotannin hexamer derivative and phlorotannin dimer derivative, respectively. Peak 10, 11, 13 and 16 exhibited similar fragment ions, among which [F-H]– at m/z 304 was characteristically matching epigallocatechin, which was reported to exist in red macroalgae (*Palmaria spp.* and *Porphyra spp.*) brown macroalgae (*Himanthalia elongata* and *Laminaria orchroleuca*)([Rodríguez-Bernaldo de Quirós, Lage-Yusty, & López-Hernández, 2010](#_ENREF_30)), therefore, the four peaks were tentatively identified as gallocatechin derivatives.

A variety of phenolic compounds isolated from terrestrial plants have been identified to show enzyme inhibition activities. p-Hydroxybenzoic acid was reported to give IC50 values of 1.94 mg/mL, 89.47 ug/mL and 1.25 mg/mL for α-amylase, α-glucosidase and lipase, respectively([Tan, Chang, & Zhang, 2017](#_ENREF_31)). (−)-epigallocatechin-3-gallate (EGCG) from green tea inhibited pancreatic lipase *in vitro* with IC50= 7.5 umol/L, while (−)-epigallocatechin, which has no galloyl ester, was ineffective([Glisan et al., 2017](#_ENREF_13)). However, few work has been done about enzyme inhibition activity of phenolic compounds from algae resources, many previous work focus on the utilization of marine polyphenols as antioxidant reagents([Chakraborty et al., 2015](#_ENREF_6); [Heffernan et al., 2015](#_ENREF_15)). Most studied phenolic compound from algae is phlorotannins, which is related to antioxidant, anti-bacterial and anti-adipogenic activities([Montero et al., 2016](#_ENREF_24)). Recently, it is reported that phlorotannins from *Ascophyllum nodosum* show α-amylase, α-glucosidase and lipase inhibition effect ([Ceri Austin, 2018](#_ENREF_5); [Pantidos et al., 2014](#_ENREF_26)). The results of this work indicated that phenolic compound from *Lessonia trabeculate* exhibited superior enzyme inhibition activity than *Ascophyllum nodosum*, suggesting the presence of some specific active compounds. It was noted that peak 4, peak 9 and peak 13 only existed in the extracts of *Lessonia trabeculate,* which are possible compounds responsible for the bioactivity, therefore, further isolation and identification of these compounds need to be carried out.



**Fig. 4**. Chromatogram of phenolic compounds (MAE fractions) from four brown algae species by liquid chromatography-diode array detection (LC-DAD)

# 4. Conclusion

In the present study, microwave assisted extraction technology was successfully applied to extract phenolic compounds from four brown algae species with higher yield and shorter time compared with conventional extraction method. According to HPLC-DAD-ESI-MS analysis, phenolic acid derivatives, phlorotannin derivatives and gallocatechin derivatives were major components in the extracts. Antioxidant test indicated that extracts from MAE of four species all exhibited higher DPPH, ABTS free radical scavenging ability and reducing power than conventional method, with the best antioxidant activities observed in the extracts of *Ascophyllum nodosum*. The extract of *Lessonia trabeculate* exhibited good α-amylase, α-glucosidase, pancreatic lipase and tyrosinase inhibition activities, especially the MAE fraction showed even better α-glucosidase inhibitory activity than acarbose. Results obtained from this study may help to exploit the use of macroalgae, especially the *Lessonia trabeculate*, as a natural resources for functional food and nutraceutical ingredients. Future work will be carried out on purification and separation of crude extract to enhance the bioactive performance and to identify the structure of individual compounds responsible for the biological activities. Moreover, the toxicity, safety, side effects and other concerned issues will be investigated in animals to facilitate the application of algae extract as real products.

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**Table 1.** **Extraction yields and total phenolic content of extracts from different brown algae**

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **Species** | **Taxonomy** | **Extraction yield (%)** | | **TPC ( GAE mg/100 g dry seaweed)** | |
| **RT** | **MW** | **RT** | **MW** |
| *Lessonia trabeculate* (LT) | Laminariales, Lessoniaceae | 4.55± 0.31f | 5.22±0.18f | 49.80±5.68ef | 74.13± 5.10cd |
| *Lessonia nigrecens* (LN) | Laminariales, Lessoniaceae | 7.35 ± 0.20e | 9.28±0.50d | 78.13 ±5.60c | 107.13± 4.41b |
| *Ascophyllum nodosum* (AN) | Fucales, Fucaceae | 10.41±0.24d | 12.46±0.76c | 51.47 ±3.28def | 139.80± 10.82a |
| *Laminaria japonica* (LJ) | Laminariales, Laminariaceae | 17.07± 0.02b | 20.93± 0.58a | 38.47 ±0.88f | 73.13± 1.67cde |

\* The results were expressed as mean value ± SD (n = 3). Different letters within the same figure mean statistical difference (p<0.05).

**Table 2. Identification of phenolic compounds of brown algae extracts**

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
| Peak | RT (min) | Proposed compound | λmax （nm） | [M-H]- (m/z) | Product ions (m/z) | Detected in | Ref |
| 1 | 2.4 | hydroxybenzoic acid derivative | ND | 343.0 | **200.7,**137.0 | LF, LN, AN, LJ | ([Agregán, Munekata, et al., 2017](#_ENREF_3)) |
| 2 | 2.9 | *p*-coumaric acid derivative | ND | 216.8 | **180.7,**162.8,118.9 | LF, LN, AN, LJ | ([Hossain, Rai, Brunton, Martin-Diana, & Barry-Ryan, 2010](#_ENREF_16)) |
| 3 | 24.9 | apigenin | 224 | 269.1 | **250.9**,170.8,154.8 | LN | ([Nagy, Solar, Sontag, & Koenig, 2011](#_ENREF_25)) |
| 4 | 27.4 | NI | 225 | 255.1 | 250.6,236.9,170.6 | LF |  |
| 5 | 28.1 | medioresinol derivative | 225 | 527.2 | 446.9,387.0,**224.7**,206.6 | AN | ([Hossain et al., 2010](#_ENREF_16)) |
| 6 | 29.3 | phlorotannin hexamer derivative | 226, 263 | 755.0 | 737.0**,712.9**,694.9,610.6,468.6 | LT, LN, AN, LJ | ([Lopes et al., 2018](#_ENREF_21)) |
| 7 | 30.8 | phlorotannin dimer derivative | 225, 263 | 297.2 | **279.0**,216.7,170.8,154.8 | LN, AN, LJ | ([Lopes et al., 2018](#_ENREF_21)) |
| 8 | 33.8 | NI | 225, 261 | 479.2 | **419.2,**396.9,255.0 | LN, LJ |  |
| 9 | 34.8 | NI | 226, 261 | 281.8 | **200.7**, 116.9 | LT |  |
| 10 | 35.6 | gallocatechin derivative | 226, 267 | 555.2 | 389.2, 304.8, 298.8, 255.0, **224.7,** | AN | ([Rodríguez-Bernaldo de Quirós et al., 2010](#_ENREF_30)) |
| 11 | 36.7 | gallocatechin derivative | 226, 267 | 555.2 | 472.8,389.2,304.8,298.8,254.9,**224.7**,164.7 | LT, LN, AN, LJ | ([Rodríguez-Bernaldo de Quirós et al., 2010](#_ENREF_30)) |
| 12 | 38.2 | NI | 225. 266 | 417.2 | **334.8**,252.6,197.6 | LT |  |
| 13 | 39.5 | gallocatechin derivative | 225, 265 | 581.2 | 414.8,298.8, **224.7** | LT | ([Rodríguez-Bernaldo de Quirós et al., 2010](#_ENREF_30)) |
| 14 | 39.5 | Dihydroxybenzoic acid | 225, 265 | 483.2 | **255.0**, 226.7,152.7 | LN, AN, LJ | ([B. Zhang et al., 2015](#_ENREF_41)) |
| 15 | 43.6 | *p*-coumaric acid derivative | 225, 265 | 339.2 | **162.8** | LT, LN, AN, LJ | ([Hossain et al., 2010](#_ENREF_16)) |
| 16 | 44.1 | gallocatechin derivative | 225, 265 | 389.2 | **304.8**, 222.7, 140.7 | LT, LN, AN, LJ | ([Rodríguez-Bernaldo de Quirós et al., 2010](#_ENREF_30)) |
| 17 | 44.5 | NI | 225, 266 | 303.1 | **259**.0, 204.9 | LT, LN, LJ |  |

RT: retention time; [M-H]-: molecular ion; NI: not identified

The most abundant ions observed in mass spectra are shown in bold.

1. \*Corresponding authors:

   Peili Shen: spl@bmscn.com +86(0)18669826108

   Yiqiang Li: liyiqiang@caas.cn +86(0)53266715597

   Chengsheng Zhang: zhangchengsheng@caas.cn +86(0)53288702115 [↑](#footnote-ref-1)