Research article

Phytochemicals, α-glucosidase and α-amylase Inhibitory Efficiency of Brittle Wort (*Chara corallina*) Extract

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Abstract

This research is the first to evaluate brittle wort (*Chara corallina*) extract for phytochemical content and inhibitory effects against α-glucosidase and α-amylase activities. An ethanolic extract of *C. corallina* had the strongest enzyme inhibitory effect. The inhibition efficiencies of all extracts against α-amylase were more potent than those against α-glucosidase, with IC_{50} values of 8.43±0.18 mg/mL against α-glucosidase and 1.78±0.07 against α-amylase. Inhibition efficiency was related to total phenolic content, DPPH radical scavenging activity, ATBS radical scavenging activity and metal-chelation activity of the extracts. Phytochemical screening of the ethanolic extract demonstrated the presence of the triterpenoid β-sitosterol. These findings provide useful supporting data for further applications of *C. corallina* extract.

1. Introduction

Algae are used as traditional foods and locally processed food products due to their many health benefits such as antioxidant, anti-inflammatory, antibacterial, antifungal, antitumour, anticancer, anti-atherosclerosis and anti-ageing activities [1-3]. Many algae, e.g., *Polyopes lancifolia* [4], *Sargassum patens*, *Fucus vesiculosus* [5] and *Ascophyllum nodosum* [6], have antidiabetic properties. Algae contain many essential minerals, vitamins, phenolics, flavonoids, tannins and other bioactive ingredients. The well-known brown seaweed *Sargassum* sp. and carrageenan from the red algae *Chondrus crispus*, were investigated for their phytochemical and pharmaceutical properties [7, 8]. However, the phytochemical and antioxidant activities of many groups of local indigenous edible algae, which may have pharmaceutical applications, have

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not yet been examined. Brittle wort or *Chara corallina* Klein ex Willdenow is the freshwater multicellular macroalgae, generally found in Krabi Province, Thailand [9]. It belongs to the Characeae family. Its thallus may be shorter or longer than 1 m and its rhizoids attach to substrates such as soil, sand, or muddy ground in shallow water [10]. *Chara corallina* has whorls of leaf-like branchlets growing from nodes [11]. It is eaten with chilli sauce or processed as an herbal tea or dried crispy furikake because of traditional belief in its many nutritional properties [12, 13]. However, data concerning its phytochemical and pharmaceutical activities are lacking. High content of essential amino acids, vitamins and minerals; especially methionine, arginine, lysine, folic acid (vitamin B9), ascorbic acid (vitamin C), potassium, magnesium, calcium, iron and selenium were reported by Chankaew *et al.* [14]. The author also noted that *C. corallina* was consumed as a traditional food to heal diabetes mellitus and ameliorate excessive accumulation of glucose in the blood. A previous study found that an ethyl acetate extract of *C. corallina* showed high content of total phenolic compounds, tannins, and DPPH, and had promising ATBS radical scavenging activity and metal-chelation activity [12]. To date, the inhibitory efficiency of *C. corallina* extract on the enzymes α-glucosidase and α-amylase to confirm diabetes treatment potential has not been examined. These enzymes play an important role by digesting starch to reduce the absorption of glucose by blood vessels, resulting in a decrease in blood glucose level [15]. The long-term use of the antidiabetic drug acarbose may have undesirable side effects and a natural enzyme inhibitor may reduce these traits. Therefore, this research investigated the antidiabetic properties, antioxidative activities and antioxidant content of *C. corallina* extract. Phytochemicals screening of the extract was also examined.

2. Materials and Methods

2.1 Algae material preparation and extraction

Chara corallina samples were collected by cutting their thallus from the substrate in clearly natural watershed in Nuea Khlong District, Krabi Province, Thailand and authenticated by the Department of Fisheries, Faculty of Agriculture, Rajamangala University of Technology Srivijaya. The samples were then cleaned, dried, ground to powder and stored at 4°C before extraction. Traditional solid-liquid extraction was employed to extract each 100 g of *C. corallina* dried samples with three different solvents: hexane extract (HE), water extract (WE) and 70%v/v ethanol extract (EE) at a ratio of 1:25 w/v dried sample:solvent. The extracts were filtered and concentrated using a rotary evaporator (for ethanolic and hexane extracts) and freeze-dryer (for water extract) to give the crude extract.

2.2 Chemicals

All chemicals used in this research were of analytical reagent grade. Aluminium chloride ($AlCl_3$), soluble starch and sodium carbonate (Na_2CO_3) were products of Univar Ajax Finechem (New Zealand). Tannic acid and *p*-nitrophenyl-α-D-glucopyranoside (PNPG) were products of Alfa-Aesar (UK). Acarbose, dinitrosalicylic acid (DNS), α-glucosidase from *Saccharomyces cerevisiae* (19.3 units/mg solid), DPPH, trolox, ATBS solution, ferrozine, ferrous chloride ($FeCl_2$), quercetin and gallic acid were purchased from Sigma-Aldrich (USA). Folin-Ciocalteu phenol reagent, benzene and α-amylase from malt-diastase (minimum activity 1:2,000 I.P.unit) were purchased from Loba Chemie (India). Sodium hydroxide (NaOH), sodium chloride (NaCl), sodium nitrite

($NaNO_2$), monosodium phosphate dihydrate ($NaH_2PO_4.2H_2O$), ethylene diamine tetraacetic acid (EDTA) and disodium phosphate (Na_2HPO_4) were obtained from Univar Ajax Finechem (Australia). Ammonia solution was product of QRëC (New Zealand). Sulfuric acid (H_2SO_4) and chloroform were purchased from RCI Labscan (Ireland). Dragendorff's reagent, methanol, ethanol, hexane and potassium persulfate ($K_2S_2O_8$) were products of Merck (Germany).

2.3 Enzyme inhibition assay

Enzyme inhibition assays were investigated in triplicate as follows:

The α-glucosidase inhibition activity was performed using PNPG as the substrate, which was an adaptation from Srimoon *et al.* [15]. The reaction mixture contained 0.20 mL of 0.1 M phosphate buffer pH 6.9, 1.0 mL of 0-5.0 mg/mL extract (HE, WE and EE) or 0-1.0 mg/mL standard acarbose or 0.1 M phosphate buffer pH 6.9 and 0.2 mL of 1.0 unit/mL α-glucosidase. Each mixture was incubated at 37ºC for 10 min and then 0.2 mL of 0.5 mM PNPG was added with incubation at 37ºC for a further 30 min. Finally, the reaction was stopped by adding 1.40 mL of 0.67 M Na_2CO_3 and the absorbance at 405 nm was measured. Percentage of inhibition and the concentration of the extract that inhibited 50% of α-glucosidase activity (IC_{50}) were calculated using graphical plot of percentage of inhibition against sample concentration. Acarbose was used as the standard.

The α-amylase inhibition activity was performed following the method of Srimoon *et al.* [15] using soluble starch as the substrate. A volume of 0.25 mL of 0.1 M phosphate buffer containing 6.7 mM NaCl (PBS) pH 6.9 was mixed with 0.25 mL of 0-1.0 mg/mL extract (HE, WE and EE) or 0-0.1 mg/mL standard acarbose or 0.1 M PBS pH 6.9. Then, 0.25 mL of 0.15 unit/mL α-amylase was added and the mixture was incubated at 37ºC for 10 min. To start the reaction, 1% soluble starch was added with incubation at 37ºC for 30 min. The reaction was stopped using 0.5 mL of DNS followed by incubation at 100ºC for 5 min. The mixture was cooled at room temperature. Finally, 1.50 mL of distilled water was added before measuring the absorbance at 540 nm. Percentage of inhibition and IC_{50} of α-amylase were calculated. Acarbose was used as the standard.

2.4 Phytochemical content and antioxidant activity assay

Phytochemical content and antioxidant activity assays were done in triplicate as follows:

Total phenolic content of the extract was determined using the Folin-Ciocalteu phenol reagent method following Wong *et al.* [16]. An aliquot of 2.0 mL of 0.1 mg/mL of each extract (HE, WE and EE) or standard gallic acid was mixed with 5.0 mL of 10% Folin-Ciocalteu phenol reagent and shaken for 3 min before adding 2.0 mL of 7.5% Na_2CO_3. The absorbance at 765 nm was measured after one hour. Total phenolic content was expressed as mg gallic acid equivalent.

Total flavonoid content of the extract was analysed using the aluminium chloride method adapted from Malla *et al.* [17]. An aliquot of 0.5 mL of 0.1 mg/mL of each extract (HE, WE and EE) or standard quercetin was mixed with 2.0 mL of distilled water and 0.15 mL of 5% $NaNO_2$. After standing for 6 min, 0.15 mL of 10% $AlCl_3$ was added and left for 6 min. Then, 2.0 mL of 4% NaOH was added and the mixture was diluted to 5.0 mL. The absorbance of the solution was read at 510 nm. Total flavonoid content was expressed as mg quercetin equivalent.

Total tannin content of the extract was estimated using the Folin-Ciocalteu phenol reagent method modified from Shad *et al.* [18]. An aliquot of 2.0 mL of 0.1 mg/mL of each extract (HE, WE and EE) or standard tannic acid was mixed with 1.0 mL of 10% Folin-Ciocalteu phenol reagent, 2.0 mL of saturated Na_2CO_3 and 5.0 mL of distilled water. After standing for 1 h, the absorbance of the solution was measured at 725 nm. Total tannin content was expressed as mg tannic acid equivalent.

DPPH was determined according to Shimada *et al.* [19]. An aliquot of 0.1 mg/mL of each extract (HE, WE and EE) or standard Trolox was mixed with 4.5 mL of 0.04 mg/mL DPPH solution and 0.5 mL of distilled water. The mixture was shaken for 20 min and then measured the absorbance at 517 nm. The IC_{50} value or the concentration of the extract that inhibited 50% of DPPH radicals was evaluated by comparison with standard trolox.

ATBS radical scavenging activity was carried out as described by Re *et al*. [20]. A 2.0 mL aliquot of 7 mM ATBS solution was mixed with 35.5 µL of 140 mM $K_2S_2O_8$ and left in the dark for 16 h to generate the blue-green ABTS radical cation. The ATBS radical stock solution was diluted to give an absorbance of 0.70±0.05 at 734 nm. An aliquot of 0.1 mL of 0.1 mg/mL of each extract (HE, WE and EE) or standard trolox was mixed with 0.9 mL of ATBS radical solution, followed by incubation for 6 min at room temperature. The absorbance at 734 nm was measured and the IC_{50} value was evaluated in comparison with standard trolox.

The metal-chelation activity was performed according to Dinis *et al*. [21]. An aliquot of 0.8 mL of 0.1 mg/mL of each extract (HE, WE and EE) or standard EDTA was mixed with 0.02 mL of 5 mM ferrozine and 0.01 mL of 2 mM $FeCl_2$. The solution mixture was rapidly shaken and incubated for 10 min at room temperature. The absorbance at 562 nm was measured and the IC_{50} value was evaluated in comparison with standard EDTA.

2.5 Phytochemical screening of the extract

The ethanolic extract of *C. corallina* was screened to determine the presence of alkaloids, anthraquinones, terpenoids, steroids and glycosides according to Gul *et al.* [22]. The presence of alkaloids was tested using Dragendorff's reagent, anthraquinones was tested using benzene and 10% ammonia solution, terpenoids was tested using chloroform and boiling with H_2SO_4, steroids was tested with chloroform and H_2SO_4, and glycosides with Liebermann's test, Keller-Kiliani test and Salkowski's test. Preliminary isolation and purification of the extract were investigated according to Yahya *et al.* [23]. The isolated and purified fraction was subjected to the ^1H NMR in $CDCl_3$ at 500 MHz to investigate the structure.

2.6 Statistical analysis

Results were evaluated by analysis of variance (ANOVA) and presented as mean±sd of triplicate. The differences among samples were estimated using Duncan's multiple range test at 0.05 level of significance of triplicate. Correlation between data was performed using Pearsons' correlation coefficient of triplicate.

3. Results and Discussion

3.1 The α-glucosidase and α-amylase inhibitory efficiency

The percentage extraction yield of hexane extract (HE), ethanolic extract (EE) and water extract (WE) were 3.69, 29.13 and 26.1, respectively. Ethanol was the most appropriate solvent for *C. corallina* extraction with the highest extraction yield while hexane gave the lowest extraction yield. Therefore, ethanol was used as the extraction solvent for the experiment.

The α-glucosidase and α-amylase inhibitory efficiencies of *C. corallina* extract are shown in Table 1. The inhibition efficiencies of the ethanolic extract were significantly higher than those of water extract and hexane extract ($p<0.05$) with IC_{50} values 8.43±0.18 and 1.78±0.07 mg/mL for

Table 1. The α-glucosidase and α-amylase inhibitory efficiency of *Chara corallina* extracts

Enzyme inhibition efficiency	IC_{50} (mg/mL)			
	Standard Acarbose	Extract		
		Hexane	Ethanol	Water
α-glucosidase	0.39±0.02[a]	11.80±1.21[b]	8.43±0.18[c]	9.54±0.79[d]
α-amylase	0.07±0.0006[a]	3.51±0.19[b]	1.78±0.07[c]	2.27±0.50[d]

Superscripts ([a,b,c,d]) in each row indicate significant differences ($p<0.05$).

α-glucosidase and α-amylase, respectively. Inhibition efficiency of *C. corallina* extract against α-amylase was found to be more potent than it was against α-glucosidase. However, the inhibition of the extract against the enzymes was lower than the synthetic drug acarbose. This finding was similar to the inhibition of *Ventilago denticulata* Willd. dried leaves extract against α-glucosidase and α-amylase reported by Srimoon *et al.* [15]. They found that the inhibitory efficiency of the extract was lower than the standard acarbose and α-amylase inhibition was stronger than α-glucosidase inhibition.

3.2 Phytochemical content and antioxidant activity

Total phenolics, total flavonoids and total tannins of *C. corallina* extract are shown in Table 2. Total phenolic and total flavonoid contents were significantly higher in 70%v/v ethanol than water or hexane extract ($p<0.05$) because solvents of different polarity can extract different compounds. Phenolic compounds contain many hydroxy groups in their structure; therefore, more polar solvents such as water, ethanol, methanol, or their mixture were suitable for the extraction of phenolic compounds. Waszkowiak *et al.* [24] reported that total phenolic and cyanogenic glycoside in flaxseed extract using 60%v/v ethanol was more selective than aqueous extract. In addition, the result revealed that total tannin content in hexane extract was a little higher than that in ethanolic extract. Tannins themselves have high molecular weight and complex structure. Although a large fraction of tannins is extracted by polar solvents such as water or ethanol, or less polar solvents such as ethyl acetate, their polarity can be altered by acetylation or methylation [25]. So, their content will be higher in low polarity solvent. This was evidenced in the study of Sultana *et al.* [26], who found that tannin content was high in n-hexane extract compared to acetone extract.

Table 2. Total phenolics, flavonoids and tannins of *Chara corallina* extracts

Antioxidant content	Extracts		
	Hexane	Ethanol	Water
Total phenolic (mgGAE/g)	6.68±0.12[a]	22.51±0.44[b]	15.52±0.01[c]
Total flavonoids (mgQE/g)	19.06±0.04[a]	23.12±0.01[b]	1.97±0.05[c]
Total tannins (mgTE/g)	30.90±0.68[a]	28.98±0.19[b]	26.37±0.11[c]

GAE=gallic acid equivalent, QE=quercetin equivalent, TE=tannic acid equivalent
Superscripts ([a,b,c,d]) in each row indicate significant differences ($p<0.05$).

Antioxidant activities of *C. corallina* extract are presented in Table 3. The DPPH radical scavenging activity, ATBS radical scavenging activity and metal-chelation activity of the ethanolic extract were significantly higher than water extract and hexane extract ($p<0.05$). Metal-chelation activity on ferrous ions was significantly stronger than other antioxidant activities ($p<0.05$), except in the hexane extract.

Table 3. Antioxidant activities of *Chara corallina* extracts

Antioxidant activities (IC_{50})	Extract		
	Hexane	**Ethanol**	**Water**
DPPH radical scavenging activity (mg Trolox/mL)	0.93 ± 0.01^{a}	0.47 ± 0.02^{b}	0.73 ± 0.06^{c}
ATBS radical scavenging activity (mg Trolox/mL)	1.97 ± 0.06^{a}	1.16 ± 0.07^{b}	1.48 ± 0.05^{c}
Metal-chelation activity (mg EDTA/mL)	1.04 ± 0.03^{a}	0.14 ± 0.00^{b}	0.52 ± 0.02^{c}

Superscripts (^{a,b,c,d}) in each row indicate significant differences ($p<0.05$)

Significant correlation was recorded between α-glucosidase and α-amylase inhibitory efficiency and total phenolics (r^2=0.9836 and 0.9900 for α-glucosidase and α-amylase, respectively). By contrast, no correlation was found between total flavonoids (r^2=0.0002 and 0.0002) and total tannins (r^2=0.3486 and 0.3221). High level of total tannins was recorded in all extracts (Table 2) but there was low correlation with the enzyme inhibition.

Antioxidant activity (DPPH, ATBS and metal-chelation activity) also showed significant correlation with total phenolic contents of all extracts (r^2=0.9946-0.9996), but there was no correlation with total flavonoid and total tannin contents (r^2=0.0035-0.0089 and r^2=0.1733-0.2768, respectively). This could have been due to only flavonoids and tannins of certain structure and hydroxy position in their molecules that influenced the antioxidant properties. This finding was consistent with the study of Othman *et al.* [27], who found no correlation between total flavonoid content and DPPH radical scavenging activity in some selected Malaysian local herb extracts. This implied that the phenolic compounds in *C. corallina* extract play a significant role in α-glucosidase and α-amylase inhibition. Strong α-glucosidase and α-amylase inhibitors also contain high concentration of total phenolic compounds and antioxidant activity. A similar trend was found in the inhibitory effect against α-amylase and α-glucosidase activities of many algal extracts such as *Ascophyllum nodosum* [6, 28], *Ecklonia stolonifera* and *Eisenia bicyclis* [29] and *Sargassum patens* [5].

Generally, three main types of enzyme inhibition mechanism are competitive, non-competitive and uncompetitive inhibition. Competitive inhibition occurs when the inhibitor and substrate have a similar structure and compete for the active site, reducing the enzyme activity. Non-competitive inhibition decreases the activity of the enzyme because the inhibitor and substrate bind to different sites on the enzyme and form an enzyme-substrate-inhibitor. Uncompetitive inhibition involves the binding of an inhibitor to the enzyme-substrate complex and inactivating it [30]. Phenolic compounds in natural inhibitors have the different behaviors against α-glucosidase and α-amylase. For example, Srimoon *et al.* [15] reported that the inhibitory efficiency of *Ventilago denticulata* Willd. dried leave extract against α-glucosidase and α-amylase was mixed-type inhibition, while the standard acarbose was competitive inhibition.

3.3 Phytochemical screening of the extract

Phytochemical screening data showed that the ethanolic extract of *C. corallina* contained terpenoids and steroids. The purified extract appeared as white needles. Preliminary data from the ^{1}H NMR indicated the presence of β-sitosterol (Figure 1), which is the triterpenoid generally found in many plants and algae [23]. From our result, an interesting challenge for future research will be the fractionation and confirmation of the structures of the compounds in *C. corallina* extract.

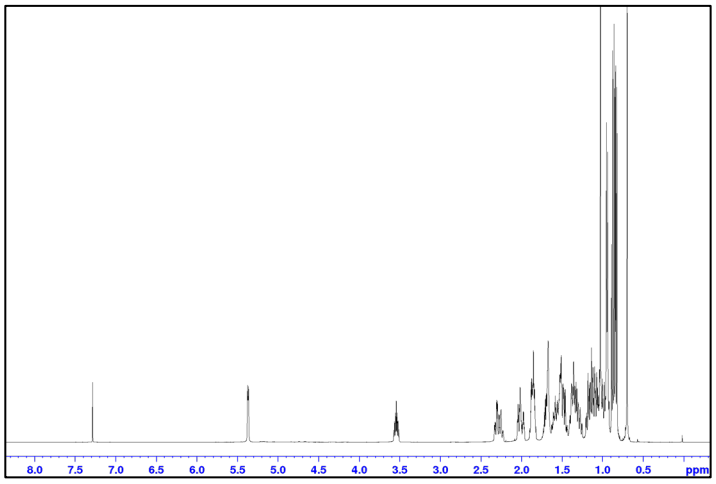

Figure 1. The ^{1}H NMR spectrum in $CDCl_3$ at 500 MHz of *Chara corallina* ethanolic extracts indicated the presence of β-sitosterol (preliminary data).

4. Conclusions

In conclusion, this is the first report describing the α-glucosidase and α-amylase inhibition efficiency of *C. corallina* extract *in vitro.* Correlation analysis showed that the phenolic compounds were responsible for inhibition activity and also antioxidant activity. The IC_{50} value of the extract against enzymes that are associated with carbohydrate digestion and assimilation was higher than standard acarbose (higher IC_{50} demonstrates lower inhibition efficiency). However, a natural inhibitor may be preferable because it should produce fewer adverse side effects. Further study should focus on the isolation, purification and identification of β-sitosterol from the extract. Additionally, the use of the extract in folk medicine or as a functional food should be investigated *in vivo*.

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