

Original Article

IN VITRO INHIBITION OF CARBOHYDRATE-HYDROLYSING ENZYMES BY MALAYSIAN SEAWEED EXTRACTS: ROLE OF EXTRACTION SOLVENT AND ACTIVE FRACTIONS

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ABSTRACT

Marine macroalgae or seaweeds, traditionally consumed in coastal regions, are rich in bioactive compounds with potential therapeutic benefits. This study investigated the inhibitory effects of ethanolic and methanolic extracts from three locally available edible seaweeds, namely *Eucheuma denticulatum*, *Sargassum polycystum*, and *Caulerpa lentillifera*, against α -amylase and α -glucosidase, key enzymes involved in postprandial glucose regulation in type 2 diabetes mellitus. Crude extracts were prepared using ethanol or methanol (1:20 w/v, 24 h maceration with stirring at room temperature), followed by *in vitro* enzyme assays using porcine pancreatic α -amylase and yeast α -glucosidase. Among the methanolic extracts, *E. denticulatum* exhibited the highest α -amylase inhibition (50% at 10 mg/mL), while the ethanolic extract inhibited 67%. *S. polycystum* and *C. lentillifera* showed moderate to low inhibition across both solvent extractions. The half maximal inhibitory concentration (IC₅₀) of ethanolic *E. denticulatum* extract was 1.99 mg/mL, indicating a potent inhibitory effect. Further fractionation of the ethanolic extract revealed that the ethyl acetate fraction had the strongest α -amylase inhibitory activity (49%), followed by acetone (42%) and hexane (36%), though all were lower than the crude extracts, suggesting possible synergistic interactions among the bioactive constituents. In contrast, none of the seaweed extracts exhibited notable α -glucosidase inhibition. These findings highlight the importance of solvent selection in extracting bioactive compounds and support the potential application of *E. denticulatum*, particularly its semi-polar constituents, as a promising candidate for functional food or nutraceutical applications that help regulate carbohydrate metabolism.

KEYWORDS: Malaysian seaweed, α -amylase inhibition, α -glucosidase inhibition, *Eucheuma denticulatum*, diabetes management

INTRODUCTION

Diabetes mellitus (DM) is a multifactorial metabolic disease that significantly contributes to global morbidity and mortality while imposing a tremendous economic and health burden on society. It is characterised by chronic hyperglycaemia resulting from defects in insulin secretion, insulin action, or both (1). Globally, DM affects over 830 million adults, with numbers continuing to rise due to rapid urbanisation, sedentary lifestyles, and unhealthy dietary habits (2). In Malaysia, recent findings from the National Health and Morbidity Survey (NHMS) 2023 showed that 15.5% of adults are living with diabetes, while another 10.2% remain undiagnosed (2,3).

Long-term treatment of DM is challenging, as it often does not result from a single causative factor but rather a constellation of environmental, lifestyle, and genetic influences. Complications of uncontrolled DM include cardiovascular diseases, nephropathy, neuropathy, retinopathy, and liver damage, highlighting the urgent need for improved strategies for disease control (3). One critical strategy in managing type 2 diabetes mellitus (T2DM) is regulating postprandial blood glucose levels through inhibiting carbohydrate-hydrolysing enzymes, such as α -amylase and α -glucosidase. While synthetic inhibitors like acarbose, miglitol, and voglibose are clinically used for this purpose, however, they are often associated with undesirable side effects, including gastrointestinal discomfort and hepatotoxicity (4).

Marine macroalgae or seaweeds have emerged as promising sources of bioactive compounds in the search for safer alternatives. These photosynthetic organisms, which are commonly found in intertidal zones, are classified into four main groups: brown (Phaeophyta), red (Rhodophyta), green (Chlorophyta), and blue-green (Cyanophyta) algae (4). In many parts of Asia, particularly coastal regions of Japan, Vietnam, and Malaysia, seaweeds have been traditionally consumed as food, reflecting a long-standing cultural connection to these potential health benefits (5). Malaysian species such as *Eucheuma denticulatum*, *Sargassum polycystum*, and *Caulerpa lentillifera* are commonly known in both food and non-food industries as a source of raw materials, stabilisers, pigments, and additives, while recently, for their health benefits, including antidiabetic, antioxidant, anti-inflammatory, and nutritive properties (6–11).

The selection of appropriate solvents for extraction is a critical determinant in recovering bioactive compounds from seaweeds and other botanical sources. Different solvents exhibit varying affinities for specific classes of phytochemicals, which influence the resulting extracts' biological activity (12). Methanol and ethanol are widely used due to their ability to extract a wide range of phenolic compounds and flavonoids, known for their antioxidant and enzyme inhibitory properties (13–15). Due to their higher phenolic content, methanolic extracts often demonstrated stronger α -amylase and α -glucosidase inhibitory activities (12,16). These enzyme inhibitory effects are of great interest in developing functional foods targeting postprandial hyperglycaemia, highlighting the practical applications of solvent choice in food science. Thus, understanding how solvent choice modulates extract composition and activity is essential for optimising extraction protocols and enhancing the therapeutic potential of seaweed-derived bioactive compounds (14).

Previous studies have shown that these seaweeds contain diverse bioactive compounds such as polyphenols, phlorotannins, phloroglucinol derivatives, carotenoids, dietary fibre, vitamins, and essential minerals, which contribute to their therapeutic potential (17,18). In particular, the red seaweed *E. denticulatum* has demonstrated significant α -amylase and lipase inhibitory activities, and contains high levels of antioxidant carotenoids like lutein and zeaxanthin (19,20). Meanwhile, *S.*

polycystum and *C. lentillifera* have shown moderate enzyme inhibition and antioxidant activities in various models (6,21).

Therefore, this study aimed to investigate the effects of different solvent extractions, namely ethanol and methanol, on the α -amylase and α -glucosidase inhibitory activities of three locally available Malaysian edible seaweeds; *E. denticulatum*, *S. polycystum*, and *C. lentillifera*. Building on our previous findings (19), the ethanolic extracts were included for comparison with newly prepared methanolic extracts to assess how the choice of solvent affects enzyme inhibition. The extract demonstrating the strongest inhibitory activity was further subjected to solvent fractionation to identify the key bioactive compounds responsible for the activity. This study provides insight into how extraction methods influence bioactivity and highlights the potential of seaweed-based extracts in developing functional foods targeting starch metabolism.

MATERIALS AND METHODS

Chemicals and reagents

Alpha-amylase enzyme, α -glucosidase and acarbose were obtained from Sigma Aldrich (St Louis, MO, USA). All other reagents and solvents were of high analytical grade unless otherwise stated. Water was obtained from arium® pro Ultrapure Water Systems (Sartorius, Goettingen, Germany).

Seaweed collection

All seaweed samples were collected from the coastal waters of Sabah with the support of the Aquaculture Division, Department of Fisheries Malaysia. Specifically, *E. denticulatum* and *C. lentillifera* were sourced from aquaculture farms in Semporna, while *S. polycystum* was harvested from wild populations. The collections were conducted during two periods, in June and October 2013, and the samples were pooled in bulk to ensure sufficient material for extraction and biological assays. The identity of each seaweed species was verified by the Department of Fisheries Malaysia and further confirmed by comparing morphological characteristics with descriptions reported in published literature (22,23). The freshly collected samples were packed dry in styrofoam boxes and transported by air to Kuala Lumpur. Upon arrival, all seaweed samples were thoroughly cleaned to remove epiphytes, salt, and sand using tap water and rinsed three times with deionised water. Cleaned samples were cut into small pieces, placed in jars, sealed with parafilm, and frozen at -80°C . The samples were then freeze-dried using a Beta 2-8 LDplus freeze dryer (Martin Christ, GMBH, Germany) at -80°C . The dried samples were ground to a fine powder (40 mesh size) and stored in airtight containers at -80°C until further analysis.

Preparation of seaweed extract

Freeze-dried seaweed powder was subjected to solvent extraction using 100% ethanol (EtOH) and methanol (MeOH) at a ratio of 1:20 (w/v). The samples were stirred at room temperature (28°C) for 24 hours in the dark, covered with aluminium foil to prevent light degradation. All solvents were of analytical grade. After extraction, the solutions were filtered through Whatman No. 1 filter paper and concentrated using a rotary evaporator (Buchi Rotavapor R-200, Switzerland) under reduced pressure at 45°C . The concentrated extracts were aliquoted into 5 mL Bijou bottles and further dried by vacuum centrifugation using MiVac (Genevac, UK) overnight at 45°C . The extraction yielded dark green crude extracts for *E. denticulatum* and *C. lentillifera*, and a dark brown extract for *S. polycystum* in both solvent extractions. The resulting dried residues were reconstituted in 50% dimethyl sulfoxide (DMSO)

and used for *in vitro* enzyme inhibition assays targeting porcine pancreatic α -amylase and yeast α -glucosidase. Crude extracts demonstrating the highest biological activity were selected for subsequent solvent fractionation.

Preparation of Seaweed Fractions Using a Prepacked Column (EXTrelut NT3)

Approximately 250 mg of crude ethanolic extract was suspended in 3 mL of 100% ethanol and sonicated for 30 minutes. The sample was then loaded onto a prepacked EXTrelut NT3 column for fractionation. Sequential elution was carried out using solvents of increasing polarity: hexane (Hex), ethyl acetate (EtOAc), acetone (Ac), butanol (BuOH), and water (H₂O). Each solvent fraction was collected separately and dried using a vacuum concentrator before being stored for further analysis.

Pancreatic α -amylase inhibition assay

As previously described, the α -amylase inhibition activity was assessed using a 96-well microplate colorimetric assay (19). Briefly, 30 μ L of seaweed extracts (10 mg/mL) in 30 mM Sorensen's phosphate buffer (pH 7) was mixed with 30 μ L of α -amylase solution (0.5 mg/mL) in the same buffer and was pre-incubated at 37°C for 10 min. After the pre-incubation, 60 μ L of 0.5% starch was added to each well. The starch solution was prepared at 0.5 % w/v in 30 mM Sorensen's phosphate (pH 7) by stirring and boiling for 15 min. The reaction mixtures were then incubated at 37°C for 10 min. After incubation, the reaction was stopped by adding 120 μ L of dinitrosalicylic acid (DNS) reagent. DNS was prepared by suspending 0.25 g of DNS in 5 mL of 2N sodium hydroxide. The solution was made up to 12 mL with deionised water. Sodium tartrate (7.5 g) was added, and the solution was made up to 25 mL. The 96-well plate was then incubated at 100°C for 5 min and cooled to room temperature. Absorbance was measured at 540 nm using a microplate reader, Elx800 (BIO-TEK Instruments Inc., Winooski, VT, USA). Acarbose (1 mg/mL), a pharmacological inhibitor, was included as a positive control. The half-maximal inhibitory concentration (IC₅₀) of each ethanolic seaweed extract against α -amylase was determined by testing a range of concentrations in the enzyme inhibition assay. Percentage inhibition was calculated for each concentration, and the data were plotted using GraphPad Prism 5.0 software (GraphPad Software, USA). A non-linear regression model (inhibitor vs. response-variable slope) was applied to fit the inhibition curve and estimate the IC₅₀ value, defined as the concentration of extract required to inhibit 50% of α -amylase activity.

Alpha-glucosidase inhibition assay

Alpha-Glucosidase (Cat. No. G 5003, Sigma Aldrich Chemical Co., USA) was assayed using a previously described method (24). Briefly, α -glucosidase was dissolved at a concentration of 0.1 U/mL in 100 mM phosphate buffer, pH 7.0, containing bovine serum albumin 2000 mg/mL and sodium azide 200 mg/mL, which was used as the enzyme source; para-nitrophenyl- α -D-glucopyranoside was used as the substrate. A 10 μ L aliquot of seaweed extracts (1, 50, and 10 mg/mL) or acarbose (1 mg/mL) was incubated for 5 min with 50 μ L enzyme source in 96-well plates at room temperature. After the pre-incubation, 5 mM *p*-nitrophenyl- α -D-glucopyranoside solution in 0.1 M phosphate buffer (pH 7.0) was added and further incubated for 5 min at room temperature. The α -glucosidase assay was carried out using 0.1 M phosphate buffer (pH 6.9) containing α -glucosidase solution (1.0 U/mL) and incubated for 10 min. Before and after incubation, absorbance readings were recorded at 405 nm using a microplate reader (Biotek Instruments Inc., USA). The reaction mixture without seaweed extract was used as a control, and the mixture without substrate was used as a blank. Acarbose was used as a positive control. The experiment was performed in triplicate.

Statistical Analysis

All data are expressed as mean $\pm$ standard error of the mean (SEM) for three independent assays ($n = 3$). Statistical comparisons were performed using one-way analysis of variance (ANOVA), followed by Bonferroni's post hoc test using GraphPad Prism version 5.0 for Windows (San Diego, California, USA). Differences were considered statistically significant at $p < 0.05$.

RESULTS

Effect of *C. lentillifera*, *E. denticulatum* and *S. polycystum* on α -amylase Inhibition

Figure 1 illustrates the inhibitory effects of acarbose, a standard antidiabetic agent, against porcine α -amylase and yeast α -glucosidase. At a 0.1 mg/mL concentration, acarbose inhibited over 80% of α -amylase and approximately 60% of α -glucosidase activity. Although the α -amylase inhibition was not statistically significant at higher concentrations, 1 mg/mL was selected as the positive control in subsequent assays to represent the maximum tested dosage to maintain consistency across both enzyme inhibition assays.

As shown in Figure 2, ethanolic extracts exhibited significantly stronger α -amylase inhibitory activity than methanolic extracts ($p < 0.05$). Among the ethanolic extracts, *E. denticulatum* demonstrated the highest inhibition (67% at 10 mg/mL), followed by *S. polycystum* (46%) and *C. lentillifera* (26%). The methanolic extract of *E. denticulatum* retained moderate inhibitory activity (50%), which was significantly lower than its ethanolic counterpart ($p < 0.05$), while methanolic extracts of *S. polycystum* and *C. lentillifera* showed minimal inhibition (15% and 11%, respectively), with no significant difference compared to the control ($p > 0.05$).

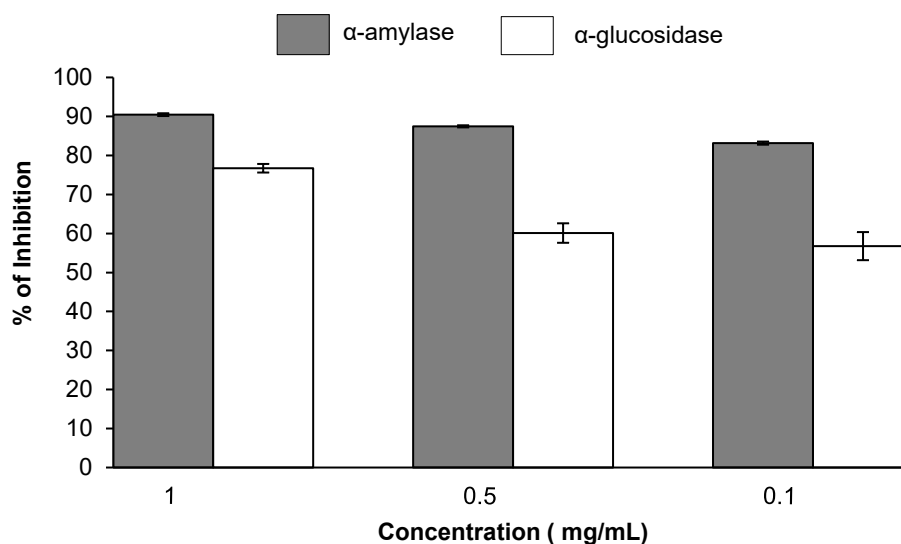


Figure 1. Inhibitory activity of acarbose against porcine α -amylase (0.5 mg/mL) and α -glucosidase (0.1 U/mL). The data represent mean $\pm$ SEM of three independent assays ($n = 3$). One-way ANOVA is followed by Bonferroni's test for post hoc analysis. Values with different superscript letters (a–c) within the same group are significantly different at $p < 0.05$.

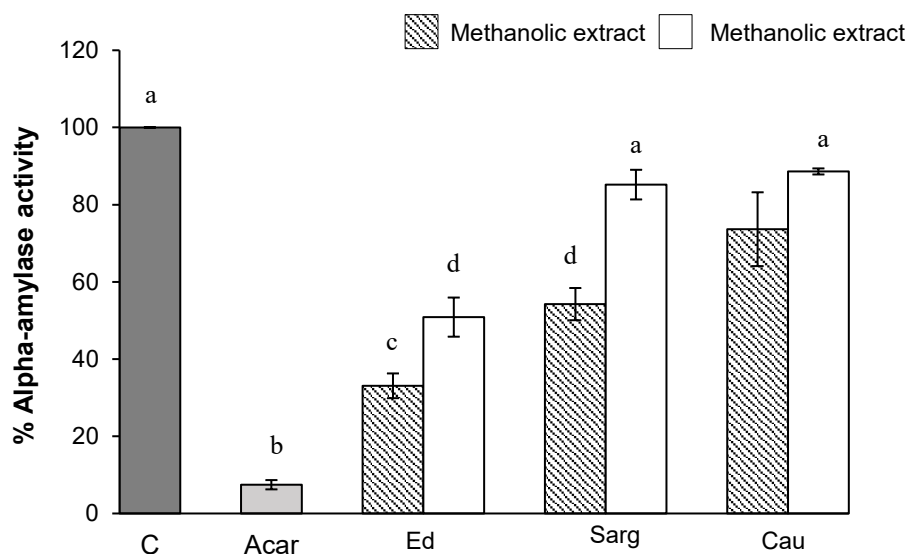


Figure 2. Effect of ethanolic and methanolic extracts of *E. denticulatum* (Ed), *S. polycystum* (Sarg) and *C. lentillifera* (Cau) at 10 mg/mL on α -amylase activity. Acarbose (Acar) at 1 mg/mL was used as a positive control. α -Amylase enzyme as a control (C) was set at 100%. The data represent mean $\pm$ SEM of three independent assays ($n = 3$). One-way ANOVA is followed by Bonferroni's test for post hoc analysis. Different alphabets (a-e), denote a significant difference ($p < 0.05$) between mean values.

Since the ethanolic extracts showed better results than methanol extracts, *E. denticulatum*, *S. polycystum*, and *C. lentillifera* ethanolic extracts were evaluated across a concentration gradient to determine their dose-dependent effects on α -amylase inhibition. Among them, *E. denticulatum* demonstrated the most potent inhibitory effect with an IC_{50} of 1.99 ± 0.09 mg/mL, significantly lower ($p < 0.05$) than the other two species, *S. polycystum* (15.95 ± 1.68 mg/mL) and *C. lentillifera* (21.4 ± 1.84 mg/mL) (Table 1). Given its consistently higher inhibitory performance across all tested concentrations, the ethanolic extract of *E. denticulatum* was selected for further fractionation and subsequent evaluation in this study.

Table 1. IC_{50} values for *in vitro* α -amylase inhibition by crude ethanolic extracts and acarbose

	IC_{50} (mg/mL)
<i>E. denticulatum</i>	1.99 ± 0.09^a
<i>S. polycystum</i>	15.95 ± 1.68^b
<i>C. lentillifera</i>	21.40 ± 1.84^c
Acarbose	0.50 ± 0.23^a

All the analyses were done in triplicate. At least four serially diluted solutions of each analyte were taken to calculate the IC_{50} values. Different alphabets (a-c), denote a significant difference between mean values within the same column

Effect of *C. lentillifera*, *E. denticulatum* and *S. polycystum* on α -glucosidase inhibition activity

None of the seaweed extracts (ethanolic and methanolic extracts) showed α -glucosidase inhibitory activity. (Data not shown).

Effect of *E. denticulatum* fractions on α -amylase activity

To determine the specific constituents contributing to this inhibitory effect, *E. denticulatum* ethanolic crude extract was fractionated using solvents of increasing polarity: hexane, ethyl acetate, acetone, butanol, and water. Each fraction was then evaluated for α -amylase inhibitory activity. The results revealed varying degrees of inhibition across the fractions. While the crude ethanolic extract demonstrated 67% α -amylase inhibition, the solvent fractions exhibited lower activities ranging from 24% to 49% (Figure 3).

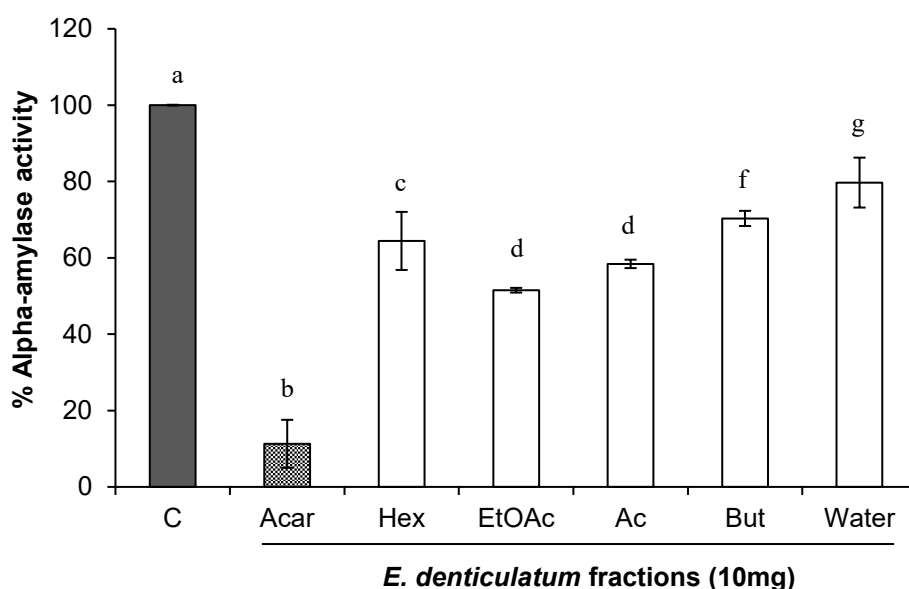


Figure 3. Effect of *E. denticulatum* fractions (Hex, *n*-hexane; EtOAc, Ethyl acetate; Ac, Acetone; But, Butanol) at 10 mg/mL on α -amylase activity. α -Amylase enzyme as a control (C) was set at 100%. The data represent mean $\pm$ SEM of three independent assays ($n=3$). Acarbose (Acar) at 1 mg/mL was used as a positive control. Different alphabets (a-g), denote a significant difference ($p < 0.05$) between mean values.

DISCUSSION

Seaweed contains a broad spectrum of secondary metabolites, and the extraction of bioactive compounds from this valuable marine source has great potential and applications. Several marine extracts have been associated with hypoglycaemia effects (25–27). Many studies demonstrated the potential of seaweeds to inhibit the starch digestive enzymes, α -amylase and α -glucosidase, an effective way to reduce postprandial blood glucose (21,28,29). The present study adds to the growing evidence supporting the potential of marine-derived compounds in managing hyperglycaemia, particularly through various extracts in inhibiting α -amylase activity. This study used ethanolic and methanolic extracts of three seaweed species from different phyla, namely *E. denticulatum*

(Rhodophyta), *S. polycystum* (Ochrophyta) and *C. lentillifera* (Chlorophyta) to evaluate their α -amylase and α -glucosidase inhibitory potential. Acarbose, a commercial inhibitor of carbohydrate digestive enzymes, was used as a positive control for both assays. α -Amylase (0.5 mg/mL) from porcine pancreas and yeast α -glucosidase (0.1 U/mL) were used as controls and represented as 100% enzymatic activity. α -Amylases (α -1,4-glucan-4-glucanohydrolase, EC 3.2.1.1) are a group of enzymes that catalyse the hydrolysis of the (α -1,4) glycosidic linkages in starch via a double displacement mechanism involving the formation and hydrolysis of a covalent β -glycosyl enzyme (30) while α -glucosidase is located in the brush border of the small intestine and acts upon 1,4- α bonds to break down starch and disaccharides to glucose (31).

Among the seaweed species evaluated, *E. denticulatum* exhibited the highest α -amylase inhibitory activity, in line with earlier findings highlighting red algae as a rich source of bioactives with antidiabetic potential (10,19,20). Notably, the ethanolic extract demonstrated a strong inhibition of α -amylase, with an IC_{50} value of 1.99 mg/mL, indicating high potency at relatively low concentrations. The inhibitory activity observed in *S. polycystum* ethanolic extracts may be linked to its fucoxanthin and phlorotannin content, compounds widely reported for their antidiabetic properties (32,33). However, moderate activity and cytotoxicity findings limited its further evaluation. *C. lentillifera* exhibited weak α -amylase inhibition in this study, although caulerpenyne, one of its terpenoid metabolites, has been reported to have such activity (34). Although methanolic extracts are often reported to yield more potent inhibition due to their efficiency in extracting polyphenols (6,35,36), the lower activity observed in all three seaweed methanolic extracts in this study (compared to the ethanolic extract) may reflect differences in compound composition or solvent selectivity. This discrepancy may be attributed to ethanol's ability to extract a broader range of semi-polar compounds, including polyphenols and pigments linked to enzyme inhibition (37). Additionally, seasonal variation, environmental growth conditions, and extraction techniques may account for the discrepancy between previous studies and current findings (6,38,39). These results underscore the need for further research in this area.

Despite their α -amylase inhibitory potential, none of the crude ethanolic and methanolic extracts from the three Malaysian seaweed species inhibited α -glucosidase. This observation may be attributed to several factors, including the nature of the extraction method and the specific bioactive constituents present in each extract. Polyphenolic compounds, such as tannins and anthocyanins, which can target different enzyme activities, with some being more effective at inhibiting α -amylase and others at inhibiting α -glucosidase (40). The variability in inhibition is primarily influenced by the molecular structure, size, polarity, and how these compounds interact with the enzymes' active sites. Structural compatibility and binding affinity are key in determining whether a compound can effectively inhibit a particular enzyme (40,41). This outcome suggests that while the extracts may help reduce the initial breakdown of starch in the digestive tract (via α -amylase inhibition), they may not effectively suppress glucose release in the small intestine, where α -glucosidase plays a critical role. As such, the extracts may have limited efficacy in controlling postprandial hyperglycaemia independently, compared to agents like acarbose, which target both enzymes. From a functional food perspective, partial enzyme inhibition is often preferred over strong dual inhibition to avoid adverse gastrointestinal side effects such as bloating and diarrhoea commonly associated with pharmaceutical inhibitors(42). Therefore, the absence of α -glucosidase inhibition may present an advantage for developing food-based interventions aimed at mild to moderate glycaemic control.

E. denticulatum was selected for further analysis as this species showed the highest anti- α -amylase activity. α -Amylase inhibitory activity among the *E. denticulatum* fractions can be attributed primarily to the differences in solvent polarity and their affinity for extracting specific bioactive compounds. Ethyl acetate, a mid-polar solvent, demonstrated the highest inhibitory effect among all the fractions tested. This is likely because ethyl acetate is particularly effective in extracting moderately polar compounds, such as phenolic acids, flavonoids, and specific sulphated polysaccharides, which are known to exhibit strong enzyme-inhibitory and antioxidant properties (43,44). Studies have reported higher phenolic content in seaweed fractions extracted with ethyl acetate, contributing to bioactivity (8,17,45). Unlike methanol or water, ethyl acetate does not extract high sugars or salts that may interfere with enzyme inhibition assays (14). However, the fractions showed lesser inhibitory activity of the α -amylase than the crude ethanolic extracts, suggesting that the potent activity of the crude extract may be due to synergistic interactions among multiple compounds, which may be partially lost during fractionation. Additionally, the fractionation process could potentially degrade or retain essential metabolites within the column, reducing efficacy.

Limitations of the study include the reliance on the DNS assay for α -glucosidase activity, which is susceptible to interference from seaweed-derived sugars, pigments, and non-carbohydrate components. These can react non-specifically with the DNS reagent and obscure actual enzyme inhibition (46). Additionally, the intense pigments found in some seaweed extracts, especially *S. polycystum*, may interfere with absorbance readings, affecting the accuracy of colorimetric assays. Optimising extraction methods to reduce pigment and interfering substances and employing more specific detection techniques may yield clearer insights into the actual inhibitory potential for both assays.

CONCLUSION

This study highlights the promising potential of *E. denticulatum* ethanolic extract and fractions in reducing starch breakdown via significant α -amylase inhibition, while no notable α -glucosidase inhibition was detected. Although this limits its function as a standalone antidiabetic agent, the extract may still offer benefits as a functional food ingredient that moderates postprandial glycaemic responses. Future studies exploring its synergistic use with other bioactive or in combination with α -glucosidase inhibitors could further enhance its nutraceutical value and broaden its application in glycaemic control.

Author Contributions: VB; study conceptualization, design, collection of data, interpretation of results, analysis and draft manuscript preparation. AI and MFN; proofreading and editing of the manuscript. All authors read and approved the final version of the manuscript for publication and agreed to be responsible for all aspects of the manuscript.

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