Journal of Agricultural Science and Technology (JAST), 29(2)
In Press, Pre-Proof Version

Evaluation of antioxidant and antidiabetic potential of protein from *Sargassum ilicifolium* extracted via conventional and enzymatic approach

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Abstract

Background & Problem: Diabetes and oxidative stress-related diseases are escalating global health concerns, driving the need for safe, natural alternatives to synthetic drugs which often have adverse side effects. However, conventional protein extraction methods from seaweed often suffer from low efficiency, high energy consumption, or degradation of heat-labile bioactive compounds. Objective: Therefore, this study aimed to extract and characterize protein from *Sargassum ilicifolium* by comparing conventional (aqueous and alkaline) extraction with enzymatic methods using five specific enzymes: Alcalase, Termamyl, Viscozyme, Celluclast, and AMG. Furthermore, the study sought to evaluate their antioxidant and antidiabetic properties to identify the most effective extraction protocol. Methods: Protein was extracted using aqueous, alkaline, and five enzymatic treatments. To optimize recovery, the extracts were precipitated at two distinct ethanol concentrations: 30% and 70%. Antioxidant activity was evaluated using DPPH, ABTS, and metal chelation assays. Antidiabetic potential was assessed through α-amylase and α-glucosidase inhibition assays. Key Results: Comparative analysis revealed that alkaline extraction yielded the highest total protein content (14.7%), but enzymatic extraction with Termamyl (30% alcohol precipitation) achieved a significant protein yield of 7.3%. Notably, enzyme-assisted extraction showed higher overall efficiency (18.4% for Alcalase) compared to conventional methods, highlighting its sustainability and selectivity. Regarding bioactivity, Termamyl (70% alcohol) exhibited the highest DPPH radical scavenging (44.32 µg Trolox/ml) and metal chelation (74.78 µg EDTA/ml). Viscozyme (70% alcohol) showed the strongest ABTS inhibition (199.57 µg Trolox/ml). In terms of antidiabetic potential, Termamyl (30% alcohol) demonstrated the highest α-glucosidase inhibition (IC_{50} =

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4.15 µg/ml), while Termamyl (70% alcohol) showed 75.93% α-amylase inhibition. Significance & Novelty: Unlike previous studies that focused on single enzymes or methods, this study provides a comprehensive comparison of multiple enzyme types (proteases vs. carbohydrases) on *S. ilicifolium*. This study demonstrates that enzymatic extraction is a promising, sustainable alternative for producing bioactive peptides with dual antioxidant and antidiabetic properties. These findings offer valuable insights for the development of nutraceuticals, functional foods, and aquaculture feeds, addressing the challenge of finding efficient, eco-friendly ways to harness marine bioactive compounds.

Keywords: Antidiabetic, Antioxidant, α-Amylase, α-Glucosidase, Bioactive peptides, Brown algae, Enzyme-assisted extraction, *Sargassum ilicifolium*.

Abbreviation

α-amylase: Alpha-amylase, **α-glucosidase:** Alpha-glucosidase, **ABTS:** 2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid), **AMG:** Amyloglucosidase, **BHT:** Butylated Hydroxytoluene, **Cc:** Celluclast, **DPPH:** 2,2-Diphenyl-1-picrylhydrazyl, **IC_{50}:** Half Maximal Inhibitory Concentration, **TE:** Trolox Equivalent, **EDTA:** Ethylenediaminetetraacetic acid, **FTIR:** Fourier Transform Infrared Spectroscopy, **PER:** Protein Efficiency Ratio, **CS:** Chemical Score, **Te:** Termamyl, **Vc:** Viscozyme.

1. Introduction

Background & Characteristics of Seaweeds: Seaweeds, widely recognized as important marine biological resources since the early 19th century, are distributed across tropical and temperate oceanic regions, particularly in the Pacific and Indian Oceans [1]. Bleakley and Hayes classified these diverse macroalgae, comprising over 168,971 species, into three main categories based on photosynthetic pigments: red seaweeds (Rhodophyta), brown seaweeds (Phaeophyta), and green seaweeds (Chlorophyta) [2]. Among these, brown seaweeds of the genus *Sargassum* are of particular interest due to their unique biochemical profile. *Sargassum ilicifolium*, a prominent brown alga, is rich in sulfated polysaccharides, fucoidans, and proteins, which contribute to its significant antioxidant and antidiabetic potential [2, 3]. Unlike other species, *S. ilicifolium* exhibits specific cell wall structures that make protein extraction challenging, necessitating optimized methods to release bound bioactive compounds. Necessity, Importance, and Challenges (Diabetes & Oxidative Stress): The global burden of diabetes mellitus and oxidative stress-related diseases is escalating, posing a major public health challenge. Synthetic antioxidants (e.g., BHT, BHA) and antidiabetic drugs (e.g., Metformin) often cause adverse side effects, driving the urgent need for natural, safe alternatives [4]. Seaweeds, containing approximately 47% protein on a dry weight basis along

with polyphenols and vitamins, are promising sources of multifunctional bioactive compounds [5, 10]. However, the commercial exploitation of seaweed proteins faces significant challenges. Conventional extraction methods, such as alkaline extraction, while yielding high protein content, often lead to the degradation of heat-labile amino acids and reduce the nutritional quality of the final product [11]. Therefore, there is a critical necessity to develop sustainable, mild, and efficient extraction techniques that preserve bioactivity. Enzyme-Assisted Extraction (EAE) & Enzyme Specificity: To address these challenges, Enzyme-Assisted Extraction (EAE) has emerged as a promising alternative. EAE utilizes specific enzymes to disrupt cell walls and solubilize proteins under mild conditions, thereby preserving bioactive properties [12]. The choice of enzyme is critical, as different enzymes target different substrates:

- Proteases (e.g., Alcalase): Hydrolyze peptide bonds, directly releasing protein fragments and peptides [13].
- Carbohydrases (e.g., Termamyl, Viscozyme, Celluclast, AMG): Degrade complex polysaccharides in the algal cell wall (such as alginate and cellulose), thereby releasing proteins and polyphenols trapped within the matrix [14]. Comparing these distinct mechanisms is essential to determine the most effective strategy for extracting bioactive peptides from *S. ilicifolium*. [Added per Reviewer 3's comment on explaining different enzymes and substrates]

Novelty of the Study: While previous studies have investigated seaweed proteins, few have comprehensively compared the effects of multiple enzyme types (proteases vs. carbohydrases) combined with different precipitation conditions on the same species. Furthermore, recent findings highlight the bioactive potential of seaweed-derived peptides. For instance, novel bioactive peptides from red seaweed proteins have been characterized [15], and comparative studies show the potential of natural proteins (like casein) in managing diabetes [4]. This study fills a critical gap by providing a detailed comparison of conventional and enzymatic methods on *S. ilicifolium*, aiming to identify the optimal protocol for producing high-value bioactive peptides. Microencapsulation and Stabilization: The successful application of these bioactive peptides in functional foods depends on their stability. Bioactive compounds are often sensitive to heat, pH, and light, which can reduce their efficacy during processing and storage. Microencapsulation is a widely used technique to protect these compounds and enhance their bioavailability. Among various methods, spray drying is one of the most common and cost-effective techniques for encapsulating bioactive peptides using carriers like maltodextrin or gum arabic [16]. While spray drying offers advantages such as continuous operation and low

moisture content, it also has disadvantages, including potential thermal degradation of heat-sensitive peptides. Other methods, such as freeze-drying, preserve bioactivity better but are expensive and have low throughput [17]. Therefore, optimizing the extraction method to yield stable, high-quality peptides is a prerequisite for successful downstream encapsulation. Objectives Therefore, the objectives of this study were to:

1. Extract and compare protein from *Sargassum ilicifolium* using conventional (aqueous and alkaline) and enzymatic methods (Alcalase, Termamyl, Viscozyme, Celluclast, and AMG).
2. Evaluate the antioxidant properties (DPPH, ABTS, metal chelation) and antidiabetic potential (α-amylase and α-glucosidase inhibition) of the extracts.
3. Determine the most effective enzyme and precipitation conditions for producing bioactive peptides with high nutritional and functional value.

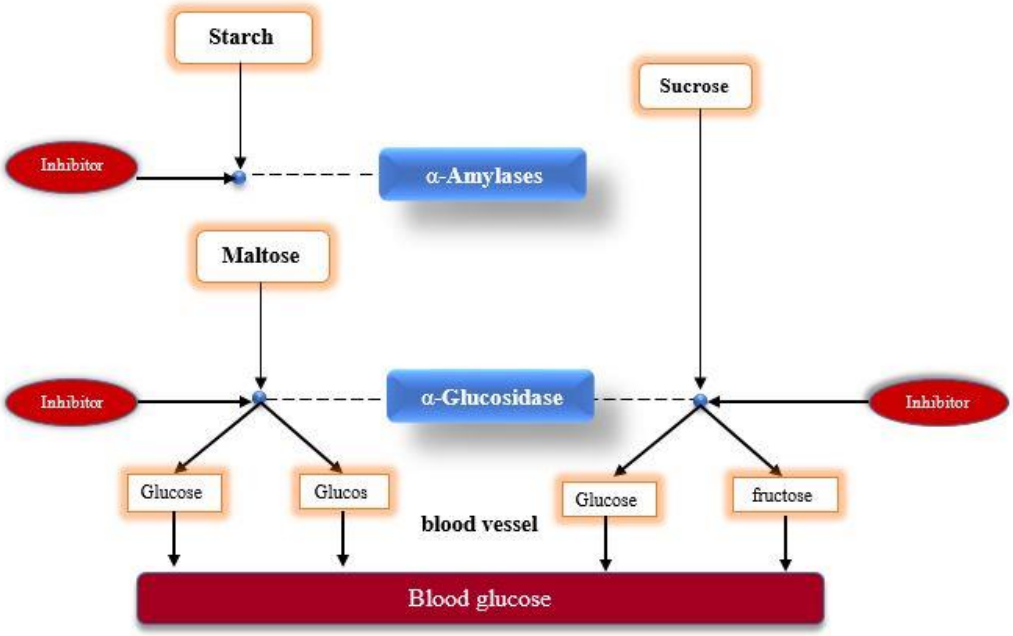

Fig. 1 Schematic of the activity of enzymes effective in starch digestion and inhibitory compounds of this group of enzymes.

2. Materials and Methods

DPPH (2,2-diphenyl-1-picrylhydrazyl), ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt), ferrozine (3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazine-4′,4″-disulfonic acid sodium salt), pNPG (4-nitrophenyl α-D-galactopyranoside), trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), acarbose, Alcalase 2.4 L (protease from *Bacillus licheniformis*, cat. no. P4860), porcine pancreatic α-amylase (cat. no. A3176), α-glucosidase from *Saccharomyces cerevisiae*, and ammonium salt of 1-anilino-8-naphthalene-

sulphonic acid (ANS) were purchased from Sigma-Aldrich (St. Louis, MO, USA). PAHBAH (4-hydroxybenzohydrazide), soluble starch (ACS reagent), and EDTA (ethylenedinitrilotetraacetic acid disodium salt dihydrate) were purchased from Merck (Darmstadt, Germany). Termamyl 120L (500 U/mL), AMG 300L (260 U/mL), Viscozyme L (100 FBGU/mL), and Celluclast BG (700 U/g) were purchased from Novozymes A/S (Bagsværd, Denmark). All other chemicals were of analytical grade and were used without further purification.

2.1. Algal collection and preparation

Seaweed *Sargassum ilicifolium* was collected from the shores of the Persian Gulf. The collected samples were taxonomically identified based on morphological characteristics using standard identification keys for brown algae and confirmed by a marine biology specialist. The samples were washed thoroughly with distilled water to remove salts and epiphytes, then dried in an oven (Model: [Memmert UFE 500], Germany) at 38 °C for 18 h and milled using an electric grinder (Iran). The powdered material was passed through a 40-mesh sieve for homogenization. The obtained algal powder was stored at −20 °C until further experiments [18].

2.2. Protein extraction

2.2.1. Aqueous extraction

To obtain bioactive peptides from the extracted seaweed, the alkali (NaOH-extracted) and aqueous extracts were adjusted to pH 7.0 and freeze-dried using a freeze dryer (Christ Alpha 2-4 LD, Germany). Then, 100 mg of dried extract was dissolved in 50 mM phosphate buffer (pH 8.0) and hydrolyzed using Alcalase (20 µL, 48 U/mL) at 50 °C for 3 h, according to the method described by Kadam et al. (2017) and Cian et al. (2012) [19, 20] with slight modifications. After hydrolysis, the enzyme was inactivated by heating at 95 °C for 10 min, and the pH was readjusted to 7.0 before freeze-drying.

2.2.2. Alkali extraction

The solid residue obtained from aqueous extraction was mixed with sodium hydroxide (NaOH) solution at a solid-to-liquid ratio of 1:15 (w/v). Different NaOH concentrations (0.1, 0.2, 0.3, and 0.4 M) were applied. The extraction pH ranged between 12.0 and 13.0 depending on the NaOH concentration. The suspension was gently stirred for 1 h at 4 °C to promote protein solubilization under alkaline conditions. The mixture was then centrifuged at 9000 rpm for 20

min at 4 °C using a centrifuge (Hettich Universal 320, Germany) to separate the supernatant from the residual solid. Following extraction, the supernatant was neutralized to pH 7.0 using 1 M HCl prior to further processing.

2.2.3. Enzymatic extraction

The experimental procedure was based on a study by Charoensiddhi et al. (2016) [21] with some modifications. In each experiment, 0.5 g of dried and ground seaweed was mixed with 50 mL of buffer (phosphate, acetate, or water), and 50 µL of different enzymes (Alcalase, Termamyl, Viscozyme, Celluclast BG, and AMG 300L) were added. The mixture was then incubated in a shaker oven (Memmert SPO 53, Germany) at 50 °C for 24 h. To stop the enzymatic reaction, the samples were heated at boiling temperature (100 °C) for 10 min and then immediately placed in an ice-water bath. The mixture was then centrifuged at 10,000 × g for 10 min at 4 °C, and the supernatant was collected. Although the optimal temperature and pH ranges of the enzymes vary, all enzymatic extractions in the present study were conducted at 50 °C for 24 h to ensure a fair comparison under identical thermal conditions. The pH conditions were adjusted according to the selected buffer system for each enzyme, as presented in Table 1.

Table 1. Optimum and experimental conditions (temperature and pH) for different enzymes used in this study.

Enzyme	Optimum Temperature (°C)	Optimum pH	Temperature used in this study (°C)	pH used in this study	Substrate specificity
Alcalase	50–70	8–10	50	8.0 (phosphate buffer)	Protease (peptide bonds)
Termamyl	90	6–7	50	7.0 (phosphate buffer)	α-1,4 glycosidic bonds (starch)
Viscozyme	35–55	3–5	50	5.0 (acetate buffer)	Cell wall polysaccharides
Celluclast	50	4–6	50	5.0 (acetate buffer)	Cellulose
AMG	60	4.0–4.5	50	4.5 (acetate buffer)	α-1,4 & α-1,6 glycosidic bonds

2.2.4. Yield Analysis

The yield of protein extracted by (aqueous, alkaline, and enzymatic) methods was calculated using the following formula:

Protein yield (%)= (Initial protein content in raw material Protein content in extract)×100

2.2.5. Production of bioactive peptides from algae

To obtain bioactive peptides from the extracted seaweed, the alkali and aqueous extracts were adjusted to a pH of 7.0 and dried using a freeze dryer. Then, 100 mg of the dried extract was mixed with 5 mL of phosphate buffer (pH 8.0) and 20 µL (48 U/mL) of Alcalase enzyme was added. Alcalase was selected for this step due to its broad specificity in hydrolyzing peptide bonds, which effectively releases bioactive peptides from the extracted proteins [20]. The mixture was placed in a shaker (Germany) at 50 °C for 3 hours. After that, the pH was adjusted back to 7.0, and the mixture was freeze-dried again. The dried extract was then dissolved in a specific amount of distilled water, and its protein content was measured using the Bradford method [22].

2.3. Determination of protein

The amount of protein during enzymatic and classical extraction was evaluated by Bradford's method [23] using a spectrophotometer (Shimadzu UV-1800], Japan) at 595 nm.

2.4. Tests related to biological properties

2.4.1. Evaluation of total phenolic compounds of the extract

To measure the total phenol content, the Folin-Ciocalteu method was used [23]. In this method, 20 µL of enzyme extract were mixed with 100 µL of Folin-Ciocalteu solution and 1.16 mL of distilled water. After resting for 5 min, 300 µL of 20% (w/v) sodium bicarbonate solution was added, and the mixture was incubated in a water bath at 60 °C for 30 min. The absorbance of the resulting solution was then measured at 760 nm using a spectrophotometer. The total phenol content was calculated using a standard Gallic acid curve formula:

$$Y=0.0166x-0.1622$$

Where Y = Sample absorption at 760 nm.

2.5. Assess antioxidant properties

2.5.1. Determination of antioxidant content by DPPH radical method

DPPH radical scavenging activity was measured by the method by Nanjo et al. (1996) [24] with slight modifications. First, concentrations of 2500, 1250, and 1000 ppm were prepared from seaweed extract with a concentration of 5000 ppm. 500 µL of the extracted sample was mixed well with 500 µL of methanolic solution (0.1 mM) of DPPH radical and kept in the dark for 30 min. One sample was considered as a control, which contained 500 µL of DPPH and 500 µL of methanol. Immediately using the spectrophotometer (Model: [Insert Model]), the

absorption of the sample was read at a wavelength of 517 nm. Then the ability to inhibit DPPH radicals was calculated using the standard Trolox curve:

Y=−0.0128x+0.6991

Where Y = Sample absorption at 517 nm.

2.5.2. Chelation ion (Fe^{2+}) activity

The effect of Fe^{2+} chelation in different samples was evaluated. Briefly, a solution with a concentration of 5000 ppm of dried extract, 2 mM solution of $FeCl_2$, and 5 mM of ferrozine was prepared. Then 500 µL of the sample was dissolved in 1850 µL of water and 50 µL of $FeCl_2$ solution and left for 3 min. Afterward, 100 µL of ferrozine was added to the solution and stored for 20 min at room temperature. The absorbance of samples was read at 562 nm. EDTA was used as a positive control. The chelating activity was calculated using the standard EDTA curve formula [25]:

Y=−0.0128x+1.0216

Where Y = Sample absorption at 562 nm.

2.5.3. ABTS radical cation inhibitory activity

The method described by Rice-Evans et al. (1999) [25] was used with minor modifications to evaluate the ABTS radical scavenging activity. Firstly, a mixture of 7 mM ABTS radical and 2.45 mM potassium persulfate at a ratio of 50:50 was prepared and left in the dark at room temperature for 12–16 h. The resulting solution was then diluted with water until the absorbance reached 0.7 ± 0.02 at 734 nm wavelength. Next, 20 µL of the sample were added to 980 µL of the diluted ABTS radical solution, and the mixture was incubated at 37 °C for 10 min. The absorbance was measured at 734 nm, and the ability of the sample to inhibit ABTS radicals was calculated using the standard Trolox curve formula:

Y=−0.0019x+0.7094

Where Y = Sample absorption at 734 nm.

2.6. Evaluation of antidiabetic effect

2.6.1. α-Glucosidase inhibition assay

To determine the inhibitory activity of the enzyme extracts against α-glucosidase enzyme, a method described by Apostolidis, Kwon, and Shetty (2007) [26] was used with slight modifications. In this method, 250 µL of seaweed enzyme extracts was mixed with 150 µL of α-glucosidase solution from *Saccharomyces cerevisiae* yeast (0.2 units per mL) and phosphate

buffer and incubated for 10 min at 25 °C. Then, 100 µL of p-nitrophenyl α-D-glucopyranoside (0.5 M) was added to the reaction mixture and the absorption of the reaction sample was measured at 410 nm. The degree of inhibition was calculated using the following equation:

Inhibition (%)=(Control absorbanceControl absorbance−Sample absorbance)×100

and the enzyme activity was defined as the amount of enzyme required to release a micromole of p-nitrophenol. The results were reported based on the IC_{50} value, which is the concentration of the sample that can inactivate 50% of the α-glucosidase enzyme.

2.6.2. α-Amylase inhibition assay

The modified method of [27] was used to investigate the alpha-amylase inhibition of seaweed enzyme extracts. In this experiment, 80 µg of the extract were combined with 20 µL of phosphate buffer (0.007 M NaCl and pH 6.8), followed by the addition of 100 µL of porcine pancreatic alpha-amylase enzyme (with an activity of 0.5 units/mL) and incubated for 5 min at 37 °C. Next, 100 µL of starch solution (0.5 g/100 mL) were added, and the mixture was incubated again at 37 °C for 20 min. After centrifugation at 13,000 × g to separate the extracts from the pellets, 20 µL of the extract were combined with 1 mL of colored solution, incubated for 10 min at 70 °C, cooled to room temperature, and measured for absorbance at a wavelength of 410 nm using a spectrophotometer.

- Control absorption = including starch and enzyme, in fact 100% enzyme activity;
- Sample absorption = including starch and enzyme and inhibitory compound;
- Background absorption 1 = starch only;
- Background absorption 2 = starch and inhibitory compound.

2.7. Statistical analysis

All experiments were carried out in triplicate and results were expressed as mean ± standard deviation (SD). Statistical significance among treatments was determined using one-way analysis of variance (ANOVA) followed by Duncan's multiple range test at a significance level of $P < 0.05$. Different letters in tables and figures indicate statistically significant differences between treatments.

3. Results and Discussion

3.1. Protein Yield and Extraction Efficiency

The protein yield obtained from water (aqueous), alkaline, and enzymatic extraction methods is presented in Table 2. Statistical analysis revealed significant differences ($p<0.05$) among the

extraction methods. The alkaline extraction method yielded the highest protein content (14.7±0.5%), which was significantly higher than both the aqueous (2.1±0.1%) and enzymatic methods. Among the enzymatic treatments, Termamyl (Te) precipitated with 30% alcohol showed the highest protein yield at 7.3±0.3%, which is approximately 50% of the yield obtained by the alkaline method. The aqueous extraction method (Hp) exhibited the lowest yield.

Table 2. Protein yield (%) obtained from different extraction methods.

Extraction Method	Protein Yield (%)
Aqueous (Hp)	2.1±0.1[a]
Alkaline	14.7±0.5[b]
Enzymatic (Te, 30% alcohol)	7.3±0.3[c]

Values are means ± SD (n=3). Different superscript letters indicate significant differences ($p<0.05$).

The superior yield of alkaline extraction can be attributed to the strong alkaline conditions (pH 10–12), which effectively disrupt peptide bonds and solubilize proteins from the seaweed biomass [27]. However, as noted by Hammed et al. (2013) [28], alkaline methods may cause the degradation of heat-labile amino acids, potentially reducing the nutritional quality of the extracted protein. In contrast, enzymatic extraction using Termamyl (Te) achieved a yield of 7.3%. While lower than the alkaline method, this yield is significantly higher than that of aqueous extraction. Termamyl, an α-amylase (carbohydrase), likely enhanced protein release by degrading the starch and polysaccharide matrix surrounding the proteins. This degradation improves the accessibility of proteins to extraction solvents without the harsh chemical conditions associated with alkaline methods. This finding is consistent with previous studies reporting that enzymatic extraction is more selective and environmentally friendly, although it may yield lower protein content compared to conventional chemical methods [29]. The low yield in aqueous extraction (2.1%) indicates that water alone is insufficient to break the complex cell wall structure of brown algae, such as *Sargassum ilicifolium*. This is in agreement with Kadam et al. (2017) [19], who reported that water-soluble proteins in seaweed are limited compared to alkali-extractable proteins.

3.2. FTIR Spectra Characteristics

The FTIR spectrum of *Sargassum ilicifolium* revealed various functional groups, as summarized in **Table 3**. The analysis confirmed the presence of characteristic peaks corresponding to the biochemical composition of the biomass:

- **O-H stretching vibrations (3200–3600 cm^{-1}):** Indicating the presence of polysaccharides and proteins.

- **C=O stretching (1700–1750 cm^{-1}):** Suggesting the presence of carbonyl compounds in proteins and polyphenols.
- **C=C stretching (2960–650 cm^{-1}):** Corresponding to various organic compounds.

Table 3. FTIR spectral characteristics of *Sargassum ilicifolium* extracts.

Wavenumber (cm^{-1})	Functional Group	Compound Type
3200–3600	O-H stretching	Polysaccharides, Proteins
1700–1750	C=O stretching	Carbonyl compounds (proteins, polyphenols)
2960–650	C=C	Various organic compounds

These functional groups are consistent with the rich composition of brown seaweeds, particularly polysaccharides (such as alginate and fucoidan) and proteins [30]. The presence of these specific groups confirms that the extraction methods employed in this study preserved the fundamental biochemical structure of the algal components. Specifically, the O-H and C=O stretches are indicative of the hydrogen bonding and secondary structures typical of protein-polysaccharide complexes in seaweed biomass, as described by Rodrigues et al. (2015) (30).

3.3. Evaluation of Antidiabetic Properties

3.3.1. α-Amylase Inhibitory Activity

Figure 2 illustrates the percentage inhibition of porcine α-amylase by different enzyme extracts at a concentration of 10 mg/ml. The highest inhibition rate was observed for Termamyl (Te) precipitated with 70% alcohol (75.93%), while the lowest inhibition rate (25.06%) was observed for Alcalase (Al) precipitated with 70% alcohol. Statistical analysis revealed no significant differences ($p > 0.05$) between some Al and Te extracts, but Te generally showed superior performance. Although the inhibition rates of the seaweed extracts were lower than that of the positive control (acarbose), they demonstrated significant bioactivity. The inhibition of carbohydrate-digesting enzymes, such as α-amylase, slows down glucose absorption, which is a key strategy for managing type 2 diabetes [16, 31]. The high inhibitory activity of Termamyl extracts can be attributed to bioactive peptides generated during enzymatic hydrolysis that interact with the active site of α-amylase [32]. The superior performance of Te (a carbohydrase) over Al (a protease) may be due to the different peptide profiles generated. Carbohydrases may produce peptides with specific chain lengths and amino acid sequences that are more effective at inhibiting α-amylase [32]. This outcome is consistent with findings by Admassu et al. (2018) [33], who reported strong α-amylase inhibition in red algae-derived proteins.

3.3.2. α-Glucosidase Inhibitory Activity

Table 4 shows the IC_{50} *values for α−glucosidaseinhibition. Thelowest* IC_{50} value (indicating the highest inhibitory activity) was observed for Termamyl (Te) precipitated with 30% alcohol at 4.15 µg/ml**,** followed by Te (70% alcohol, 5.3 µg/ml**).** Statistical analysis revealed significant differences ($p < 0.05$) between Al and Te extracts, with Te showing superior inhibitory potential. α-Glucosidase is a key enzyme in the final stage of starch digestion. The superior activity of Termamyl extracts, particularly those precipitated at 30% alcohol, suggests that smaller peptides generated by this method have better accessibility to the α-glucosidase active site [16]. This supports the hypothesis that peptide size and hydrophobicity influence enzyme inhibition. These results are comparable to other studies on brown seaweeds. For instance, Pangestuti et al. (2021) [34] reported IC_{50} values of 3838.6 mg/L (approximately 3.8 mg/ml) for *Halimeda macroloba*, which indicates weaker activity compared to the enzymatic extracts in this study. Furthermore, crude aqueous extracts of brown seaweeds such as *Padina sulcata* and *Sargassum binderi* have been shown to exhibit significant inhibitory effects against carbohydrate-digesting enzymes [17]. Additionally, Davan et al. (2013) [35] demonstrated that combined extracts of *Ascophyllum nodosum* and *Fucus vesiculosus* inhibited α-glucosidase activity by nearly 100%. Collectively, these findings suggest that *Sargassum* species are promising sources of potent α-glucosidase inhibitors. Given that the synthetic drug acarbose, commonly used for type 2 diabetes management, can cause adverse side effects such as abdominal distention and bloating [14, 31], natural bioactive peptides from seaweed offer a viable alternative with fewer side effects and similar therapeutic potential.

Table 4. IC_{50} values for α-glucosidase inhibition by different enzyme extracts.

Type of enzyme	Extraction condition	IC_{50} (µg/ml)
Alcalase	Seasoned with 70% alcohol	36.54 ± 1.2^{d}
	Alcohol precipitated only 70%	25.0 ± 0.8^{c}
	Complete extract	21.65 ± 0.9^{c}
	Precipitated with 30% alcohol	4.15 ± 0.2^{a}
Termamyl	Seasoned with 70% alcohol	8.77 ± 0.4^{b}
	Alcohol precipitated only 70%	5.3 ± 0.3^{a}
	Complete extract	8.2 ± 0.5^{b}

Values are means ± SD (n=3). Different superscript letters indicate significant differences ($p<0.05$). Lower IC_{50} indicates higher inhibitory activity.

3.3.3. DPPH Radical Scavenging Activity

The DPPH radical scavenging activity of the various enzyme extracts is presented in Figure 2**.** Statistical analysis revealed significant differences ($p < 0.05$) among the different enzymes and precipitation methods. The assay showed a wide range of activity, with values varying from

44.32 µg Trolox/ml for Termamyl (Te) precipitated with 70% alcohol to 3.53 µg Trolox/ml for Viscozyme (Vc) precipitated with 30% alcohol. Generally, Te extracts exhibited the highest antioxidant potential, particularly the fraction precipitated at 70% alcohol. The DPPH radical scavenging activity relies on the ability of bioactive compounds, such as peptides and polyphenols, to donate hydrogen atoms or electrons to neutralize free radicals [36]. The superior performance of Te (a carbohydrase) compared to Al (a protease) can be attributed to the distinct peptide profiles generated during hydrolysis. Specifically, carbohydrases may produce peptides with amino acid sequences and chain lengths that are more effective at radical scavenging. These findings align with previous studies on *Sargassum* species, which reported high radical-scavenging capacities after enzymatic treatment with Te, Vc, and AMG [37]. For instance, Ganesan et al. (2018) [38] observed significant free radical scavenging in *S. angustifolium* and *S. boveanum* following hydrolysis. Variations in activity among treatments are likely influenced by enzyme specificity, the resulting amino acid sequences, and the total peptide concentration [39].

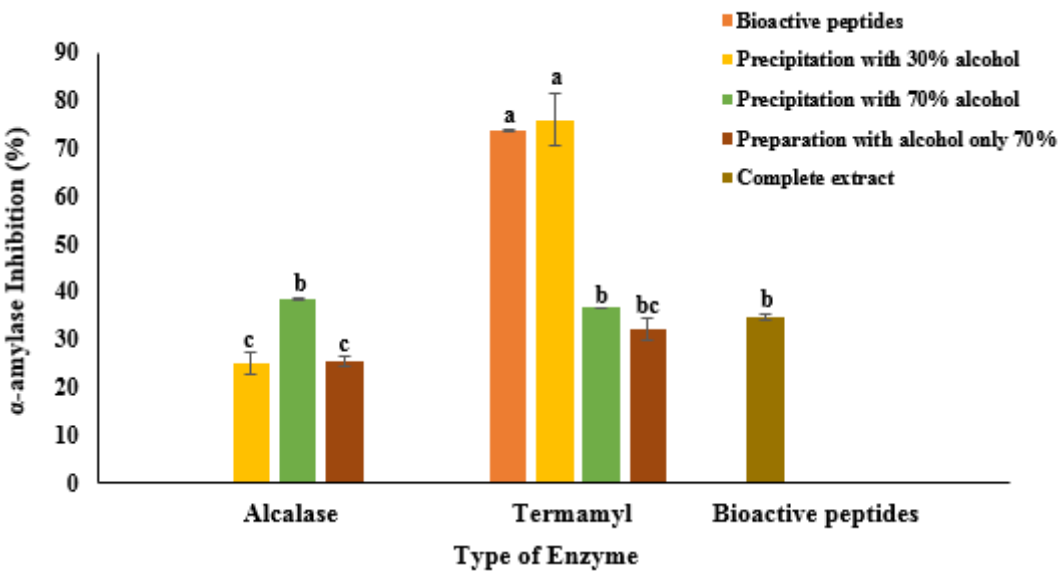

Fig. 2. DPPH radical scavenging activity of different enzyme extracts from *Sargassum ilicifolium* (µg Trolox/ml). Different letters indicate statistically significant differences ($P < 0.05$). Each experiment was performed in triplicate. The bars show the mean ± SD of three separate studies.

3.3.4. Metal Chelating Activity (Fe^{2+})

Figure 3 presents the metal chelating activity of the enzyme extracts, expressed in µg EDTA/ml. The highest chelating activity was observed in the Termamyl (Te) extract precipitated with 70% alcohol, reaching 74.78 µg EDTA/ml. Conversely, the

Amyloglucosidase (AMG) extract precipitated with 30% alcohol exhibited the lowest activity at 23.57 µg EDTA/ml. Statistical analysis revealed significant differences ($p < 0.05$) among the different enzyme types and precipitation methods. The enhanced metal chelation ability is likely associated with low-molecular-weight peptides capable of binding metal ions. It has been reported that smaller proteins and peptides, resulting from the breakdown of larger protein structures, often possess superior metal-chelating properties [40]. The superior performance of Te extracts (particularly at 70% alcohol) may be attributed to the presence of specific amino acid residues, such as histidine, cysteine, and methionine, which have a high affinity for metal ions [41]. In contrast, the lower chelating capacity observed in AMG extracts (at 30% alcohol) may result from the extensive breakdown of polysaccharides into simple sugars and oligosaccharides, which have reduced molecular weight and consequently lower chelating potential [42].

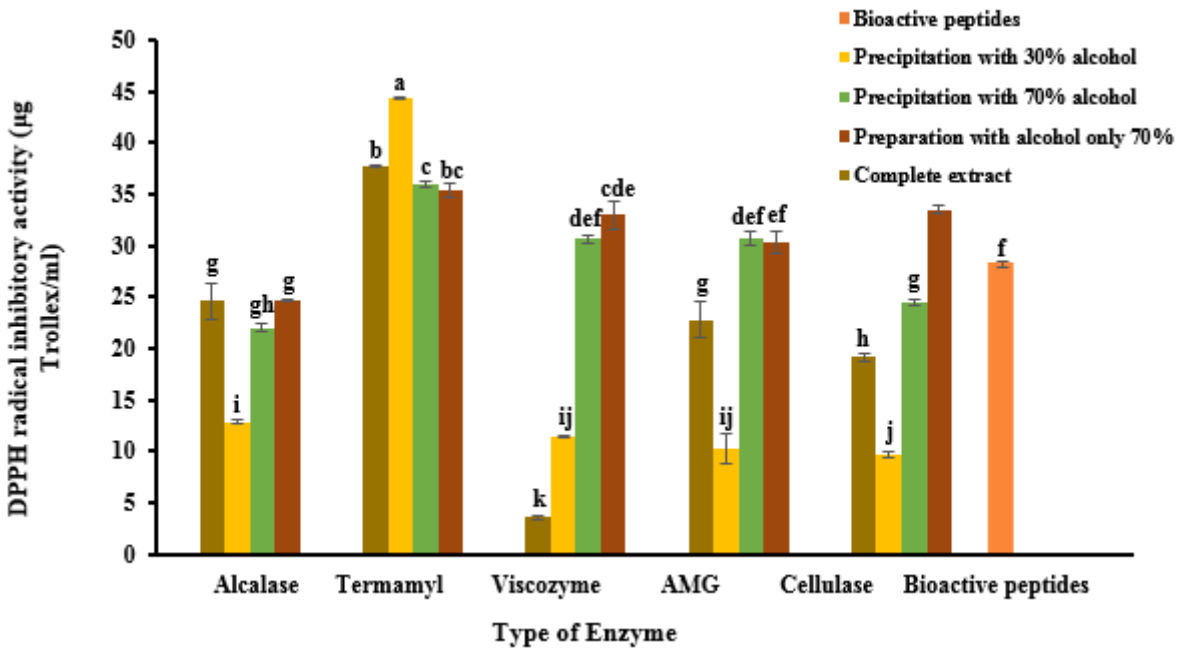

Fig. 3. Metal chelating (Fe^{2+}) activity of different enzyme extracts from *Sargassum ilicifolium* (µg EDTA/ml). Different letters indicate statistically significant differences ($P < 0.05$). Each experiment was performed in triplicate. The bars show the mean ± SD of three separate studies.

3.3.5. ABTS Radical Scavenging Activity

Figure 4 presents the ABTS radical scavenging activity of the enzyme extracts from *Sargassum ilicifolium*. The highest activity was observed for Viscozyme (Vc) precipitated with 70% alcohol (199.57 µg Trolox/ml), while the lowest activity was recorded for Vc precipitated with 30% alcohol (54.67 µg Trolox/ml). Alcalase (Al) hydrolysates and other peptide fractions showed intermediate values (approximately 171–173 µg Trolox/ml). Statistical analysis

revealed significant differences ($p < 0.05$) among the different enzyme types and precipitation methods. The strong ABTS inhibitory activity observed in *S. ilicifolium* extracts is consistent with earlier research on *Sargassum* species, including *S. ilicifolium* [43] and *S. angustifolium* [37]. The high concentration of antioxidant compounds, particularly polyphenols and sulfated polysaccharides, contributes significantly to this potent antioxidant capacity [18]. The marked difference in ABTS activity between Vc extracts at different alcohol concentrations can be attributed to the solubility of compounds with varying molecular weights. Higher alcohol concentrations (70%) tend to precipitate larger molecular weight antioxidants, including highly polymerized polyphenols, whereas lower concentrations (30%) are more effective at retaining smaller molecular weight compounds [42].

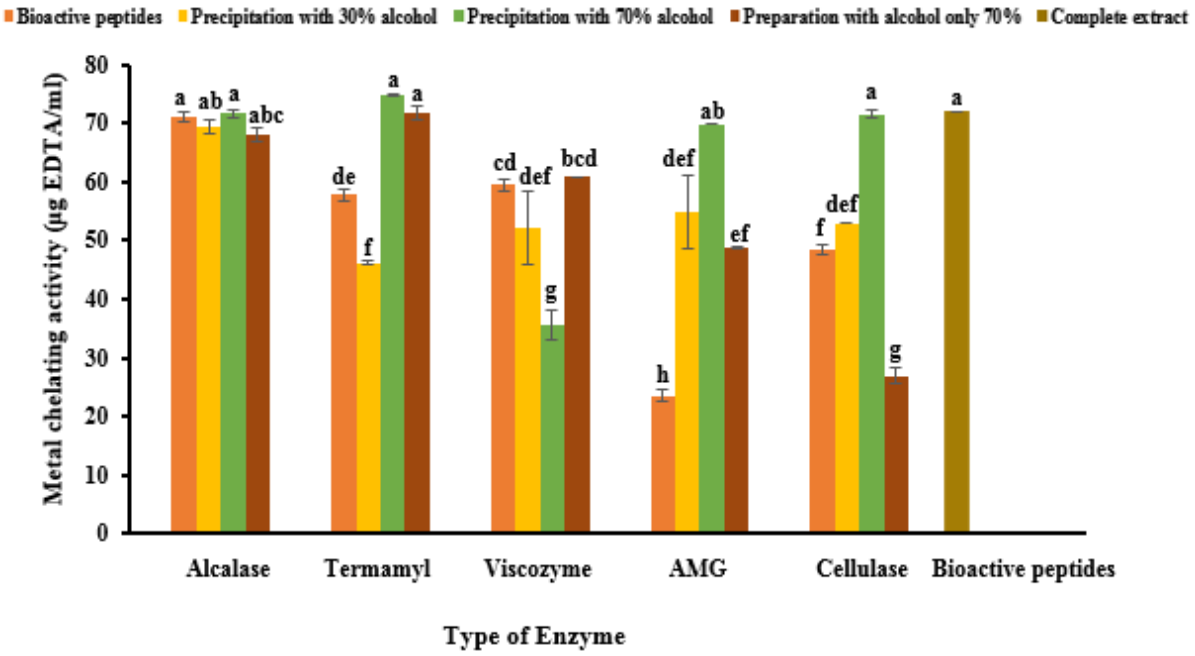

Fig. 4. ABTS radical scavenging (µg Trolox/ml) Same letter indicate no statistically significant differences (P < 0.05). Each experiment was performed three times. The bars show the mean SD of three separate studies.

3.4. Investigation of Enzyme Extraction Efficiency

3.4.1. Effect of Different Enzymes on Extraction Efficiency

Figure 5 illustrates the extraction efficiency of the different enzymes used. Alcalase (Al) and Termamyl (Te) exhibited the highest extraction efficiencies at 18.4% and 18%, respectively, when precipitated with 30% alcohol. However, when precipitated with 70% alcohol, both Al and Viscozyme (Vc) showed significantly lower efficiencies. Among the carbohydrases tested, Te yielded the highest extraction efficiency. In contrast, Al, a protease, demonstrated superior efficiency due to its ability to hydrolyze peptide bonds within the protein polypeptide chains,

thereby enhancing protein solubilization [44]. This finding is consistent with Naseri et al. (2020), who reported that Alcalase achieved the highest protein extraction rate from red algae (*Palmaria palmata*) under varying pH and temperature conditions. Enzyme-assisted extraction (EAE) is a promising alternative to conventional solvent extraction (CSE), which often requires harsh conditions that may degrade heat-labile compounds and reduce biological activity [45]. Furthermore, the study revealed that the use of phosphate buffer significantly enhanced extraction efficiency compared to using Alcalase alone, suggesting that buffer composition plays a crucial role in carbohydrate extraction. Finally, there was a significant difference in extraction efficiency between 30% and 70% alcohol precipitation. This can be attributed to the solubility differences of polysaccharides based on their molecular weight; larger molecular weight polysaccharides have lower solubility in ethanol, leading to their precipitation at lower ethanol concentrations (e.g., 30%), whereas smaller molecular weight compounds remain in solution or precipitate at higher concentrations [46].

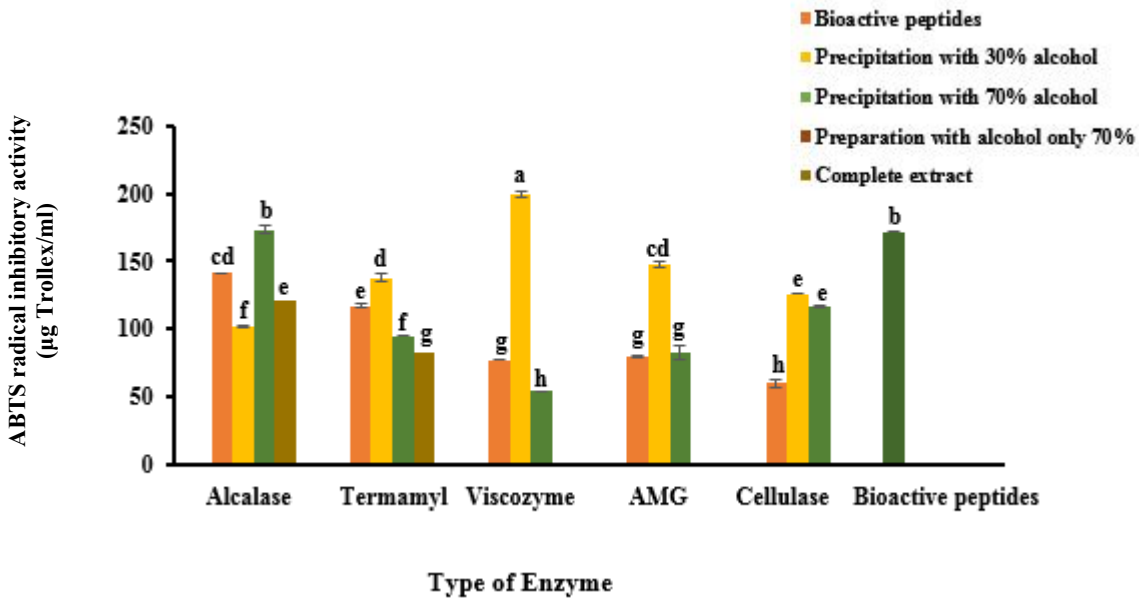

Fig. 5. Enzyme extraction efficiency (%) of different enzymes from *Sargassum ilicifolium*. Different letters indicate statistically significant differences ($P < 0.05$). Each experiment was performed in triplicate. The bars show the mean ± SD of three separate studies.

3.4.2. Comparison of Aqueous and Alkali Extraction Methods

Figure 6 compares the classical aqueous and alkali extraction methods. As the NaOH concentration increased, the extraction efficiency also increased, reaching a maximum of 9.13% at 0.4 M NaOH. The lowest efficiency (1.4%) was observed at 0.1 M NaOH. In contrast,

the aqueous extraction method showed a significantly lower efficiency of approximately 5.8%. The higher efficiency observed at increased NaOH concentrations can be attributed to the enhanced solubility of water-insoluble proteins in high pH environments [19]. Alkaline conditions effectively disrupt the cell wall structure and solubilize proteins that are otherwise inaccessible. This finding is consistent with Cermeño et al. (2020) [29], who reported that alkaline extraction yields higher protein concentrations from *Ulva rigida* compared to acid or water extraction.

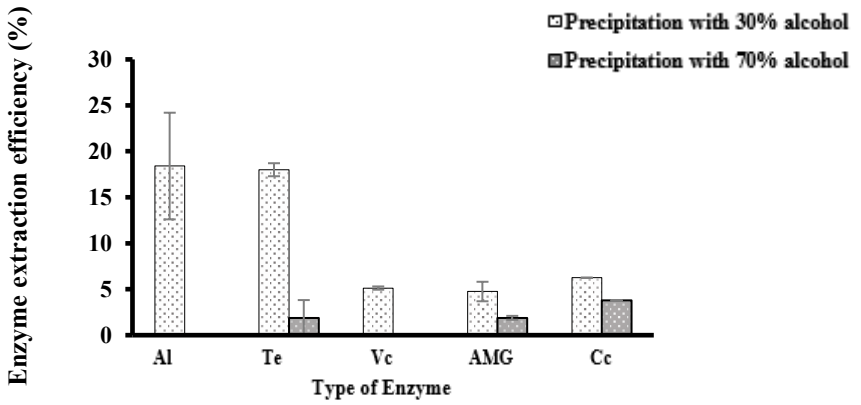

Fig. 6. Conventional extraction efficiency (%) using aqueous and alkaline methods from *Sargassum ilicifolium*. Different letters indicate statistically significant differences ($P < 0.05$). Each experiment was performed in triplicate. The bars show the mean ± SD of three separate studies.

3.4.3. Protein Production in Enzymatic Extraction (Detailed Mechanism)

The protein content was determined using the Bradford method (1976) [22]. As detailed in the materials and methods, the extraction process involved conventional alkaline treatment followed by enzymatic hydrolysis and subsequent alcohol precipitation. Conventional Alkaline Extraction, the conventional alkaline method yielded the highest protein content at 14.7%. This superior yield can be attributed to the strong alkaline conditions, which effectively disrupt peptide bonds and non-selectively solubilize proteins from the biomass [25]. However, as noted by Hammed et al. (2013) [28], such aggressive chemical methods may cause the degradation of heat-labile amino acids, potentially compromising the nutritional quality. In contrast, enzymatic treatments yielded lower protein contents, with Termamyl (Te) precipitated with 30% alcohol extracting the highest amount among enzymatic groups at 7.3%. The lower yield in enzymatic extraction is due to the specificity of enzymes, which target only specific bonds.

However, the effect of enzyme precipitation using alcohol is critical. As discussed by Cermeño et al. (2020) [29], while enzymatic yields are lower, the precipitation step is essential for isolating bioactive peptide fractions and removing non-protein contaminants such as sugars and phenolics. This results in a product with higher purity and preserved biological activity, making it more suitable for functional food applications despite the lower total yield. The variation in yield between 30% and 70% alcohol precipitation further highlights the role of solvent polarity in selectively precipitating specific peptide sizes."

3.4.4. Polyphenolic Compound Content

Figure 7 illustrates the total polyphenolic content obtained from the various enzyme treatments and precipitation conditions. Viscozyme (Vc) precipitated with 70% alcohol yielded the highest polyphenol content at 19.04 µg gallic acid/ml, followed by Celluclast (Cc) precipitated with 70% alcohol at 17.91 µg gallic acid/ml. In contrast, the complete extracts of AMG and Cc exhibited the lowest polyphenol levels (approximately 14 µg gallic acid/ml). The results indicate that carbohydrases generally produced higher phenolic yields compared to the protease (Alcalase). This can be attributed to the ability of carbohydrate-active enzymes to effectively degrade algal cell wall polysaccharides, thereby releasing bound phenolic compounds that are covalently linked to carbohydrates [30]. This finding is supported by Habeebullah et al. (2020) [47], who reported that carbohydrate-hydrolyzing enzymes released significantly more phenolic compounds from seaweed than proteolytic enzymes. Regarding the precipitation step, 70% alcohol generally yielded higher polyphenol content than 30% alcohol. This observation can be explained by the solubility properties of different molecular weight compounds; higher ethanol concentrations are more effective at precipitating larger molecular weight compounds, including highly polymerized polyphenols [42]. This is consistent with Tierney et al. (2013) [48], who demonstrated that ethanol concentration significantly affects the recovery of polymeric polyphenols.

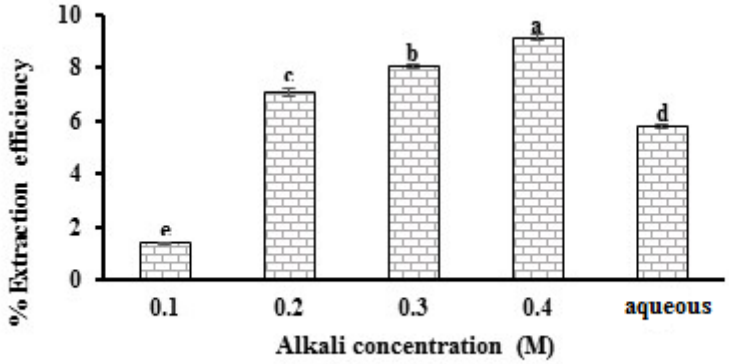

Fig. 7. Total polyphenol content of *Sargassum ilicifolium* after extraction using various enzymes (µg gallic acid/ml). Different letters indicate statistically significant differences ($P < 0.05$). Each experiment was performed in triplicate. The bars show the mean ± SD of three separate studies.

3.4.4.1. Effect of Enzyme Type and Precipitation on Yield

The yield of enzymatic extracts varied significantly depending on the specific enzyme used and the alcohol concentration for precipitation. As shown in Figure 8, Viscozyme (Vc) precipitated with 70% alcohol (Vc-70%) yielded the highest amount of polyphenols among all treatments at 19.04 µg gallic acid/ml, which was considerably higher than other samples. This was followed by Celluclast (Cc) precipitated with 70% alcohol (Cc-70%) at 17.91 µg gallic acid/ml. In contrast, the complete extracts of AMG (AMG-C) and Cc (Cc-C) exhibited the lowest polyphenol contents, at 14.26 and 14.46 µg gallic acid/ml, respectively.

3.4.4.2. Effect of Alcohol Precipitation Concentration

It is noteworthy that 70% alcohol precipitation generally yielded higher polyphenol content compared to 30% precipitation across most enzyme treatments. This can be attributed to the solubility differences of compounds based on molecular weight; higher ethanol concentrations are more effective at precipitating larger molecular weight compounds, including highly polymerized polyphenols [42]. Conversely, 30% alcohol precipitation primarily retains low molecular weight phenolic compounds, while 70% precipitation allows for the recovery of both medium and high molecular weight polyphenols [49].

3.4.4.3. Comparison with Previous Studies

The results indicate that carbohydrases produced higher phenolic yields than the protease (Alcalase). This finding is consistent with previous studies on *Chondria cornuta* and *Chondria dasyphylla* [40], which also reported that Vc extracts had the highest polyphenolic content. The

superior performance of Vc and Cc (carbohydrases) over Al (protease) can be explained by the ability of carbohydrate-active enzymes to effectively degrade algal cell wall polysaccharides, thereby releasing bound phenolic compounds that are covalently linked to carbohydrates [30]. This mechanism is supported by Habeebullah et al. (2020) [47], who reported that carbohydrate-hydrolyzing enzymes released more phenolic compounds from seaweed than proteolytic enzymes. Furthermore, the observation that higher ethanol concentrations improve polyphenol recovery aligns with Tierney et al. (2013) [48], who demonstrated that ethanol concentration significantly affects the recovery of polymeric polyphenols.

4. Conclusions

This study demonstrated that enzyme-assisted extraction is a viable and efficient alternative to classical methods for processing *Sargassum ilicifolium*. The highest overall extraction efficiency (18.4%) was achieved using Alcalase precipitated with 30% alcohol. While conventional alkaline extraction yielded the highest protein content (14.7%), enzymatic extraction with Termamyl (Te) at 30% alcohol provided a significant yield (7.3%) while better preserving bioactivity. The research highlighted the superior bioactivity of enzymatic extracts. Termamyl (Te) extracts, particularly those precipitated with 70% alcohol, exhibited the strongest antioxidant properties, including DPPH radical scavenging (44.32 µg Trolox/ml) and metal chelating activity (74.78 µg EDTA/ml). Additionally, Te extracts precipitated with 30% alcohol demonstrated the highest antidiabetic potential, showing significant inhibition of α-amylase and α-glucosidase. This suggests that seaweed-derived peptides could serve as natural alternatives to synthetic drugs like acarbose, which is associated with adverse side effects. Furthermore, Viscozyme (Vc) precipitated with 70% alcohol yielded the highest polyphenol content (19.04 µg gallic acid/ml), underscoring the effectiveness of carbohydrases in releasing bound phenolic compounds. Similarly, ABTS radical scavenging activity was highest for Te precipitated with 70% alcohol (199.57 µg Trolox/ml). Overall, Termamyl (Te) emerged as the most versatile enzyme for producing high-value protein components and bioactive peptides. These findings suggest that *S. ilicifolium* is a promising source of functional ingredients suitable for applications in aquaculture, animal feed, and human nutrition.

5. Future Perspectives

While this study successfully demonstrates the efficacy of enzymatic extraction using Termamyl and other enzymes to obtain bioactive peptides from *Sargassum ilicifolium* with promising antioxidant and antidiabetic properties, several avenues remain for future

investigation to enhance the commercial and therapeutic applicability of these extracts. Future research should prioritize *in vivo* studies to evaluate the bioavailability, metabolic stability, and actual hypoglycemic effects of the optimized Termamyl-derived peptides in animal models of diabetes and oxidative stress. Furthermore, detailed structural characterization, such as mass spectrometry (LC-MS/MS) and amino acid sequencing, is necessary to identify the specific peptide sequences responsible for the observed bioactivities. Understanding the structure-activity relationship (SAR) will facilitate the design of synthetic analogs or standardized extracts. The stability of these bioactive peptides under various storage conditions (temperature, pH, light) and during simulated gastrointestinal digestion should also be assessed. Additionally, exploring microencapsulation techniques could enhance their shelf-life and delivery efficiency. To transition from laboratory scale to industrial application, future studies should focus on scaling up the enzymatic extraction process. A comprehensive techno-economic analysis is required to assess the cost-effectiveness of using specific enzymes like Termamyl compared to conventional methods, ensuring economic viability for large-scale production. Finally, investigating the synergistic effects of these peptides when combined with other natural bioactive compounds or conventional antidiabetic drugs could provide insights into enhanced therapeutic outcomes and potential drug interactions.

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