Antioxidant Profiling of *Botryocladia leptopoda* Based on *In Silico* and *In Vitro* Studies

Fenny Crista Anastasia Panjaitan*, Huey-Jine Chai

Abstract

Seaweed has long been recognized as an excellent source of antioxidants. The presence of antioxidant activity from *Botryocladia leptopoda* was investigated. The study aims to estimate the potential antioxidative peptides released by *B. leptopoda* sequences using an in-silico approach and to explore the antioxidant capacities revealed by different extracts. *In silico* studies using the ProtParam and BIOPEP-UWM databases showed that *B. leptopoda* proteins are potential sources of antioxidative peptides. Glycine and leucine were identified as the primary amino acids detected in the sequences. The antioxidant capacities of various seaweed extracts at different drying temperatures (60 °C and 70 °C) and solvents (alkaline-ethanol, ethanol, and water) were further observed *in vitro*. The fraction from alkaline-ethanol extraction of seaweed dried at 60 °C (BL-A, 1 mg/mL) produced a high phenolic concentration of 6.44 mg GA/g ($p < 0.05$). It exhibited superior reducing power activity, with an absorbance of 0.29 at 700 nm ($p < 0.05$) and ferrous ion chelating activity of 99.64% ($p < 0.05$). In comparison, significant DPPH radical scavenging activities were observed in the ethanol extracts of seaweed dried at 60 °C (BL-B) and 70 °C (BL-C), with 46.08% and 33.74%, respectively, at a 1 mg/mL concentration ($p < 0.05$). At a 10 mg/mL concentration, BL-B and BL-C optimally scavenged DPPH radicals (96.61% and 94.79%, respectively). Overall, this study showed that *in silico* and *in vitro* analyses yielded similar results. Thus, *B. leptopoda* can be considered a promising source of antioxidative products in the functional food and pharmaceutical industries.

Keywords: BIOPEP-UWM database, extraction, phenolic, red seaweed

[1] Marine Product Processing Study Program, Marine and Fisheries Polytechnic of Jembrana, Bali 82218, Indonesia
[2] Seafood Technology Division, Fisheries Research Institute, Council of Agriculture, Keelung 20246, Taiwan

*Corresponding Author:
fennycap@gmail.com

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Introduction

Seaweed is increasingly recognized for its ecological significance within marine ecosystems and its potential as a natural source of diverse bioactive compounds. It is considered a cost-effective and sustainable source of various biologically active components including polysaccharides, proteins, phycocyanin, vitamins, carotenoids, polyphenols, fatty acids, amino acids, tocopherols, and sterols, which exhibit a wide range of biological activities (Sapatinha *et al*., 2022). These bioactivities include antioxidant, antiviral, antimicrobial, anti-inflammatory, antihypertensive, anti-cancer, and anti-diabetic activities (Cermeño *et al*., 2020; Cotas *et al*., 2020; Sharanagat *et al*., 2019). Due to these unique metabolites and activities, numerous investigations and studies have been conducted to explore and to optimize the utilization of seaweed in comparison to other marine organisms. Consequently, seaweed has found broad application in the food, fertilizer, animal feed, skincare, and pharmaceuticals industries (Kalasariya *et al*., 2024; Lomartire & Gonçalves, 2022). Moreover, the application of natural resources as therapeutic treatments has gained emerging interest compared to synthetic drugs due to health issues and side effects (Chaachouay & Zidane, 2024). Among these various biological activities, antioxidant activity has received particular attention due to its critical role in combating oxidative stress and related chronic diseases.

Antioxidants play a crucial role in mitigating oxidative stress in cells, which is linked to various diseases, including chronic illnesses such as cancer, neurodegenerative diseases, and cardiovascular disease (Abu-Baih *et al*., 2024; Pradhan & Ki, 2023; Yamagishi *et al*., 2023). Pintaningrum & Butsainah (2023) emphasized that daily intake of antioxidants is widely recognized for its importance in protecting against free radical-induced damage and is essential for maintaining optimal health, thereby contributing to the prevention of serious diseases. Chen *et al*. (2019) reported that

Isochrysis zhanjiangensis hydrolyzed with gastrointestinal proteases exhibited a potential antioxidative peptide identified as Asn-Asp-Ala-Glu-Tyr-Gly-Ile-Cys-Gly-Phe (NDAEYGICGF; MW: 1088.16 Da). Similarly, Zhang *et al.* (2019) identified a peptide (Glu-Leu-Trp-Lys-Thr-Phe) isolated from *Gracilariopsis lemaneiformis* hydrolysates could significantly scavenge DPPH free radicals. These finding suggest that consuming seaweed may offer enhanced health benefits and improve quality of life. Numerous studies have evaluated seaweed's antioxidant activity through bioinformatics approaches and *in vitro* analysis. The combination of *in silico* and *in vitro* studies is preferred, as preliminary insights from *in silico* analyses can reduce the time and cost associated with subsequent *in vitro* experiments (Bermejo *et al*., 2024).

Bioinformatics, often referred to as *in silico* analysis, is used to predict the potential bioactivities exhibited by peptides and proteins based on their amino acid sequences. Amino acid sequences refer to the specific order of amino acids in a polypeptide chain (Dewangan *et al*., 2023). *In silico* tools are primarily used for identifying bioactive peptides from specific food proteins, such as the NCBI database (Geer *et al*., 2010), the BIOPEP-UWM database (Minkiewicz *et al*., 2019), and the ProtParam tool (ProtParam, 2017). These tools reduce the cost and time spent during analysis by enabling the rapid identification and characterization of proteins from complex materials (Panjaitan *et al*., 2018; Tejano *et al*., 2019). These computational tools have mainly been applied to detect the antioxidant and other bioactivities from various seaweed species, such as brown seaweed *Padina boergesenii* (Cholaraj & Venkatachalam, 2024), *Caulerpa racemosa* (Dissanayake *et al*., 2022), red seaweed *Eucheuma spinosum* (Damongilala *et al*., 2023), green seaweed *Ulva lactuca* (Amin *et al*., 2022) and *Sargassum ilicifolium* (Lakshmanan *et al*., 2022). Amin *et al*. (2022) investigated potential antioxidative peptides in the RuBisCO protein of *U. lactuca* using several computational tools, including the UniProtKB database, BIOPEP-UWM, PeptideRanker and ToxinPred. Moreover, the effectiveness of these compounds can also be influenced by post-harvest processing methods such as drying and extraction.

Drying is the most common method of preserving seaweed (Blikra *et al*., 2021). Moreover, thermal drying affects the antioxidant capacity of preserved seaweed, and this effect must be determined for specific types of seaweed. Charles *et al*. (2020) reported that the oven drying method is preferred and recommended due to its cost-effectiveness compared to vacuum and freeze-drying techniques. This method provides an economical alternative to sun drying for the production of seaweed-enriched functional foods. Moreover, various extraction methods have been employed to optimize the antioxidant capacity of seaweed. Previous studies have shown that different solvent systems and extraction techniques significantly influence the antioxidant activity of seaweed (Sadeghi *et al*., 2024; Subbiah *et al*., 2023a, 2023b). Considering the influence of drying and extraction on antioxidant activity, this study investigates the antioxidative potential of the red seaweed *Botryocladia leptopoda*.

Botryocladia leptopoda, also known as *B. leptopoda* (J. Agardh) Kylin, is alternatively classiefied as *Chrysymenia uvaria* var. *leptopoda* J. Agardh and is a red seaweed species distributed in Taiwan (Guiry, 2013). Research on this species and its bioactive activities are still limited (Gajalakshmi et al., 2018; Lakshmi *et al*., 2004). Therefore, this study was conducted to predict the antioxidative peptides of *B. leptopoda* using the BIOPEP-UWM database and to evaluate the antioxidant capacities of various extracts obtained from different drying temperatures. The objectives of this study were to estimate the potential antioxidative peptides released by *B. leptopoda* sequences through an *in silico* approach and to explore the antioxidant capacities revealed by different extracts.

Material and Methods

Materials

Fresh *B. leptopoda* was obtained from the coastal area of Southern Taiwan in May 2022 during the low tide. The seaweed sample was freshly obtained and put in a box containing seawater before being transferred to the laboratory. The samples were transferred to the laboratory of the Taiwanese Fisheries Research Institute (FRI) and stored for further analysis. The sample was then cleaned and rinsed using tap water prior to further analysis. All reagents and chemicals utilized in this study were of analytical-grade quality.

Protein sequences and amino acids identification of *B. leptopoda*

Protein sequences of *B. leptopoda* were acquired from the UniProtKB database (https://www.uniprot.org), showing the accession number, protein name, number of amino acid residues, molecular weight, and sequences. The amino acid composition was observed using the ProtParam tool (https://web.expasy.org/protparam). These sequences were then used to estimate the number of antioxidant peptides expressed in *B. leptopoda* proteins.

BIOPEP-UWM database-based antioxidative peptides analysis

Bioactive peptides of *B. leptopoda* were identified using the BIOPEP-UWM database (http://www.uwm.edu.pl/biochemia/index.php/en/biopep) to estimate the number of antioxidant biopeptides listed in the protein sequences. The process involved selecting the "Bioactive peptides" menu, followed by navigating to the "Analysis" section. Additionally, the protein sequences were analyzed using the "Profiles of potential biological activity" tool to display the BIOPEP ID, peptide name, activity, total number of peptides, sequences, and the location of bioactive peptides within the sequences (Panjaitan *et al.*, 2018). The concurrence of bioactive fragments (A) within the protein sequences was determined using the formula A = a/N, where "a" represents the number of fragments with a specific activity, and "N" represents the total number of amino acid residues (Minkiewicz *et al.*, 2019).

Dried *B. leptopoda* preparation

The raw materials underwent a thorough washing process, being rinsed three times with tap water to eliminate any seawater residues and foreign particles. Subsequently, the cleaned red seaweed was drained at room temperature. Following this, the seaweed was placed in a drying oven (Memmert, Germany) at 60 °C and 70 °C for 48 hours and then finely pulverized using a dry blender (Mill Powder Tech, Taiwan). The sample was then subjected to extraction systems using various methods, as depicted in Figure 1.

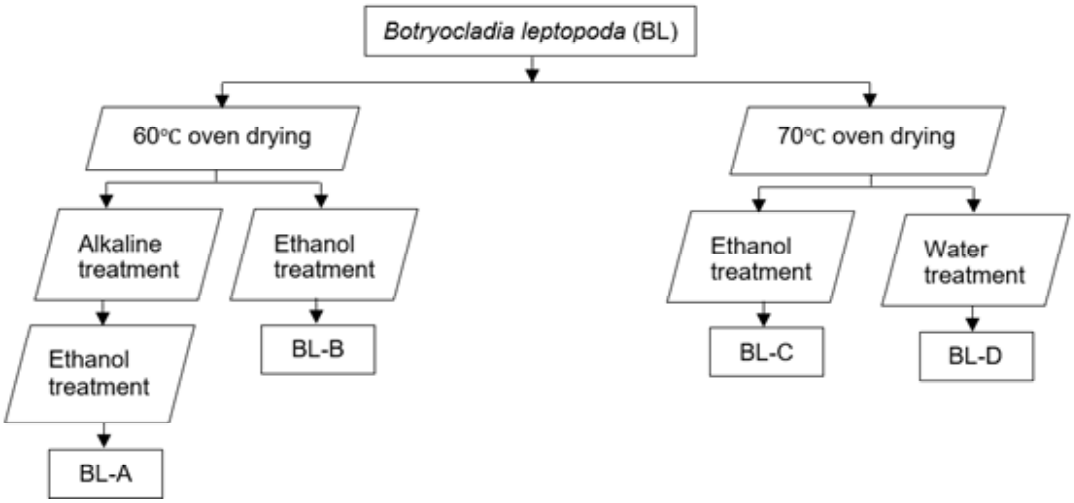


Figure 1. Various methods of *Botryocladia leptopoda* extraction.

Extraction of *B. leptopoda*

Alkaline-ethanol extraction: This method followed the procedure outlined by Kadam *et al.* (2017) with some adjustments to the seaweed-to-distilled water ratio, centrifugation speed, and the tiered level of NaOH extraction, as well as the use of EtOH and a rotary evaporator. Initially, 80 g of dried seaweed was diluted in 500 mL of distilled water for 3 hours. The resulting mixture was homogenized using a homogenizer (Yuchengtech, China) and then centrifuged at 15,000 xg, 4 °C for 20 minutes. The precipitate was diluted into 400 mL of 0.4 M NaOH and 0.5% β-mercaptoethanol (v/v) for 10 minutes at 4 °C, followed by a second centrifugation to obtain a second precipitate. Subsequently, 20 g of precipitate was subjected to extraction using 95% ethanol (200 mL) for 3 hours. The solution was then filtered using Whatman No. 1 filter paper and concentrated using a rotary evaporator (Rotavapor® R-100, Buchi, Switzerland) at 40°C to remove the ethanol. The concentrated sample was stored in a dry cabinet (Patron, Ampore House Co., Ltd., Taichung, Taiwan) at 27°C and 40% relative humidity for further use.

Ethanol extraction: In this method, the dried seaweed (20 g) was mixed with 100 mL ethanol for 3 hours. The mixture was filtered using Whatman No. 1 filter paper and concentrated using a rotary evaporator. The residues were stored in a dry cabinet for subsequent analysis.

Water extraction: The sample (20 g) was diluted into distilled water (1:10, w/v). The mixture underwent sterilization (121°C, 15 atm) for 15 minutes and was then filtered. The filtrate was then lyophilized and ground into a fine powder.

The yield of all seaweed extracts was measured and calculated in percentage (%) using the following formula:

$$\text{Yield (\%)} = \frac{\text{Final weight of extracts}}{\text{Initial weight of dry seaweed}} \text{ x } 100\%$$

Total phenolic content (TPC) analysis

The total phenol content (TPC) was determined using a plate reader (Multiskan Go, Thermo Fisher Scientific, Waltham, MA, USA) according to a protocol described by Badmus et al., (2019) employing the Folin-Ciocalteu technique. The seaweed extract (5 mg) was dissolved in methanol to make a stock solution. The solution was then diluted into appropriate concentrations. The sample (20 µL) was mixed with Folin-Ciocalteu reagent (100 µL) and then left for 5 min to stand prior to the addition of 80 µL of Na_2CO_3 (7.5%). The mixture was incubated for 2 hours in a dark room. The sample was read using a UV-VIS spectrophotometer (Multiskan Go, Thermo Fisher Scientific, Waltham, MA, USA) at 740 nm. Water was the blank, and gallic acid was used to make a standard curve. The TPC was expressed as gallic acid equivalents in milligrams per gram of dried sample (mg GAE/g).

DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging assay

The DPPH (2,2-diphenyl-1-picrylhydrazyl) radical analysis was conducted following a modified method as described by Giriwono *et al*. (2020) using a 96-well microplate. The sample was dissolved using methanol to achieve various final concentrations. Briefly, DPPH was dissolved in methanol to achieve a concentration of 0.1 mM. The sample (100 µL), methanol (as control), and L-ascorbic acid (positive control) were combined with DPPH (100 µL) in a microplate. The mixture was incubated for 30 min in a dark room before absorbance measurement at 517 nm using a Multiskan Go (Thermo Fisher Scientific, Waltham, MA, USA). The calculation of DPPH radical scavenging activity was determined as below:

$$\text{DPPH radical scavenging activity (\%)} = \frac{(\text{Abs. control} - \text{Abs. sample})}{Abs.control} \; x \; 100\%$$

Reducing power assay

The reducing power activity was assessed using the method outlined by Kumar *et al*. (2020), with modifications to the overall analysis volume to accommodate the use of a microplate reader (Multiskan Go, Thermo Fisher Scientific, Waltham, MA, USA). The seaweed extracts were dissolved in 0.2 M sodium phosphate buffer (PBS; pH 6.6). Seaweed dilution or distilled water (the negative control), at 250 µL, was mixed with 250 µL of 1% potassium ferricyanide solution and allowed to stand for 20 min at 50 °C. Subsequently, 250 µL of 10% trichloroacetic acid (TCA) was added, and the sample was centrifuged (Thermo Fisher Scientific, Waltham, MA, USA) for 10 min at 3000 rpm. Then, 250 µL of solution mixture was combined with 200 µL of distilled water and 50 µL of 0.1% ferric chloride, followed by incubation at room temperature for 10 min. Finally, the sample (200 µL) was placed into a microplate and the absorbance at 700 nm.

Ferrous ion chelating assay

The ferrous ion chelating activity was analyzed using the method described by Chakraborty *et al*. (2017), with adjustments made to the overall analysis volume to accommodate the use of a microplate reader (Multiskan Go, Thermo Fisher Scientific, Waltham, MA, USA). An aliquot of 500 µL, respectively, of seaweed fractions and EDTA-Na were mixed with 25 µL of 2 mM $FeSO_4$ and 1.85 mL of deionized water, followed by 50 µL of 5 mM ferrozine. The solution was equilibrated for 10 minutes at room temperature. A 200 µL aliquot of sample was transferred to a 96-well plate, and the absorbance was read at 562 nm. Deionized water was used as a control group. The metal ion chelating activity assay was determined using the following formula:

$$\text{Ferrous ion chelating activity} = \left(1 - \frac{\text{Abs. sample}}{\text{Abs. control}}\right) \text{x } 100\%$$

Statistical Analysis

Statistical analysis was performed using a one-way analysis of variance (ANOVA) followed by Duncan's post hoc test ($p < 0.05$) with Statistical Program for the Social Sciences 22.0 (SPSS Inc., Chicago, IL, USA, version 22.0). All measurements were taken in triplicate. The results were expressed as mean ± standard deviation (SD). A Pearson correlation test was used to assess correlations between means of total phenolic contents (TPC) and antioxidant activities of different solvent extracts of seaweed. Principal component analysis (PCA) was used to examine the dataset's mean-variance and determine the correlation between TPC and antioxidant activities in different solvent fractions.

Results and Discussion

Protein sequences and amino acid composition of *B. leptopoda*

B. leptopoda is a type of red seaweed that is abundantly distributed in Taiwan (Guiry, 2013). In addition, this species has also been detected across Asian regions, including South India (Gajalakshmi *et al*., 2018), Pakistan (Afzal Rizvi & Shameel, 2005), and Iran (Moein *et al*., 2015). Pangestuti and Kim (2015) summarized that red seaweeds have higher protein levels and comprise almost 50% of the dry weight compared to green and brown seaweeds. However, fewer studies and less information regarding protein analysis have been obtained from *B. leptopoda*.

Protein sequence screening of *B. leptopoda* was conducted using the UniProtKB database accessed on 30 April 2024. Table 1 presents the list of proteins from *B. leptopoda* selected from the database, along with their characteristics, including accession number, cellular location, number of amino acids, and molecular weight. The proteins included cytochrome oxidase subunit 1 (mitochondrion), ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (plastid), and ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (chloroplast).

Additionally, ribulose-1,5-bisphosphate carboxylase/oxygenase (RubisCo) is the primary protein found abundantly in seaweed due to its crucial role in photosynthesis, as observed in green seaweed such as Caulerpa (Agirbasli & Cavas, 2017). The proteins identified from *B. leptopoda* exhibit different molecular weights corresponding to the number of amino acids in their sequences. The amino acid composition in the protein sequences was calculated using the ProtParam tool to determine the amino acid concentration (Panjaitan *et al*., 2022).

Results showed that glycine (G) and leucine (L) were the top residues identified in cytochrome oxidase subunit 1 (mitochondrion), RubisCo large subunit (plastid), and RubisCo large subunit (chloroplast), as depicted in Figure 2. Glycine is the major amino acid in seaweed proteins, followed by arginine, alanine, and glutamic acid (Pangestuti & Kim, 2015). The amino acid composition varies depending on the seaweed species, habitat, and harvesting season (Tanna *et al*., 2019).

Table 1. List of proteins from *Botryocladia leptopoda* and their characteristics

No.	Protein name	Accession number	Location in cell	Number of amino acids	Molecular weight
1.	Cytochrome oxidase subunit 1, partial	APZ75448.1	Mitochondrion	221	23,930 Da
2.	Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit, partial	AER42223.1	Plastid	444	49,174 Da
3.	Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit, partial	AER42223.1	Chloroplast	452	50,056 Da

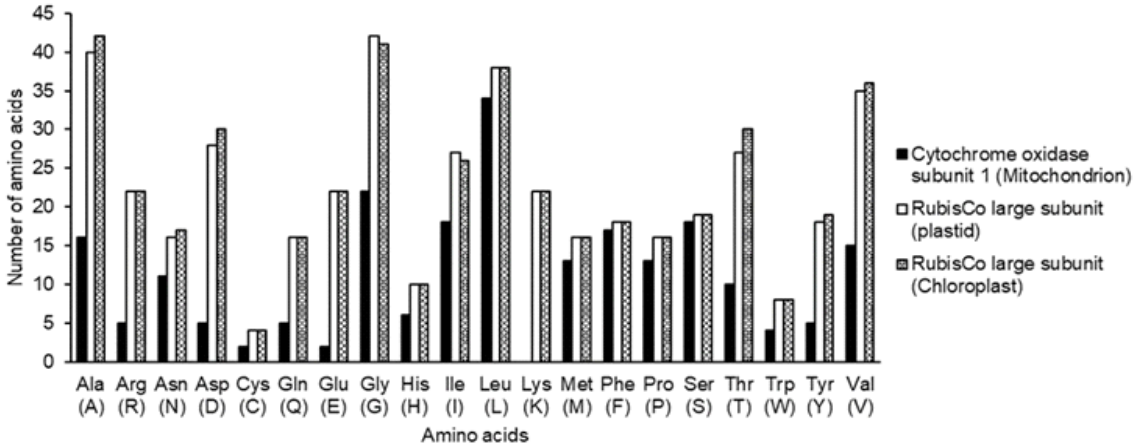


Figure 2. Composition of amino acid identified in *Botryocladia leptopoda* proteins. Ala (A): Alanine, Arg (R): Arginine, Asn (N): Asparagine, Asp (D): Aspartate, Cys (C): Cysteine, Gln (Q): Glutamine, Glu (E): Glutamate, Gly (G): Glycine, His (H): Histidine, Ile (I): Isoleucine, Leu (L): Leucine, Lys (K): Lysine, Met (M): Methionine, Phe (F): Phenylalanine, Pro (P): Proline, Ser (S): Serine, Thr (T): Threonine, Trp (W): Tryptophan, Tyr (Y): Tyrosine, Val (V): Valine.

Amino acid composition in protein sequences correlates with antioxidant activity. Li & Li (2013) stated that the physicochemical properties of amino acid residues contribute to antioxidative potency, with the C-terminal regions of amino acids making a stronger contribution than those at the N-terminal for estimating antioxidant capacity. Moreover, hydrophobic amino acids, such as alanine, glycine, leucine, isoleucine, valine, proline, tryptophan, methionine, and phenylalanine, which are present in peptide sequences, significantly contribute to the antioxidant potential (Pangestuti & Kim, 2015; Zhai *et al*., 2020). Therefore, *B. leptopoda* is predicted to have potent antioxidative peptides due to the significant presence of amino acids such as alanine, glycine, valine, and leucine.

Prediction of potential antioxidant activity

The antioxidative biopeptides of *B. leptopoda* were predicted using the BIOPEP-UWM database (accessed on 30 April 2024). Antioxidative peptide profiling from each protein is illustrated in Figure 3. The BIOPEP-UWM database has been widely utilized to identify potential bioactivities in food proteins (Minkiewicz *et al*., 2019; Panjaitan *et al*., 2018; Panjaitan *et al*., 2022). Selected protein sequences, such as cytochrome oxidase subunit 1 (mitochondrion), RubisCo large subunit (plastid), and RubisCo large subunit (chloroplast), were analyzed to observe the potential antioxidant activity that might be released from these sequences. These sequences accounted for 16, 39,

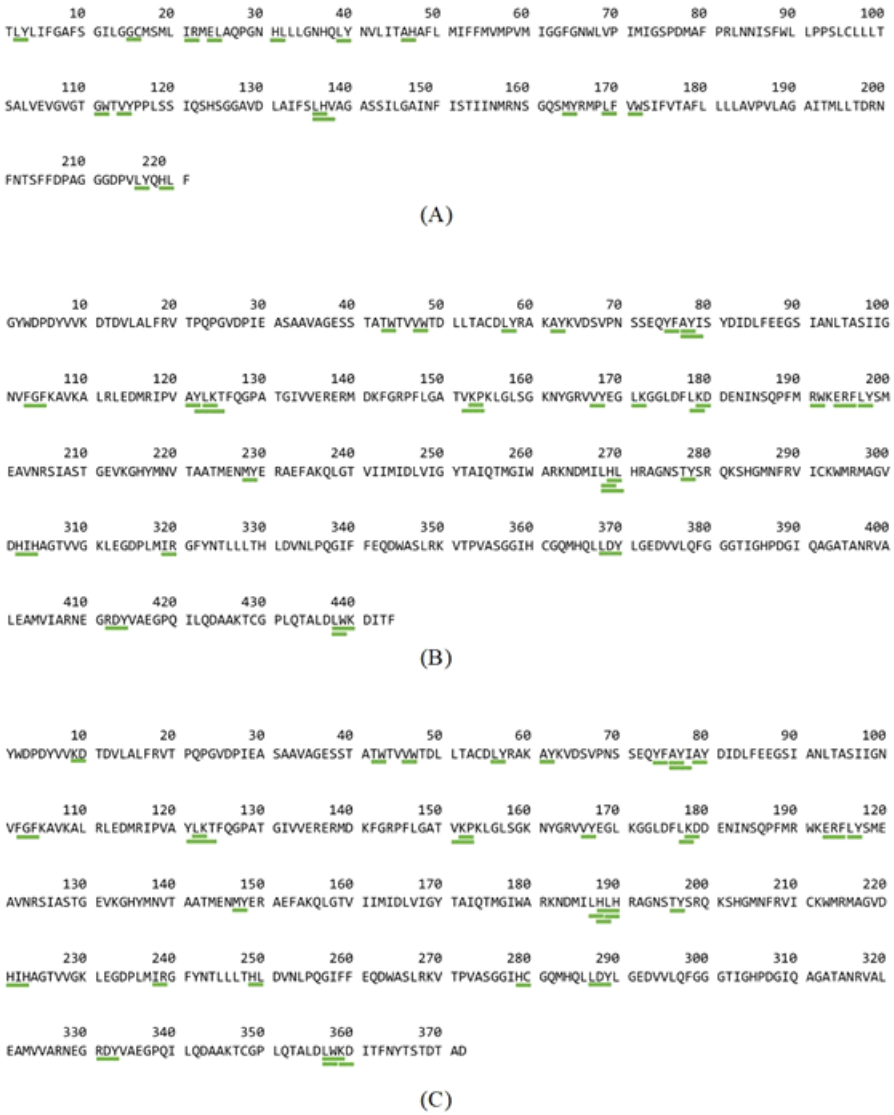


Figure 3. Antioxidant activity profiling from (A) Cytochrome oxidase subunit 1 (Mitochondrion), (B) RubisCo large subunit (plastid), (C) RubisCo large subunit (Chloroplast). Green underlines ( ) presented antioxidative biopeptides.

and 40 peptides, respectively. The occurrences of antioxidative fragments (A) in the cytochrome oxidase subunit 1 (mitochondrion), RubisCo large subunit (plastid), and RubisCo large subunit (chloroplast) sequences were determined to be 0.07, 0.09, and 0.09, respectively.

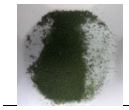
Most biopeptides were described as dipeptides, and only some as tripeptides. Similar peptides were observed from each protein sequence, for example, LH, HL, and LY. From those results, L is a dominant amino acid generating strong antioxidant activity. Some fractions could even possess antioxidant activity in different structures, such as LH (dipeptides) or LHV (tripeptides). Moreover, the structure of amino acid fractions influenced their bioactivities. Xu *et al.* (2024) reported that the amino acid L contributed significantly to enhancing the antioxidant activity instead of isoleucine in the peptide sequence (either C-terminus or N-terminus). Amino acids, including W, E, L, I, M, V, and Y, are suitable in the C-terminus and have some effect on antioxidant activity (Wen *et al*., 2020). According to the QSAR (quantitative structure-activity relationship) method, hydrophobic amino acids at the C-terminal played a vital role in the radical scavenging system (Du *et al*., 2022). In line with this finding, further analysis should be conducted to determine the antioxidant capacity of *B. leptopoda*. Results from the BIOPEP-UWM database predicted potential antioxidative peptides based on the structure of intact amino acids in the sequence.

Total phenolic concentration (TPC) and yield of *B. leptopoda* extracts

The TPC and yield of *B. leptopoda* extracts are listed in Table 2. Results showed that the seaweed extract obtained from the combined alkaline and ethanol extraction had the highest phenolic concentration at 6.44 ± 0.26 mg GA/g sample ($p < 0.05$); however, it had the lowest yield at 1.84%. Alkaline extraction can rupture polysaccharide structures in seaweed cells and exploit functional ingredients (Sun *et al*., 2018). Alkaline extraction (BL-A) can solubilize proteins, thereby contributing to a high total protein content (TPC). Additionally, some components might be lost during the combined extraction process, resulting in a lower final yield.

On the other hand, water extraction (BL-D) revealed the lowest concentration of total phenols and the highest yield, at 1.75 ± 0.01 mg GA/g sample ($p < 0.05$) and 41.32%, respectively. The heat treatment during water extraction might reduce the phenolic compounds in seaweed. Chan *et al.* (2015) reported similar results, indicating that the water extract (80°C, 2 hours) had the lowest TPC at 6.06 mg PGE/g. Moreover, using pressurized hot water, seaweed extraction increased the yield by solubilizing polysaccharides. Saravana *et al.* (2016) revealed that increasing temperature and pressure (°C/bar) elevated the residue obtained after extracting brown seaweed due to lower surface tension, mass transfer, and higher solubility of various components.

Table 2. Total phenolic and yield of *Botryocladia leptopoda* obtained from various extraction methods

Fresh seaweed	Dried seaweed	Extracts*	Total phenolic (mg GA/g)	Yield (%)
Botryocladia leptopoda 	Dried seaweed at 60 °C 	BL-A 	6.44 ± 0.26[d]	1.84
		BL-B 	3.16 ± 0.08[c]	6.42
	Dried seaweed at 70 °C 	BL-C 	2.75 ± 0.07[b]	7.72
		BL-D 	1.75 ± 0.01[a]	41.32

*Extracts: BL-A (alkaline extracts), BL-B (ethanol extracts from dried seaweed at 60°C), BL-C (ethanol extracts from dried seaweed at 70°C), BL-D (water extracts)

The drying temperature also affected the phenolic concentration of the seaweed. The present study showed that ethanol extracts from dried seaweed at 60°C (BL-B) had a higher total phenolic content (TPC) than those from seaweed dried at 70°C (BL-C), at 3.16 ± 0.08 and 2.75 ± 0.07 mg gallic acid (GA)/g, respectively. These findings were similar to the study of Ling *et al*. (2015), showing that oven-dried *Kappaphycus alvarezii* at 40 °C had a higher total phenolic content than oven-dried seaweed at 80 °C. Drying is a crucial step in seaweed processing; however, this method can reduce the phytochemical components in the seaweed. Additionally, seaweed preserved through oven drying showed a higher TPC (mg GAE/g) than other drying methods, such as solar drying, freeze-drying, and vacuum drying (Charles *et al*., 2020).

DPPH radical scavenging activity

The DPPH (1,1-Diphenyl-2-picryl-hydrazil) scavenging activity of seaweed fractions was analyzed in a dose-dependent manner (Table 3). The results indicated that the radical scavenging activity of various seaweed extracts was concentration-dependent, with the antioxidant capacity increasing as the concentration of extracts increased. Overall, the scavenging activity of BL-B and BL-C, at a concentration of 10 mg/mL, was not significantly different from that of L-ascorbic acid ($p < 0.05$). Specifically, seaweed extracts (BL-B and BL-C) could optimally scavenge free radicals, achieving 96.61 ± 0.59% and 94.79 ± 1.02%, respectively, at 10 mg/mL. Chan *et al*. (2015) also reported that the antioxidant activity of ethanol, methanol, and acetone extracts of *Gracilaria changii* increased with concentration, reaching a maximum plateau of 10 mg/mL.

Potent DPPH radical scavenging activities were observed in ethanol extracts (BL-B and BL-C) from *B. leptopoda* despite their total phenolic values being lower than those of BL-A (alkaline coupled with ethanol treatment). Moreover, BL-D exhibited a better radical scavenging system than BL-A. Based on this result, TPC did not always show a positive correlation with DPPH activity in seaweed extracts. Airanthi et al., (2011) also noted the inconsistencies between TPC and DPPH activity in methanol extracts from various types of brown seaweeds may be partly attributed to structural differences in phenolic compounds. These phenolics include a diverse array of compounds with varying degrees of polymer subunits, which are associated with the polarity of these compounds. Hence, this finding implies that alkaline treatment is particularly effective in extracting phenolic compounds from seaweed samples. Furthermore, the presence of antioxidants other than polyphenols in red seaweed could contribute to scavenging DPPH radicals, such as phycobiliproteins, terpenoids, flavonoids, and alkaloids (Carpena *et al*., 2023). This result aligns with *in silico* studies, which predict that *B. leptopoda* possesses potent antioxidative peptides due to the significant presence of alanine (A), glycine (G), leucine (L), and valine (V). In addition, L is a dominant amino acid exhibiting potent antioxidant properties, as seen in dipeptides (LH) and tripeptides (LHV).

Table 3. DPPH scavenging activity of *Botryocladia leptopoda* extracts at various concentrations

Sample	DPPH radical scavenging activity (%)							
	0.06 mg/ml	0.13 mg/ml	0.25 mg/ml	0.5 mg/ml	1 mg/ml	2 mg/ml	5 mg/ml	10 mg/ml
L-asorbic acid	94.05 ± 0.45^{d}	94.51 ± 0.52^{d}	94.74 ± 0.83^{e}	95.04 ± 0.63^{e}	95.33 ± 0.70^{e}	95.34 ± 0.30^{e}	95.39 ± 0.21^{e}	97.42 ± 0.80^{cd}
BL-A	16.43 ± 1.19^{a}	16.54 ± 1.22^{a}	16.59 ± 1.59^{a}	18.73 ± 1.67^{a}	20.28 ± 0.85^{a}	21.17 ± 0.50^{a}	22.61 ± 0.31^{a}	25.43 ± 0.19^{a}
BL-B	21.49 ± 0.54^{c}	24.55 ± 0.98^{c}	27.53 ± 1.28^{d}	33.76 ± 1.30^{d}	46.08 ± 0.27^{d}	59.03 ± 0.42^{d}	93.21 ± 0.45^{d}	96.61 ± 0.59^{d}
BL-C	22.12 ± 0.78^{c}	23.58 ± 1.32^{c}	25.11 ± 1.05^{c}	28.71 ± 1.08^{c}	33.74 ± 0.50^{c}	45.72 ± 1.06^{c}	72.72 ± 1.24^{c}	94.79 ± 1.02^{c}
BL-D	19.24 ± 1.16^{b}	19.41 ± 1.33^{b}	21.32 ± 1.44^{b}	22.90 ± 1.04^{b}	24.99 ± 1.12^{b}	26.35 ± 1.32^{b}	33.47 ± 0.47^{b}	43.35 ± 0.84^{b}

Sample: BL-A (alkaline extracts), BL-B (ethanol extracts from dried seaweed at 60°C), BL-C (ethanol extracts from dried seaweed at 70°C), BL-D (water extracts). Statistical analysis was performed using SPPS with One-way ANOVA, and the differences were calculated using Duncan's method. Values are given as mean ± SD from triplicate determinations. Values in the same column with different letters are significantly different ($p < 0.05$).

Reducing power activity

The reducing power assay measures the Fe^{3+} reduction, which is linked to the indicator of electron-donating activity in antioxidant mechanisms (Chakraborty *et al.*, 2017). Antioxidant agents contained in seaweed extracts reduce the Fe^{3+}/ ferricyanide complex to the ferrous form, as evidenced by the emergence of Perl's Prussian blue at 700 nm (Lin *et al*., 2012).

The reducing power capacity of *B. leptopoda* extracts was evaluated at various concentrations, as illustrated in Table 4. Findings revealed that the

reduction activity of *B. leptopoda* extracts ranged from 0.20 to 0.29 (absorbance at 700 nm), notably lower than the overall reduction capacity of L-ascorbic acid. The reduction potentials of seaweed extracts rose with escalating concentrations. However, augmenting the concentration of seaweed extracts up to 10 mg/mL did not result in a significant enhancement in reduction activity. A previous study by Chakraborty *et al*. (2015) using methanolic extracts (1 mg/mL) from three red seaweeds (*Hynea musciformis*, *H. valentiae*, and *Jania rubens*) also reported low absorbance values at 700 nm, ranging from 0.33 to 0.74, which were considerably higher than *those of B. leptopoda* extracts. Landa-Cansigno *et al*. (2023) likewise observed a similar pattern in *Sargassum pallidum* extracts. The reductive capacity of the aqueous fraction from *S. pallidum* reached 0.505 (absorbance at 700 nm) at 0.2 mg/mL, which was twice that in aqueous extracts of *B. leptopoda* at 0.25 mg/mL. The result suggests that *B. leptopoda* fractions were not particularly effective as reducing agents.

B. leptopoda showcased robust antioxidant characteristics as identified through *in silico* and *in vitro* analyses. The computational study suggested that *B. leptopoda* proteins could potentially produce antioxidative peptides, particularly those rich in glycine (Gly) and leucine (Leu), which significantly contributed to the antioxidant efficacy. Paiva *et al*. (2017) also reported a strong correlation between amino acid profiles and antioxidant activity in the protein hydrolysate of the brown seaweed *Fucus spiralis*. Furthermore, the antioxidant activities of various extracts demonstrated a dose-dependent pattern, with effectiveness escalating alongside concentration increments. Overall, BL-A (a blend of alkaline and ethanol extracts) displayed notably high total phenolic content and efficiently chelated Fe^{2+} ions.

Table 4. Reducing power activity of *Botryocladia leptopoda* extracts at various concentrations

Sample	Reducing power (absorbance 700 nm)							
	0.06 mg/ml	**0.13 mg/ml**	**0.25 mg/ml**	**0.5 mg/ml**	**1 mg/ml**	**2 mg/ml**	**5 mg/ml**	**10 mg/ml**
L-asorbic acid	0.98 ± 0.03^{c}	2.51 ± 0.03^{c}	2.59 ± 0.00^{d}	2.60 ± 0.01^{c}	2.60 ± 0.00^{d}	2.60 ± 0.02e	2.60 ± 0.01^{d}	2.60 ± 0.01^{d}
BL-A	0.22 ± 0.00^{ab}	0.23 ± 0.00^{ab}	0.24 ± 0.00^{b}	0.26 ± 0.00^{b}	0.29 ± 0.00^{c}	0.32 ± 0.01d	0.42 ± 0.01c	0.52 ± 0.01c
BL-B	0.20 ± 0.00^{a}	0.21 ± 0.00^{a}	0.21 ± 0.00^{a}	0.22 ± 0.01^{a}	0.23 ± 0.01^{a}	0.27 ± 0.00a	0.35 ± 0.01a	0.44 ± 0.01a
BL-C	0.22 ± 0.00^{b}	0.25 ± 0.00^{b}	0.25 ± 0.00^{c}	0.25 ± 0.00^{b}	0.27 ± 0.00^{b}	0.30 ± 0.01c	0.38 ± 0.01b	0.47 ± 0.01b
BL-D	0.20 ± 0.01^{a}	0.23 ± 0.01^{ab}	0.24 ± 0.00^{b}	0.25 ± 0.00^{b}	0.28 ± 0.00^{b}	0.29 ± 0.01b	0.35 ± 0.01a	0.44 ± 0.00a

Sample: BL-A (alkaline extracts), BL-B (ethanol extracts from dried seaweed at 60°C), BL-C (ethanol extracts from dried seaweed at 70°C), BL-D (water extracts). Statistical analysis was done using SPPS with One-way ANOVA, and the differences were calculated using Duncan's method. Values are given as mean ± SD from triplicate determinations. Values in the same column with different letters are significantly different (P<0.05).

Ferrous ion chelating activity

Ferrous ions are primarily found in the food system and serve as the most effective pro-oxidants (Landa-Cansigno *et al*., 2023). Therefore, the chelating ability of *B. leptopoda* fractions was determined. The chelating effects are shown in Table 5. Results indicate that BL-A was the most effective in chelating ferrous ions compared with the other fractions. At a concentration of 1 mg/mL, BL-A exhibited insignificant chelating activity compared to EDTA-Na ($p > 0.05$). On the other hand, BL-B, BL-C, and BL-D demonstrated nearly similar chelating activity at 10.72%, 11.59%, and 10.42%, respectively ($p > 0.05$). Additionally, the chelating activity rose with the increasing concentration from 0.06 to 10 mg/mL. The findings were consistent with TPC compounds, indicating that BL-A had the highest total polyphenolic content (TPC) among the other extracts. This study aligns with previous research, which has reported that polyphenols derived from seaweed exhibit metal chelating potency, dependent on the phenolic structure, the number of -OH groups, and their location (Subbiah, Duan *et al*., 2023). Moreover, the presence of hydrophobic amino acids, such as leucine (L), found in peptides like LH, HL, and LY, as previously identified through *in silico* analysis, contributed to strong antioxidant activity. The amino acid profile also influenced the ferrous ion chelating capacity in *F. spiralis* seaweed (Paiva *et al*., 2017).

Correlation of phenolic content and antioxidant activities of different solvent extracts

The relationship between phenolic contents and various antioxidative assays of seaweed extracts was evaluated through Pearson correlation analysis. According to Table 6, ferrous ion chelating activity demonstrated a positive correlation with TPC at 0.958, suggesting that the ferrous ion chelating capacity is

dependent on TPC. This finding suggests that the phenolic components of red seaweed can effectively chelate metal ions, which aligns with a study by Chakraborty *et al*. (2017) that utilized methanol extracts from various species of red seaweeds, including *J. rubens*, *H. valentiae*, and *H. musciformis*. Likewise, a positive correlation was also identified between TPC and reducing power activity at 0.344, indicating that phenolic compounds contribute to electron donation. Similarly, Murugan & Iyer (2014) observed this result in extracts of red and brown seaweed, showing a correlation between reducing power capacity and phenolic content.

Conversely, a negative relationship between DPPH radical scavenging activity and TPC, at -0.408, suggests that phenolic compounds do not significantly influence DPPH scavenging. This finding suggests that antioxidant activity is not solely dependent on TPC, and other components and metabolites, besides phenolics, contribute to the DPPH scavenging capacity, such as polysaccharides (Airanthi *et al*., 2011; Carpena *et al*., 2023). Agar and carrageenan are the most abundant carbohydrates in red seaweed, accounting for 40-50% of the dry weight (Torres *et al*., 2019). Many studies have reported various bioactivities attributed to carrageenans, including antioxidant properties (Premarathna *et al*., 2024; Sokolova *et al*., 2011).

Principal component analysis (PCA) was then performed to examine the similarities and differences among the TPC and antioxidant properties of seaweed extracted using different solvents. The PCA biplot showed 74.06% (F1) and 25.33% (F2) of the variance, respectively (Figure 4). F1 was primarily influenced by the TPC of all extracts, as well as the DPPH scavenging activity (DPPH), reducing power activity (RP), and ferrous ion chelating activity (FIC). Additionally, BL-A, BL-B, and BL-C extracts had a notable impact on F1, while BL-D predominantly contributed to F2. The similarity observed between

Table 5. Ferrous ion chelating activity of *Botryocladia leptopoda* extracts at various concentrations

Sample	Ferrous ion chelating activity (%)							
	0.06 mg/ml	0.13 mg/ml	0.25 mg/ml	0.5 mg/ml	1 mg/ml	2 mg/ml	5 mg/ml	10 mg/ml
EDTA-Na	97.70 ± 0.60^{d}	98.27 ± 0.98^{d}	99.49 ± 0.04^{d}	99.64 ± 0.14^{d}	99.82 ± 0.18^{b}	99.89 ± 0.05^{c}	99.89 ± 0.05^{c}	99.90 ± 0.01^{d}
BL-A	15.41 ± 0.58^{c}	35.87 ± 0.57^{c}	66.87 ± 0.59^{c}	98.63 ± 0.60^{c}	99.64 ± 0.13^{b}	99.81 ± 0.13^{c}	99.79 ± 0.07^{c}	99.72 ± 0.08^{d}
BL-B	5.71 ± 0.22^{ab}	5.75 ± 0.40^{a}	7.71 ± 0.71^{a}	8.87 ± 0.59^{a}	10.72 ± 0.45^{a}	11.24 ± 0.40^{a}	11.40 ± 0.82^{a}	14.47 ± 0.55^{a}
BL-C	4.80 ± 1.22^{a}	6.21 ± 0.19^{a}	7.57 ± 0.25^{a}	9.13 ± 0.70^{b}	11.59 ± 0.31^{a}	12.56 ± 0.05^{b}	14.48 ± 0.83^{b}	18.41 ± 0.19^{b}
BL-D	6.20 ± 0.66^{b}	8.93 ± 0.69^{b}	9.22 ± 0.70^{b}	9.36 ± 0.66^{b}	10.42 ± 1.38^{a}	10.98 ± 0.52^{a}	16.11 ± 1.78^{b}	21.99 ± 1.90^{c}

BL-A (alkaline extracts), BL-B (ethanol extracts from dried seaweed at 60°C), BL-C (ethanol extracts from dried seaweed at 70°C), and BL-D (water extracts). Statistical analysis was done using SPPS with One-way ANOVA, and the differences were calculated using Duncan's method. Values are given as mean ± SD from triplicate determinations. Values in the same column with different letters are significantly different ($P<0.05$).

Table 6. Pearson correlation test between TPC and antioxidant activities of different solvent extracts

Variables	DPPH radical scavenging activity	Reducing power activity	Ferrous ion chelating activity	TPC
DPPH radical scavenging activity	1	-0.978	-0.645	-0.408
Reducing power activity	-0.978	1	0.571	0.344
Ferrous ion chelating activity	-0.645	0.571	1	0.958
TPC	-0.408	0.344	0.958	1

TPC, RP, and FIC with BL-A extracts suggests a close association among them. Furthermore, BL-A also indicates that the alkaline coupled with ethanol extraction methods resulted in high TPC, RP, and FIC.

Moreover, DPPH, BL-B, and BL-C showed a strong correlation, suggesting that the ethanol system enhances DPPH scavenging capacity. Correspondingly, BL-B and BL-C exhibited good DPPH scavenging activity. In addition, BL-D is not directly related to TPC and any antioxidant properties, indicating that water extraction might not yield high TPC and antioxidative agents from seaweed. In addition, the presence of amino acids in seaweed extracts also contributes to their antioxidant capacity, as previously identified through *in silico* studies, for example, LH, HL, and LY. Xiong (2010) reported that peptides containing leucine or proline have the ability to chelate transition metal ions and quench active oxygen species.

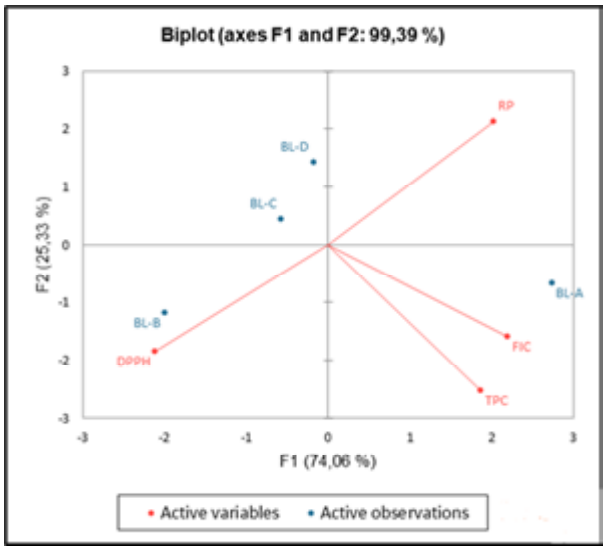


Figure 4. Biplot (F1xF2) for the PCA of TPC (Total phenolic concentration) and antioxidant capacity (DPPH: DPPH radical scavenging activity, RP: Reducing power activity, FIC: Ferrous ion chelating activity) from seaweed extracted by various solvents (BL-A, BL-B, BL-C, and BL-D).

Conclusion

This study conducted antioxidant profiling of *B. leptopoda* using both *in silico* and *in vitro* analyses. Screening with BIOPEP-UWM suggested that *B. leptopoda* is an excellent source of antioxidative properties. *In vitro* analysis was performed to demonstrate the antioxidant capacities of different extraction systems, showing a dependence on seaweed extracts. As the seaweed extract concentration increased, both phenol and antioxidant capacity increased. Red seaweed extracted using a combination of alkaline and ethanol (BL-A) exhibited higher total phenolic compound content, as well as increased reducing power and ferrous ion chelating activity. Ethanol extractions (BL-B and BL-C) resulted in potent DPPH radical scavenging activity. In contrast, water extraction (BL-D) did not optimally produce strong antioxidant activity in any of the assays, even as the dose increased. PCA analysis revealed that the total phenolic content present in red seaweed does not always correlate with potential antioxidant properties. This study indicates that *B. leptopoda* has the potential to be used as a candidate for developing antioxidative supplements in the functional food industry.

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References

Abu Baih, D. H., Abd El Mordy, F. M., Saber, E. A., Ali, S. F. E. S., Hisham, M., Alanazi, M. A.,…Abdelmohsen, U. R. (2024). Unlocking the potential of edible *Ulva* sp. seaweeds: Metabolomic profiling, neuroprotective mechanisms, and implications for Parkinson's disease management. *Archiv der Pharmazie*, e2400418.

Afzal Rizvi, M., & Shameel, M. (2005). Pharmaceutical biology of seaweeds from the Karachi coast of Pakistan. *Pharmaceutical Biology*, *43*(2), 97-107.

Agirbasli, Z., & Cavas, L. (2017). *In silico* evaluation of bioactive peptides from the green algae *Caulerpa*. *Journal of Applied Phycology*, *29*, 1635-1646.

Airanthi, M. W. A., Hosokawa, M., & Miyashita, K. (2011). Comparative antioxidant activity of edible Japanese brown seaweeds. *Journal of Food Science*, *76*(1), C104-C111.

Amin, M. A., Chondra, U., Mostafa, E., & Alam, M. M. (2022). Green seaweed *Ulva lactuca*, a potential source of bioactive peptides revealed by *in silico* analysis. *Informatics in Medicine Unlocked*, *33*, 101099.

Badmus, U. O., Taggart, M. A., & Boyd, K. G. (2019). The effect of different drying methods on certain nutritionally important chemical constituents in edible brown seaweeds. *Journal of Applied Phycology*, *31*, 3883-3897.

Bermejo, M., Camara-Martinez, I., Sanchez-Dengra, B., Ruiz-Picazo, A., Gonzalez-Alvarez, I., & Gonzalez-Alvarez, M. (2024). *In silico*, *in situ*, *in vitro*, and *in vivo* predictive methods for modeling formulation performance. In *From Current to Future Trends in Pharmaceutical Technology* (pp. 67-116). Elsevier.

Blikra, M. J., Altintzoglou, T., Løvdal, T., Rognså, G., Skipnes, D., Skåra, T.,…Fernández, E. N. (2021). Seaweed products for the future: Using current tools to develop a sustainable food industry. *Trends in Food Science & Technology*, *118*, 765-776.

Carpena, M., García-Pérez, P., García-Oliveira, P., Chamorro, F., Otero, P., Lourenço-Lopes, C.,…Prieto, M. (2023). Biological properties and potential of compounds extracted from red seaweeds. *Phytochemistry Reviews*, *22*(6), 1509-1540.

Cermeño, M., Kleekayai, T., Amigo Benavent, M., Harnedy Rothwell, P., & FitzGerald, R. J. (2020). Current knowledge on the extraction, purification, identification, and validation of bioactive peptides from seaweed. *Electrophoresis*, *41*(20), 1694-1717.

Chaachouay, N., & Zidane, L. (2024). Plant-derived natural products: a source for drug discovery and development. *Drugs and Drug Candidates*, *3*(1), 184-207.

Chakraborty, K., Joseph, D., & Praveen, N. K. (2015). Antioxidant activities and phenolic contents of three red seaweeds (Division: *Rhodophyta*) harvested from the Gulf of Mannar of Peninsular India. *Journal of Food Science and Technology*, *52*, 1924-1935.

Chakraborty, K., Maneesh, A., & Makkar, F. (2017). Antioxidant activity of brown seaweeds. *Journal of Aquatic Food Product Technology*, *26*(4), 406-419.

Chan, P. T., Matanjun, P., Yasir, S. M., & Tan, T. S. (2015). Antioxidant activities and polyphenolics of various solvent extracts of red seaweed, *Gracilaria changii*. *Journal of Applied Phycology*, *27*, 2377-2386.

Charles, A. L., Sridhar, K., & Alamsjah, M. A. (2020). Effect of drying techniques on color and bioactive potential of two commercial edible Indonesian seaweed cultivars. *Journal of Applied Phycology*, *32*, 563-572.

Chen, M.-F., Zhang, Y. Y., Di He, M., Li, C. Y., Zhou, C. X., Hong, P. Z., & Qian, Z.-J. (2019). Antioxidant peptide purified from enzymatic hydrolysates of *Isochrysis Zhanjiangensis* and its protective effect against ethanol-induced oxidative stress of HepG2 cells. *Biotechnology and Bioprocess Engineering*, *24*, 308-317.

Cholaraj, R., & Venkatachalam, R. (2024). Investigation of antioxidant and anticancer potential of fucoidan (*in-vitro* & *in-silico*) from brown seaweed *Padina boergesenii*. *Algal Research*, *79*, 103442.

Cotas, J., Leandro, A., Pacheco, D., Gonçalves, A. M., & Pereira, L. (2020). A comprehensive review of the nutraceutical and therapeutic applications of red seaweeds (*Rhodophyta*). *Life*, *10*(3), 19.

Damongilala, L. J., Dotulong, V., Apriyanti, E., & Kurnia, D. (2023). Antioxidant and antibacterial activities of the tropical red alga *Eucheuma spinosum*: *in silico* study. *Natural Product Communications*, *18*(7), 1934578X231187467.

Dewangan, Y., Berdimurodov, E., & Verma, D. K. (2023). Amino acids: Classification, synthesis methods, reactions, and determination. In *Handbook of biomolecules* (pp. 3-23). Elsevier.

Dissanayake, I. H., Bandaranayake, U., Keerthirathna, L. R., Manawadu, C., Silva, R. M., Mohamed, B.,…Peiris, D. C. (2022). Integration of *in vitro* and *in-silico* analysis of *Caulerpa racemosa* against antioxidant, antidiabetic, and anticancer activities. *Scientific Reports*, *12*(1), 20848.

Du, Z., Wang, D., & Li, Y. (2022). Comprehensive evaluation and comparison of machine learning methods in QSAR modeling of antioxidant tripeptides. *ACS omega*, *7*(29), 25760-25771.

Gajalakshmi, D., Shettu, N., & Murugesan, S. (2018). Phytochemical screening of marine red alga Botryocladia leptopoda (J. Agardh) Kylin. *International Journal of Interdisciplinary Research and Innovations*, *6*(2), 463-470.

Geer, L. Y., Marchler-Bauer, A., Geer, R. C., Han, L., He, J., He, S.,…Bryant, S. H. (2010). The NCBI biosystems database. *Nucleic Acids Research*, *38*(suppl_1), D492-D496.

Giriwono, P. E., Iskandriati, D., Tan, C. P., & Andarwulan, N. (2020). In-vitro anti-inflammatory activity, free radical (DPPH) scavenging, and ferric reducing ability (FRAP) of Sargassum cristaefolium lipid-soluble fraction and putative identification of bioactive compounds using UHPLC-ESI-ORBITRAP-MS/MS. *Food Research International*, *137*, 109702.

Guiry, M. (2013). AlgaeBase. World-wide electronic publication. *http://www. algaebase. org*.

Kadam, S. U., Álvarez, C., Tiwari, B. K., & O'Donnell, C. P. (2017). Extraction and characterization of protein from Irish brown seaweed *Ascophyllum nodosum*. *Food Research International*, *99*, 1021-1027.

Kalasariya, H. S., Maya-Ramírez, C. E., Cotas, J., & Pereira, L. (2024). Cosmeceutical Significance of Seaweed: A Focus on Carbohydrates and Peptides in Skin Applications. *Phycology*, *4*(2), 276-313.

Kumar, L. R., Treesa Paul, P., Anas, K., Tejpal, C., Chatterjee, N., Anupama, T.,…Mathew, S. (2020). Screening of effective solvents for obtaining antioxidant rich seaweed extracts using principal component analysis. *Journal of Food Processing and Preservation*, *44*(9), e14716.

Lakshmanan, A., Balasubramanian, B., Maluventhen, V., Malaisamy, A., Baskaran, R., Liu, W.-C., & Arumugam, M. (2022). Extraction and characterization of fucoidan derived from *Sargassum ilicifolium* and its biomedical potential with *in silico* molecular docking. *Applied Sciences*, *12*(24), 13010.

Lakshmi, V., Kumar, R., Gupta, P., Varshney, V., Srivastava, M., Dikshit, M.,…Misra-Bhattacharya, S. (2004). The antifilarial activity of a marine red alga, *Botryocladia leptopoda*, against experimental infections with animal and human filariae. *Parasitology Research*, *93*, 468-474.

Landa-Cansigno, C., Serviere-Zaragoza, E., Morales-Martínez, T., Ascacio-Valdes, J., Morreeuw, Z., Gauyat, C.,…Reyes, A. (2023). The antioxidant and anti-elastase activity of the brown seaweed *Sargassum horridum* (*Fucales*, *Phaeophyceae*) and their early phenolics and saponins profiling for green cosmetic applications. *Algal Research*, *75*, 103271.

Li, Y.-W., & Li, B. (2013). Characterization of structure–antioxidant activity relationship of peptides in free radical systems using QSAR models: Key sequence positions and their amino acid properties. *Journal of Theoretical Biology*, *318*, 29-43.

Lin, H.-C., Tsai, W.-S., & Chiu, T.-H. (2012). Antioxidant properties of seven cultivated and natural edible seaweed extracts from Taiwan. *Journal of Aquatic Food Product Technology*, *21*(3), 248-264.

Ling, A. L. M., Yasir, S., Matanjun, P., & Abu Bakar, M. F. (2015). Effect of different drying techniques on the phytochemical content and antioxidant activity of *Kappaphycus alvarezii*. *Journal of Applied Phycology*, *27*, 1717-1723.

Lomartire, S., & Gonçalves, A. M. (2022). An overview of potential seaweed-derived bioactive compounds for pharmaceutical applications. *Marine Drugs*, *20*(2), 141.

Minkiewicz, P., Iwaniak, A., & Darewicz, M. (2019). BIOPEP-UWM database of bioactive peptides: Current opportunities. *International Journal of Molecular Sciences, 20*(23), 5978.

Moein, S., Moein, M., Ebrahimi, N., Farmani, F., Sohrabipour, J., & Rabiei, R. (2015). Extraction and determination of protein content and antioxidant properties of ten algae from Persian Gulf. *Int. J. of Aquatic Science, 6*(2), 29-38.

Murugan, K., & Iyer, V. V. (2014). Antioxidant and Antiproliferative Activities of Extracts of Selected Red and Brown Seaweeds from the M andapam Coast of T amil N adu. *Journal of Food Biochemistry, 38*(1), 92-101.

Paiva, L., Lima, E., Neto, A. I., & Baptista, J. (2017). Angiotensin I-converting enzyme (ACE) inhibitory activity, antioxidant properties, phenolic content and amino acid profiles of Fucus spiralis L. protein hydrolysate fractions. *Marine Drugs, 15*(10), 311.

Pangestuti, R., & Kim, S.-K. (2015). Seaweed proteins, peptides, and amino acids. In *Seaweed sustainability* (pp. 125-140). Elsevier.

Panjaitan, F. C. A., Gomez, H. L. R., & Chang, Y.-W. (2018). *In silico* analysis of bioactive peptides released from giant grouper (*Epinephelus lanceolatus*) roe proteins identified by proteomics approach. *Molecules, 23*(11), 2910.

Panjaitan, F. C. A., Pratama, F., Panjaitan, R., & Sitepu, G. S. B. (2022). Identifikasi Potensi Kandungan Peptida Bioaktif Ace Inhibitor pada Lemuru (*Sardinella lemuru*) dengan Teknik in Silico. Prosiding Seminar Nasional Ikan,

Pintaningrum, Y., & Butsainah, N. F. Z. (2023). Daily seaweed intake effect on coronary heart disease and stroke prevention: A systematic review and meta-analysis. AIP Conference Proceedings, Mataram, Indonesia.

Pradhan, B., & Ki, J. S. (2023). Antioxidant and chemotherapeutic efficacies of seaweed derived phlorotannins in cancer treatment: a review regarding novel anticancer drugs. *Phytotherapy Research, 37*(5), 2067-2091.

Premarathna, A. D., Ahmed, T. A., Kulshreshtha, G., Humayun, S., Darko, C. N. S., Rjabovs, V.,…Hincke, M. T. (2024). Polysaccharides from red seaweeds: Effect of extraction methods on physicochemical characteristics and antioxidant activities. *Food Hydrocolloids, 147*, 109307.

ProtParam, E. (2017). ExPASy-ProtParam tool. *SIB: Lausanne, Switzerland*.

Sadeghi, A., Rajabiyan, A., Nabizade, N., Meygolinezhad, N., & Ahmady, A. Z. (2024). Seaweed-derived phenolic compounds as diverse bioactive molecules: A review on identification, application, extraction and purification strategies. *International Journal of Biological Macromolecules*, 131147.

Sapatinha, M., Oliveira, A., Costa, S., Pedro, S., Gonçalves, A., Mendes, R.,…Pires, C. (2022). Red and brown seaweeds extracts: A source of biologically active compounds. *Food Chemistry, 393*, 133453.

Saravana, P. S., Choi, J. H., Park, Y. B., Woo, H. C., & Chun, B. S. (2016). Evaluation of the chemical composition of brown seaweed (*Saccharina japonica*) hydrolysate by pressurized hot water extraction. *Algal Research, 13*, 246-254.

Sharanagat, V. S., Singla, V., & Singh, L. (2019). Bioactive compounds from marine sources. In *Technological Processes for Marine Foods, From Water to Fork* (pp. 23-60). Apple Academic Press.

Sokolova, E., Barabanova, A., Bogdanovich, R., Khomenko, V., Solov'eva, T., & Yermak, I. (2011). In vitro antioxidant properties of red algal polysaccharides. *Biomedicine & Preventive Nutrition, 1*(3), 161-167.

Subbiah, V., Duan, X., Agar, O. T., Dunshea, F. R., Barrow, C. J., & Suleria, H. A. (2023a). Comparative study on the effect of different drying techniques on phenolic compounds in Australian beach-cast brown seaweeds. *Algal Research, 72*, 103140.

Subbiah, V., Ebrahimi, F., Agar, O. T., Dunshea, F. R., Barrow, C. J., & Suleria, H. A. (2023b). Comparative study on the effect of phenolics and their antioxidant potential of freeze-dried Australian beach-cast seaweed species upon different extraction methodologies. *Pharmaceuticals, 16*(5), 773.

Sun, Y., Hou, S., Song, S., Zhang, B., Ai, C., Chen, X., & Liu, N. (2018). Impact of acidic, water and alkaline extraction on structural features, antioxidant activities of *Laminaria japonica* polysaccharides. *International Journal of Biological Macromolecules, 112*, 985-995.

Tanna, B., Brahmbhatt, H. R., & Mishra, A. (2019). Phenolic, flavonoid, and amino acid compositions reveal that selected tropical seaweeds have the potential to be functional food ingredients. *Journal of Food Processing and Preservation, 43*(12), e14266.

Tejano, L. A., Peralta, J. P., Yap, E. E. S., Panjaitan, F. C. A., & Chang, Y.-W. (2019). Prediction of bioactive peptides from *Chlorella sorokiniana* proteins using proteomic techniques in combination with bioinformatics analyses. *International Journal of Molecular Sciences, 20*(7), 1786.

Torres, M. D., Flórez-Fernández, N., & Domínguez, H. (2019). Integral utilization of red seaweed for bioactive production. *Marine Drugs, 17*(6), 314.

Wen, C., Zhang, J., Zhang, H., Duan, Y., & Ma, H. (2020). Plant protein-derived antioxidant peptides: Isolation, identification, mechanism of action and application in food systems: A review. *Trends in Food Science & Technology, 105*, 308-322.

Xiong, Y. L. (2010). Antioxidant peptides. *Bioactive proteins and peptides as functional foods and nutraceuticals*, 29-42.

Xu, B., Dong, Q., Yu, C., Chen, H., Zhao, Y., Zhang, B.,…Chen, M. (2024). Advances in Research on the Activity Evaluation, Mechanism and Structure-Activity Relationships of Natural Antioxidant Peptides. *Antioxidants, 13*(4), 479.

Yamagishi, K., Sun, W., & Iso, H. (2023). Seaweed in Traditional Diets and Relationship with Cardiovascular Disease Incidence and Mortality. In *Ancient and Traditional Foods, Plants, Herbs and Spices used in Cardiovascular Health and Disease* (pp. 65-73). CRC Press.

Zhai, Y., Chen, Y., Teng, Z., & Zhao, Y. (2020). Identifying antioxidant proteins by using amino acid composition and protein-protein interactions. *Frontiers in cell and developmental biology, 8*, 591487.

Zhang, X., Cao, D., Sun, X., Sun, S., & Xu, N. (2019). Preparation and identification of antioxidant peptides from protein hydrolysate of marine alga *Gracilariopsis lemaneiformis*. *Journal of Applied Phycology, 31*, 2585-2596.