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Original Article

Antioxidant and Antimicrobial Activities of Peptide Fractions Derived from Enzymatic Digestion of *Desmodesmus* sp. Protein Extract

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HIGHLIGHTS

- *Desmodesmus* sp. protein extract was digested with different types of protease.
- Digestion of protein extract by pepsin and bacterial protease resulted in enhanced antioxidant activity.
- Digestion by the bacterial protease from *Bacillus licheniformis* resulted in bioactive peptides.

ABSTRACT

Cyanobacteria and microalgae are promising sources of valuable bioactive compounds for nutraceutical and pharmaceutical applications. Proteins and peptides derived from these microorganisms have been shown different biological actions, including antioxidant and antimicrobial activities. In this study, *Desmodesmus* sp. protein extract was digested using two proteases, including bacterial protease with optimum activity in alkaline conditions and pepsin with optimum activity in acidic pH. The peptide mixtures derived from protease hydrolysis were evaluated by DPPH assay for antioxidant activity and microdilution antimicrobial assay. The results showed that peptides derived from both pepsin and bacterial protease digestions enhanced the antioxidant activity and these samples had between 80-100% antioxidant activities. In addition, pepsin digestion could reduce the MIC against *S. aureus* and methicillin-resistant *S. aureus* (MRSA) twofold compared to the initial protein extract before digestion. In conclusion, the peptides derived from enzymatic digestion of *Desmodesmus* sp. protein extract had promising biological activities that need further studies to identify the most bioactive peptide.

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Introduction

Recently, the discovery rates of traditional antimicrobial drugs have been declining, while the challenge of antibiotic-resistant microbial infections has raised. Besides, microalgae, as a promising natural source of bioactive compounds, have attracted the scientists' focus toward discovering new drugs (Singh et al., 2020). Microalgae are a diverse group of photosynthetic microorganisms, in the form of single cells or organized cell masses (colonies) in marine and freshwater ecosystems. The ability of microalgae to multiply or survive in a wide range of environmental conditions leads to the production of secondary metabolites that are of considerable value in biotechnological fields, such as aquaculture, health, food, and pharmaceutical industries (Martínez-Francés and Escudero-Oñate, 2018).

Most cyanobacteria, such as *Spirulina*, *Anabena*, *Nostoc*, and *Oscillatoria* can produce various types of secondary metabolites and bioactive compounds, including bioactive peptides and proteins. The presence of peptide synthetase enzyme in cyanobacteria can lead to the biosynthesis of peptide-based active compounds, such as microcystins. A number of microalgae and cyanobacteria, such as *Chlorella* sp. and *Spirulina* sp., contain high protein content (more than 50% of dry weight). Therefore, the extraction of protein content and their enzymatic digestion products can lead to bioactive mixtures containing various peptides. Studies have shown that some of these peptides have potentially strong biological activities (Agyei et al., 2016; Rengasamy et al. 2020).

Peptides are valuable biomacromolecules with various promising therapeutic and diagnostic applications. Natural peptides with less than 50 amino acids can be considered as small molecules, and have been discovered as important bioactive metabolites in almost all animals, plants, marine organisms, fungi, and bacteria. The natural peptides can be extracted directly or be derived from the proteolytic breakdown of extracted proteins (Ovando et al., 2018; Gogineni and Hamann, 2018). The bioactivities of cyanobacterial peptides have been reported in different fields, such as anti-oxidant (Niknam et al., 2022), anti-inflammatory (Tabarzad et al., 2020), anti-cancer (Skjånes et al., 2021), etc.

Antimicrobial compounds have also been reported from cyanobacteria and microalgae, which include alkaloids, fatty acids, indoles, macrolides, peptides, phenols, pigments, and terpenes. Among them, antimicrobial peptides isolated from cyanobacteria and microalgae are promising candidates for drug discovery. Antibacterial cyanopeptides were mainly reported from Oscillatoriales and Nostocales, with a variety of structures, however, they are primarily cyclic peptides. They can be divided into ribosomal and non-ribosomal peptides that all could be active against pathogenic Gram-negative and Gram-positive bacteria. As another example, bacteriocins have been found in cyanobacteria with antimicrobial activity, which have been introduced to be as a food preservative agent, or as an antibiotic. Despite extensive cyanobacterial therapeutic discoveries so far, few of them have been gone in the nutritional or pharmaceutical markets, however, it is growing fast (Saad et al., 2022; Raja et al., 2016).

The antimicrobial activity described for microalgae peptides has been rare and mostly limited to protein hydrolysates. Despite the promise of antimicrobial peptide applications and the importance of finding new natural sources of antibiotics, several limitations remain in their pharmaceutical applications (Rojas et al., 2020).

The most commonly used method for protein digestion and functional peptide production is enzymatic hydrolysis, where the enzymes can be non-digestive (non-GI) proteases (such as papain, alcalase, and thermolysin) from bacteria, fungi, or plants, or can be digestive (GI) proteases (such as pepsin, trypsin and chymotrypsin) of animal origin (Barati et al., 2020). For peptide production with a potential impact on health or food quality, it is important to choose the appropriate proteolytic enzyme, as well as, to perform the process under optimal physicochemical conditions (pH, temperature, incubation time), in order to maximize yield (degree of hydrolysis). The activity of the proteolytic enzyme can have a significant effect on the distribution of peptides' molecular weights and their bioactivities (Olvera-Rosales et al., 2023; Han et al., 2021, Niknam et al., 2022). In addition, in this hydrolysis process, if necessary, two or more enzymes can be used simultaneously or sequentially to generate bioactive peptides (Ovando et al., 2018). For food and pharmaceutical industries, the use of enzymatic hydrolysis in the production of bioactive peptides has been preferred, since this process gives better yield and purity than extraction with organic solvent (Zambrowicz et al., 2013).

In this study, peptides derived from enzymatic digestions using two types of enzymes on *Desmodesmus* sp. protein extract were evaluated considering the antioxidant and anti-microbial activities of produced hydrolysates.

Materials and Methods

Cells and chemicals

Desmodesmus sp. (GenBank accession No. OR381686.1) was cultured in BG11 medium and its biomass was

separated using centrifugation at 8000 × g, 20 °C for 10 min. Then, the biomass was lyophilized to achieve a dry powder for further purification. Ascorbic acid (A920902) and Trichloroacetic acid (TCA) were supplied from Merck Chemical (Germany). O-phthalaldehyde (OPA) (P1378) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) (D9132) were supplied from Sigma Aldrich (USA). All other chemicals were supplied from Merck Chemical (Germany). Bacterial protease from *Bacillus licheniformis* (P3910) and pepsin from porcine gastric mucus (#1.07185.0010) were from BioBasic (Canada). Peptone A (#G213) was supplied from BioBasic (Canada).

Protein extraction of Desmodesmus sp. *biomass*

To extract the protein content of *Desmodesmus* sp. biomass, a similar method to Sun *et al.* was run (Sun et al., 2016). In brief, 5 g of *Desmodesmus* sp. powder was dispersed in 100 mL of deionized water and alkaline water (pH 10 adjusted by NaOH 0.1 N), and a dispersion was made. The cell was disrupted by an ultrasonic homogenizer (30 s-10 s cycles of and 200W-off, for 30 min) (Topsonic, 20KHz, 400W, Farasout, Iran). Then, *Desmodesmus* sp. extraction mixture was centrifuged for 20 min at 8000 g and 22 °C (Sigma refrigerated ultracentrifuge, Sigma, Germany). Two similar rounds of extraction were run. The supernatants of all extractions that contained proteins, were separated from residual biomass and mixed. In the next step, the protein was precipitated using ammonium sulfate (43% w/v) and TCA (10%) precipitation methods at -20 °C and overnight. Next centrifugation at 15000 g, -4 °C for 20 minutes was run to separate the precipitated proteins. The precipitate was washed with cold acetone (100%) two times to remove contaminations. Finally, the precipitate was dried and a part of it was dissolved in 50 mM Tris-HCl buffer pH 8.5 or 50 mM phosphate buffer pH 8 (for digestion with bacterial protease) and another part in 50 mM phosphate buffer pH 6.6 (for digestion with pepsin). Finally, total protein content was determined using the Bradford assay (TaKaRa Bradford Protein Assay Kit, T9310, Japan).

Enzymatic digestion of protein extract

The ratio of 2% (w/w) of enzyme: protein was applied according to the results of the Bradford assay. Thus, the calculated volume of each enzyme stock (pepsin and bacterial protease stock solution with a concentration of 10 $mg.mL^{-1}$) was added to each sample. The enzyme digestion was performed in 50 mM Tris-HCl buffer pH 8.5 for bacterial enzyme and 50 mM phosphate buffer pH 6.6 for pepsin. The digestion was run for 3 h at 37 °C in Shaker Water Bath (WNB 7-45, Memmert, Germany), and then, placed at 95 °C for 30 min to stop the digestion process.

In the following step, the digested samples were evaluated by the OPA test to determine the yield of enzyme digestion. The small peptide fraction of the enzymatic digestion mixture was separated using dialysis at 4°C for 72 h in BPS (dialysis membrane with cut-off 14 kDa, #D0405, sigma, USA).

Determination of peptide content by OPA test

The OPA reagent was composed of 99 mL of boric acid 3% with pH 10.5, 1 mL of 50 $mg.mL^{-1}$ of O-phthaldehyde (OPA) in ethanol and 50 µL of 2-mercaptoethanol. To draw the calibration curve, a serial dilution of peptone from 1 $mg.mL^{-1}$ was prepared. In the first step, 20 µL of standard peptone or test sample and 200 µL of OPA reagent were mixed and incubated at room temperature for one hour. Then, the fluorescence (excitation at 340 nm and emission at 455 nm) absorption values were recorded by multi-mode microplate reader (Cytation3, Agilent, USA). Finally, the peptide concentrations were calculated based on the calibration curve for standards using the calibration curve.

Evaluation of antioxidant activity using DPPH assay

First, all enzymatic digestion samples were diluted 1:10. Four solutions of ascorbic acid at 1 to 0.125 $mg.mL^{-1}$ were used as a positive control. Then, 50 µL of each sample was mixed with 50 µL of DPPH methanolic solution (0.1 mM). As the negative control, 50 µL of absolute ethanol and 50 µL DPPH reagent were mixed. One blank sample for each test sample was also tested. Finally, the absorbance of the samples at 520 nm was recorded by an ELISA reader (Garni Medical Engineering Co., Iran). Then, the antioxidant activity was calculated using the following formula:

$$Antioxidant\ activity = [1 - \frac{(A_s - A_b)}{(A_c)}] \times 100$$

Antibacterial activity assay

Antibacterial activity was assessed using agar well diffusion and microdilution methods. For the agar well diffusion method, a 0.5 McFarland suspension of bacterial strains was prepared and spread on the surface of Muller-Hinton agar medium plates that had previously been punched to produce wells. Finally, after 24 h incubation at 37 °C, the bacterial inhibition zone for each sample was determined. For the microdilution

method, an initial 100 µL of Muller-Hinton broth medium containing $CaCl_2$ (20 µg.mL^{-1}) and $MgCl_2$ (10 µg.mL^{-1}) was added to each well of a 96-well microplate. The initial peptide concentration was determined by the OPA test. In triplicate, serial dilutions of the peptide were prepared. Then, 10 µL of a 1:20 dilution of 0.5 McFarland suspension (5×10^6 CFU.mL^{-1}) was added to each well. The bacteria used in this study included *Escherichia coli* (PTCC 1276), *Klebsiella oxytoca* (PTCC 1402), *Streptococcus pyogenes* (PTCC 1522), *Staphylococcus aureus* (PTCC 1337) and methicillin-resistant *S. aureus* (MRSA). The growth of bacteria was assessed after 24 h incubation at 35 ± 2 °C in ambient air.

Statistical analysis

All tests were performed in triplicate. The values of mean ± SD were calculated using Excel 2016. A comparison of the means was performed with the analysis of variance (ANOVA) test by GraphPad Prism 9.0.0. The *p-value* less than 0.05 was considered as significant.

Results and Discussion

Yield of protein extraction from Desmodesmus sp. *biomass*

Two sequential steps of extraction were run to improve the protein extraction yield. Using alkaline water as an extraction solvent improved the yield of protein extraction (Fig. 1). The concentration of total protein in each sample after protein precipitation by ammonium sulfate and TCA was reported in Table 1. The results showed that the yield of total protein extraction was around 20% (w/w) of dry biomass.

Table 1: protein content of extraction samples.

Sample*	Protein conc. µg.mL^{-1}
N (Crude2)	106.734 ± 5.64
NAC	35.46816 ± .38
NAD	133.7175 ± 9.20
NBC	32.76981 ± 3.1
NBD	215.5144 ± 11.3
NA-Supernatant	49.22975 ± 3.62
NB-Supernatant	10.82323 ± 1.3
W (Cude1)	92.34278 ± 6.32
WAD	125.1727 ± 5.41
WBAD	61.55221 ± 9.21
WBD	63.89078 ± 4.53
WA-Supernatant	51.83815 ± 2.21
WB-Supernatant	17.83894 ± 1.89

*W: extraction with deionized water; N: extraction with alkaline water; A: ammonium sulfate precipitation; B: TCA precipitation; C: dissolution in phosphate buffer, pH 6.6; D: dissolution in Tris buffer, pH 8.5.

Comparison of the total protein contents of different samples showed more than the importance of alkaline conditions in the enhancement of protein extraction, that TCA precipitation and resolution in the Tris buffer pH 8.5 improved the protein concentration in the final solution (Fig. 1). It can be concluded that the extracted proteins are more soluble in alkaline conditions than a neutral or acidic buffer.

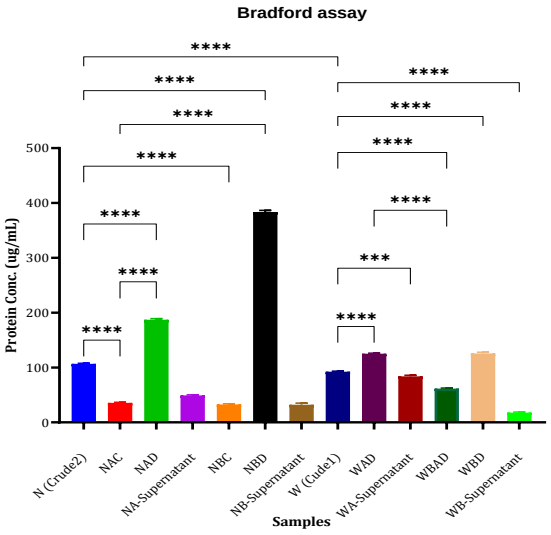

Figure 1: Comparison of protein concentration in the final solution using different methods of precipitation and resolution buffer. The means of protein concentrations in different samples were compared using ANOVA by GraphPad Prism 9.0.0. ****p-value* <0.001; *****p-value* <0.0001. W: extraction with deionized water; N: extraction with alkaline water; A: ammonium sulfate precipitation; B: TCA precipitation; C: dissolution in phosphate buffer pH 6.6; D: dissolution in Tris buffer, pH 8.5.

A significant difference (*p-value* <0.0001) between the protein concentrations of supernatant and solubilized precipitated samples showed that using TCA precipitation methods was more efficient than ammonium sulfate precipitation in the purification of protein content. More efficiency of TCA precipitation method compared to the other ones in protein purification from cyanobacteria was also previously approved for cyanotoxins purification from *Coscinodiscus granii* (SCCAP K-1831), *Chaetoceros socialis* (SCCAP K-0550), *Skeletonema marinoi* (SCCAP K-0669) and *Phaeodactylum tricornutum* (SCCAP K-1280) (Lage et al., 2016).

Comparison of the yield of enzymatic digestion of protein extract

The change in free amine concentration in extracts can be considered as an index for protein digestion and the presence of short-length peptides in the final mixture. Digestion with different enzymes, pepsin or bacterial protease, resulted in different yields of protein digestion. (Fig. 2).

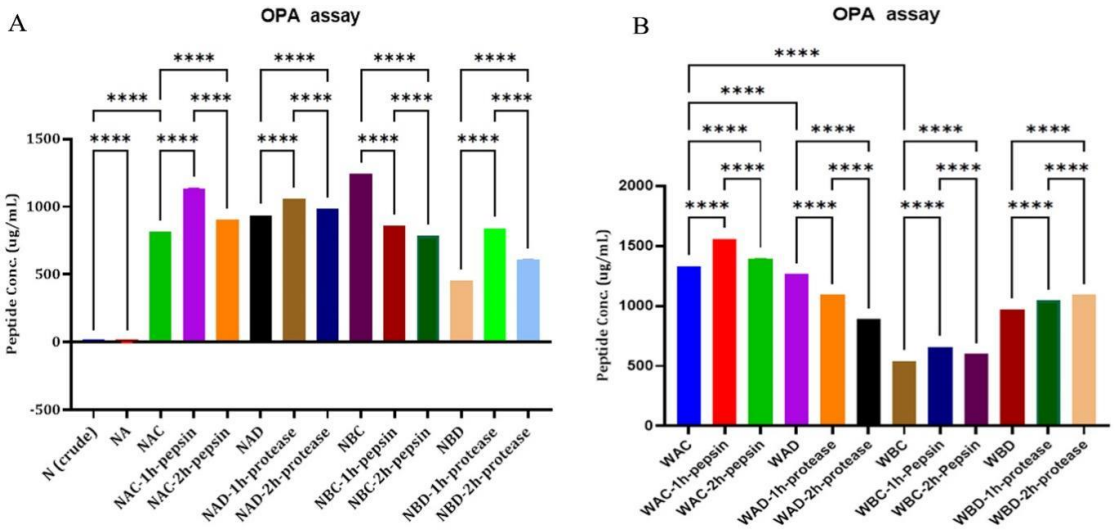

Figure 2: Peptide concentration assay using OPA Test. The concentration means were compared using ANOVA by GraphPad Prism 9.0.0. ****p-value* <0.001; *****p-value* <0.0001. W: extraction with deionized water; N: extraction with alkaline water; A: ammonium sulfate precipitation; B: TCA precipitation; C: dissolution in phosphate buffer pH 6.6; D: dissolution in Tris buffer, pH 8.5; E: dissolution in phosphate buffer, pH 8, NA: dissolution in water after ammonium sulfate precipitation.

As seen in Fig. 2, peptide concentration after 1 h digestion by pepsin on all water-extracted samples was the highest value; while 1 h digestion with bacterial protease on the same extracts except those precipitated with TCA and re-solubilize in Tris buffer pH 8.5, could not be effective in increasing peptide concentration. For alkaline water extracted samples, both 1 h pepsin and protease digestion significantly increased the peptide concentration (*p-value* <0.0001), but increasing the time of digestion to 2 h could not improve this value.

Our previous study on *Spirulina platensis* indicated that the digestion of protein extract by bacterial protease was more efficient compared to the digestion by pepsin or papain, while the hydrolysate obtained from pepsin digestion exhibited the highest value of antioxidant activity (80%), especially the peptide fraction with molecular weights less than 14 kDa (Niknam et al., 2022).

Comparison of the antioxidant activity of different enzyme

The antioxidant activity of different samples was analyzed using DPPH assay. The results showed that compared to ascorbic acid 1 mg.mL^{-1} that showed antioxidant activity of 92.72± 2.43%, some of the peptide mixtures derived from pepsin and bacterial protease digestion had near 85% and 100% antioxidant activities, respectively. The comparison of the antioxidant activity of different samples showed that in most cases, 2 h enzymatic digestion improved the antioxidant activity, significantly (*p-value* <0.0001) (Fig. 3). Increasing the time of enzymatic digestion may result in single amino acids or dipeptides with high antioxidant activities, similar to the antioxidant activity of mycosporine-like amino acids extracted from cyanobacteria (Rastogi et al., 2016; Salehian et al., 2023).

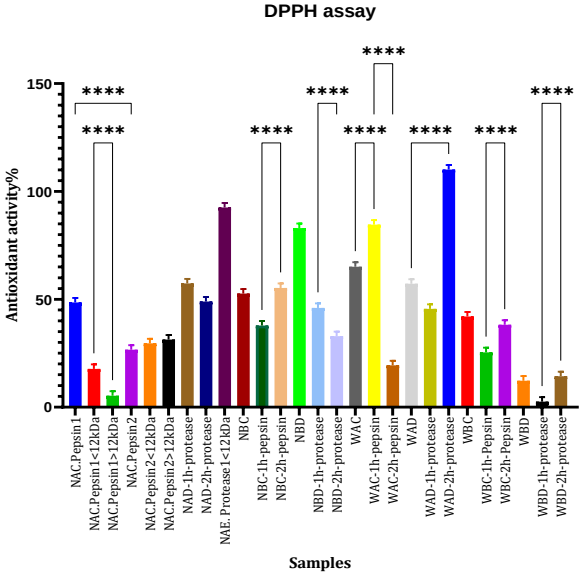

Figure 3: Antioxidant activities of different extracts before and after enzymatic digestion, using DPPH assay. W: extraction with deionized water; N: extraction with alkaline water; A: ammonium sulfate precipitation; B: TCA precipitation; C: dissolution in phosphate buffer, pH 6.6; D: dissolution in Tris buffer, pH 8.5; E: dissolution in phosphate buffer pH 8. The comparison of means was performed using the ANOVA method by GraphPad Prism 9.0.0. ****p-value* <0.001; *****p-value* <0.0001.

Table 2: The antimicrobial activity of different samples determined by agar well-diffusion method.

Sample*	Growth inhibition zone (mm)				
	S. aureus	*MRSA*	*P. aeruginosa*	*E. coli*	*K. pneumonia*
Supernatant [a].N	22	21	-	-	-
NA[a]	20	20	-	-	-
NAE[b]	23	22	-	-	-
NAE- 1h-bacterial protease [b]	20	20	-	-	-
NBC[c]	10	-	3	12	-
NBC-2h-pepsin[c]	-	-	-	10	-
NAC-1h-pepsin[c]	12	-	-	-	-
NAC-1h-pepsin[c]	11	-	-	-	-
NBD[c]	24	-	-	-	-
NBD-1h-bacterial protease [c]	14	-	-	-	-
NBD-2h-bacterial protease [c]	12	-	-	-	-
NAD[c]	14	-	-	-	-
NAD-1h-bacterial protease [c]	17	-	-	-	-
NAD-2h-bacterial protease [c]	13	-	-	-	-

*W: extraction with deionized water; N: extraction with alkaline water; A: ammonium sulfate precipitation; B: TCA precipitation; C: dissolution in phosphate buffer, pH 6.6; D: dissolution in Tris buffer, pH 8.5; superscript a: protein concentration of 30 ± 3.5 µg.mL^{-1}; b: peptide concentration of 1 ± 0.3 µg.mL^{-1}, and c: peptide concentration of 1000 ± 100 µg.mL^{-1}.

Antimicrobial activity of extracts and their related enzymatic digestions

The antimicrobial activity of protein and peptide samples was evaluated using agar well diffusion and microdilution methods. The best antimicrobial activity was observed against *S. aureus* and MRSA (Table 2). Therefore, the peptides derived from enzymatic digestion of *Desmodesmus* sp. could be considered as more effective compounds against Gram-positive bacteria. However, they did not show promising activity against *P. aeruginosa*.

As this method might face some challenges regarding the peptide diffusion into the agar, the microdilution method was also performed to determine the MIC of positive samples against *S. aureus* and MRSA. The results are shown in Table 3.

Table 3: The MIC of different samples against *S. aureus* and MRSA determined by microdilution method.

Sample*	MIC (µg.mL^{-1})	
	MRSA	*S. aureus*
NA (protein)	>29.551	>14.78
NAE-1h-Bacterial protease	>0.4713	>0.2356
NAE	>0.03885	>0.01942

*W: extraction with deionized water; N: extraction with alkaline water; A: ammonium sulfate precipitation; B: TCA precipitation; C: dissolution in phosphate buffer, pH 6.6; D: dissolution in Tris buffer, pH 8.5.

Previously, a group of researchers studied the methanolic extract of *Desmodesmus* sp. cultured in BG 11 culture medium, and they found that this extract had significant antimicrobial activity. A remarkable antimicrobial activity against Gram-positive bacteria, *S aureus* with MIC and MBC of 31.25 and 125 µg.mL^{-1}, respectively. This methanolic extract was relatively active against *Listeria monocytogenes*, MRSA and *P. aeruginosa* (with MIC equal to 250 µg.mL^{-1}), and *Aeromonas hydrophila* (MIC of 1 mg.mL^{-1}). The minimum antibacterial activity of 1 mg.mL^{-1} was observed against *L. monocytogenes*, MRSA, *A. hydrophila*, and *P. aeruginosa* (Arguelles et al., 2017).

The strain of *Scenedesmus* is highly similar to *Desmodesmus* sp. A study evaluated *Scenedesmus obliquus* regarding antimicrobial activity. Dried *S. obliquus* biomass (20 g) was extracted by various organic solvents, such as methanol, ethanol, acetone, chloroform, diethyl ether, ethyl acetate, and hexane. Antimicrobial activity of extracts and crude fractions of *S. obliquus* was evaluated on six species of pathogenic bacteria, *Bacillus cereus*, and *S. aureus* as Gram-positive and *Salmonella typhi*, *Escherichia coli*, *P. aeruginosa*, and *Klebsiella pneumoniae* as Gram-negative bacteria. Results showed that the aqueous, chloroform, and diethyl ether extracts had promising antibacterial activity against all pathogenic bacteria except for *P. aeruginosa*, followed by ethyl acetate extract. Acetone extract had no antibacterial activity against all bacteria (TÜney et al., 2006).

Moreover, it was reported that the methanolic extract of *Scenedesmus quandricanda* had good antibacterial activity against *E. coli*, *B. cereus* and *S. aureus*, while ethanolic, acetone and diethyl ether extracts were only active against *B. cereus* and *S. aureus.* They also found that *P. aeruginosa* showed resistance to ethanol, acetone, diethyl ether and methanolic extract of *S. quandricanda* (Arguelles and Sciences, 2018). A study reported that acetone, methanol, diethyl ether and hexane extracts of *Scenedesmus* sp. had modest antibacterial activity against *Pseudomonas*, while water extract showed significant antibacterial properties compared to others (Ishaq et al., 2015). Besides, Najdensky and colleagues reported that the ethanolic extract of *Scenedesmus obliquus* has antibacterial activity against *S aureus*, *B. cereus*, *E. coli*, *P. aeruginosa* and *S. typhi* (Marrez et al., 2019). Moreover, the extracts of *Scenedesmus* sp. and *S. quadricauda*, harvested in 5-day-old and 10-day-old cultures exhibited antibacterial activity against *S. aureus* and *E. coli*, and *P. aeruginosa*, respectively. The different extracts generally showed potent antimicrobial activity with MIC values less than 1 $mg.mL^{-1}$ (Aremu et al., 2014).

Conclusion

In this study, the protein content of *Desmodesmus* sp. was extracted using deionized water at pH 7.4 and alkaline water at pH~10, and then, the extracts were enzymatically digested using a bacterial protease and pepsin. Results showed that using alkaline conditions could enhance the yield of protein extraction and the samples studied here could inhibit the growth of *S. aureus* and MRSA more efficiently than other bacterial strains including Gram-negative strains and *P. aeruginosa*. In addition, using bacterial protease to prepare bioactive peptides from protein extract resulted in more increase in antioxidant activity. Moreover, pepsin digestion enhanced the antimicrobial activity of the extract against *S. aureus* and MRSA, while bacterial protease digestion did not reduce the MIC against these two species.

Ethical Statement

This work did not contain any animal or human studies performed by the authors.

Competing Interests

The authors have no conflicts of interest to declare.

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Data Availability

All relevant data has been reported in the manuscript. Any additional essential data can be provided by the request from the corresponding author.

Authors Contribution

M. Tabarzad designed and supervised the project. M. Ehsani and B. Alizadeh performed the experiments and analyzed the results. T. Hosseinabadi and A. Mahboubi counseled the methods and data analysis. M. Tabarzad and M. Ehsani contributed to the manuscript drafting. The final manuscript was reviewed and confirmed by all authors.

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