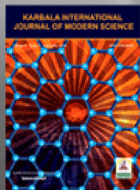
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Evaluation of MCF-7 Breast Cancer Cell Cytotoxic and Antioxidant Activities of Peptide Fractions from Symbiotic Bacteria of Jellyfish Catostylus sp.

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Abstract

Marine-derived symbiotic microorganisms are recognized as a promising source of bioactive compounds with potential therapeutic applications, yet research on jellyfish-associated bacteria remains limited. This study examines the bioactivity of peptide fractions derived from symbiotic bacteria isolated from the jellyfish *Catostylus* sp., collected from the coastal waters of South Sulawesi, Indonesia, with a focus on their anticancer and antioxidant properties. Following sample collection, the symbiont bacteria were isolated, enzymatically hydrolyzed, and purified before their biological activity was evaluated. Preliminary cytotoxicity screening using the brine shrimp lethality assay revealed that the extracellular peptide fraction (5–10 kDa) and intracellular peptide fraction (3–5 kDa) exhibited the highest cytotoxic effects. Subsequent testing against MCF-7 breast cancer cells demonstrated potent anticancer activity, with IC_{50} values of 19.83 μg/mL (5–10 kDa) and 6.43 μg/mL (3–5 kDa). Antioxidant assays using the DPPH and ABTS methods showed that the 5–10 kDa fraction achieved IC_{50} values of 254.25 μg/mL (DPPH) and 41.30 μg/mL (ABTS), whereas the 3–5 kDa fraction recorded IC_{50} values of 218.02 μg/mL (DPPH) and 37.89 μg/mL (ABTS). These findings suggest that peptide fractions from jellyfish-associated symbiotic bacteria exhibit significant in vitro anticancer and antioxidant properties. Although the present study is limited to in vitro analysis, it provides novel evidence supporting the potential of *Catostylus* sp.-derived bacterial peptides as candidates for the development of antioxidant-based therapies for cancer prevention and treatment, particularly breast cancer

Keywords

Anticancer; Antioxidant; Bioactive peptides; Jellyfish; Symbiotic bacteria.

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Cover Page Footnote

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RESEARCH PAPER

Evaluation of MCF-7 Breast Cancer Cell Cytotoxic and Antioxidant Activities of Peptide Fractions From Symbiotic Bacteria of Jellyfish *Catostylus* sp

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Abstract

Marine-derived symbiotic microorganisms are recognized as a promising source of bioactive compounds with potential therapeutic applications, yet research on jellyfish-associated bacteria remains limited. This study examines the bioactivity of peptide fractions derived from symbiotic bacteria isolated from the jellyfish *Catostylus* sp., collected from the coastal waters of South Sulawesi, Indonesia, with a focus on their anticancer and antioxidant properties. Following sample collection, the symbiont bacteria were isolated, enzymatically hydrolyzed, and purified before their biological activity was evaluated. Preliminary cytotoxicity screening using the brine shrimp lethality assay revealed that the extracellular peptide fraction (5−10 kDa) and intracellular peptide fraction (3−5 kDa) exhibited the highest cytotoxic effects. Subsequent testing against MCF-7 breast cancer cells demonstrated potent anticancer activity, with IC_{50} values of 19.83 μg/mL (5−10 kDa) and 6.43 μg/mL (3−5 kDa). Antioxidant assays using the DPPH and ABTS methods showed that the 5−10 kDa fraction achieved IC_{50} values of 254.25 μg/mL (DPPH) and 41.30 μg/mL (ABTS), whereas the 3−5 kDa fraction recorded IC_{50} values of 218.02 μg/mL (DPPH) and 37.89 μg/mL (ABTS). These findings suggest that peptide fractions from jellyfish-associated symbiotic bacteria exhibit significant in vitro anticancer and antioxidant properties. Although the present study is limited to in vitro analysis, it provides novel evidence supporting the potential of *Catostylus* sp.-derived bacterial peptides as candidates for the development of antioxidant-based therapies for cancer prevention and treatment, particularly breast cancer.

Keywords: Anticancer, Antioxidant, Bioactive peptides, Jellyfish, Symbiotic bacteria

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1. Introduction

Cancer is a pathological condition characterized by abnormal cell growth, uncontrolled proliferation, and the ability to invade surrounding tissues and organs [1]. According to the International Agency for Research on Cancer (IARC), nearly 20 million new cancer cases were reported worldwide in 2022, with breast cancer in women being the most frequently diagnosed (11.6 %) and the leading cause of cancer-related deaths (approximately 6.9 % of total cancer deaths) [2]. Conventional therapies such as surgery, radiotherapy, and chemotherapy can prolong patient survival but often lead to adverse effects and drug resistance, which limit long-term treatment efficacy [3,4]. These challenges highlight the urgent need for new drugs that are highly selective and specific, particularly those derived from natural sources [5].

Marine organisms are recognized as major producers of bioactive compounds due to their high biodiversity and are considered important sources of active peptides [6,7]. Marine-derived peptides possess diverse pharmacological and nutraceutical properties, including antimicrobial, antitumor, antihypertensive, anticancer, and antioxidant activities [8]. Recent reviews have provided a comprehensive overview of the chemistry and biological activities of peptides derived from fungi, further emphasizing the vast potential of marine environments as reservoirs of bioactive peptides for therapeutic development [9].

In addition to marine fauna, symbiotic bacteria associated with marine organisms are increasingly acknowledged as valuable producers of bioactive metabolites and peptides, yet they remain relatively underexplored [6,7]. Jellyfish are key components of aquatic ecosystems, functioning in the food web and as hosts for diverse symbiotic bacteria. These bacteria contribute to biological processes such as digestion, pathogen resistance, and the production of bioactive compounds, while also participating in carbon, sulfur, phosphorus, and nitrogen cycles, forming a mutually beneficial relationship with their jellyfish hosts [10].

Previous studies have reported the bioactive properties of jellyfish protein hydrolysates, including antioxidant activity in *Rhizostoma pulmo* [11], *Nemopilema nomurai* [12], and *Lobonema smithii* [13]. However, compared with symbiotic bacteria from other marine organisms such as algae, sponges, and corals, research on antioxidant and anticancer peptides derived from jellyfish-associated bacteria is still in its early stages.

This study aims to utilize symbiotic bacteria from the jellyfish *Catostylus* sp. as a source of bioactive peptide fractions through enzymatic hydrolysis. The resulting peptides were evaluated for anticancer activity using the Brine Shrimp Lethality Test (BSLT) and MTT assays, and antioxidant activity using the DPPH and ABTS methods, to assess their potential as novel antioxidant and anticancer therapeutic agents.

2. Experiments

2.1. Materials

Jellyfish samples were obtained from the Lantebung coast of Maros Regency, South Sulawesi, Indonesia. Auxiliary materials included nutrient broth media (Merck, Darmstadt, Germany), nutrient agar media (Merck, Darmstadt, Germany), pepsin enzyme (Sigma−Aldrich, St. Louis, USA), ammonium sulfate ($(NH_4)_2SO_4$) (Merck, Darmstadt, Germany), Tris−HCl 0.1M (Merck, Darmstadt, Germany), sodium chloride (NaCl) 2 M (Merck, Darmstadt, Germany), calcium chloride ($CaCl_2$) 0.01 M (Merck, Darmstadt, Germany), β-mercaptoethanol 1 % (Merck, Darmstadt, Germany), triton X-100 0.5 % (Merck, Darmstadt, Germany), mtt reagent (Merck, Darmstadt, Germany), abts reagent (Merck, Darmstadt, Germany), dpph Reagent (Merck, Darmstadt, Germany), bovine serum albumin (Merck, Darmstadt, Germany), folin-ciocalteu reagent (Sigma−Aldrich, St. Louis, USA), *Artemia salina Leach*, and distilled water (double-distilled laboratory grade).

2.2. Collection and preparation in the nutrient broth medium

Jellyfish *Catostylus* sp. samples (morphologically identified by the Faculty of Marine Science and Fisheries Laboratory, Universitas Hasanuddin, Makassar, Indonesia) were collected from the coastal area of Lantebung, Maros District, South Sulawesi, Indonesia. The collected samples were rinsed with seawater and stored in a cool box at approximately 4 °C. 5 g of the sample was weighed and transferred into an Erlenmeyer flask containing 45 mL of sterile seawater. The flask was then incubated in a shaker incubator at 37 °C and 180 rpm for 2 h. Subsequently, 5 mL of the suspension was inoculated into 45 mL sterile Nutrient Broth (NB) medium and incubated for 24 h under the same conditions. The process was then continued to the bacterial isolation stage.

2.3. Determination of optimal growth time and extraction of bioactive proteins from symbiotic bacterial isolates

The bacterial isolates (1−3 colonies) were placed into 100 mL of sterile inoculum medium and incubated in a shaker at 37 °C and 180 rpm for 24 h. Afterwards, 10 % of the active culture was transferred to a sterile production medium and maintained under the same conditions for 60 min. Cell growth was measured every 6 h using a UV-Vis spectrophotometer by measuring optical density (OD) at 600 nm. Subsequently, the culture was centrifuged at 3500 rpm for 30 min to obtain the crude extracellular protein.

The pellet (cells) obtained was ground and mixed with 50 mL of Buffer A. In an ice bath, the cell suspension was frozen and thawed three times, and then sonicated for 3 × 10 min to facilitate complete cell disruption. After that, a crude intracellular protein extract was obtained by centrifuging the cell lysate for 30 min at 4 °C and 3500 rpm.

2.4. Purification of bioactive proteins

Intracellular and extracellular protein extract filtrates are fractionated using ammonium sulfate at the following saturation levels: 0−20 %, 20−40 %, 40−60 %, and 60−80 %. The mixture is left at 4 °C for 24 h. Residues were separated from the filtrate, and the sample was centrifuged at 13,000 rpm for 15 min at 4 °C. The residue obtained was then dissolved in approximately 5 mL of Buffer B. The protein content of each fraction was determined, and further purification was achieved through dialysis [14]. The dialyzed fractions were again measured for protein content using the Lowry method [15].

2.5. Protein hydrolysis process

Protein hydrolysis is carried out using a method developed by Ref. [16], with some modifications. The fraction with the highest protein content is diluted to 3 %. Furthermore, hydrolysis was done using pepsin enzyme with a substrate-to-enzyme ratio of 3:100 at 37 °C and pH 2. The hydrolysis process is designed by varying the hydrolysis time (0, 0.5, 1, 2, 3, 4, 5, and 6 h) to determine the optimal conditions for protein hydrolysis. The enzyme was inactivated by heating in boiling water for 10 min, and then the mixture was centrifuged at 10,000 rpm, 4 °C for 20 min. The peptide hydrolysate obtained was then used to determine the degree of hydrolysis (DH). The degree of hydrolysis (DH) was determined by calculating the percentage of protein precipitated in 10 % TCA compared to the soluble protein. Fractions with a high degree of hydrolysis were then further separated using ultrafiltration, followed by anticancer testing, the Brine Shrimp Lethality Test (BSLT) technique, and further testing against MCF-7 cancer cells.

2.6. Ultrafiltration of peptide hydrolysates

The ultrafiltration of peptide hydrolysates was performed using membranes with molecular weight cut-offs of 10 kDa, 5 kDa, and 3 kDa. The process began with a 10 kDa membrane to separate larger molecules, followed by a 5 kDa membrane for further separation, and finally, a 3 kDa membrane to purify smaller peptides. Subsequently, the Lowry method was used to determine the protein content [17].

2.7. Toxicity properties test using the bslt method

The BSLT approach was used to conduct toxicity testing as modified by Ref. [18]. Peptide fractions were prepared at concentrations of 100 ppm, 10 ppm, and 1 ppm, each in triplicate. Sterile seawater containing 10 shrimp larvae was added to each tube to a final volume of 5 mL and incubated for 24 h at room temperature under a 50-W incandescent lamp. Subsequently, the number of dead and surviving larvae was counted using a magnifying glass, and the LC_{50} value (μg/mL) was determined using the Bliss Method program [19,20]. Toxicity (LC_{50}) calculations were performed using probit analysis.

2.8. Anticancer assay

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) Assay was used to evaluate anticancer activity. Cytotoxicity testing was conducted using the MTT method. A 100 μL suspension of cells in a complete medium (density of 10000 cells/well) was added to a plate and incubated for 24 h in a 5 % CO_2 incubator. The cell condition was checked, and, if suitable, treatment sample concentrations of 0.63, 1.25, 2.5, 5, 10, and 20 μg/mL were generated. Each well's media was discarded after incubation, followed by a 100 μL 1× PBS wash. Then, 100 μL of MTT solution (5 mg/mL in 1× PBS) was added.

An additional 4 h were spent incubating the plate at 37 °C. MTT interacted with viable cells, producing purple formazan crystals. The formazan formation reaction was stopped by adding 10 % SDS

(Sodium Dodecyl Sulfate) in 0.01 M HCl, followed by overnight incubation at room temperature. At the end of incubation, absorbance was measured with an ELISA reader at 500−600 nm. The percentage of viable cells was calculated from absorbance data, and the IC_{50} value was calculated by creating a plot of log concentration against the proportion of viable cells [21].

2.9. Antioxidant activity assay using the DPPH (2,2-diphenyl-1-picrylhydrazyl) method

The antioxidant activity of peptide fractions was assessed by their ability to scavenge DPPH free radicals, as described in a modified protocol by Ref. [22]. A 1 mL volume of 0.4 mM DPPH solution was combined with 0.5 mL of peptide fraction at varying concentrations. After the mixture was well-blended, it was allowed to sit at room temperature for 15−20 min. A UV-Vis spectrophotometer was then used to detect the absorbance at 517 nm. The positive control was vitamin C. Antioxidant activity was determined by comparing the percentage of DPPH radical scavenging with that of the control.

2.10. Antioxidant activity assay using the ABTS (2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)) method

To generate ABTS radicals, 140 mM potassium persulfate was combined with a 7 mM ABTS solution, and the mixture was incubated for 12−16 h. After that, the solution was diluted until its absorbance at 734 nm was 0.7 ± 0.02. Absorbance was measured at 734 nm using a spectrophotometer after combining 0.5 mL of the sample solution with 2 mL of ABTS solution. The mixture was then allowed to sit at room temperature for 20 min. Ascorbic acid served as a positive control, and the percentage of ABTS radical inhibition compared to the control was utilized to calculate antioxidant activity [23].

2.11. Qualitative analysis or screening using Liquid Chromatography-High-Resolution Mass Spectrometry (HRMS)

Qualitative analysis was conducted using a Thermo Scientific Orbitrap Exploris 120 instrument equipped with a ZORBAX Eclipse Plus C18 RRHD column (2.1 × 100 mm, 1.8 μm). The mobile phase consisted of water containing 0.1 % formic acid (eluent A) and acetonitrile containing 0.1 % formic acid (eluent B) with a 43-min gradient elution system at a flow rate of 0.25 mL/min. The sample injection volume was 3 μL, and the column temperature was maintained at 40 °C. Mass detection was performed using an electrospray ionization (ESI) source in both positive and negative modes over a mass range of 100−1500 *m*/*z*. Data acquisition and processing were conducted using Thermo Xcalibur 4.4 software (Thermo Scientific, Bremen, Germany) to identify compounds based on accurate mass and MS/MS fragmentation patterns. The measurement conditions and testing parameters are provided in the supplementary file (Table S1 (https://kijoms.uokerbala.edu.iq/cgi/editor.cgi?article=3442&window=additional_files&context=home)).

3. Results and discussion

3.1. Sample collection and preparation in nutrient broth (NB) medium

The sampling of the jellyfish *Catostylus* sp. was conducted along the coastal area of Lantebung Beach, Maros District, South Sulawesi, Indonesia. The samples were placed in sterile Erlenmeyer flasks and stored in a laboratory freezer at a minimum temperature of −17 °C to preserve their quality before further analysis. Morphological identification confirmed that the collected jellyfish species was *Catostylus* sp. This species plays an essential ecological role in maintaining the balance of the marine ecosystem. However, the local community has not yet fully realized its economic potential due to its relatively low market value and limited knowledge of developing bioactive compounds from *Catostylus* sp.

3.2. Isolation of protein from symbiont bacteria and determination of optimal growth time from symbiotic bacterial isolates

Bacterial isolation was performed using a serial dilution method from *Catostylus* sp. jellyfish samples. The number of microorganisms suspended in the liquid sample was a reference for determining the appropriate dilution level. The pure isolate obtained was then analyzed using biochemical tests and molecular identification through sequencing of the 16S rRNA gene. Based on the analysis, the isolate was identified as *Enterobacter baumannii*. Following this, the optimal bacterial growth time was determined to be 36 h, as assessed by optical density (OD) and protein concentration measurements. As incubation time increased, the protein concentration also increased, with the highest concentration recorded at 4.165 mg/mL and an absorbance of 0.849.

3.3. Purification of protein from symbiont bacteria

The purification process was carried out using ammonium sulfate fractionation. The protein precipitate obtained from ammonium sulfate fractionation is semi-purified and may contain residual salts and other simple impurities. Therefore, further purification is necessary to remove residual salts or other interfering ions. One of the methods commonly used for purification is dialysis [24]. In this study, the protein precipitates from fractions F1, F2, F3, and F4 were dissolved in buffer B, with equal volumes for each fraction. The dialyzed protein fractions were then quantified using the Lowry method [25]. The measurement results are presented in Table 1.

Table 1 shows that protein concentrations differ between intracellular and extracellular fractions, which is attributed to differences in protein solubility in water, leading to variations in the amount of protein precipitate obtained. This finding is supported by the theory proposed by Ref. [26], suggesting that different protein types can influence the concentration of each fraction. Proteins with lower solubility tend to precipitate more rapidly, whereas proteins with higher solubility exhibit better resistance to precipitation.

The results indicate that the intracellular protein fraction F4 had the highest protein concentration after dialysis, at 11.552 mg/mL. Meanwhile, the extracellular protein fraction F2 had the highest protein concentration after dialysis, reaching 8.486 mg/mL. Based on optimization of the purification process via ammonium sulfate fractionation and dialysis, the two fractions with the highest protein concentrations were selected for further enzymatic hydrolysis with pepsin.

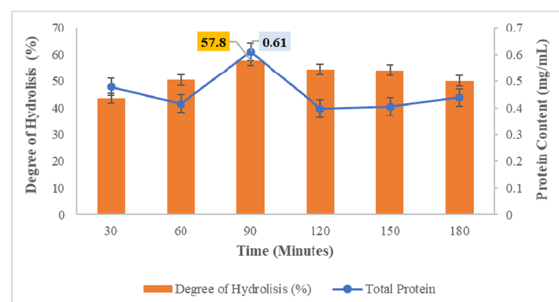
3.4. Enzymatic hydrolysis of proteins

The hydrolysis process in this study was carried out using the pepsin enzyme with incubation times of 0, 30, 90, 120, 150, and 180 h at 37 °C. This variation aimed to determine the optimal time required for the enzyme to hydrolyze proteins [27]. The enzyme was inactivated to terminate hydrolysis by immersing the hydrolysate in a preheated water bath, which prevented excessive hydrolysis and enzyme denaturation [28]. Enzymatic hydrolysis was chosen because it efficiently breaks down substrates without causing degradation, yielding various peptides containing specific amino acids, such as di- and tripeptides. Hydrolyzed proteins are converted into peptides and amino acids, collectively known as hydrolysates [29,30]. The obtained hydrolysates were then evaluated for their degree of hydrolysis by adding 20 % trichloroacetic acid (TCA) and subsequently centrifuging. The determination of the degree of hydrolysis aimed to measure the rate of protein breakdown into short-chain compounds [31]. The analysis indicated hydrolysis occurred during the enzymatic process, as shown in Fig. 1a and b.

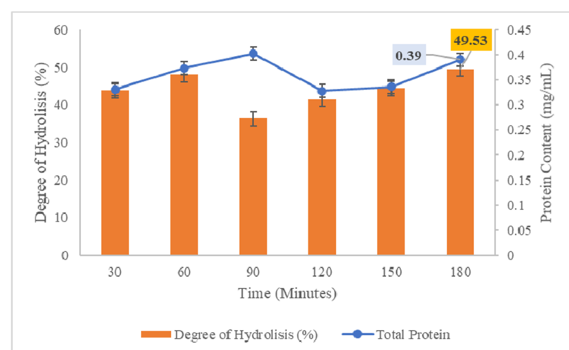
Fig. 1 illustrates the degree of hydrolysis (DH) obtained during enzymatic treatment of the intracellular (F1) and extracellular (F2) protein fractions. Specifically, Fig. 1a presents the measurement data of protein hydrolysate concentration and the percentage DH for the intracellular fraction (F1), whereas Fig. 1b shows the corresponding data for the extracellular fraction (F2). To ascertain the DH, the trichloroacetic acid (TCA) precipitation method was employed. The concentration of protein dissolved in TCA reflects the number of peptides generated from amino acids as a result of peptide-bond cleavage during hydrolysis. Variations in hydrolysis time influenced the increase in free amino acid content and DH values. As the degree of hydrolysis increased, the percentage of cleaved peptide bonds also increased. The pepsin enzyme achieved the highest hydrolysis level at 90 min in the intracellular fraction (F1), with a DH value of 57.799 % (Fig. 1a), and at 180 min in the extracellular fraction (F2), with a DH value of 49.534 % (Fig. 1b). These durations were established as the optimal conditions for producing bioactive peptides.

Table 1. Protein concentration measurement results before and after dialysis.

Types of proteins	Protein fraction	Fractionations	After dialysis
		Protein content (mg/mL)	Protein content (mg/mL)
Extracellular protein	F1	2.39	6.33
	F2	1.97	8.48
	F3	1.73	1.73
	F4	1.40	1.40
Intracellular protein	F1	14.77	10.21
	F2	14.63	7.00
	F3	13.79	8.90
	F4	14.92	11.55



(a)



(b)

Fig. 1. (a) Protein hydrolysate concentration measurement data and percentage degree of hydrolysis for F1 fraction (intracellular); (b) Measurement data of protein hydrolysate concentration and percentage degree of hydrolysis for F2 fraction (extracellular).

3.5. Ultrafiltration

The next step involves ultrafiltration using a molecular weight cut-off (MWCO) membrane on the extracellular protein hydrolysate (F1) and the intracellular protein hydrolysate (F2) obtained by enzymatic hydrolysis. Ultrafiltration is a separation technique based on differences in the molecular weight of peptide hydrolysates. Several studies have shown a strong correlation between peptide molecular weight and biological activity, with lower molecular weight generally associated with higher biological activity. Therefore, purifying peptide hydrolysates through the ultrafiltration process can be used to obtain small-sized peptide hydrolysates, as it is expected to enhance their biological activity and facilitate their absorption in the body [32].

The ultrafiltration process was carried out using three MWCO membranes: 10 kDa, 5 kDa, and 3 kDa (Vivaspin 20). This process yielded four peptide fractions based on their ability to pass through the membranes. The fraction that could not pass through the 10 kDa membrane was labeled >10 kDa, the fraction that passed through the 10 kDa membrane but was retained by the 5 kDa membrane was labeled 5–10 kDa, and the fraction that passed through the 5 kDa membrane but was retained by the 3 kDa membrane was classified as 3–5 kDa. In comparison, the fraction that could pass through the 3 kDa membrane was labeled <3 kDa. The protein content analysis of peptide fractions derived from the symbiotic bacteria of the jellyfish *Catostylus* sp. is presented in Table 2.

The results showed that the highest protein content and *in vitro* cytotoxicity were observed in fractions 5–10 kDa and 3–5 kDa, respectively, indicating that these fractions contained greater amounts of peptides with molecular weights of 5–10 kDa and 3–5 kDa, respectively. Based on the ultrafiltration results, each peptide fraction was subsequently subjected to a cytotoxicity assay using the Brine Shrimp Lethality Test (BSLT) as a simple, preliminary screening method for anticancer bioactivity.

3.6. Toxicity properties test using the bslt method

The cytotoxicity of anticancer peptide fractions was evaluated using the Brine Shrimp Lethality Test (BSLT) as an initial screening method, widely recognized for assessing bioactive compounds and toxicity. This preliminary screening was followed by an in vitro cytotoxicity assay against MCF-7 breast cancer cells, focusing on the most potent fractions identified from the BSLT results. The cytotoxicity test results of peptide fractions against *Artemia salina* Leach larvae are presented in Fig. 2.

Table 2. The results of the protein content of the protein hydrolysate and peptides.

Types of peptide fraction	Peptide fraction	Protein content (mg/mL)	Total volume (mL)	Total protein (mg/mL)
Extracellular hydrolysate	>10 kDa	10.92	23	251.16
	5–10 kDa	14.78	20	295.6
	3–5 kDa	9.46	15	141.9
	<3 kDa	8.86	20	177.2
Intracellular hydrolysate	>10 kDa	10.18	25	254.5
	5–10 kDa	10.84	20	216.8
	3–5 kDa	14.04	20	280
	<3 kDa	10.64	15	159.60

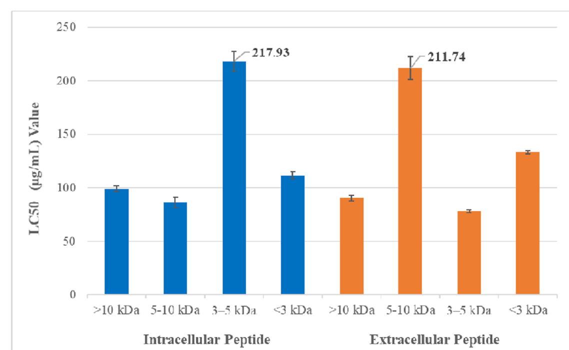


Fig. 2. Results of LC_{50} testing using the brine shrimp lethality test (BSLT).

Based on the cytotoxicity assay results shown in Fig. 2, the LC_{50} values indicate varying levels of toxicity across the peptide fractions. Fraction 3−5 kDa exhibited the lowest LC_{50} value (78.019 μg/mL), suggesting the highest cytotoxic activity compared to other fractions. Additionally, fraction 5−10 kDa demonstrated a relatively low LC_{50} value, indicating that this fraction possesses considerable toxicity against *Artemia salina larvae* and warrants further evaluation for its bioactivity as a potential anticancer agent in specific cancer cell lines and in antioxidant assays. Conversely, fractions 3−5 kDa and 5−10 kDa exhibited the highest LC_{50} values (>200 μg/mL), indicating lower toxicity. These differences suggest that specific peptide fractions exhibit more substantial toxic potential and can be considered candidates for further investigation. On the other hand, fractions with high LC_{50} values may contain bioactive compounds with a safer profile, which could be utilized in pharmaceutical or biotechnological research. This finding is considered favorable compared to previous studies on other marine biota types by Ref. [33]. The LC_{50} values of *Eucheuma cottonii* and *Eucheuma spinosum* extracts were 165.88 μg/mL and 337.21 μg/mL, respectively. Likewise, the research [34] obtained natural extracts from marine sponges, which showed a toxicity level of 30.94−179.41 μg/mL. These comparisons suggest that the peptide fractions tested in the present study exhibit relatively strong cytotoxic activity compared to previously reported marine-derived extracts.

3.7. Anticancer assay against MCF-7 breast cancer cells

The anticancer activity was evaluated using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay to assess the ability of the samples to inhibit cancer cell proliferation. The cytotoxic assay results are presented in Table 3. Sample 5−10 exhibited an IC_{50} value of 19.83 μg/mL, whereas sample 3−5 kDa demonstrated a lower IC_{50} value of 6.4 μg/mL, indicating a higher efficacy in inhibiting 50 % of MCF-7 breast cancer cell growth. As a positive control, Doxorubicin showed an IC_{50} value of 0.12 μg/mL. MCF-7 cells are used because human breast cancers are generally ER-

Table 3. Cytotoxicity assay of the sample against MCF-7 cells at six concentration ranges.

Fraction peptide	Concentration (μg/mL)	Log concentration	% Proliferation inhibition (PI)			% PI Average	Standard deviation (SD)	IC_{50} (μg/mL)
			Replicate 1	Replicate 2	Replicate 3			
Doxorubicin	0.16	−0.81	22.20	22.99	20.51	21.90	1.27	0.12
	0.31	−0.50	63.28	59.18	60.34	60.94	2.11	
	0.63	−0.20	88.62	88.97	93.07	90.22	2.48	
	1.25	0.09	85.33	91.55	93.59	90.16	4.31	
	2.5	0.40	92.44	91.64	92.79	92.29	0.59	
	5	0.70	95.02	94.84	93.51	94.46	0.82	
5−10 kDa	0.63	−0.20	−3.85	−3.14	−9.19	−5.39	3.31	19.83
	1.25	0.098	1.04	4.06	9.39	4.83	4.23	
	2.5	0.40	17.57	14.64	10.99	14.40	3.30	
	5	0.70	20.78	24.60	16.78	20.72	3.91	
	10	1.00	38.12	34.91	36.07	36.37	1.62	
	20	1.30	55.54	58.21	53.41	55.72	2.41	
3−5 kDa	0.62	−0.20	1.84	2.02	−4.39	−0.18	3.65	6.43
	1.25	0.10	−6.34	−0.03	−7.94	−4.77	4.18	
	2.5	0.40	−3.32	−6.17	−1.81	−3.76	2.21	
	5	0.70	−0.65	−2.07	0.06	−0.89	1.09	
	10	1.00	83.28	85.95	87.64	85.63	2.20	
	20	1.30	101.24	101.33	101.42	101.33	0.09	

Note:
PI = Proliferation Inhibition.
SD ≤ 10 % indicates that the variation between replicates still meets the criteria.

positive; using MCF-7 provides an in vitro model that closely mimics clinical conditions, particularly when testing compounds with potential therapeutic effects on hormonal pathways. Additionally, MCF-7 is one of the most well-characterized and widely used breast cancer cell lines, offering extensive genomic, transcriptomic, and pharmacological data for cross-study comparisons. The cytotoxicity test results against MCF-7 cells can be summarized in Table 3.

In the Cytotoxicity test against MCF-7 cells, Doxorubicin was used as a positive control. Doxorubicin is widely known as a chemotherapeutic agent and is used as a positive control. It is an antibiotic that can be used to treat various types of cancer, including breast cancer. Doxorubicin is a comparator to evaluate the test compound's inhibition of cancer cell proliferation. Based on Table 3, cytotoxic testing is conducted at six concentrations. The first test was on Doxorubicin, which has a significant cytotoxic effect on MCF-7 cells. At concentrations of 2.5−5 μg/mL, the inhibition value of cell proliferation was greater than 90 % with a low standard deviation. Concentrations of 0.16−1.25 μg/ml showed greater variation between replicates, so they can be ignored to obtain a strong correlation in statistical analysis. Therefore, the use of doxorubicin in this study was to ensure the validity and evaluation of the cytotoxic potential of the peptide hydrolysate samples tested.

Cytotoxicity testing of 5−10 kDa fraction samples was conducted at six concentration levels (0.63, 1.25, 2.5, 5, 10, and 20 μg/mL). The percentage inhibition of MCF-7 cancer cell proliferation is shown in Table 3. The %PP results at low concentrations (0.63 μg/mL) showed an average inhibition of −5.39 %, indicating an insignificant inhibitory effect. While at a concentration of 1.25 μg/mL, it can be seen that it began to increase with an average value of 4.83 %, at a concentration of 2.5 μg/mL, the inhibition increased to 14.40 %, and at 5 μg/mL, it reached 20.72 %, indicating a cytotoxic effect. At high concentrations (10−20 μg/mL), the average proliferation inhibition reached 36.37 % and 55.72 %, respectively, indicating a significant increase and a strong cytotoxic effect. This means a potent cytotoxic effect of the 5−10 kDa fraction.

Additionally, cytotoxicity testing of the 3−5 kDa fraction was conducted at six concentration levels, as outlined in Table 3. At concentrations of 0.63−2.5 μg/mL, inhibition of cell proliferation was relatively low or negative, with mean %PI values ranging from −7.94 % to −1.81 %. At intermediate concentrations (5 μg/mL), proliferation inhibition began to show a positive value of 0.059 %. Meanwhile, proliferation inhibition increased significantly at high concentrations (10−20 μg/mL), with mean percentage of inhibition (PI) values of 85.63 % and 101.33 %, respectively. This indicates a strong cytotoxic ability of the 3−5 kDa fraction. Based on the IC_{50} values calculated from these data (6.43 μg/mL for the 3−5 kDa fraction and 19.83 μg/mL for the 5−10 kDa fraction), both peptide fractions exhibit pharmacologically relevant anticancer activity. However, their potency remains lower than that of the standard chemotherapeutic agent Doxorubicin (IC_{50} = 0.12 μg/mL). This difference reflects the more potent cytotoxicity of Doxorubicin, which intercalates DNA and inhibits topoisomerase II, while suggesting that the peptide fractions may offer advantages, such as lower systemic toxicity and distinct modes of action. These IC_{50} values also fall within the range reported for natural peptide-based anticancer agents (typically 5−50 μg/mL), supporting the potential of the 3−5 kDa fraction, in particular, as a promising candidate for further pharmacological investigation and possible combination therapy with standard chemotherapeutics. The microscopic images of cells treated with the test compound are shown in Fig. 3.

Fig. 3 shows that the MTT assay results determine the effectiveness of inhibiting the proliferation of MCF-7 cancer cells. Inhibition can be observed through changes in cell shape, cell density, and color. In Fig. 3, Media + Cell (without treatment) and the addition of dimethyl sulfoxide (DMSO) serve as a negative control, both showing high cell density. This indicates that the cells are still alive and have a normal survival rate. There is no significant indication of cell proliferation inhibition, as color changes occur only when cells are still alive and can reduce MTT to formazan.

In contrast, in the samples treated with doxorubicin (DOXO), a decrease in cell density (fewer live cells) and a paler color were observed, indicating the inhibition of cell proliferation. That is, the number of living cells decreased after doxorubicin treatment. In line with its cytotoxic properties against cancer cells, these results indicate that doxorubicin inhibits cell growth. This is consistent with its role as a potent chemotherapeutic agent and supports its use as a positive control in this study.

In Fig. 3A, treatment with the 5−10 kDa peptide fraction resulted in a lower cell density compared to the media with cells and the negative control with dimethyl sulfoxide (DMSO), with more empty spaces and visibly damaged cells at various sample concentrations. Meanwhile, in Fig. 3B, treatment with the 3−5 kDa peptide fraction also showed

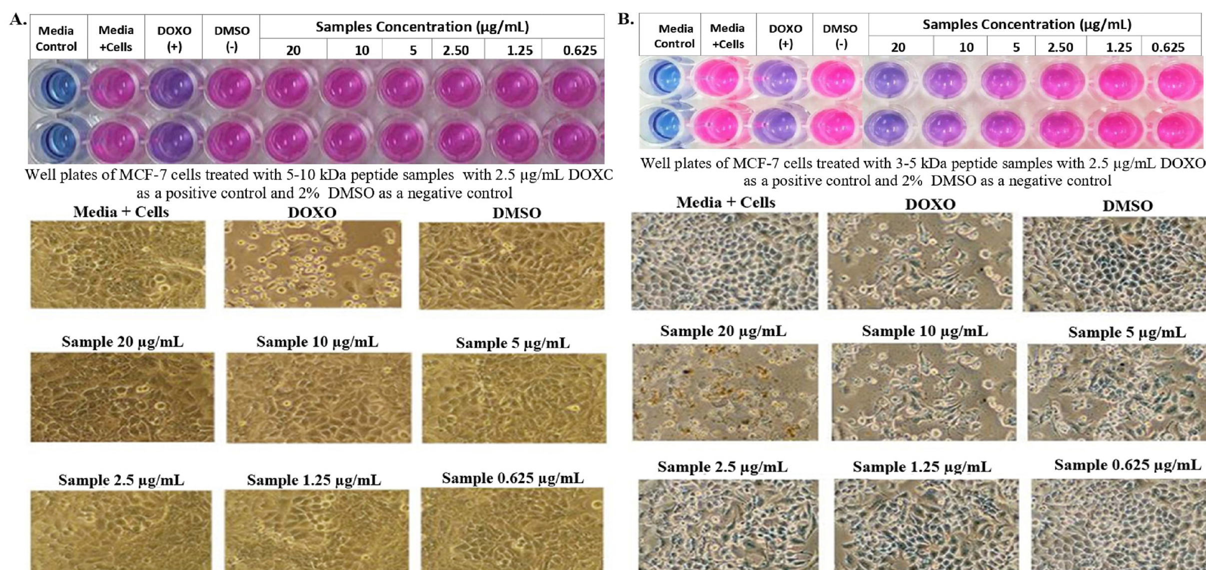


Fig. 3. MTT Assay (3-(4.5-dimethylthiazol-2-yl)-2.5-diphenyltetrazolium bromide) results.

fewer living cells. In addition, the cells appeared smaller and irregularly shaped. This suggests that the treatment may have a cell-killing effect, inhibiting the growth of cancer cells, and also demonstrates high effectiveness in reducing cell proliferation. Based on the research, the 3−5 kDa peptide fraction shows a more potent cytotoxic effect than the 5−10 kDa fraction.

The results obtained were better than those of previous studies conducted by Ref. [35] assessing the anticancer activity of *Turbo brunneus*, *Cypraea annulus*, and *Babylonia spirata*, showing vigorous cytotoxic activity against MCF-7 cell lines with IC_{50} values of 135.59 μg/mL, 412.2 μg/mL, and 222.92 μg/mL, respectively. A study conducted by Ref. [36] found that a fraction of less than 3 kDa hydrolysate of swamp eel protein (*Monopterus* sp.) had an IC_{50} value of 6.5 μg/mL against breast cancer cells (MCF-7). The lower IC_{50} values in the 3−5 kDa fractions are likely due to differences in organism sources, amino acid composition, hydrolysis conditions, and longer incubation times. In previous studies, the hydrolysates obtained by ultrafiltration were tested for peptides with molecular weights less than 3 kDa. In contrast, the 3−5 kDa peptides in this study may have retained more complex structures or bioactive motifs, thereby potentially enhancing their cytotoxic effects. These factors likely explain the more potent cytotoxicity compared to previous reports.

3.8. Antioxidant assay

The antioxidant activity test conducted on two peptide fractions showed antioxidant activity, as determined by comparison between the DPPH and ABTS methods. The IC_{50} values for each fraction are also listed in Table 4, indicating that the two peptide fractions exhibit different free radical-scavenging abilities. The antioxidant mechanism produced by the symbiotic bacteria of *Catostylus* sp. involves strengthening the host defense system by enhancing natural killer cell activity and activating the nonspecific immune system, as well as inducing thermally induced differentiation and apoptosis, which collectively act as inhibitory factors [36]. Bioactive antioxidant compounds produced by symbiotic bacteria have the potential to combat oxidative stress, a factor that contributes to the development of various chronic diseases, including cancer. Bioactive

Table 4. IC_{50} values obtained from DPPH and ABTS radical scavenging activity assays.

Types of Proteins	Fractions	Antioxidant test using the DPPH method	Antioxidant test using the ABTS method
		IC_{50} Average (μg/mL)	IC_{50} Average (μg/mL)
Peptide hydrolysate	5−10 kDa	254.25	41.30
	3−5 kDa	218.02	37.89
Control (+)	Ascorbic acid	47.87	15.23

compounds, including peptides produced by marine symbiotic bacteria, are recognized for their potential as anticancer agents and immune system regulators [37]. Several studies also show that increasing intake of natural sources of bioactive compounds, particularly those with high antioxidant content, reduces the risk of degenerative diseases and cancer [38]. The results are presented in the supplementary file (Fig. S1 (https://kijoms.uokerbala.edu.iq/cgi/editor.cgi?article=3442&window=additional_files&context=home)), showing the radical scavenging activities of DPPH and ABTS for the 5−10 kDa and 3−5 kDa peptide fractions at concentrations of 10, 20, 40, 80, and 160 ppm.

Based on Table 4, the antioxidant activity of two peptide fractions, 5−10 and 3−5 kDa, was evaluated using two comparative methods: DPPH and ABTS. The data in the table show the IC_{50} values (mg/mL), which indicate the sample concentration required to inhibit 50 % of free radical activity. Antioxidant activity testing using DPPH and ABTS methods showed the most vigorous activity in the 3−5 kDa peptide fraction, with IC_{50} values of 218 mg/mL and 37.891 mg/mL, respectively. This indicates that the 3−5 kDa fraction exhibits more potent antioxidant activity than the 5−10 kDa peptide fraction, as it requires a lower concentration to achieve 50 % radical inhibition.

A comparison of antioxidant assay methods is evident from the IC_{50} values, which show that the DPPH method yields significantly higher values than the ABTS method for both fractions. This finding suggests that the ABTS method is more sensitive to the antioxidant activity of these fractions than the DPPH method. This result aligns with the study conducted by Ref. [39], which reported that the ABTS method is more sensitive in detecting the antioxidant capacity of moringa leaf extract than the DPPH method. The significant difference in IC_{50} values between the two methods is due to differences in measurement principles and the sensitivity of each method to the types of antioxidants being tested. The results obtained were better than those of the previous study conducted by Ref. [40]. Antioxidant testing was performed on two marine bivalve mollusk *Tergillarca granosa* peptide hydrolysates using the DPPH method, with IC_{50} values of 530 μg/mL and 360 μg/mL, respectively. In contrast, using the ABTS method, IC_{50} values of 960 μg/mL and 540 μg/mL were obtained, respectively.

Such differences may stem from variations in peptide sources, amino-acid composition, hydrolysis conditions, and assay parameters, all of which can influence the antioxidant potential of peptide fractions.

3.9. Liquid chromatography high-resolution mass spectrometry (HRMS) analysis

Bioactive compounds in the sample were characterized using Liquid Chromatography-High-Resolution Mass Spectrometry (LC-HRMS). The predominant compounds were identified by comparing their mass spectra with reference data from the Library of Natural Products database. The identified compounds, along with their corresponding total peak areas, are presented in Table 5. We have also attached the completed profiles for all compounds based on the LC-HRMS results to the supplementary file (Table S2 (https://kijoms.uokerbala.edu.iq/cgi/editor.cgi?article=3442&window=additional_files&context=home)).

Liquid chromatography-high-resolution mass spectrometry (LC-HRMS) analysis revealed the presence of various bioactive peptides in both the extracellular (5−10 kDa) and intracellular (3−5 kDa) fractions (Table 5). Identification was performed by comparing the sample mass spectra with the Library of Natural Products database. The results showed several predominant compounds in both fractions, with the highest peak areas indicating their relative abundance. In addition, other oligopeptides such as L-methionyl-L-asparaginyl-L-proline and L-leucyl-glycyl-threonyl-isoleucyl-prolyl-glycine were also detected, with mass-to-charge (m/z) values and retention times consistent with reference data.

The presence of peptides containing hydrophobic amino acid residues, such as leucine, tyrosine, and methionine, is known to contribute to antioxidant and cytotoxic activities through free radical scavenging and the inhibition of cancer cell proliferation. These findings support the previously observed antioxidant and anticancer activities, suggesting that the peptide fractions from this hydrolysate have strong potential as bioactive compounds.

4. Conclusion

Peptide fractions obtained through ultrafiltration from the symbiotic bacteria of *Catostylus* sp. demonstrated clear anticancer and antioxidant activities, with the 3−5 kDa fraction showing the most potent effects in both cytotoxic and radical-scavenging assays. The 5−10 kDa fraction also exhibited notable activity. These findings provide initial evidence that these peptide fractions could be developed as natural candidates for cancer prevention and therapy.

However, this study is limited by its reliance solely on in vitro assays, which do not fully represent the complexity of biological systems. The

Table 5. List of identified compounds in the bioactive peptide sample identified using the library of natural products.

No	Types of peptide fraction	Compound	Pattern	Annot. Deltamass [ppm]	Calculated molecular weight	Mass-to-charge ratio (*m/z*)	Retention time (RT) [min]	Area (Max)
1	Extracellular hydrolysate (5–10 kDa)	L-Alanyl-L-alanyl-L-prolyl-L-alanine	$C_{14}H_{24}N_4O_5$	−1.58	328.1741	329.1814	1.67	1.3E+07
		L-Methionyl-L-asparaginyl-L-proline	$C_{14}H_{24}N_4O_5S$	−1.43	360.1462	361.1535	2.48	9.2E+06
		L-Threonyl-L-alanyl-L-valin	$C_{12}H_{23}N_3O_5$	−1.57	289.1633	331.1971	2.58	1.7E+06
		L-Leucyl-L-leucylglycylglycine	$C_{16}H_{30}N_4O_5$	−1.57	358.2210	359.2283	2.77	3.1E+06
		L-Leucyl-L-glutaminyl-L-leucine	$C_{17}H_{32}N_4O_5$	−1.34	372.2367	373.2440	4.09	6.1E+06
		L-α-Glutamyl-L-leucyl-L-leucylglycylglycine	$C_{21}H_{37}N_5O_8$	−1.17	487.2636	488.2709	4.62	2.6E+07
		L-Leucyl-L-asparaginyl-L-tyrosine	$C_{19}H_{28}N_4O_6$	−1.65	408.2002	409.2074	6.51	8.2E+07
2	Intracellular hydrolysate (3–5 kDa)	Leu-Leu-Tyr	$C_{21}H_{33}N_3O_5$	−1.42	407.2414	408.2487	1.75	5.7E+06
		L-Methionyl-L-asparaginyl-L-proline	$C_{14}H_{24}N_4O_5S$	−1	360.1462	361.1536	2.46	1.2E+07
		L-Lysyl-L-α-glutamyl-L-threonine	$C_{15}H_{28}N_4O_7$	−1.33	376.1953	377.2025	3.52	4.3E+06
		Leu-thr-ser	$C_{13}H_{25}N_3O_6$	−1.41	319.1738	320.1811	4.09	4.4E+07
		L-Leucyl-L-leucyl-L-glutamic acid	$C_{17}H_{31}N_3O_6$	−1.03	373.2209	374.2282	4.94	2.2E+07
		L-Leucyl-L-asparaginyl-L-tyrosine	$C_{19}H_{28}N_4O_6$	−1.48	408.2002	409.2075	6.51	5.9E+07
		L-Leucylglycyl-L-threonyl-L-isoleucyl-L-prolylglycine	$C_{25}H_{44}N_6O_8$	−0.96	556.3215	557.3288	6.88	1.3E+07

specific amino acid sequences and structural characteristics of the active peptides have not been elucidated, and their mechanisms of action remain undetermined.

Future research should focus on comprehensive amino acid sequencing, in vivo efficacy and toxicity evaluations, and in silico molecular interaction analyses to strengthen these findings. Such studies are crucial for validating the therapeutic potential and safety of these peptide fractions, thereby supporting their advancement toward clinical applications.

Ethics information

This study involved no human or animal subjects. Therefore, ethical approval was not required.

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Conflict of interests

There is no conflict of interest among the authors.

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