RESEARCH ARTICLE

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Isolation of Protein and Peptides from Symbiont Bacteria of Green Algae, *Caulerpa lentillifera* and Their Potency as Anticancer

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Abstract

Objective: Algae contain many symbiotic bacteria, often considered pollutants in algal cultivation. Recent studies indicate that these connections enhance the longevity of both organisms. Researching the bioactive metabolites of marine bacteria has emerged as a promising strategy for drug discovery. Green algae, including Caulerpa lentillifera, have anticancer activity and possess antioxidant qualities. The research emphasizes the isolation and identification of beneficial proteins from symbiotic bacteria, particularly Caulerpa lentillifera. **Methods:** The stages of the research included isolation and identification of the endophytic bacteria of the green alga symbiont *C. lentillifera*, isolation of protein from the bacterial symbiont, fractionation, hydrolysis, ultrafiltration of protein into peptides, and testing for activity. Screening activities used the BSLT to obtain the value of LC_{50}, and the mitotic test of the sea urchin zygote cell *Tripneustes gratilla* Linn to find the value of IC_{50}. **Results:** The results of this study indicated that the bacterial symbiont of the algae *C. lentillifera* was a species of Cobetia marina strain CL2-2. The peptide with molecular weight < 3 kDa from Cobetia marina strain CL2-2 was active. The peptide was from protein deposited with 40-60% saturated ammonium sulfate and hydrolyzed using pepsin enzyme. BSLT toxicity tests indicate that peptides with a molecular weight of less than 3 kDa showed significant toxicity, indicated by an LC_{50} value of 4.061 ppm. In a mitotic cytotoxicity test involving sea urchin zygote cells, peptides with a molecular weight of less than 3 kDa indicated significant cytotoxic activity, resulting in an IC50 value of 7.236 µg/mL. **Conclusion:** The bioactive peptide with molecular weight of less than 3 kDa resulting from protein hydrolysis isolated from the green algae symbiont *C. lentillifera* has the potential as an anticancer agent.

Keywords: Green algae- symbiont bacteria- bioactive peptides- Cobetia marina

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Introduction

Algae, a type of marine organism, harbor a diverse array of symbiotic bacteria [1], whereas bacteria are traditionally considered contaminants in algae cultures [2]. Recent research has shed light on the beneficial interactions between algae and bacteria, particularly in biotechnology [3]. Their interactions span a spectrum from commensalism to parasitism and mutualism [4], with numerous examples highlighting the importance of mutualistic and symbiotic relationships, often essential for the survival of both organisms [5]. Studying marine bacteria's bioactive metabolites has become a viable approach to finding new drugs.

Some algae are cytotoxic to cancer cell development during anticancer research [6]. The green algae may have anticancer activities [7] and antioxidant properties [8]. Based on earlier studies, more investigation into green algae species is necessary to determine whether algae may be an anticancer agent. Asmi et al. [9] have revealed that symbiotic bacteria associated with algae produced proteins with antibacterial and anticancer properties. Kasturiasih et al. [10] stated that symbiotic bacteria from the green algae, *Caulerpa lentillifera*, are capable of producing the enzyme L-Asparaginase, with significant toxicity (LC_{50} < 30 µg/mL). Furthermore, because *Caulerpa lentillifera* is high in protein, minerals, fiber, vitamins, and saturated and unsaturated fatty acids, it is not only utilized as food

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but also offers medical benefits for humans [11].

The Brine Shrimp Lethality Test (BSLT) is a method widely recognized for its potential for toxicity tests using Artemia salina, a species endorsed by the United States Environmental Protection Agency (US-EPA) for toxicity analysis [12]. The method is a favorite due to its affordability, quick turnaround time, accessibility, and sensitivity to toxic substances [13]. More studies have demonstrated a correlation between toxicity tests using Artemia salina and in vivo experiments in mice [14].

Since the 1970s, sea urchins have been an effective bioassay model for evaluating the influence of xenobiotics on early life stages and cellular function [15]. The model is a well-known marine organism model for studying biological evolution. It is the mostly model used in drug discovery research to understand the process of cell proliferation produced by natural chemicals with antimytotic action [16, 17]. Sea urchin zygotic cells are selectively sensitive to medications and divide in the same way that cancer cells do. Therefore, they are commonly used in anti-cancer research [18]. This research has led to the discovery of molecular targets for chemotherapy prevention and treatment [19]. According to Galasso et al. [20], sea urchin and human cells respond similarly to cancer cells in terms of cell death [21]. The study aims to evaluate the toxicity and cytotoxicity of peptides from the symbiotic bacteria *Caulerpa lentillifera*, both tests give useful information. The tests were done on Artemia salina larvae and sea urchin zygotic cells to find out more about their safety and possible uses. For example, the Artemia salina larvae check whether the compound is potentially harmful, while the sea urchin zygotic cells check how it affects the cell division process. The combination of both allows for the discovery of compounds relevant for further anticancer discovery.

Materials and Methods

Chemicals and tools

The materials used in this study included *Caulerpa lentillifera* sourced from Takalar, South Sulawesi, Indonesia, and various media and chemicals such as Nutrient Agar, Mac Conkey, Nutrient Broth, Mueller-Hinton Agar, Sea urchins, Artemia salina leach, Buffer A (comprising Tris–HCl 0.1 M pH 8.3, Triton X-100 0.5%, β-mercaptoethanol 1%, NaCl 2 M, CaCl2 0.01 M), Bovine Serum Albumin, distilled water, Lowry B (containing Na2CO3 2%, NaOH 0.1 N, CuSO4.5H2O 1%, sodium potassium tartrate 2%), Lowry A (consisting of Follin ciocalteus, phosphotungstate phosphomolybdate acid solution mixed with distilled water at a 1:1 ratio), and HCl 1 M. These chemicals were sourced from Merck Company, Germany, ensuring the highest purity standards. The study utilized several laboratory instruments including a UV-visible spectrophotometer T60 from PG Instruments, an Incubator from Memmert, and a Centrifuge model Hermle Z 366 K. These instruments were essential for conducting various experimental procedures and analyses throughout the study.

Isolation and identification of bioactive proteins from symbiotic bacteria

The *Caulerpa lentillifera* sample underwent a preparation process involving smoothing using a mortar and pestle, followed by filtration through a filtering cloth and rinsing with 45 mL of sterile seawater. The filtered material was then transferred into an Erlenmeyer tube containing 45 mL of sterile Nutrient Broth (NB) medium. The mixture was shaken in an incubator shaker at 180 rpm and 37 °C overnight. Subsequently, the material was subjected to progressive dilution from 10-1 to 10-10. Dilutions ranging from 10-5 to 10-8 were then cultured on Nutrient Agar (NA) medium and incubated for 2 x 24 hours at 37 °C. Following incubation, bacterial colonies that developed in each petri dish were observed.

Identification of a single bacterial colony was conducted using morphological tests to determine the genus of bacteria. Gram staining and biochemical testing were employed for further characterization. Biochemical assays were particularly utilized to ascertain the species of symbiotic bacteria from *Caulerpa lentillifera*, referring to Bergey's Manual of Determinative Bacteriology for identification protocols [20].

The molecular identification is done using the MEGA-X software program. The sequence alignment analysis is done by comparing the sequence obtained (query) with that already existing on Gene Bank with the database searches of the NCBI website (http//www.ncbi.nlm.nih.gov) using BLAST (Basic Local Alignment Search Tool).

Production and purification of bioactive proteins from symbiosis bacteria

Bioactive protein production occurred during the optimal growth conditions at 37 °C, with inoculation of up to 10% of the medium volume into sterile Nutrient Broth (NB). The mixture was then incubated in a shaker incubator at 180 rpm for an appropriate duration at 37 °C. After incubation, the resulting mixture was centrifuged at 5000 rpm and 4 °C for 30 minutes to separate the cell debris and bacterial cells. The centrifuged cells were diluted, and 50 mL of buffer A was added. Subsequently, the cell fragments underwent three cycles of freeze-thawing and sonication three times every ten minutes in ice water to facilitate precise cell disruption. The fragmented cells were then centrifuged at 5000 rpm for 30 minutes at 4 °C to obtain an intracellular protein filtrate [22]. A defined volume of the intracellular filtrate was subjected to ammonium sulfate precipitation at a specific saturation level. The mixture was stirred with a magnetic stirrer until completely dissolved and then left for 24 hours at 4 °C. The precipitate formed was separated from the filtrate by centrifugation at 13000 rpm for 15 minutes at 4 °C. The resulting pellet was dissolved in 5 mL of buffer, and the protein levels were analyzed. The fractionated solution was then purified using dialysis to obtain the desired bioactive protein.

Bioactive Protein Hydrolysis

The protein fraction with the highest activity is diluted into a 3% protein solution and digested using pepsin

enzymes under ideal conditions. After being inactive at 85 °C, the protein solution is hydrolyzed with pepsin and then kept at 4 °C [23]. The Lowry method can be used to determine total protein contents.

The solution is incubated at 4 ˚C for 30 minutes. The sample was then centrifuged for 25 minutes at 3000 rpm and 4 ˚C temperature [24, 25]. The degree of hydrolysis (DH) can be calculated by dividing the percentage of dissolved protein in 10% TCA by the amount of dissolvable protein.

Ultrafiltration of Bioactive Protein Hydrolysate

The hydrolysates with the highest activity are ultrafiltered using separating membranes based on differences in molecular size (Millipore Co., Bedford, USA), in sequence with their respective component sizes (<3 kDa, 5 kDa, and 10 kDa). The produced peptides are kept at -20 °C before proceeding to the next step [26].

Protein Content Determination

The Lowry method was used to determine protein levels, with Bovine Serum Albumin (BSA) as the standard solution and distilled water as the blank solution. A series of reaction tubes, each with a volume of 2 mL, were prepared with standard BSA. To each tube, 2.75 mL of Lowry B reagent was added and stirred evenly before being left at room temperature for 15 minutes. After incubation, 0.25 mL of Lowry A reagent was added to each tube and mixed evenly for 30 minutes or until the blue color stabilized. The absorption of each solution was then measured at its maximum wavelength (λ max) to generate a standard curve. This standard curve was used to establish the relationship between protein concentration and absorbance. For the protein fraction solution, 2 mL of the solution was added to a reaction tube, and the same treatment procedure was followed as with the standard BSA solutions. The absorption data obtained from the protein fraction solution was then plotted on the standard curve to determine the protein concentration. This method allows for accurate quantification of protein levels in the sample.

Toxicity test with Brine Shrimp Method Lethality Test (BSLT)

To assess the toxicity of the protein bioactive sample, various concentrations (1; 10; and 100 µg/mL) were prepared and added to separate tubes. Sterile seawater containing 10 shrimp Artemia salina Linn larvae was then added to each tube to reach a final volume of 5 mL. The tubes were then illuminated for 24 hours. After the incubation period, the number of dead and surviving larvae in each tube was counted using magnifying glasses. The percentage of mortality for each protein fraction concentration was calculated using the formula provided by Khairunnur et al. [22]. The LC_{50} (lethal concentration, 50%) value, which represents the concentration at which 50% of the larvae died, was determined using Ms. Excel. Probit analysis was performed to determine the LC_{50} value and evaluate the toxicity of the protein bioactive sample. The toxicity criteria provided in Table 1 were used to assess the toxicity level of the protein bioactive sample based on its LC_{50} value.

Antimytotic Activity Test

The BSLT results are used to determine the fraction with the highest level of toxicity. The fraction is dissolved with saltwater in an Eppendorf tube at concentrations of 1 µg/mL, 10 µg/mL, and 100 µg/mL, with a final volume of 1 mL. Following 10 minutes of fertilization, 100 µL of zygotic cells are introduced to the tube. Each sample was examined three times. The sample is kept at a temperature of 15-20 °C, with folding intervals of 10 minutes. Cell division was observed under a microscope after two hours of incubation. Zygotic cells that do not divide are identified and counted [18].

Probit analysis is used to determine the IC_{50} (µg/mL) value. The IC_{50} value is then evaluated using the cytotoxicity criteria in Table 2.

Results

Symbiotic Bacterial Isolation and Identification

The bacteria were isolated using a dilution procedure ranging from 10-1 to 10-8. Isolates that exhibited a clear zone around the colony were further purified using the quadrant streak method. Among the purified isolates, isolate CL2-2 obtained from the inner section of the green algae *Caulerpa lentillifera* (as shown in Figure 1) was selected for further research based on the stability of the developed clear zone. This isolate was chosen for

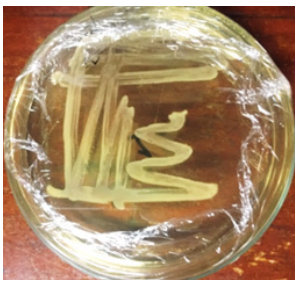

Figure 1. CL2-2 Bacterial Isolate Obtained from the Green Algae *Caulerpa lentillifera*

Table 1. Classification of LC_{50} Toxicity Values [27]

LC_{50} value (µg/mL)	Toxicity
< 20	Very Toxic
20 - 100	Toxic
100 - 500	Medium
500 - 1000	Weak
> 1000	Non Toxic

Table 2. Cytotoxicity Parameters based on IC_{50} Values. [28]

IC_{50} value (µg/mL)	Cytotoxic Activity
< 20	Toxic
20 - 200	Medium
200 - 500	Weak
> 500	Non Toxic

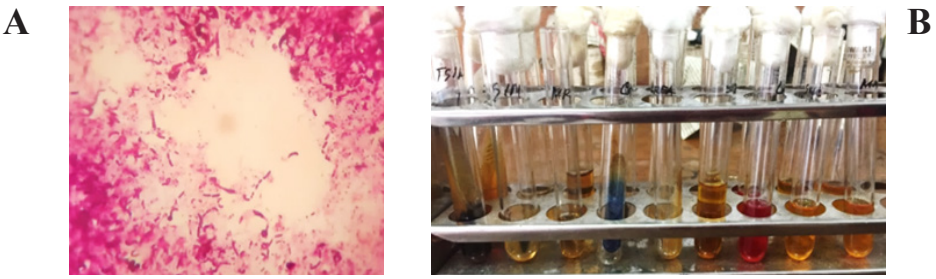

Figure 2. A) Morphological examination of a CL2-2 isolate using a 100x magnification microscope. B) CL2-2 isolate biochemical testing

Table 3. Identification Results of CL2-2 Symbiotic Bacteria

Test		Bacterial Isolate CL2-2
TSIA	Butt	Acid
	Slant	Acid
	Gas	-
	H2S	-
MR-VP	VP	-
	MR	+
SIM	Motility	-
	Indol	-
Urea	-	
Citric	-	
Carbohydrate test	Sucrose	+
	Mannitol	+
	Sucrose	+
	Lactose	+

subsequent identification and extraction of bioactive proteins.

Based on various tests, including gram staining and simple biochemical assays, as well as the morphological and sequencing gene 16s rRNA on the CL2-2 isolate has the closest homology of 99% with Cobetia marina strain 0402 as seen in Figure 2. Consequently, this isolate was identified as C. marina CL2-2 (Figure 3, Table 3).

Production and purification of bioactive proteins from symbiotic bacteria

The fermentation product of the C. marina CL2-2 isolate was obtained after 36 hours of fermentation in a production medium. To prevent protein denaturation, which can occur at high temperatures and in the presence of high concentrations of acid or base, the fermentation product was separated from bacterial cells using centrifugation at 5000 rpm for 30 minutes at 4 °C with buffer A. The buffer A solution, which contains Triton X-100, aids in chemically disrupting the cells by stretching the plasma membrane and facilitating cell lysis upon physical agitation. Additionally, the freezing technique was employed to ensure complete cell disruption (Figure 4).

The separation of bioactive proteins from other protein types began with fractionation using ammonium sulfate. This process aimed to separate proteins based on their differences in water solubility. The protein fraction obtained after purification with ammonium sulfate during the fractionation step is considered a semi-pure protein, as it may still contain salt residues and other impurities that need to be further purified to eliminate interfering ions. Dialysis is a commonly used method for this purpose (Table 4).

Enzymatic Protein Hydrolysis

Enzymatic hydrolysis was used in this research, with pepsin enzymes serving as the catalyst. The resultant hydrolysate is then centrifuged to determine the degree

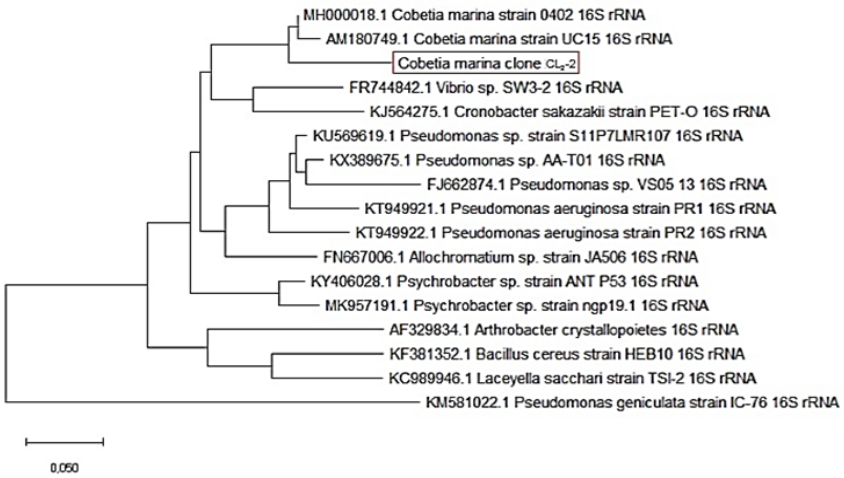

Figure 3. The Phylogenetic Tree Isolates Bacteria CL2-2

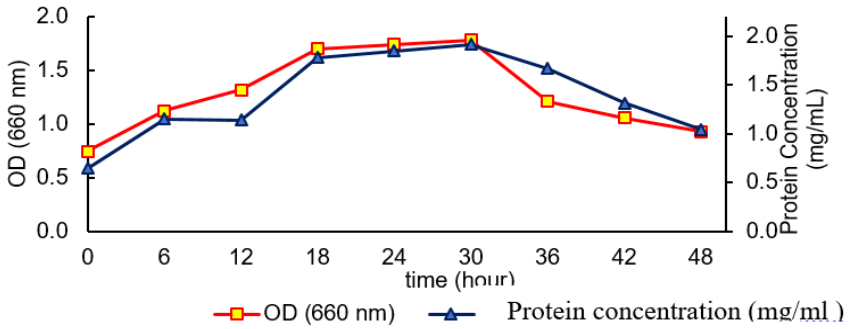

Figure 4. The Effect of Fermentation Duration on Intracellular Protein Production and Bacterial Growth. CL2-2 *C. marina*

Table 4. Protein Concentration of Bioactive Protein from Symbiotic Bacteria

Protein Extract	The volume of each fraction (mL)	Protein Concentration (mg/mL)
F1	10	9.78
F2	10	7.87
F3	10	10.77
F4	10	7.43

of hydrolysis (DH) after adding 20% TCA. The degree of hydrolysis (DH) is used to quantify the rate of protein degradation into short-chain molecules [29]. Based on the analysis, Figure 5 shows the enzymatic hydrolysis process.

The hydrolysis degrees (DH) achieved from this investigation ranged between 8% and 35%. The results show that the higher the DH value, the more peptide bonds have been disrupted. Figure 6 shows the highest DH value of 35.28 percent at 90 minutes of incubation. Then, the activity of toxicity to protein hydrolysates was tested to determine the best timing to proceed to the next step. Figure 6 depicts the activity screening findings, which demonstrate that the incubation time of 90 minutes has the most hazardous activity, thus this time is chosen as the best time to continue with the ultrafiltration phase utilizing MWCO.

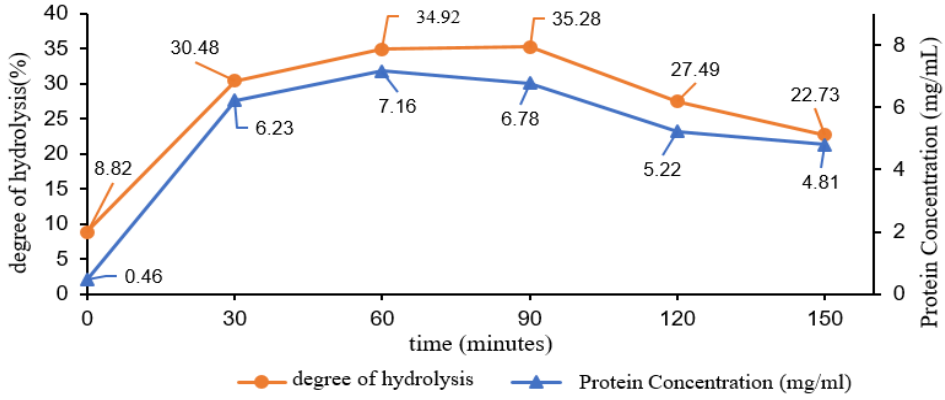

Figure 5. Data from Measurements of Hydrolyzed Protein Content and DH Percentage at 3% Enzyme and Substrate Concentrations, pH 2, and 37 °C.

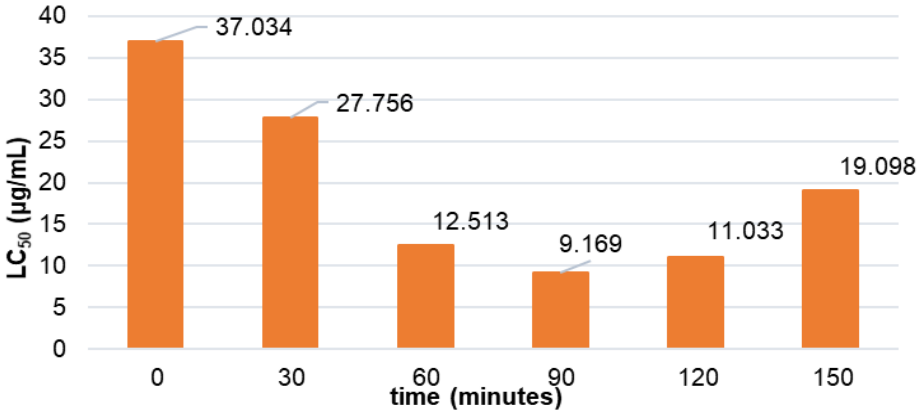

Figure 6. Screening Results of Activity Toxicity to Incubation Time Variations

Table 5. The Peptide Ratio of Each Fraction is based on Its Molecular Size

Peptide Fraction	Peptide Concentration (mg/mL)
> 10 kDa	4.65
5 - 10 kDa	3.65
3 – 5 kDa	2.50
< 3 kDa	1.53

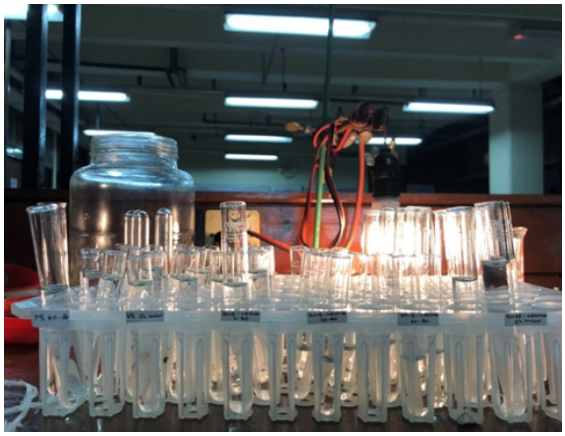

Figure 7. Toxicity Test with BSLT Method

The used MWCO separator media is divided into three types: MWCO with 10 kDa, 5 kDa, and 3 kDa sizes, thus obtaining four types of peptides with different molecular sizes: > 10 kD, 10-5 kD, 5-3 kD, and < 3 kD (Table 5).

Toxicity test with Brine Shrimp Lethality Test (BSLT) Method

The LC_{50} value, as indicated in the probit table, serves as a measure of sample toxicity, representing the concentration at which 50% of the test organisms would perish. A lower LC_{50} value indicates a higher level of toxicity for the sample. For instance, if the LC_{50} value of a test substance is 20 ppm, it would be classified as very toxic, while values ranging from 30 to 100 ppm would be considered toxic, and values greater than 1000 ppm would be deemed non-hazardous. This assessment of toxicity provides insight into the potential anticancer activity of the sample. In this study, the peptide <3 kDa showed the highest toxicity, with an LC_{50} value of 4.061 g/mL, as shown in Table 6, Figure 7.

Cytotoxicity testing of peptide fractions with mitotic methods against sea urchin Zigot cells Tripneustes gratilla Linn

The sperm and egg cells that have been extracted

Table 6. LC_{50} Values for Each Fraction of the Intracellular Protein of *C. marina* CL2-2

Peptide Fraction	LC_{50} Value (µg/mL)	Toxicity
> 10 kDa	32.702	Toxic
5 - 10 kDa	23.879	Toxic
3 – 5 kDa	8.006	Very toxic
< 3 kDa	4.061	Very Toxic

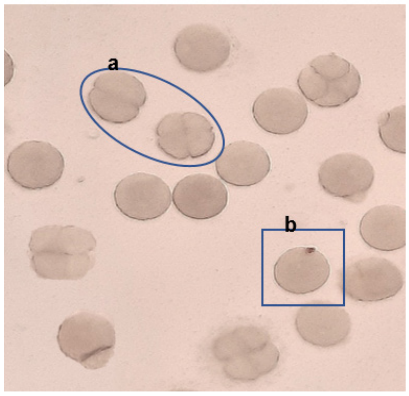

Figure 8. The Results of the Visualization of the Observance of the Sea Urchin Zygote Cell Division Tripneutes Gratina Linn. against the peptide fraction. Description: a = zygote cells divide; b = non-splitting zygotes

with KCl 10% are combined for fertilization. Following fertilization, the peptide fraction in the concentration sharing is induced within the zygotic cell. The observations were made after two hours of incubation by counting the number of seagull zigot cells that divided and did not divide. Figure 8 shows the outcomes of the observations. Table 7 shows the findings of the percentage inhibition of sea urchin zygote cells by peptide fractions.

Antimytotic testing measures cell growth inhibition as IC50 (Inhibitory Concentration 50%). The IC50 value was calculated to establish the lowest rate/concentration

Table 7. Data Obtained from the Calculation of the Percentage Inhibition of the Peptide Fraction against the Zygote Cells of the Tripneustes gratilla Linn.

Peptide Fraction	Concentration (µg/mL)	Inhibition Percentage (%)
> 10 kDa	1	27
	10	34
	100	44
5 - 10 kDa	1	30
	10	39
	100	48
3 – 5 kDa	1	37
	10	47
	100	54
< 3 kDa	1	44
	10	50
	100	59

Table 8. The IC_{50} Values of the Peptides of Each Fraction are Based on the Molecular Size.

Peptide Fraction	IC_{50} Value (µg/mL)	Cytotoxic Activity
> 10 kDa	496.195	Weak
5 - 10 kDa	160.521	Medium
3 – 5 kDa	30.232	Medium
< 3 kDa	7.236	Very Toxic

of the test drug that might be utilized as a half-division inhibitor in sea urchin zygote cells. [30]. The IC50 value is calculated from the percentage value of inhibition after applying the probit-log graphic method to the concentration of the test drug. The IC50 value can be seen in Table 8.

Probit analysis indicates that peptides of molecular size < 3 kDa have an IC50 of 7.236 µg/mL. The study found that the peptide fraction with a molecular weight of less than 3 kDa inhibits sea urchin egg cells. It should be emphasized that peptides < 3 KDa in this investigation were not further purified. Despite this, the peptide has the potential to be used as an anticancer drug.

Discussion

Based on the research findings. it can be concluded that the symbiotic bacteria isolated from the green algae *Caulerpa lentillifera* identified as Cobetia marina has the capability to produce bioactive proteins. The Cobetia marina strain CL2-2 symbion bacterium can create anti-cancer peptides with molecular weights of less than 3 kDa. BSLT toxicity experiments indicate that peptides of a molecular size of <3 kDa are very toxic, with an LC_{50} value of 4.061 ppm. In a mitotic cytotoxicity test against sea urchin zygote cells, peptides with molecular size < 3 kDa showed high cytotoxic activity, with an IC50 value of 7.236 µg/mL.

Author Contribution Statement

The research was conducted collaboratively by a team, with each author contributing to various aspects such as developing the research strategy, conducting practical experiments, collecting data, and drafting the manuscript. Furthermore, it was ensured that all authors reviewed and approved the final version of the manuscript before submission.

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Conflict Of Interests

The authors declare there is no conflict of interest among the authors.

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