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Anticancer Peptides Isolated from Symbiotic Bacteria Against Lung Cancer: Potential and Opportunity

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Abstract. This research aimed to evaluate the bioactivity of the peptide fragment from symbiotic bacteria in the lung cancer LK-2 cell line compared to the normal M5S cell line. Protein hydrolyzates were separated using Molecular Weight Cut Off (MWCO) to isolate peptide fragments. Fragments were categorized as ≤ 5 kDa, > 5-10 kDa, and > 10 kDa to evaluate their toxicity through the Brine Shrimp Lethality Test (BSLT) method and to study cytotoxicity effects and proliferation in LK-2 lung cancer cells against normal M5S cells. MTT assay was used to analyse the percentage of living cells and cell growth. The Selectivity Index (SI) equation was applied to determine selectivity. Findings indicated that the peptide fragment ≤ 5 kDa had the highest toxicity, with an LC_{50} of 8.15 µg/ml. The number of LK-2 cells that died increased when exposed to peptide fragments of sizes ≤ 5 kDa, > 5-10 kDa, and > 10 kDa over 16 hours. Moreover, cell growth in LK-2 cells decreased after 16 hours, while there was no significant decline ($P < 0.05$) in M5S cells compared to the controls. The peptide fragments with molecular weights ≤ 5 kDa and > 5-10 kDa demonstrated the highest selectivity, resulting in an SI value of 3. These results suggest that the selected peptide fragments may serve effectively as anticancer agents. This study highlights the potential of peptides from symbiotic bacteria as possible treatments for cancer, particularly lung cancer.

Introduction

Generative diseases, such as cancer, are a double burden in the world of health. Cancer is a disease that causes death globally (Amin et al., 2025). The diagnosis, treatment, and prevention of cancer in the last decade have made significant progress. Currently, the most common cancer treatment involves surgery, chemotherapy, and radiation. However, the main problem with this treatment is the emergence of resistance and adverse side effects (Deslouches & Di, 2017; Maksymowicz et al., 2023). This study has made researchers interested in developing anticancer compounds with specific and selective targets with fewer side effects (Kao et al., 2024; Li & Jin, 2024a).

Lung cancer remains the most prevalent malignancy and the leading cause of cancer mortality globally(Cao et al., 2024). LK-2 is a human squamous cell carcinoma lung cancer cell line, representing the primary subtype of NSCLC. NSCLC accounts for approximately 85% of all lung cancers, with squamous cell carcinoma (SCC) being a common variant that is difficult to treat clinically (Hijazo-Pechero et al., 2023). LK-2 is a stable human cell line, easy to grow in vitro. It has been widely used as a preclinical model in studies of cytotoxicity, proliferation inhibition, and apoptosis mechanisms of anticancer agents.

Nowadays, bioactive peptides are getting more attention because research findings indicate that these substances positively influence human health. Anticancer peptides (ACPs) have selective activity against cancer cells. ACPs, compared to proteins and other small organic molecules, have several advantages, such as small size, high activity,

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sequence diversity, and more site modification for functional molecules (Hwang et al., 2022; Kumar & Li, 2017; Li & Jin, 2024b).

Natural products are now trending alternatives used in treating different cancer types and offer valuable opportunities for evaluation not only as anticancers but also with other relevant action mechanisms (Chan et al., 2023). Marine has enormous potential in the searching active compound effort. Many bioactive compounds have been isolated from marine biota, including marine algae. Although marine algae were popularly used as a source of bioactive compounds, symbiont bacteria are an alternative source of bioactive materials to prevent the overexploitation of marine biota.

In this study, the protein hydrolyzate from previous research that was isolated from symbiotic bacteria in *Sargassum* sp, which has been identified as *Enterobacter hormaechei* strain SG-A1 (Asmi et al., 2020, 2021), was hydrolyzed using pepsin enzyme and separated based on molecular weight. The peptide fragments were tested for their toxicity and Cellular proliferation using LK-2 lung cancer cells compared to normal M5S cells. The selectivity index was also calculated in this study.

Experimental

Material and Methods

The materials used in this study were *Artemia salina* Leach, seawater, lung cancer LK-2 cell line, mouse skin fibroblast M5S cell line Dulbecco's modified Eagle's medium (DMEM), 3-(4.5-dimethylthiazol-2-y/)-2.5diphenyl-tetrazolium bromide (MTT), DMSO, fetal bovine serum (FBS), penicillin, streptomycin, Alpha modified Eagle's medium (αMEM), BCA Protein Assay Kit (Thermo Scientific Cat.# 23225.

Procedures

Ultrafiltration

Hydrolysate with the highest anticancer activity from previous studies (Asmi et al., 2021)was ultrafiltrated using Vivaspin 3, 5, and 10 kDa molecular weight cutoff (MWCO) membranes, respectively (Sartorius, Goettingen, Germany). Liophyliz (≤ 5 kDa, > 5-10 kDa, and > 10 kDa) obtained and stored at -20 ºC (He et al., 2013; Wang & Zhang, 2013).

Determination of protein concentration

Protein concentration was measured using PierceTM BCA Protein Assay Kit (Thermo Scientific Cat.# 23225).

Toxicity test

Artemia Salina eggs (0.1 g) were inoculated into 100 mL of seawater and maintained at 30 °C, under vigorous aeration and persistent incandescent illumination, for 24 hours. Upon hatching, 10 larvae were carefully transferred into a vial. The extract concentrations included 0 mg/mL (as a control), 10 mg/mL, 100 mg/mL, and 1000 mg/mL. Each concentration was evaluated in triplicate. After a 24-hour exposure period, the count of deceased individuals was recorded (Tahar & Hariani Soekamto, 2023).

MTT assay

Cytotoxicity and cellular proliferation were conducted utilizing the 3 (4,5-dimethylthiazol-2-yl) 2, 5 diphenyltetrazolium bromide (MTT) assay. The lung cancer cell line, LK-2, was kept in Dulbecco's modified Eagle's medium (DMEM) that contained heat-inactivated 100 ml/l fetal bovine serum, 100 U/ml penicillin, and 100 mg/ml streptomycin, for the mouse skin fibroblast cell line M5S, alpha-modified Eagle's medium (αMEM) was used. The cells were maintained in a humid environment with 5% CO_2 at 37ºC, while the culture medium was refreshed every two days.

The MTT assay investigated how peptide fragments impacted cell survival and growth. A total of 1 x 10^4 cells were placed in a 96-well plate. After an incubation period of 24 hours, the cells were exposed to different doses of fractions and incubated for another 16 hours. Following this, 50 ml of MTT in FBS (0. 5mg/ml) was introduced to each well, and the cells continued to incubate for an additional 4 hours. To dissolve the MTT formazan crystals, 100 ml of DMSO was added to each well. The absorbance was measured at 570 nm using a Microplate Reader. The control cells' absorbance was considered to be 100% viability. The results are presented as the percentage of viable cells compared to their respective controls (Mazlan et al., 2020; Zafari et al., 2015).

Selective index

The selectivity of a compound can be determined using the selectivity index (SI) parameter with the equation (Peña-Morán et al., 2016).

$$SI = \frac{IC_{50} \text{ normal cancer cells}}{IC_{50} \text{ cancer cells}} \quad (1)$$

Result and Discussion

Ultrafiltration

Protein hydrolysis has been carried out using the pepsin

enzyme in previous studies. The results were followed by ultrafiltration, separating the peptide fragments based on their molecular weight. Many studies have shown that the biological activity of peptides is related to their molecular weight. Peptides with small molecular weights often present a strong biological activity. So, separating the protein hydrolyzate obtained into small peptides is necessary, hoping to increase its activity or facilitate its absorption in the body.

Table 1. The protein content of peptide fragment.

No	Peptide (kDa)	Protein content (mg/ml)
1	≤ 5	0.71
2	> 5-10	1.21
3	> 10	1.60

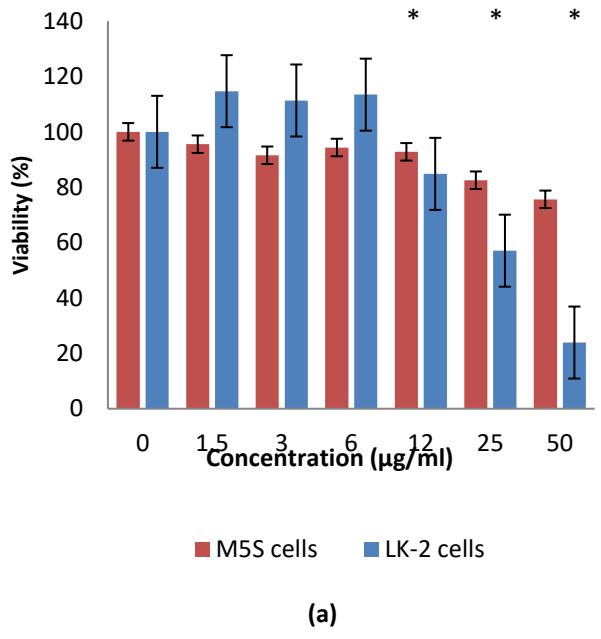
In this study, the separation used two membranes, namely the MWCO Vivaspin 20 membrane from Fisher Scientific, with a size of 5 kDa and 10 kDa. The final results obtained three fractions: peptides with molecular weights of less than 5 kDa, 5-10 kDa, and more than 10 kDa. The protein content of the peptide fragments was measured using a BCA kit. The results of the calculation of protein content are shown in Table 1. This method allows for identifying the most biologically active peptide molecular weight range.

Toxicity test

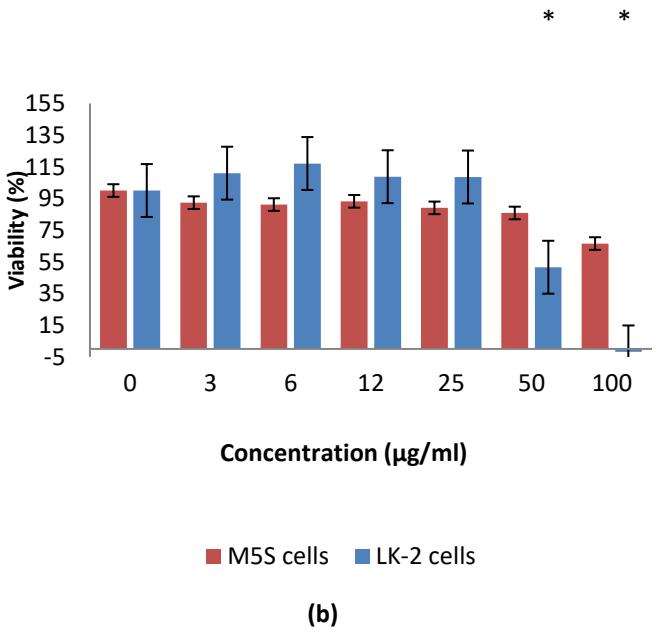
Table 2 shows the F4h1 peptide fragment, a peptide with a size of ≤ 5 kDa, has the highest toxicity of 8.15 µg/ml compared to the F4h2 peptide (5-10 kDa) and F4h3 (>10 kDa). According to Thundimadathil (2012) and Ali et al. (2013), peptides with smaller sizes can penetrate the membranes of bacterial cells and cancer cells. Peptides with a molecular size of ≤ 5 kDa are included in the category of small oligopeptides, which have a high ability to diffuse passively or actively penetrate cell membranes. Small size facilitates penetration through plasma membrane pores or non-specific endocytosis pathways. Small molecules experience faster transmembrane transport, especially in cancer cells that often have increased membrane permeability due to metabolic stress and changes in membrane lipid structure.

Table 2. The LC_{50} of peptide fragment.

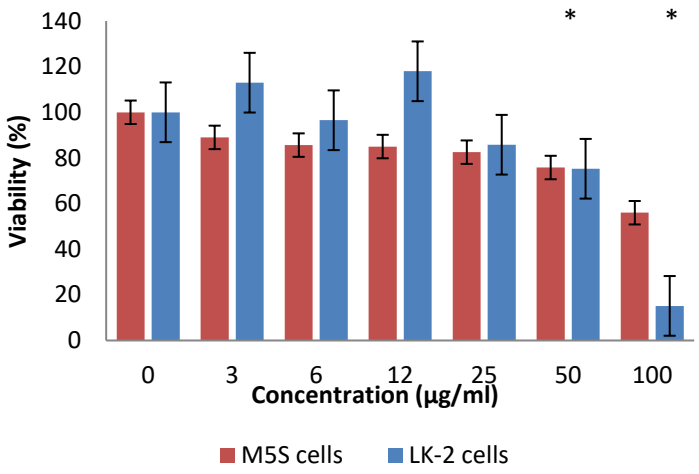
No	Peptide (kDa)	LC_{50} (µg/ml)
1	≤ 5	8,15
2	> 5-10	24,12
3	> 10	19,71



(a)

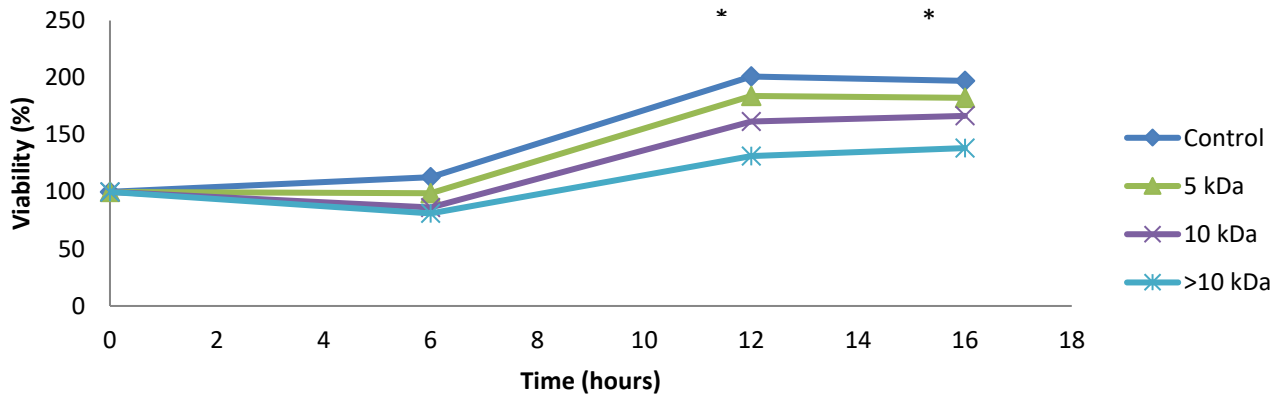


(b)

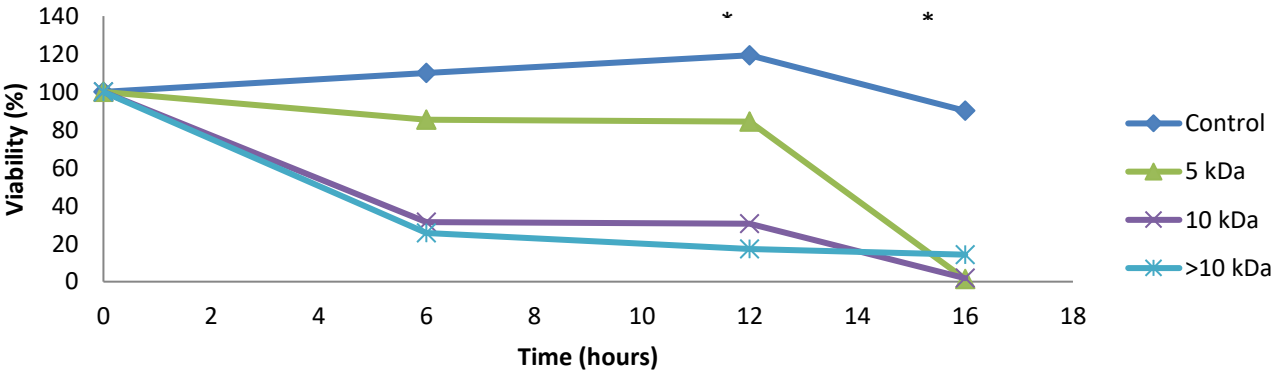


(c)

Figure 1. MTT cytotoxicity evaluation. Cell viability bar graphs that represent LK-2 and M5S cell lines were exposed to varying amounts of peptide fragments: (a) ≤ 5 kDa; (b) > 5-10 kDa; and (c) > 10 kDa for 16 hours. The total number of viable cells was determined using the MTT assay. Each data point is represented as the mean ± SD, and results are statistically significant with P < 0. 05 in comparison to the control.



(a)



(b)

Figure 2. Cell proliferation test**.** The impact on the survival of LK-2 cells (A) and M5S cells (B) was measured after treatment with various concentrations of peptide fragments, taking place at 0, 6, 12, and 16 hours of incubation using the MTT assay. Each value shows the mean from three independent experiments. P < 0. 05 in comparison to the control.

MTT assay

The MTT assay assessed the cytotoxic impacts of peptide fragments on the LK-2 and M5S cell lines. Different concentrations of the fractions were introduced to both types of cells. Cell viability was then calculated as a percentage. The results of the analysis are illustrated in a visual format and can be found in Figure 1.

The data indicated that the LK-2 cells displayed an increase in death when treated with peptide fragments of sizes ≤ 5 kDa, > 5-10 kDa, and > 10 kDa over the 16-hour treatment duration, with more than 50% of the cells being dead. In contrast, M5S cells did not experience a significant rise in cell death. After treatment with peptide fragments, the LK-2 cells exhibited a reduction in the proliferation rate at the 16-hour mark, while the M5S cells did not show any notable decline (Figure 2). The majority of anticancer drugs are unable to differentiate between cancerous and normal cells (Blaurock et al., 2016). This finding suggests that peptide fragments are not only effective in exerting anticancer activity but also demonstrate a notable degree of selectivity toward cancer cells over normal cells. Laterosporulin 10 (LS10) peptide from Brevibacillus sp has cytotoxic activity on lung carcinoma (H1299) while showing no cytotoxicity towards normal cells (Baindara et al., 2017). Leodoglucomides A and B, which are compounds isolated from *Bacillus licheniformis*, have also been shown to exhibit cytotoxic activity against lung cancer cells (Tareq et al., 2012). Cancer cells have high proliferative activity but are closer to the cell cycle regulation failure threshold and cell death (apoptosis). Peptides can exploit the physiological vulnerabilities of cancer cells and induce cell death more efficiently without causing significant damage to normal cells. In this case, the bioactive peptide is increasingly considered a candidate for cancer therapy.

Selective index (SI)

The selectivity of the peptide fraction of epiphytic bacteria was evaluated using SI parameters. The findings of the selectivity index revealed that the peptide fragments with a size of ≤ 5 kDa, > 5-10 kDa showed a high level of selectivity against LK-2 lung cancer cells because they had a SI value of 3 (Table 3). A compound is generally considered to possess strong selectivity when its Selectivity Index (SI) value is equal to or higher than 3. In contrast, it is regarded as having low selectivity if the SI value falls below this value (Njoya et al., 2020). SI is considered biologically selective, meaning these compounds are more toxic to cancer cells than normal cells, which is crucial for developing safe drugs.

Table 3. Selective index of fraction peptide.<

No	Peptide (kDa)	IC_{50} M5S cells	IC_{50} LK-2 cells	SI
1	≤ 5	105.14	35.66	3
2	>5-10	164.64	60.54	3
3	>10	116.86	71.56	2

Conclusion

This study reported that peptide fragments have the potential to be used as new anticancer agents in cancer therapy, especially lung cancer. The results of this study should encourage further research to identify the composition of amino acids produced by bioactive peptide fragments and understand the mechanism of the compounds produced.

Conflict of Interest

The authors declare that there is no conflict of interest.

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