RESEARCH PAPER

Recombinant Brevinin-1 E8.13 Peptide: Biotechnological Production And Anti-Lung Cancer Properties

Quynh Bach Thi Nhu[1], Huyen Ngo Thi[2], Trang Thu Nguyen[2], Manh Hai Tran[2], Cuong Khac Bui[3,4], Minh Tri Duong Le[5], Dung Bui Tri[6], Huyen La Thi[2,7*]

[1] Faculty Of Medical Technology, Hai Phong University Of Medicine And Pharmacy, Hai Phong 180000, Vietnam.
[2] Institute Of Biology, Vietnam Academy Of Science And Technology, 18-Hoang Quoc Viet, Cau Giay, Hanoi, Vietnam.
[3] Laboratory Animal Research Center, Vietnam Military Medical University, Hanoi, Vietnam.
[4] Vietnamese-German Center For Medical Research (Vg-Care), Hanoi, Vietnam.
[5] Nguyen Sieu School, 35 Mac Thai To Street, Yen Hoa, Hanoi, Vietnam.
[6] Hanoi Medical University, 01 Ton That Tung Street, Kim Lien Ward, Hanoi, Vietnam.
[7] Graduate University Of Science And Technology, Vietnam Academy Of Science And Technology, 18-Hoang Quoc Viet Street, Cau Giay, Hanoi, Vietnam.
Corresponding Author: Huyen La Thi, Email: huyenla@ib.ac.vn

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Abstract

Brevinin peptides belong to a family of antimicrobial peptides (amps) known for their strong antibacterial and anticancer activities. Among them, brevinin-1 e8.13 has emerged as a promising candidate for therapeutic development. This study aimed to establish an efficient recombinant expression system for brevinin-1 e8.13 in escherichia coli bl21(de3) and to evaluate its anticancer efficacy and safety profile. The gene encoding brevinin-1 e8.13 was cloned into the pet32a(+) vector and expressed as a thioredoxin (trx)-his-tagged fusion protein. Expression conditions were optimized by varying iptg concentrations (0.1–1.0 mm) and induction times (4–8 h). The recombinant protein was purified using ni^{2+} affinity chromatography, followed by factor xa cleavage and high-performance liquid chromatography (hplc). Antitumor activity was assessed in a murine lung cancer model, while toxicity was evaluated through hematological and biochemical analyses. Optimal expression was achieved with 0.5 mm iptg for 6 – 8 h, yielding 30.2 mg/l of fusion protein with a purification efficiency of 63%. The cleaved peptide reached over 90% purity as determined by hplc. In vivo experiments demonstrated that recombinant brevinin-1 e8.13 significantly reduced tumor volume without affecting body weight or causing damage to major organs, and no systemic toxicity was observed. These findings indicate that brevinin-1 e8.13 can be efficiently produced in e. Coli and exhibits potent anticancer activity with a favorable safety profile, supporting its potential as a novel therapeutic peptide for cancer treatment.

Keywords: Brevinin-1 E8.13, Tumor Suppression, Cancer Therapy, Recombinant Peptide, Antimicrobial Peptide.
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I. Introduction

Antimicrobial peptides (AMPs) are naturally occurring peptides with the ability to kill bacteria, fungi, and viruses. They play a crucial role in the immune systems of many animals, especially amphibians like frogs, where they serve as an effective first line of defense. AMPs are typically small (10–50 amino acids), positively charged, and hydrophobic, with structures that include α-helix, β-sheet, or random coil formations. AMPs kill bacteria through two main mechanisms: pore formation and metabolic inhibition. In pore formation, AMPs interact with bacterial cell membranes, disrupt their structure, form pores, and cause loss of membrane permeability, ultimately leading to cell death. In metabolic inhibition, some AMPs penetrate the cell and interfere with intracellular processes, such as inhibiting

protein, DNA, or RNA synthesis. The difference between the bacterial membrane (negatively charged) and the host cell membrane (neutral) plays a key role in the selectivity of AMPs. However, factors such as pH, salt concentration, and the presence of protease enzymes can influence AMP activity. AMPs hold promise as a new class of antibiotics, particularly in light of growing bacterial resistance. Yet, challenges remain: they are susceptible to degradation by proteases, may lose their activity in harsh environments, some can be toxic to host cells at high concentrations, and large-scale synthesis is costly (Brogden 2005; Epand and Vogel 1999; Jenssen, Hamill, and Hancock 2006; Yeaman and Yount 2003).

Kwon et al. (1998) analyzed brevinin 1E, an antibacterial peptide found in the skin of the frog *Rana esculenta*, consisting of 24 amino acids. This peptide demonstrates effective antibacterial activity against both Gram-positive and Gram-negative bacteria, as well as antifungal properties. Brevinin 1E holds promise as a potential new antibacterial drug, particularly in light of the growing issue of antibiotic resistance. However, further research is required to enhance its selectivity and reduce potential toxicity (Kwon, Hong, and Lee 1998).

Similarly, Ju et al. (2021) reported that brevinin-1RL1 is an antimicrobial peptide extracted from the skin secretions of frogs from the species *Rana limnocharis*. Brevinin-1RL1 demonstrates the ability to inhibit the growth of various types of cancer cells, including breast, liver, and lung cancer. This anticancer activity is dose-dependent, with higher efficacy at sufficiently high concentrations. Brevinin-1RL1 kills cancer cells through two main mechanisms: apoptosis and necrosis. In apoptosis, the peptide damages the mitochondrial membrane, causing mitochondrial imbalance, releasing apoptosis-inducing factors (such as cytochrome c), and activating caspases, leading to DNA fragmentation. In necrosis, the peptide directly attacks the cancer cell membrane, disrupting membrane integrity, causing leakage of intracellular substances, and resulting in rapid cell destruction. However, at high concentrations, the peptide can be toxic to non-cancerous cells. Brevinin-1RL1 is not only a potent antimicrobial peptide but also a promising candidate for cancer therapy due to its ability to induce apoptosis and necrosis in cancer cells. Further research is necessary to optimize the peptide's efficacy and safety for clinical applications . Several other studies have also demonstrated that brevinin-1BYa, brevinin-1GHa, brevinin-1, and brevinin-2, isolated from the skin of *Rana dybowskii*, as well as peptides from the brevinin-1 family isolated from *Rana boylii*, exhibit strong antifungal, antibacterial, and anticancer activities .

Brevinin-2R exhibits strong activity against various cancer cell lines, including T-cell leukemia (Jurkat), B-cell lymphoma (BJAB), breast cancer (MCF-7), and lung cancer (A549). Its mechanism of action involves mitochondrial damage, reduction of mitochondrial membrane potential, increased production of reactive oxygen species (ROS), and decreased intracellular ATP levels, ultimately leading to caspase-independent cell death. Notably, this peptide demonstrates selectivity, with minimal effects on non-cancerous cells. The study by Ju et al. (2021) explored the mechanism by which brevinin-2R1, a member of the brevinin family, induces cancer cell death. The results showed that brevinin-2R1 activates a cell death pathway involving both lysosomes and mitochondria, including an autophagy-like process. Unlike traditional apoptosis, this peptide triggers cell death through a unique mechanism associated with mitochondrial damage and the involvement of BNIP3, a pro-apoptotic protein.

Brevinin-1 E8.13 was previously synthesized and shown to exhibit cytotoxic activity against cancer cells (La, H.T. et al., 2025). To enable large-scale production and address the translational challenges associated with brevinin peptides and antimicrobial peptides (AMPs) in general, recombinant brevinin-1 E8.13 was produced in this study. The antitumor efficacy and safety of the recombinant peptide were subsequently evaluated in a mouse model. The findings suggest that brevinin-1 E8.13 has potential as a candidate for further preclinical development as an anticancer agent, while also supporting its prospective application as an antimicrobial peptide.

II. Material and Methods

1. Material

The brevinin-1 E8.13 gene (Genbank: UVJ64031.1) was provided by Animal Cell Biotechnology (VAST, Vietnam). *E. coli* BL21(DE3) (Thermo Fisher Scientific, USA) was used as the host for recombinant protein expression. Luria-Bertani (LB) medium was used for bacterial culturing.

Restriction enzymes *Bam*HI, *Xho*I, and T4 ligase were purchased from Thermo Fisher Scientific, and the pET-32a(+) vector (Thermo Fisher Scientific, USA) was used.

A549 cell line was provided by ATCC (American Type Culture Collection) and cultured and maintained at Animal Cell Biotechnology.

2. Methods

2.1. Construction of the recombinant expression vector

The gene coding for brevinin-1 E8.13 was provided by the Animal Cell Technology Department (IBT, VAST) with a size of 93 bp and was inserted into the pET32a(+) vector through the cutting sites of *Bam*HI and *Xho*I under the effect of T4 ligase, transformed into the *E. coli* BL21(DE3). *E. coli* BL21(DE3)/pET32a(+)/brevinin-1 E8.13 cells were grown on selection carried out on LB agar plates containing 100 µg/mL ampicillin. Selected clones were selected using the colony PCR method. The selected plasmids were isolated and checked by sequencing the gene segment inserted into the vector.

2.2. Expression and optimization of expression conditions

During the process of expressing the protein, two key factors IPTG concentration and induction time were found to influence how well the recombinant strain *E. coli* BL21(DE3)/pET32a+/brevinin-1 E8.13 expressed the protein.

The *E. coli* BL21(DE3)/pET32a+/brevinin-1 E8.13 was cultured in LB medium (pH = 7) supplemented with 100 µg/mL ampicillin, at 37°C, shaking at 250 rpm, with a 2% inoculum ratio. When the optical density at 600 nm (OD_{600}) reached 0.6 – 0.8, IPTG was added at the concentrations and induction durations shown in **Table 1**.

Table 1: Experimental matrix with 2 factors IPTG and Induction time

Run	IPTG (mM)	Induction time (h)
1	0.1	4
2	0.1	6
3	0.1	8
4	0.5	4
5	0.5	6
6	0.5	8
7	1	4
8	1	6
9	1	8

The pET32a+/brevinin-1 E8.13 plasmid was transformed into the *E. coli* BL21(DE3)**.** A single clone was grown at 37°C and 250 rpm in 100 µg/mL ampicillin presence until the optical density (OD_{600}) reached 0.6 – 0.8. Protein expression was induced with isopropyl-β-D-1-thiogalactopyranoside (IPTG) concentration and induction time as shown in **Table 1**. Then the cells were harvested by centrifugation at 4,000×g for 20 minutes and re-suspended in PBS 1X. Sonication in 10 repeating cycles of 15s on/20s off on ice to cell disruption and the cell pellet was stored at -20°C for further use. The expression of the recombinant protein was examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

2.4. Method recombinant peptide purification

The bacteria pellet was resuspended with the native binding buffer and purified followed by the manufacturer's instruction (Ni-NTA Purification System, Thermo Fisher Scientific, USA). The 6xHis/Ni-NTA system offers a fast and versatile method for affinity purification of recombinant proteins and antigenic peptides. It relies on the strong binding affinity between six consecutive histidine residues (6xHis tag) and immobilized nickel ions, facilitating highly selective interactions that enable purification of tagged proteins or protein complexes from less than 1% purity to more than 95% in a single-step.

The recombinant-induced cells were resuspended in 60 mL binding buffer. Then, the cells were lysed by sonication at 400 W for 30 minutes (15 s working, 15 s free) in an ice water bath. Centrifuge at 10,000 g for 20 min at 4°C, collect the supernatant and load it through HisPur™ Ni-NTA Spin Columns, 3 mL to purify the His-tag target protein. Wash the column with lysis buffer at 2 times the volume of protein lysate and lyse the target protein bound on the column with a solution containing 25-250 mM Imidazole. The samples from the fractions were analyzed using 12% SDS-PAGE with a discontinuous buffer system as described by Laemmli (1970) (Laemmli 1970).

2.5. Method for evaluating the efficiency of brevinin-1 E8.13 protein purification

To calculate the purification efficiency, the protein content before and after purification is determined using the Bradford method (Bradford, n.d.). Prepare a standard curve with albumin solution diluted to concentrations of 0, 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100 µg/mL (**Table 2**). Measure the optical density of the solution at 595 nm. Draw a standard curve.

Table 2. Build albumine standard curve

Variable name	Control	1	2	3	4	5	6	7	8	9
Concentrate	0	10	20	30	40	50	60	70	80	90

(µg/ml)										
Standard albumin (µL)	0	10	20	30	40	50	60	70	80	90
Deionized (µL)	1000	990	980	970	960	950	940	930	920	910
Reagents (mL)	2	2	2	2	2	2	2	2	2	2

2.6. Method for recombinant peptide release and purification by HPLC

To obtain single peptides from fusion proteins, fusion proteins were dialyzed through a membrane in a factor Xa cutting buffer solution. The cutting reaction was optimized for cutting conditions and collected on a small scale with a cutting volume of 3-5 mL: factor Xa content 10 U/1mg protein, protein concentration 1.2 mg/ml, 16 – 20 h, 22 – 25ºC. Finally, the purity and the molecular weight of peptide was analyzed by HPLC.

2.7. Evaluation of brevinin-1 E8.13 activity on lung tumors and cytotoxicity

The brevinin-1 E8.13 peptide was tested for its anti-cancer activity on an experimental tumor model. Mice were divided into 4 groups, each group of 10 mice, injection concentration 10 µg/g/dose, 6 doses, for 2 weeks/dose:

Group 1: Control

Group 2: Buffer

Group 3: Peptide

Group 4: Combined (Buffer + Peptide)

A healthy volunteer peripheral blood sample was provided 2 ml for the experiment to analyze the hemolytic activity of peptides. The red blood cells were centrifuged, the number of red blood cells was counted and divided into wells with 10^6 cells/well, peptide was added at concentrations of 2.5, 5, 20, 30, 40 and 50 µg/mL, and incubated at room temperature for 1 hour. Then, the samples were collected and measured over a wavelength range of 410 – 600 nm on a BioTek Synergy HT.

3. Result and Discussion

3.1. Construction of the recombinant expression vector

Plasmid pET32a(+)/brevinin-1 E8.13 was transformed into *E. coli* BL21(DE3) was grown on LB agar medium supplemented with 100 µg/mL ampicillin.

Electrophoresis results showed that the PCR product had a single band with a size equivalent to the theoretically predicted size of the *brevinin-1* E8.13 gene (93 bp)

The results confirmed that the gene segment coding for the brevinin-1 E8.13 peptide was inserted in the correct orientation and reading frame, contained complete Factor Xa cleavage sites, and included a stop codon at the end of the peptide sequence (**Fig. 1**).

```
 1  D  I  G  S  I  E  G  R  F  L  G  A  L  F  K  V  A  S  K  L
 1 TGATATCGGATCCATTGAAGGTCGTTTTTTGGGAGCGTTGTTCAAGGTGGCTTCTAAATT
 1          10         20         30         40         50
 1 ACTATAGCCTAGGTAACTTCCAGCAAAAAACCCTCGCAACAAGTTCCACCGAAGATTTAA

21  V  P  A  A  I  C  S  F  S  K  K  C  *  L  E
61 AGTACCAGCAGCTATTTGTTCATTTTCTAAAAAATGTTGACTCGAG
61          70         80         90         100
61 TCATGGTCGTCGATAAACAAGTAAAAGATTTTTTACAACTGAGCTC
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Fig. 1. Sequencing results of the gene segment coding for brevinin-1 E8.13 peptide

3.2. Expression and optimization of expression conditions

The recombinant cell line was cultured in LB medium supplemented with 100 µg/ml ampicillin. The fusion protein was expressed under the following conditions: induction at an OD_{600} of 0.6–0.8, with 0.5 mM IPTG, at a culture temperature of 37°C, and sample collection 2 hours post-induction. The results are presented in **Fig. 2**.

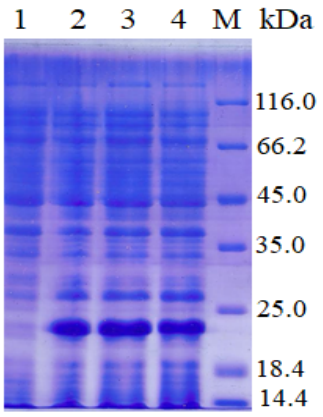

Fig. 2. Electrophoretic result of brevinin-1 E8.13 fusion protein expression level of *E. coli* BL21(DE3)/pET32a/brevinin-1 E8.13 cell line. 1: *E. coli* BL21(DE3)/pET32a/brevinin-1 E8.13 before induction; 2- 4: *E. coli*

BL21(DE3)/pET32a/brevinin-1 E8.13 after induction; M. Marker protein

After induction, a prominent new protein band appeared in the samples, which was absent in the pre-induction samples. This band is likely the fusion protein containing the target peptide. To confirm this, the cell line was cultured in 100 ml of medium, and the solubility of the expressed protein was assessed. The results indicated that the protein was in a soluble form. The fusion protein was then purified using a Ni-resin affinity chromatography column under native conditions, following the manufacturer's instructions. The purification results are shown in **Fig. 3**.

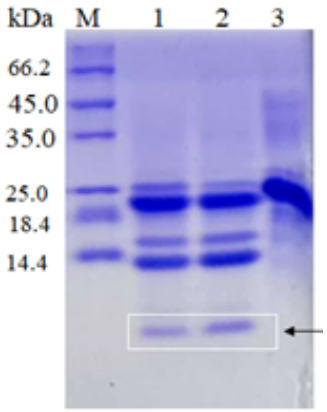

Fig. 3. Image of purified and cleaved fusion protein obtained brevinin-1 E8.13
M. Marker; 1-2. Fusion protein 8.13 cleaved with factor Xa; 3. Fusion protein 8.13 not cleaved

The predicted peptide fragment was identified as brevinin-1 E8.13. To confirm this, the sample was analyzed using mass spectrometry, and the results indicated that the expressed peptide was indeed brevinin-1 E8.13, with the sequence FLGALFKVASKLVPAAICSFSKKC (**Fig. 1**). Based on these findings, the *E. coli* BL21(DE3)/pET32a+/brevinin-1 E8.13 cell line was further utilized for large-scale fermentation to produce and collect substantial amounts of peptide 8.13 for subsequent biological activity analysis.

During the expression process, the IPTG concentration and sample collection time were the two main factors influencing the protein expression efficiency of the recombinant *E. coli* BL21(DE3)/pET32a+/brevinin-1 E8.13 strain. The statistical significance of the model was tested using Fisher's F-test, and the significance level was expressed as a p-value. The results showed the minimum and maximum ranges of the investigated variables and the full experimental design for their actual and coded values. With these variable factors, the number of experiments required to be conducted in this study was 9 experiments (**Table 1**). Each experiment was repeated three times.

The results also showed that at a concentration of 0.5 mM IPTG, after 4 – 6 hours of induction, the protein expression level was the highest at 57.84%. SDS-PAGE analysis assessed the expression levels across all nine experiments (**Fig. 4**).

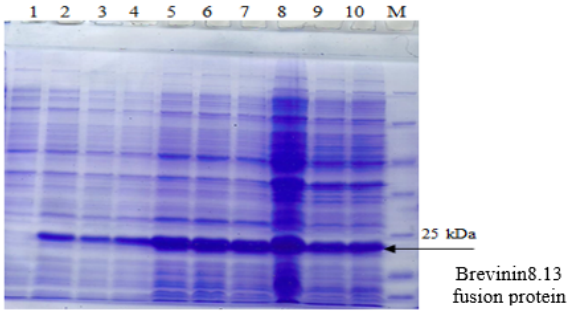

Fig. 4. Optima IPTG concentration a *E. coli* BL21(DE3.

Reconbinant brevinin-1 E8.13

M. Marker, Lane 1. Pre-induction sample; Lane 2: 0.1 mM, 4 h; Lane 3: 0.1 mM, 6 h; Lane 4: 0.1 mM, 8 h; Lane 5: 0.5 mM, 4 h; Lane 6: 0.5 mM, 6 h; Lane 7: 0.5 mM, 8 h; Lane 8: 1.0 mM, 4 h; Lane 9: 1.0 mM, 6 h; Lane 10: 1.0 mM, 8 h..

The electrophoresis results revealed a strong brevinin-1 E8.13 fusion protein band, while the uninduced sample in lane 1 showed no such band. To optimize the expression of recombinant brevinin-1 E8.13, *E. coli* BL21(DE3)/pET32a+ cells were induced with different IPTG concentrations (0.1, 0.5, and 1.0 mM) and harvested at 4, 6, and 8 hours post-induction. The expression levels of the recombinant protein were evaluated using SDS-PAGE and analyzed by Dolphin 1D software (**Fig. 5**).

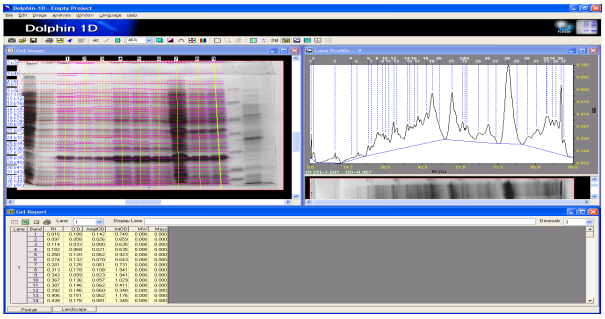

Fig. 5. SDS-PAGE analysis of recombinant Brevinin-1 E8.13 expression under different IPTG concentrations and induction durations.

E. coli BL21(DE3)/pET32a+ cells were induced with 0.1, 0.5, or 1.0 mM IPTG for 4, 6, or 8 hours. Lane 1: 0.1 mM, 4 h; Lane 2: 0.1 mM, 6 h; Lane 3: 0.1 mM, 8 h; Lane 4: 0.5 mM, 4 h; Lane 5: 0.5 mM, 6 h; Lane 6: 0.5 mM, 8 h; Lane 7: 1.0 mM, 4 h; Lane 8: 1.0 mM, 6 h; Lane 9: 1.0 mM, 8 h. The left panel shows the SDS-PAGE gel image with corresponding protein bands, and the right panel displays densitometric analysis generated by Dolphin 1D software.

These findings align with general principles outlined in Terpe (2006), which emphasize that optimal expression often occurs at moderate inducer concentrations and carefully timed induction to balance yield and host viability. Our observation that 1.0 mM IPTG resulted in reduced expression efficiency and potential metabolic stress is consistent with the reported drawbacks of overexpression systems described by Terpe, including formation of inclusion bodies and impaired host cell growth (Terpe 2006).

Furthermore, a more specific comparison can be made with the study by Aleinein et al. (2013), who cloned and expressed Ranalexin, another antimicrobial peptide from Rana catesbeiana, in *E. coli* BL21(DE3). Similar to our findings, they found that expression efficiency was strongly dependent on IPTG concentration and induction time. However, while they used 0.4 mM IPTG for 5 hours, our results suggest that a slightly higher IPTG concentration (0.5 mM) and longer induction time (6–8 hours) was more effective for brevinin-1 E8.13. This variation may reflect differences in gene sequence, codon usage, peptide hydrophobicity, or protein folding characteristics between ranalexin and brevinin-1 E8.13 (Aleinein et al. 2013).

Similarly, Zhou et al. (2009) isolated Brevinin-2 from the skin of *Hylarana guntheri* and cloned it into the pET32a(+) vector for expression as a Trx-fusion protein in *E. coli*. Their results showed that the fusion protein accounted for up to 45% of the total cellular protein when induced with 0.5 mM IPTG for 4.5 hours. These findings are largely consistent with those observed in our study (Zhou, Li, and Li 2009a).

In summary, our findings reinforce and extend the insights from previous studies, confirming that fine-tuning IPTG concentration and induction time is crucial for optimal expression of antimicrobial peptides in *E. coli*, and that system-specific factors such as vector design and peptide sequence must be empirically optimized.

To evaluate the impact of induction condition optimization, SDS-PAGE was performed to compare the expression levels of brevinin-1 E8.13 fusion protein before and after applying the optimal IPTG concentration and induction duration (**Fig. 6**).

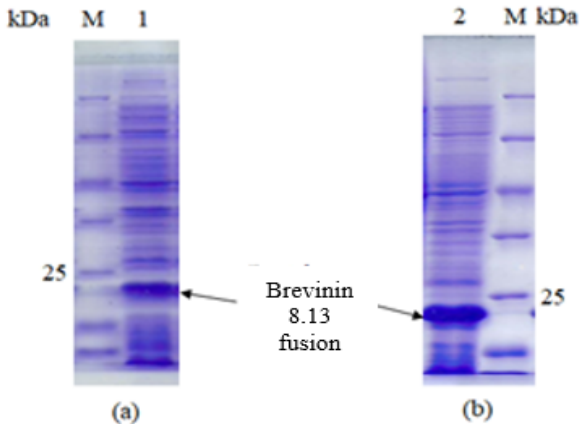

Fig. 6. Brevinin-1 E8.13 fusion protein before (a) and after (b) optimizing conditions of *E. coli* BL21(DE3)/pET32a+/brevinin-1 E8.13.

M. Marker (Thermo); 1. Sample before optimizing conditions; 2. Sample after optimizing conditions

As shown in **Fig. 6**, lane 1 (a) represents the crude protein extract before optimization, where a faint band corresponding to the Brevinin-1 E8.13 fusion protein (~25 kDa) is visible, indicating low expression levels. In contrast, lane 2 (b) displays the sample obtained under optimized conditions (0.5 mM IPTG, 6–8 h induction), showing a significantly more intense band at the same molecular weight, confirming enhanced expression.

This improvement in band intensity highlights the effectiveness of condition optimization in increasing the yield of the target fusion protein. These results are consistent with earlier densitometric analysis (**Fig. 5**), validating the selection of 0.5 mM IPTG and 6–8 h induction as optimal parameters for high-level expression of brevinin-1 E8.13 in *E. coli* BL21(DE3).

3.3. Recombinant peptide purification

Purification was performed under Invitrogen's native conditions. The brevinin-1 E8.13 fusion protein was initially purified on a small scale using 2 ml of nickel resin. The binding buffer was optimized per the manufacturer's instructions, with the addition of 10 mM imidazole to minimize non-specific binding. The wash solution contained 30 mM imidazole, while the elution

solution used 250 mM imidazole. These buffer conditions were later scaled up for larger purifications using 20 ml of nickel resin and a chromatographic column across five columns (**Fig. 7**).

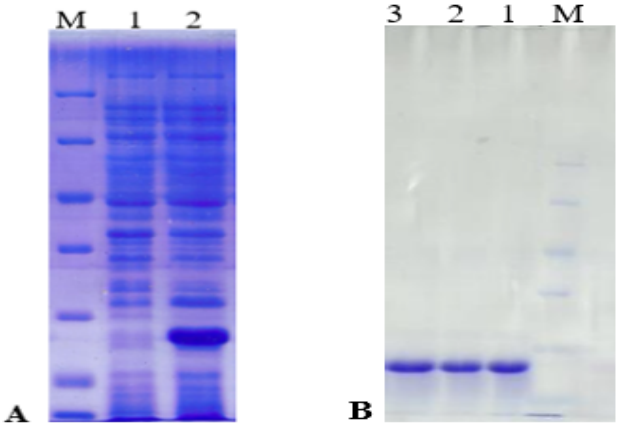

Fig. 7. Recombinant protein products before and after washing on Ni^{2+} column

A: Expressed protein. M: Marker, Lane 1: *E. coli* BL21/pET32a+, Lane 2: *E. coli* BL21(DE3)/pET32a+/brevinin-1 E8.13

B: Brevinin-1 E8.13 purification. M. Marker, Lane 1 – 3: Wash with 30 mM imidazole

The expression of recombinant brevinin-1 E8.13 was confirmed by SDS-PAGE analysis (**Fig. 7A**). A distinct band corresponding to approximately 25 kDa was observed in *E. coli* BL21(DE3) cells harboring the pET32a(+)/brevinin-1 E8.13 plasmid (Lane 2), while no such band was present in the control strain transformed with the empty pET32a(+) vector (Lane 1). This indicates that the brevinin-1 E8.13 fusion protein was successfully expressed in the host cells under the optimized induction conditions.

To purify the recombinant protein, Ni^{2+} affinity chromatography was applied, and eluted fractions using 30 mM imidazole were analyzed (**Fig. 7B**, Lanes 1–3). Electrophoresis results showed a distinct protein band at approximately 24.7 kDa in the purified fractions, confirming the successful purification of the fusion protein containing the peptide. Post-purification analysis on a polyacrylamide gel revealed that, compared to the pre-purification sample, the purified samples exhibited a single prominent band corresponding to the expected molecular weight of brevinin-1 E8.13. All collected fractions displayed high purity, with no detectable contamination from other proteins. These results confirm that the recombinant protein was purified with high efficiency and specificity using Ni^{2+} affinity chromatography.

Together, the data demonstrate both effective expression of brevinin-1 E8.13 in *E. coli* and successful downstream purification, making this approach suitable for further functional or structural characterization of the peptide.

3.4. Evaluating the efficiency of brevinin-1 E8.13 protein purification

Purification efficiency was calculated by determining the protein content before and after purification. The expression level of the brevinin-1 E8.13 fusion protein was 57.84%, yielding 30.2 mg of fusion protein per liter of fermentation medium. For a purification batch using 20 ml of nickel resin across five columns, a total of 5 liters of fermentation medium was processed, resulting in 151 mg of protein. The protein content after purification was measured using the Bradford method.

The concentration of the brevinin-1 E8.13 fusion protein obtained after purification was 0.64 mg/ml, resulting in a total yield of 96 mg per purification batch. Therefore, the purification efficiency of the brevinin-1 E8.13 fusion protein was calculated as:

Purification efficiency = (96:151) x 100% = 63%

Zhou et al. (2009) successfully expressed brevinin-2GU in E. coli. The recombinant brevinin-2GU, after purification and enzymatic processing, demonstrated the ability to stimulate insulin release from pancreatic β-cells (Zhou, Li, and Li 2009b). However, its expression yield, accounting for approximately 45% of total cellular protein, was lower compared to brevinin-1 E8.13 in this study.

Currently, there is still limited research focusing on the recombinant expression of brevinin peptides, as well as their in vitro efficacy against tumors.

3.5. Recombinant peptide release and purification by HPLC

Isolate single peptides from the fusion protein, the protein was dialyzed using a membrane in a factor Xa cleavage buffer. The cleavage reaction was optimized on a small scale with a reaction volume of 3–5 ml, as detailed in Section 3.4. The optimized conditions included a factor Xa concentration of 10 U per mg of protein, a protein concentration of 1.2 mg/ml, an incubation time of 16–20 hours, and a temperature of 22–25°C. For larger-scale processing, the reaction volume was increased to 50–60 ml per tube per cut. The results, as shown in **Fig. 8**,

indicate that runs 11 and 12 of the fusion protein were cleaved effectively, producing distinct peptide bands. However, the cleavage efficiency did not reach 100%.

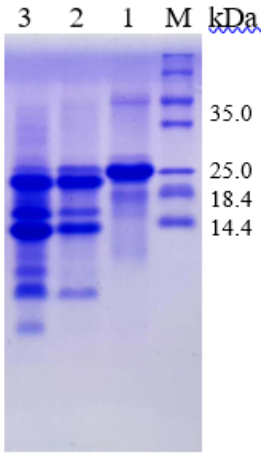

Fig. 8. Electrophoretic images of peptides excised from fusion proteins
M: Marker; Lane 1: Sample 8.13 before cutting with Factor Xa; Lane 2: Sample 8.13 cut with Factor Xa 2 Lane 3: Sample 8.13 cut with Factor Xa 1

Fig. 8 shows that the cleaved samples contain peptide bands ranging from 2–3 kDa, consistent with the calculated size of brevinin-1 E8.13 (2.64 kDa). These results confirm the successful expression and isolation of the brevinin-1 E8.13 peptide. To further purify the desired peptide fragment, the cleavage mixture was passed through a fresh nickel-resin column. The collected solution was stored at -20°C and subsequently freeze-dried for preservation. The purified peptide solution was analyzed by polyacrylamide gel electrophoresis, with the results presented in **Fig. 9**.

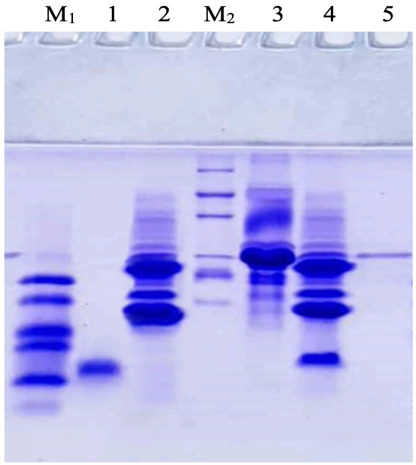

Fig. 9. Electrophoresis image of brevinin-1 E8.13 peptide cut from fusion proteins and removing other proteins using Ni-resin column
M1: Marker peptide; Lane 1: recombinant brevinin-1 E8.13 peptide; Lane 2: Sample bound to Nickel-resin column; M2: Marker protein; Lane 3: Uncut sample, Lane 4: Fusion protein sample cut with factor Xa; Lane 5: Small amount of uncut fusion protein sample.

The results showed that after cleavage and transfer to the nickel column, other protein bands, including factor Xa (a recombinant protein with a His-tag), were retained on the column. This allowed the isolation of a relatively pure brevinin-1 E8.13 protein band. HPLC analysis demonstrated that recombinant brevinin-1 E8.13 reached a purity of 90.092%, making it appropriate for further investigation.

3.6. Evaluation of brevinin-1 E8.13 activity on lung tumors and cytotoxicity

Yu et al. (2021) evaluated the cytotoxicity of brevinin-1RL1 on six human cancer cell lines and three non-cancer cell lines. They found that brevinin-1RL1 exhibited a dose-dependent inhibitory effect on cancer cells across various lines (HCT116, MDA-MB-231, SW480, A549, SMMC-7721, and B16-F10), with IC50 values ranging from 5 to 10 μM. Moreover, brevinin-1RL1 demonstrated lower toxicity towards non-cancer cell lines, including NCM460, BEAS-2B, and HaCaT, with the highest IC50 observed in HaCaT cells (28.67 μM) (Ju et al. 2021). Several studies have demonstrated that certain brevinin-1 analogues, such as brevinin-1CEa, exhibit anti-cancer effects by inhibiting the growth of MCF-7 and HeLa cells (Yu et al. 2009). Additionally, researchers have investigated the anti-cancer properties of brevinin-1EMa derivatives across seven tumor cell lines, including A498, A549, HCT116, MKN45, PC-3, SK-MEL-2, and SK-OV-3 (Kang, Ji, and Lee 2012).

After being obtained, the recombinant peptide brevinin-1 E8.13 was tested for its anti-cancer activity on cultured cell lines, and the results demonstrated strong activity, even at larger scales. To explore its potential further, the peptide was then tested in an experimental tumor model. Due to limited funding and the impact of COVID-19, the experiment was conducted using a normal mouse model. Although inducing tumors in a normal mouse model is challenging due to the strong immune system, which complicates the evaluation of drug efficacy, the results were promising. After one month of treatment, the tumor mass in mice treated with the peptide was significantly reduced, indicating the peptide's potential for further development (**Fig. 11** and **Fig. 12**).

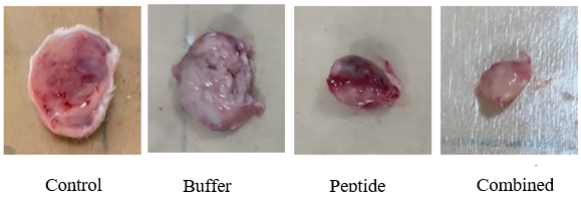

Fig. 11. Images of some mouse tumors after treatment with brevinin-1 E8.13 peptide after one month

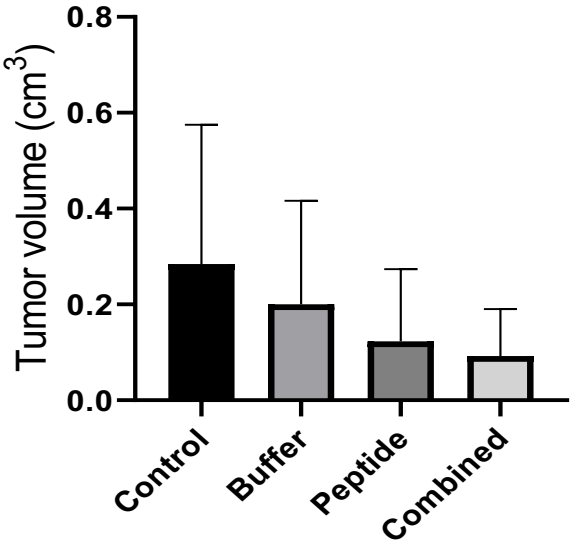

Fig. 12. The graph summarizes the results of average tumor volume measurements in different experimental and control groups

Treatment with the buffer, peptide, or their combination demonstrated an inhibitory effect on lung cancer tumor growth in mice, with significant tumor size reduction observed in the groups treated with the peptide or its combination. In the control group, 75% of the mice showed no reduction in tumor size, while 25% experienced a slight decrease. In contrast, the peptide-treated group achieved much better results, with 75% of the mice showing a clear reduction in tumor size. Of these, 62.5% exhibited a gradual decrease over time, and 12.5% showed a substantial reduction followed by partial recovery, though the tumor size remained smaller than the original size before treatment (**Fig. 13**) and the weight of the mice was not reduced (**Fig. 14**).

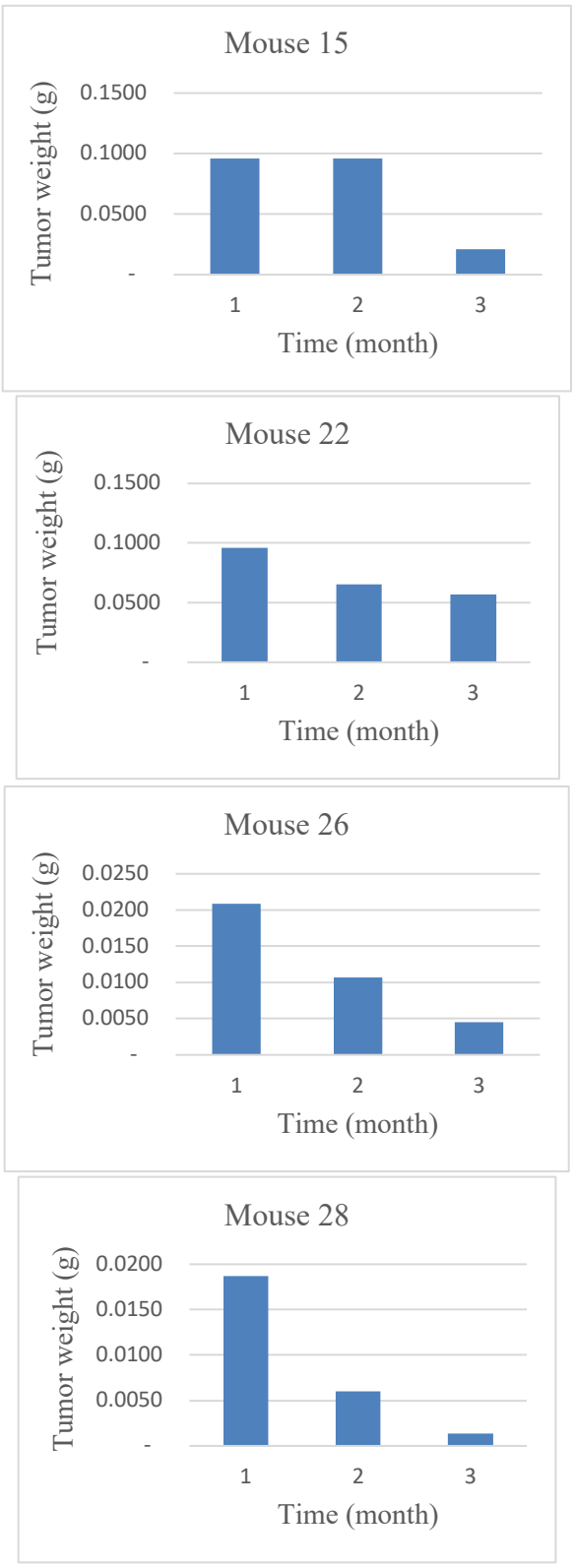

Fig. 13. Tumor weight results of mice in the experimental group using peptide

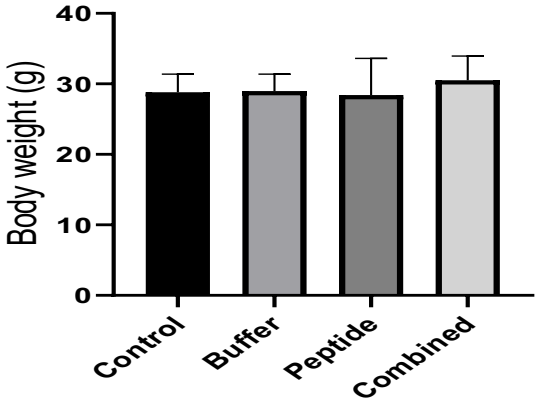

Fig. 14. Results of measuring the average body weight of mice in different experimental and control groups

Treatment with the buffer, peptide, or their combination did not affect the animal's weight throughout the treatment period. This indicates that the doses used in this study were safe and exhibited minimal to no toxicity in the experimental animals. In 2011, Hossain et al. reported the anticancer activity of modified brevinin, such as dicarba-brevinin-1BYa, which showed enhanced cytotoxicity against human erythroleukemia cells, MDA-MB-231, and HepG2 following treatment (Hossain et al. 2011).

Evaluation of blood parameters in mice showed that treatment with the buffer, peptide, or their combination had no significant effect on the blood albumin levels of the experimental animals, indicating that liver albumin synthesis remained unaffected across groups. Additionally, while there was a slight increase in blood cholesterol levels in the treatment groups, this increase was not statistically significant (**Fig. 15**).

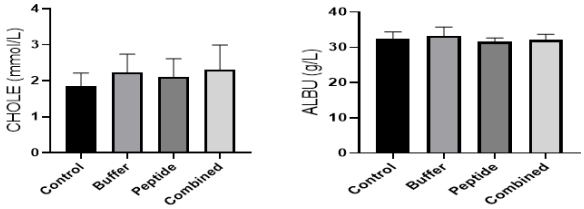

Fig. 15. Results of blood parameters measurements of mice in different experimental and control groups

To further ensure the safety of the treatment, the research team evaluated its effects on several indicators of liver function. The results of liver enzyme tests showed no significant changes between the experimental animal groups, indicating that the treatments using the buffer, peptide, or their combination did not cause liver cell damage or affect liver function in the animals (**Fig. 16**).

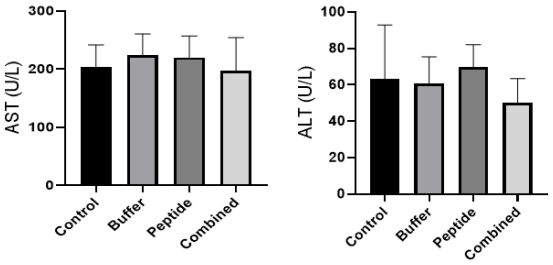

Fig. 16. Results of liver function indices in different experimental and control groups of mice

The derivative Brevinin-1BYa-3, in which the disulfide bond is replaced by a thioether bridge, has been reported to exhibit anti-tumor activity against human prostate cancer (C4-2B), non-small cell lung cancer (A549), and liver cancer (HuH-7) cell lines. This derivative demonstrates greater stability in a reductive environment, a more stable secondary structure, enhanced resistance to protease degradation, and superior anti-tumor activity compared to the original peptide. However, due to the similarity between cancer cell membranes and normal cell membranes particularly in cells with a high content of anionic phospholipids – the peptide may exhibit cytotoxicity toward both cell types (Xiong et al. 2021). Lin et al. (2021) reported three peptides derived from the skin secretion of *Hylarana latouchii*, brevinin-1HL, temporin-HLa, and temporin-HLb, exhibiting potential antimicrobial and anticancer activities. However, these peptides may be cytotoxic to both cancer and normal cells due to their non-specific mechanism of action, primarily through membrane disruption. This lack of selectivity could lead to undesirable side effects, limiting their clinical applications (Lin et al. 2021).

Similarly, peptides such as Caerin 1.1, derived from the skin of Litoria frogs, exhibit strong inhibitory effects on HeLa cervical cancer cells at low concentrations. Other peptides, including Brevinin, Mastoparan-C (MP-C), Tryptophyllin L, Bombesin, and Luteinizing Hormone-Releasing Hormone (LHRH), have also shown potential for cancer therapy due to their targeted mechanisms and reduced side effects compared to conventional treatments (Chen et al. 2023).The anticancer activity of Brevinin-2R has been demonstrated through its ability to inhibit cell proliferation, induce apoptosis, and disrupt the G2/M phase of the cell cycle in K562 leukemia cells (Asoodeh et al. 2013). Similarly, Brevinin-1H, isolated from the skin secretion of Amolops hainanensis, and its analogues have shown promise for

both antimicrobial and anticancer applications (Pei et al. 2021).

While all these peptides exhibit potential in antimicrobial and anticancer therapies, their cytotoxicity toward normal cells remains a major concern for clinical safety. However, our experiment demonstrates that Brevinin-1 E8.13 not only effectively inhibits tumor growth but also shows safety in normal cells, highlighting its potential as a promising peptide for further development.

Creatinine is an indicator of kidney function, with elevated levels potentially indicating impaired kidney function. The Buffer, Peptide, and Combined groups had slightly lower CREA levels compared to the Control group, but the differences among the experimental groups were not significant (**Fig. 17**).

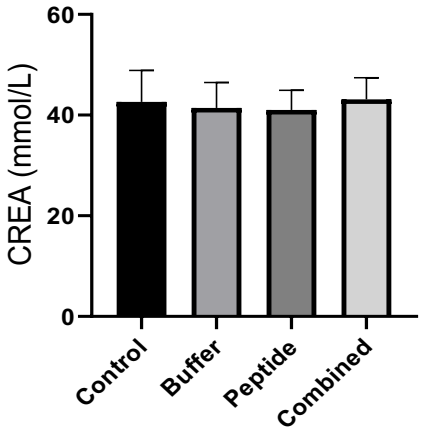

Fig. 17. Crea index results in relation to kidney function in different experimental and control groups of mice

The creatinine test results showed minimal variation between the experimental animal groups, suggesting that treatments with the buffer, peptide, or their combination did not affect renal function in the animals. In conclusion, recombinant peptide 8.13 demonstrated strong antitumor activity in animal models. After treatment with 6 doses of 10 µg/g/dose body weight, the mice exhibited a clear reduction in tumor size, with no changes in body weight and normal liver and kidney function.

Evaluating blood indices in mice treated with the peptide showed an increase in lymphocyte count, while monocyte and granulocyte counts decreased (**Fig. 18**).

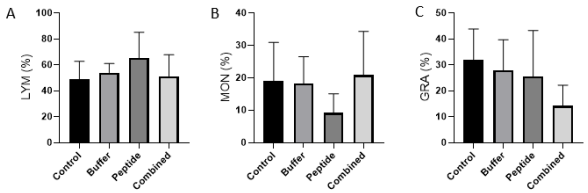

Fig. 18. Blood white blood cell count test results

This suggests that peptide treatment may enhance cell-mediated immune responses through T lymphocytes. By the end of the experiment, the tumors in the treatment group were also observed to be smaller on average compared to the control group. In this cancer model, mice still retained an immune response to the tumor, meaning that larger tumors could potentially trigger a stronger immune reaction.

Additionally, the red blood cell (RBC T/L), hemoglobin (HGB g/L), and hematocrit (HCT %) levels were largely unaffected (**Fig. 19**).

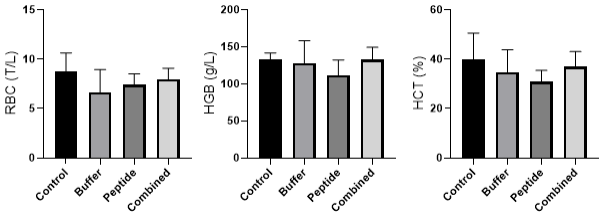

Fig. 19. Blood test results

RBC and HGB levels in the peptide-treated and combination groups showed minimal changes compared to the control group. Although HCT levels showed a slight decrease, the difference was not significant and remained within the margin of error. This suggests that the peptide brevinin-1 E8.13 has potential for therapeutic use without causing severe side effects on the hematopoietic system.

However, platelet count showed a decreasing trend (**Fig. 20A**), while blood glucose levels increased during the examination (**Fig. 20B**).

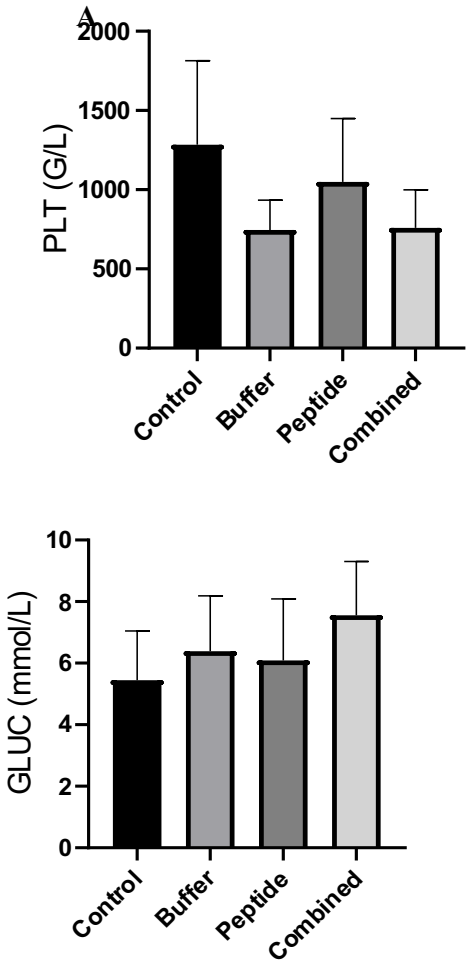

Fig. 20. Blood platelet test results

Treatment with buffer and the combined method showed a decreasing trend in red blood cell count, possibly due to a component in the buffer affecting platelet formation and function. It may be worth considering reducing the concentration of buffer and combined treatment in future experiments. Blood glucose levels in the buffer, peptide, and combined groups also showed an increasing trend; however, this increase was not significant. In this experiment, blood glucose was measured in mice without an 8-hour fasting period prior to blood collection.

4. Conclusion

This study successfully established an effective bacterial expression system for the recombinant production of brevinin-1 E8.13. The use of a Trx-His fusion system in *E. coli* BL21(DE3), coupled with Ni^{2+} affinity chromatography and optimized induction conditions, resulted in high-yield and high-purity production of the peptide. Functional analyses demonstrated that the recombinant peptide possesses significant antitumor activity in a lung cancer mouse model, while maintaining a favorable safety profile, including minimal cytotoxicity to normal tissues and no adverse effects on organ function. These findings provide a solid foundation for further development of brevinin-1 E8.13 as a therapeutic agent in cancer treatment, and suggest that recombinant AMP production via microbial systems is a viable strategy for drug development.

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Conflict of Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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