Erzincan Üniversitesi
Fen Bilimleri Enstitüsü Dergisi
2024, 17(2), 505-522
ISSN: 1307-9085, e-ISSN: 2149-4584
Araştırma Makalesi

Erzincan University
Journal of Science and Technology
2024, 17(2), 505-522
DOI: 10.18185/erzifbed.1500259
Research Article

Enhanced Production and Therapeutic Evaluation of TP4 Peptide in *Pichia pastoris* Against Hepatocellular Carcinoma HUH-7 Cells

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Received: 12/08/2024, **Revised:** 08/08/2024, **Accepted:** 13/08/2024, **Published:** 31/08/2024

Abstract

Tilapia piscidin 4 (TP4), a cationic antimicrobial peptide, is recognized for its diverse biological roles, including antibacterial, wound-healing, and anticancer properties. Herein, the codon-optimized sequence of TP4 peptide was expressed using the pPICZαA expression vector containing the *AOX*1 promoter, a strong and inducible promoter, in the *Pichia pastoris* KM71H expression system. Recombinant TP4 peptide was purified by Ni-NTA affinity chromatography. After purification, the anticancer activity of TP4 was assessed in HUH-7 hepatocellular carcinoma cells, and the underlying mechanisms were determined. In the present study, it was demonstrated for the first time that recombinant TP4 displayed strong anticancer activity in the human HUH-7 cell line. The TP4 antimicrobial peptide can be used as a competitive candidate for the treatment of cancer cells due to its anticancer effects.

Keywords: Tilapia piscidin 4, recombinant peptide, *Pichia pastoris*, HUH-7

Pichia pastoris'te TP4 Peptidinin Artırılmış Üretimi ve Hepatoselüler Karsinom HUH-7 Hücrelerine Karşı Terapötik Değerlendirmesi

Öz

Bir katyonik antimikrobiyal peptiti olan Tilapia piscidin 4 (TP4), anti-bakteriyel, yara iyileştirici ve antikanser özellikleri de dahil olmak üzere çeşitli biyolojik rolüyle tanınmaktadır. Burada, TP4 peptitinin kodon optimizasyonlu dizisi, *Pichia pastoris* KM71H ekspresyon sisteminde güçlü ve indüklenebilir bir promotor olan *AOX1* promotoru altında pPICZαA ekspresyon vektörü kullanılarak eksprese edilmiştir. Rekombinant TP4 peptiti, Ni-NTA afinite kromatografisi ile saflaştırılmıştır. TP4'ün saflaştırılmasından sonra TP4'ün antikanser aktivitesi hepatosellüler karsinoma hücrelerinde değerlendirilmiş ve altta yatan mekanizmalar belirlenmiştir. Bu çalışmada ilk kez rekombinant TP4'ün insan HUH-7 hücre hattında güçlü antikanser aktivite gösterdiği belirlenmiştir. TP4 antimikrobiyal peptiti, antikanser etkileri nedeniyle kanser hücrelerinin tedavisinde rekabetçi bir aday olarak kullanılabilir.

Anahtar Kelimeler: Tilapia piscidin 4, rekombinant peptit, *Pichia pastoris*, HUH-7

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1. Introduction

Hepatocellular carcinoma (HCC) is the most prevalent type of primary liver cancer. According to World Health Organization statistics in 2022, HCC is the sixth most common type of cancer worldwide. It is also the third most common cause of cancer death after lung and colorectal cancer [1]. Current treatments for cancers in general include radiation, chemotherapy, immunotherapy, and surgical methods. However, limited selectivity in targeting tumor cells and the emergence of resistance in tumor cells are challenges of such treatments. Therefore, new therapeutic methods are necessary [2].

Cancer cells often have negatively charged phosphatidylserine (PS) or anionic structures in their outer membranes, unlike healthy cells that are normally zwitterionic. Among therapeutic peptides, antimicrobial/pore-forming peptides (AMPs) attract attention due to their anticancer effects. AMPs occur naturally in all living organisms and are part of the immune defense mechanism. AMPs have cationic charges and form amphipathic structures in nonpolar solvents. Antimicrobial cationic peptides target cancer cell membranes and induce cell death through necrosis or apoptosis [3-5].

Tilapia piscidin 4 (TP4), a marine antimicrobial peptide and a cationic antimicrobial peptide identified from Nile Tilapia (*Oreochromis niloticus*), is known to exhibit multiple biological functions, including antibacterial, wound-healing, immunomodulatory, and anticancer activities [6-8]. Furthermore, TP4 has attracted attention for its potent anticancer activity in glioblastoma cells, human non-small cell lung cancer cells, and triple-negative breast cancer cells [9,10].

Although AMPs have many features such as scientific research and industrial use, the main factors that limit studies on AMPs are that obtaining AMPs is time-consuming and quite costly. There are three main production methods for obtaining AMPs: direct AMP extraction from natural sources, chemical synthesis, and recombinant techniques. The recombinant method is preferred over chemical synthesis and extraction in many aspects. For example, it is more cost-effective and provides an endless source of peptide production for both research and large-scale production for pharmaceutical use [11-13]. *Pichia pastoris*, a methylotrophic yeast, has recently become the most frequently used expression system for eukaryotic protein production. This is because *P. pastoris* can make post-translational modifications such as correct polypeptide folding, disulfide bond formation, and glycosylation, which are very important for eukaryotic proteins and which bacteria cannot do [14,15].

TP4 peptide, which is the subject of this study, was first produced recombinantly in *P. pastoris* in a study conducted by Neshani et al. (2018). In addition, in studies conducted by Huang et al. (2020) and Tai et al. (2021), TP4 peptide was produced recombinantly in *P. pastoris* and was aimed to be used for different purposes by taking advantage of the high antimicrobial effect of TP4 [16,17].

Recent studies have increasingly focused on the anticancer effects of the TP4 peptide. Su et al. (2019) on glioblastoma cells (U87MG, U251) determined that TP4 increased reactive oxygen

species, decreased ATP, and significantly increased the release of cyclophilin A, a necrotic biomarker [10]. Su et al. (2021) also showed that TP4 can cause necrotic cell death in human synovial sarcoma AsKa-SS and SW982 cell lines [18]. In another study, it was reported that the toxic effect of TP4 on non-small cell lung cancer cells primarily led to necrosis. Additionally, while cell membrane disruption was observed at high concentrations of TP4, it was determined that it resulted in controlled cell death at low concentrations [9]. Furthermore, the cytotoxicity of TP4 was also tested in triple-negative breast cancer (TNBC) cell lines under both in vitro and in vivo conditions. TNBC cells have been shown to activate the FBJ murine osteosarcoma viral oncogene homolog B (*FOSB*), which is counteracted by *TP4* through selective binding to mitochondria and induction of cytosolic Ca^{2+} accumulation. *FOSB* overexpression results in TNBC cell death, whereas inhibition of calcium signaling abrogates *FOSB* induction and blocks TP4-induced TNBC cell death. In conclusion, this study showed that *TP4* strongly induces *FOSB*, especially in TNBC, providing a new therapeutic approach for TNBC through *FOSB* induction [19,20].

Synthetic TP4 peptide was used in studies investigating the anticancer effect of TP4. However, there is no study yet to examine the anticancer effect of recombinant TP4 produced. Additionally, within the scope of this study, TP4 peptide was studied for the first time in HUH-7 hepatocellular carcinoma cells. This study aimed to recombinantly produce the TP4 peptide in the *P. pastoris* expression system. Additionally, the study evaluated the therapeutic potential and anticancer activity of the produced TP4 on HUH-7 hepatocellular carcinoma cells.

2. Material and Methods

Strains and Chemicals

Escherichia coli XL1-Blue strain was used for subcloning and amplification of expression vectors. For the development of *E. coli* cells, LB medium (0.5% yeast extract, 1% peptone and 1% NaCl) was prepared with appropriate antibiotic additions. After cloning, zeocin was added to the LB agar medium to select positive *E. coli* XL1-Blue cell transformants. *P. pastoris* KM71H strain was used as the host system for protein expression. YPD (1% yeast extract, 2% peptone, 2% dextrose) medium was used as preculture to grow transformant *P. pastoris* cells. BMGY (2% glycerol, 1% yeast extract, 2% peptone, 1.34% yeast nitrogen base w/o amino acids, 4×10^{-5} % biotin and 100 mM phosphate buffer, pH 6), BMMY (contained 1% methanol instead of glycerol in BMGY) culture media were used for protein expression studies.

HUH-7 (human hepatocellular carcinoma cell line) was obtained from ATCC (American Type Culture Collection, USA). Phosphate buffer saline (PBS), Heat-inactivated fetal bovine serum, Trypsin–EDTA, Dulbecco's modified Eagle's medium (DMEM), L-glutamine, penicillin-streptomycin and MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) were purchased from Biological Industries (Biological Industries Co, Beth-Haemek, Israel). Qiagen RNeasy Mini Kit (Qiagen, Hilden, Germany) was used to isolate RNA, and Biorad iScript™ cDNA Synthesis Kit (Bio-Rad Laboratories Inc., Hercules, CA, USA) was used to synthesize cDNA. Human Bax, Bcl-2, Casp3 ELISA kits were from Elabscience Company (Elabscience

Biotechnology, Wuhan, Chine). All other chemicals were purchased from Sigma Aldrich (Merck KGaA, Darmstadt, Germany).

HUH-7 cell line was cultured in DMEM supplemented with 10 % fetal bovine serum, 1% pen-strep solution, 1% L-Glutamine at 37 °C in a 5% CO_2 incubator and 95% humidity.

2.1. Recombinant Production of TP4

Gene Synthesis and Plasmid Construction

The nucleic acid sequence encoding the TP4 peptide was obtained from NCBI GenBank (accession number XM_002491337). After the DNA sequence encoding the TP4 peptide was determined, restriction enzyme cutting sites, stop codon, G-TEV Protease region and C-terminal 6x His sequence (His-tag, for purification of the peptide) was added to the ends of the sequence. For effective protein production, codon optimization according to the characteristics of host system is very important [21]. Codon optimization of the obtained nucleotide acid sequence was performed according to the *P. pastoris* codon usage frequency table by Genscript (NJ, USA). To obtain the *TP4* gene sequence, 6 primers (Table 1) that were partially complementary to each other were ordered by Sentebiolab Company (Ankara, Türkiye), and these primers were combined by the overlap PCR method, respectively, and a 141 bp length DNA sequence was obtained. PCR products of the 141 bp DNA sequence encoding the TP4 peptide were purified using a commercial purification kit (Hydra, Gel and PCR Purification Kit, Türkiye). TP4 DNA sequence: 5-CTC GAG CAT CAC CAC CAT CAT CAC GAG AAC CTA TAC TTT CAA GGG TTT ATT CAC CAT ATT ATA GGC GGT CTC TTC AGC GCC GGA AAA GCG ATC CAT AGA CTG ATC CGA CGC CGG AGG CGT TGA TCT AGA-3.

Table 1. Overlap primers for synthesis to *TP4* gene.

Primer	Sequence
TP4F1	AAACTCGAGAAAAGACATCACCACCATCATCACG
TP4R1	CCCTTGAAAGTATAGGTTCTCGTGATGATGGTG
TP4F2	ACTTTCAAGGGTTTATTCACCATATTATAGGCGG
TP4R2	CGCTTTTCCGGCGCTGAAGAGACCGCCTATAATA
TP4F3	CCGGAAAAGCGATCCATAGACTGATCCGACGCCG
TP4R3	TTTTCTAGATCAACGCCTCCGGCGTCGGAT

TP4 gene was cloned into the pPICZαA vectors from *XhoI-XbaI* restriction sites to construct pPICZαA-TP4 expression vector. The schematic representation of the vector was given in Fig. 1.

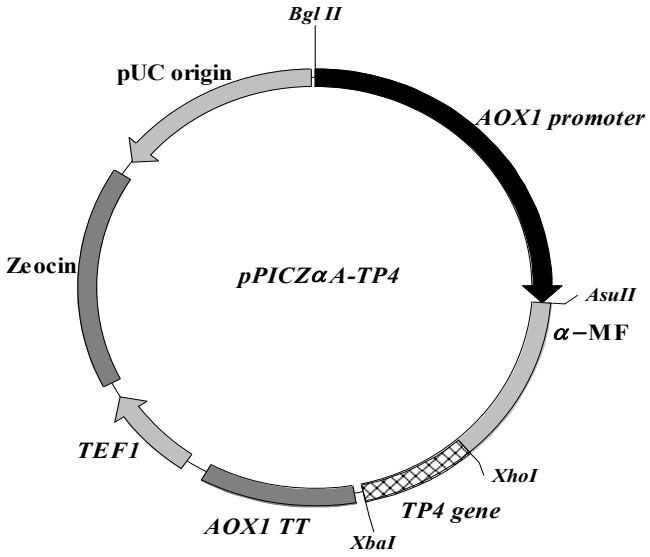

Figure 1. Schematic representation of the constructed expression vector pPICZαA-TP4

Cloning of the *TP4* gene in *P. pastoris*

The expression vector was linearized by digestion with restriction enzymes *SacI* at the *AOX1* promoter site. The expression cassette obtained was transformed into the electrocompetent *P. pastoris* KM71H strain by electroporation using an electroporator (Bio-rad, Gene Pulser Xcell Electroporation System) at 2000 V, 5 ms [22]. The selection of transformant cells was performed on YPD agar plates containing 100 µg/mL zeocin.

Recombinant TP4 Production in Shake-flasks

The recombinant protein production studies were carried out in shake-flask conditions. Selected clone was cultured in 5 mL of YPD medium overnight. The culture was used as inoculum for BMGY medium and incubated for 18 hours for cell accumulation. The cells were harvested by centrifugation at 1200 ×g and transferred to BMMY medium, which is the protein production medium. TP4 production was induced by adding methanol to a final concentration of 1% every 24 hours. The induction phase was continued at 28°C for 216 hours.

Purification of the Recombinant TP4

In this study, the recombinant protein was synthesized with a polyhistidine (6xHis) tag attached to the C-terminus. The use of a histidine tag, particularly the 6×His tag (0.84 kDa), is advantageous due to its small size and lack of charge at physiological pH, which typically does not interfere with the folding, structure, or function of the protein [24]. Moreover, the purification method employed for histidine-tagged proteins relies on immobilized metal affinity chromatography (IMAC), offering efficient purification with high yields. In this technique, the protein of interest selectively binds to transition metal ions (nickel, in this instance) immobilized on a resin matrix via the histidine tag, effectively separating it from other unlabeled proteins in the supernatant. Subsequently, the recombinant protein is eluted from the resin using imidazole, which competes with the histidine tag for binding sites [25].

The produced 6×His labeled TP4 peptide was purified using Ni-NTA resin. The resin was washed with 1×PBS pH 7.4 containing 20 mM imidazole, and then the TP4 peptide attached to the resin was treated 3 times with 1×PBS pH 7.4 elution buffer containing different concentrations of imidazole (50, 100 and 200 mM) [26]. Protein samples in different fractions of the purification process were analyzed by SDS-PAGE. The total protein amount was determined using the Coomassie Bradford Plus Protein Assay Kit according to the kit protocol.

SDS-PAGE Analysis

For the analysis of protein samples taken from the purification steps analyzed by the tricin SDS-PAGE protocol described by Schägger (2006) [23]. The samples were mixed with 4×SDS gel loading buffer in a 20 µL and incubated at 70°C for 10 min. denatured. Then, peptides were separated by electrophoresis in the SDS-PAGE gel, which consisted of two phases: a loading gel containing 4% polyacrylamide and a separation gel containing 16% polyacrylamide.

Electrophoresis was performed in Anode (0.1 M Tris, 0.0225 M HCL pH 8.9) and Cathode Buffer (0.1 M Tris, 0.1 M Tricine, 0.1% SDS, pH 8.25) buffer at 80V for 30 min. and 120V 100 min. was carried out. Then, the gel was stained with Coomassie Brilliant Blue dye and then treated with a decolorizing solution (10% acetic acid, 50% methanol and 40% pure water) to remove the dye, and the resulting SDS-PAGE gel was imaged on ChemiDoc Touch Imaging System (Bio-Rad Laboratories Inc., Hercules, CA, USA).

Antimicrobial Activity

The minimum inhibitory concentration test (MIC) and the minimum bactericidal concentration test (MBC) are important methods to assess the antimicrobial activity levels of the recombinant TP4. MIC and MBC values were determined on *Escherichia coli* (gram-negative) and *Bacillus subtilis*-ATCC 23857 (gram-positive).

The bacteria were activated through batch cultivation in shake-flasks containing sterile Tryptic Soy Broth (TSB) with at 37 °C 200 rpm. Subsequently, 100 µL of the culture was inoculated onto tryptone soy agar (TSA) plates and incubated for 24 hours at 37 °C. Then, a single colony was selected and inoculated into shake-flasks containing sterile TSB, and incubated for 12 hours at 37 °C.

To determine MIC and MBC, Microtitre Broth Dilution Method was used. Seven decreasing serial dilutions of TP4 peptide (50-25-12.5-6.25-3.125-1.56-0.78 µg/mL) applied two bacteria on 96-well plates with 5 replicates. Following incubation at 37 °C for varying durations (6-12-18-24 hours), bacterial growth was assessed by measuring absorbance at 600 nm using a microplate reader. As a negative control, bacterial cultures without TP4 were included, while serial dilutions of 0.01 mg/mL kanamycin used as positive control. Starter culture was prepared in 5 mL TSB and incubated for 18 hours at 37 °C, then the starter culture was inoculated at a ratio of 1/100 into the flask, it was incubated at 37 °C in a shaker at 200 rpm until Optic Density was 0.5. 150 µl of this culture was transferred to each well of the 96-well plate under aseptic

conditions. Following incubation, viable cell counts were calculated based on absorbance values at 600 nm.

2.2. Cell Culture

Cell Viability Assay

The anticancer effects of TP4 against growth of cells was determined using the MTT assay. Firstly, the cancer cells were seeded in 96-well culture plate at $5x10^3$ cells per well for 24 hours of incubation. In the literature, TP4 has been applied to various cancer cells at different concentration ranges. Ting et al. (2016) observed that treatment with 15 µg/mL TP4 was sufficient to kill more than 50% of breast cancer cells within 6 hours. Su et al. (2019) determined that the 50% lethal dose (LD50) of TP4 for glioblastoma cells was 20 µg/mL. In a subsequent study, Su et al. (2021) reported IC50 values of 29.20 and 32.41 µg/mL for synovial sarcoma cells. The TP4 concentration range for this study was determined by considering the IC50 concentrations reported in previous studies. In this study, TP4 at concentrations of 100, 50, 25, 12.5, 6.25, 3.125, 1.56, and 0.78 µg/mL was applied to HUH-7 cells for 3, 6, 12, and 24 hours at 37°C, with 5% CO_2 and 95% humidity. At the end of the 3, 6, 12, and 24-hour incubations, 100 µL of MTT reagent was added to each well to determine cell viability. After 3 hours, the absorbance was measured using a microplate reader at 570 nm to calculate the percent cell viability.

RT-PCR Analysis

Total RNA was extracted from HUH-7 cells treated by TP4 (3.5-4.5-5.5 µg/mL) at the end of the 3-h incubation. RT-PCR was performed using specific primers for *BAX*, *BCL-2*, *CASP3* and first strand cDNA synthesis was carried out according to the manufacturer's protocols. *ACTB* was used as the reference gene. Gene expression levels of *BAX*, *BCL-2*, *CASP3*, and *ACTB* were analyzed using primers (Table 2) synthesized by Sentebiolab Company (Ankara, Türkiye).

PCR was performed using a SYBR Green Master Mix (Qiagen, Hilden, Germany) with a reaction mix containing 1 µL cDNA, 2 µL each of primer and 12,5 µl SYBR Green Master Mix (total volume 25 µL). PCR conditions were as follows for one cycle: 95°C for 10 min followed by 45 cycles of 95°C for 15 sec, 64°C for 30 sec and 72°C for 15 sec. Each reaction was run in triplicate. Analysis of relative expression of these genes was performed using the $2^{-\Delta\Delta Ct}$ method.

Table 2. Primers for detection of genes expression.

Gene	Primer sequence	Reference
BAX	F: 5-AGCAGATCATGAAGACAGGG R: 5-GAAGTTGCCGTCAGAAAACA	[27]
CASP3	F: 5-GCGCTCTGGTTTTCGTTAAT R: 5-ACCCATCTCAGGATAATCCATTT	[27]

BCL2	F: 5-TATCTGGGCCACAAGTGAAG R: 5-ATTCGACGTTTTGCCTGAAG	[27]
ACTB	F: 5-CACCATGGATGATGATATCGC R: 5-GAATCCTTCTGACCCATGCC	[27]

ELISA Tests

Total protein was extracted from HUH-7 cells treated by TP4 (3.5-4.5-5.5 µg/mL) at the end of the 3-h incubation. To determine protein expression levels of the Bax, Bcl-2, Casp3 on HUH-7 cell treated by TP4 commercial sandwich ELISA kits.

TAS and TOS Analysis

TAS (Total Antioxidant Level) and TOS (Total Oxidant Level) tests measure the total capacity of total antioxidants and oxidants present in a biological sample. Commercially purchased Rel Assay Diagnostic TAS-TOS Assay Kits (Türkiye) were used to determine TAS and TOS status. Proteins purified from TP4-treated cells and stored at -80 ºC for use were used as samples. Experiments were carried out in 4 replicates according to the kit protocols.

3. Results and Discussion

Cloning of the *TP4* Gene into Expression Vector

In this study, 141 bp DNA sequence encoding the TP4 peptide was obtained by using the overlap PCR method and sequence was confirmed by agarose gel (Figure 2). After, DNA sequence containing the TP4 *gene* was digested with *Xho*I-*Xba*I enzymes and ligated to the pPICZαA plasmid digested from the *Xho*I-*Xba*I enzyme recognition site. The generated expression vector was named pPICZαA-TP4. Ligation mixture was transferred to competent *E. coli* XL1-Blue cells prepared using the $CaCl_2$ method, and selection was performed on LB Lennox agar medium containing 25 µg/mL zeocin antibiotic. Three colonies grown on antibiotic-containing medium were selected, and the accuracy of the expression vector and the cloned gene was verified *Asu*I ve *Sal*I restriction enzymes. The verified expression vector was linearized using the *Sac*I restriction endonuclease. Then linearized plasmid was purified by using PCR Purification Kit and it was transferred to competent *P. pastoris* KM71H cells by electroporation technique.

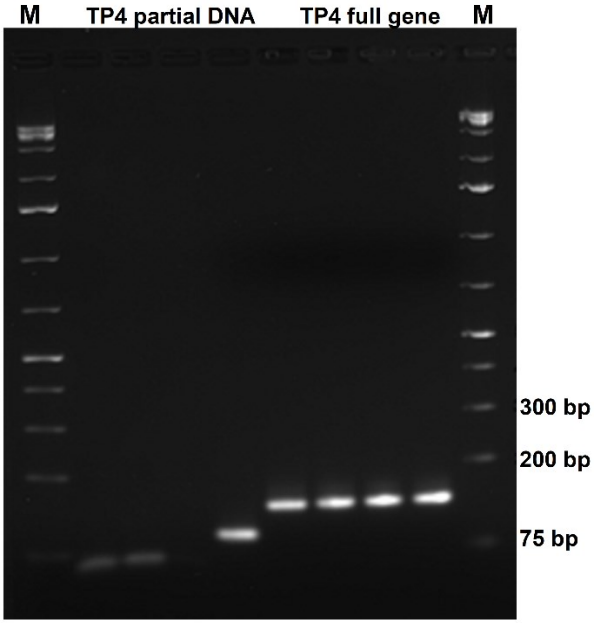

Figure 2. Obtaining the *TP4* gene by overlap PCR. (M: 1kb DNA ladder, Fermentas; Line 1-4: Partial TP4 DNA fragments; Line 5-8: Full construct of *TP4* gene)

Expression Studies of the *TP4* Gene in *P. pastoris*

After the transformation of the expression vector into the *P. pastoris* KM71H strain made competent by the Lithium-acetate method, the selected clone was first developed in YPD medium at 28°C overnight. The cells grown in YPD medium were inoculated into shake-flask containing 200 mL of BMGY medium and incubated for 18 hours at 28°C and 250 rpm. At the end of the cell accumulation period, the culture was centrifuged and the resulting cell pellet was resuspended in 200 mL of BMMY medium. During the induction phase, methanol, which acts as both a carbon source and inducer, was added to the medium every 24 hours to a final concentration of 1% for 216 h. At the end of the induction phase, cell density of the culture (OD_{600} nm) was measured and the supernatant sample was collected for purification analysis.

The literature indicates that the recombinant production of TP4 peptide in the P. pastoris expression system has been performed using similar methods [11,16,17]. Neshani et al. (2018) cloned the TP4 gene into the pPIC9 vector and expressed it in the *P. pastoris* GS115 strain. Tai et al. (2021) and Huang et al. (2020) both cloned the TP4 gene into the pPICZαA vector and expressed it in the *P. pastoris* X-33 strain. In this study, the TP4 gene was cloned into the pPICZαA vector and expressed in the *P. pastoris* KM71H strain, unlike previous studies. Most *P. pastoris* strains are Mut^+ (methanol usage positive phenotype) and grow rapidly with methanol induction. However, the KM71H strain is Mut^s, with the AOX1 gene deleted, resulting in slower methanol metabolism due to reliance on the AOX2 gene. Strains with deleted AOX genes can sometimes outperform wild-type strains in recombinant protein production [28]. Additionally, the Mut^s strain offers advantages such as lower costs in large-scale fermentations and reduced contamination risk due to requiring less methanol for induction.

Purification of Recombinant TP4

The supernatant sample collected was used for recombinant TP4 purification. The sample was concentrated and equilibrated with 1×PBS pH 7.4 containing 10 mM imidazole and loaded into the column containing Ni-NTA resin. For elimination of the non-specific binding the column was washed using 1×PBS buffer containing 20 mM imidazole. Then, the column was treated 1×PBS buffer containing different concentrations (50, 100 and 200 mM) of imidazole for elution of the his-tagged TP4 protein. The presence of histidine-tagged TP4 and purified TP4 were determined by the tricine SDS-PAGE gel analysis (Figure 3). Elutions containing recombinant TP4 protein were collected and dialyzed against 1×PBS buffer and the resulting purified protein was used to further analysis.

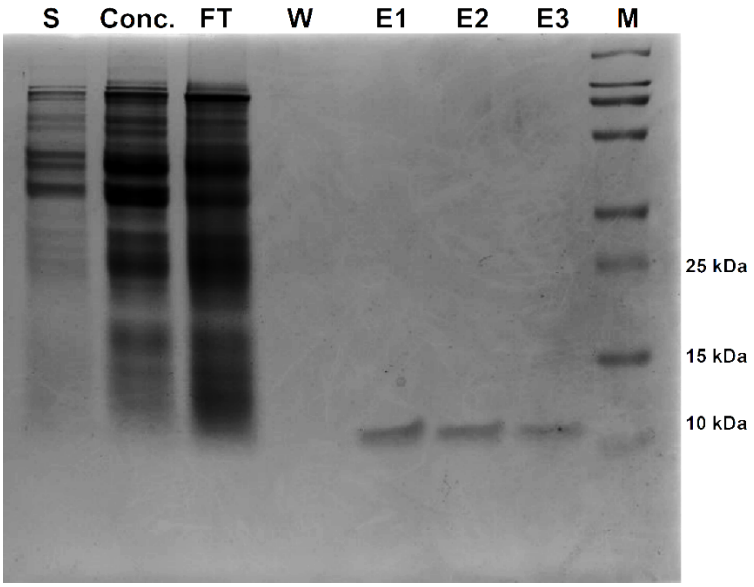

Figure 3. SDS-PAGE analysis of the purification steps of the TP4 protein (M: Protein marker; S: Supernatant sample; Conc.: Concentrated sample; FT: Flow Through; W: Wash (1×PBS containing 20 mM imidazole), E1-3: Elution (1×PBS containing 50, 100, 200 mM imidazole, respectively).

There are three main production methods for obtaining AMPs. These are direct AMP extraction from natural sources, chemical synthesis, and recombinant techniques. The recombinant method is preferred over the chemical method in many respects and has been increasingly considered for peptide production in recent years. Direct extraction of AMPs from natural sources is lengthy and costly, and it also poses a threat to ecosystems by harming many species. The chemical synthesis method involves multiple complex chemical steps, increasing the risk of errors in peptide sequences. Recombinant DNA technology is preferred over chemical and enzymatic synthesis methods in many respects and has been increasingly used for peptide production in recent years [13, 29].

Recombinant TP4 Expression in *P. pastoris* Showed Antimicrobial Activity

First, the antibacterial effect of the recombinant TP4 was determined by MIC and MBC methods. As seen in the results shown in Table 3 and Figure 4, it was determined that TP4 peptide had an antibacterial effect on *E. coli* and *B. subtilis.*

Bacteria have anionic properties, bind to cationic AMP, and disrupt its structure. They bind to negatively charged bacterial cell membranes through electrostatic interactions, disrupting their functions and causing death [30]. In this study, the accuracy of the activity of TP4 produced was examined on gram positive and negative bacteria. As a result, it was determined that even a small amount of TP4 had a very high antibacterial activity.

Table 3. MIC and MBC values of TP4 on strains

Hour	*E. coli* MIC (µg/mL)	*B. subtilis* MIC (µg/mL)	*E. coli* MBC (µg/mL)	*B. subtilis* MBC (µg/mL)
6	3.125	6.25	12.5	12.5
12	3.125	3.125	12.5	12.5
18	6.25	1.56	25	25
24	6.25	1.56	50	25

In the literature, the antibacterial activity of recombinantly produced TP4 was investigated using a similar method [11]. Although the results obtained are partially compatible with the literature, it is thought that the higher MIC and MBC values even in the same bacteria compared to the literature are due to the differences in the parameters during the experiment.

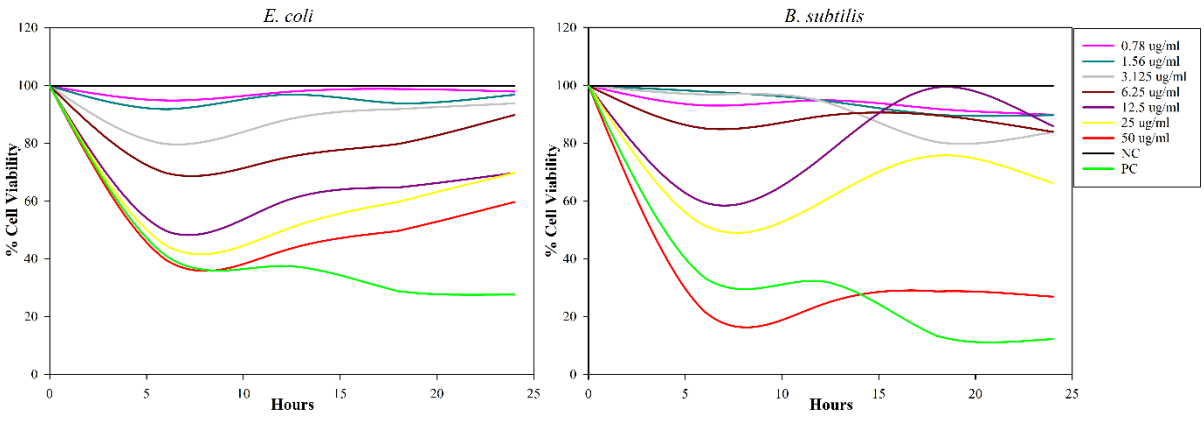

Figure 4. MIC and MBC of TP4 peptide on *E. coli* and *B. subtilis*

TP4 Decreases Cell Viability in HUH-7 Hepatocellular Carcinoma Cells

In this study was investigated anticancer effects of TP4 on HUH-7 hepatocellular carcinoma cells. For this aim, firstly, the cytotoxic effects TP4 on cancerous cells were determined by

MTT cell proliferation assay. Different concentrations of TP4 (100-50-25-12,5-6,25, 3,125, 1,56, 0,78 µg/mL) were applied on HUH-7 cells for 3-, 6-, 12- and 24-hours at 37 °C, 5% CO_2 and 95% humidity. IC50 value was found to be 4.5 µg/mL for 3-hour incubation by using AA Bioquest IC50 calculator tool. When cell proliferation at different concentrations was examined, it was observed that more than 80% of the cells died with the application of 100 µg/mL TP4, and 65% of the cells died with 50 µg/mL TP4. In contrast, the proliferation of cells gradually increased in the control group without treatment. Cytotoxic effects of TP4 are given in Figure 5.

Cancer cells often have negatively charged phosphatidylserine (PS) or anionic structures in their outer membranes, unlike healthy cells that are normally zwitterionic. Antimicrobial cationic peptides target cancer cell membranes and induce cell death through necrosis or apoptosis [4]. In the literature, TP4 has been investigated in various cell lines including SW982 and Aska-SS human synovial sarcoma cells, MDA-MB231, MDA-MB453 and MCF7 breast cancer cells, U87MG and U251 glioblastoma cells [10,18,19]. However, its anticancer effect was investigated for the first time on HUH-7 hepatocellular carcinoma cells. According to the MTT experiment results in HUH-7 cell line, the IC50 value was determined as 4,5 µg/ml. The results obtained were mostly consistent with the literature and the IC50 value was found to be lower than the literature results. This situation is thought to be due to the difference in cell type and protocol.

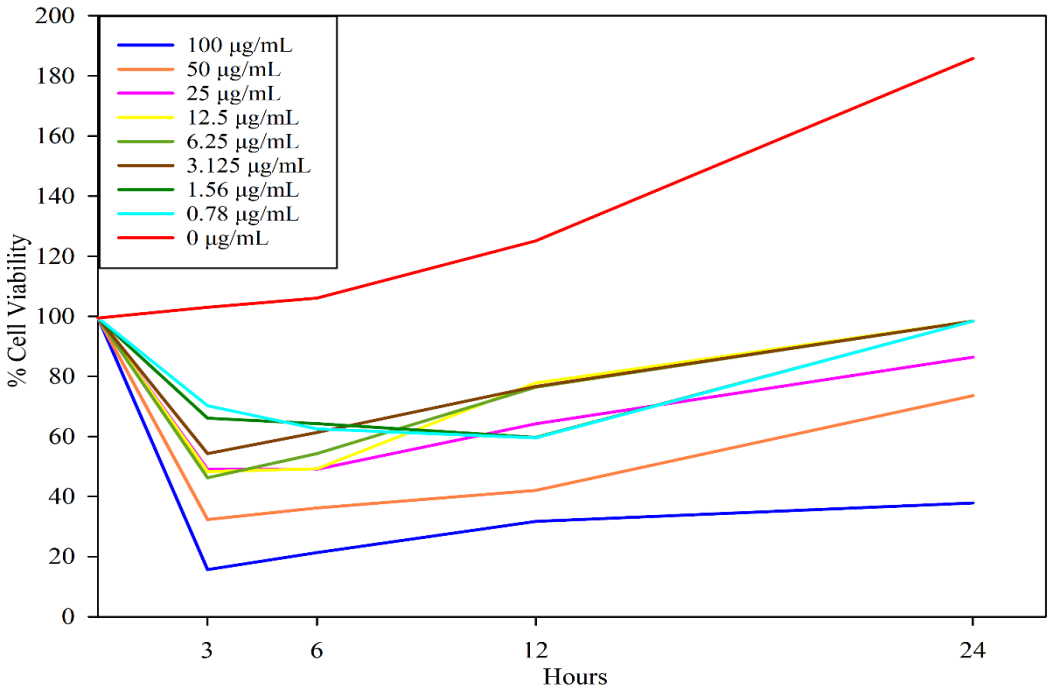

Figure 5. TP4 selectively kills HUH-7 cancer cells. Cell viability in HUH-7 was determined by MTT assay following treatment with varying doses of TP4 (0.78-100 µg.mL^{-1}) at the indicated time-points (3-24h).

TP4 Importantly Effects mRNA and Protein Levels of Cells

Antimicrobial peptides target cancer cells through various mechanisms such as cell membrane disruption, mitochondrial dysfunction, ROS production, and DNA damage, and induce cell death through necrosis or apoptosis [10,31,32]. When the cell is damaged, pro-apoptotic (*BAX and CASP3*) or anti-apoptotic (*BCL-2*) genes in the cell are activated, and these genes play an important role in the control of apoptosis [33,34].

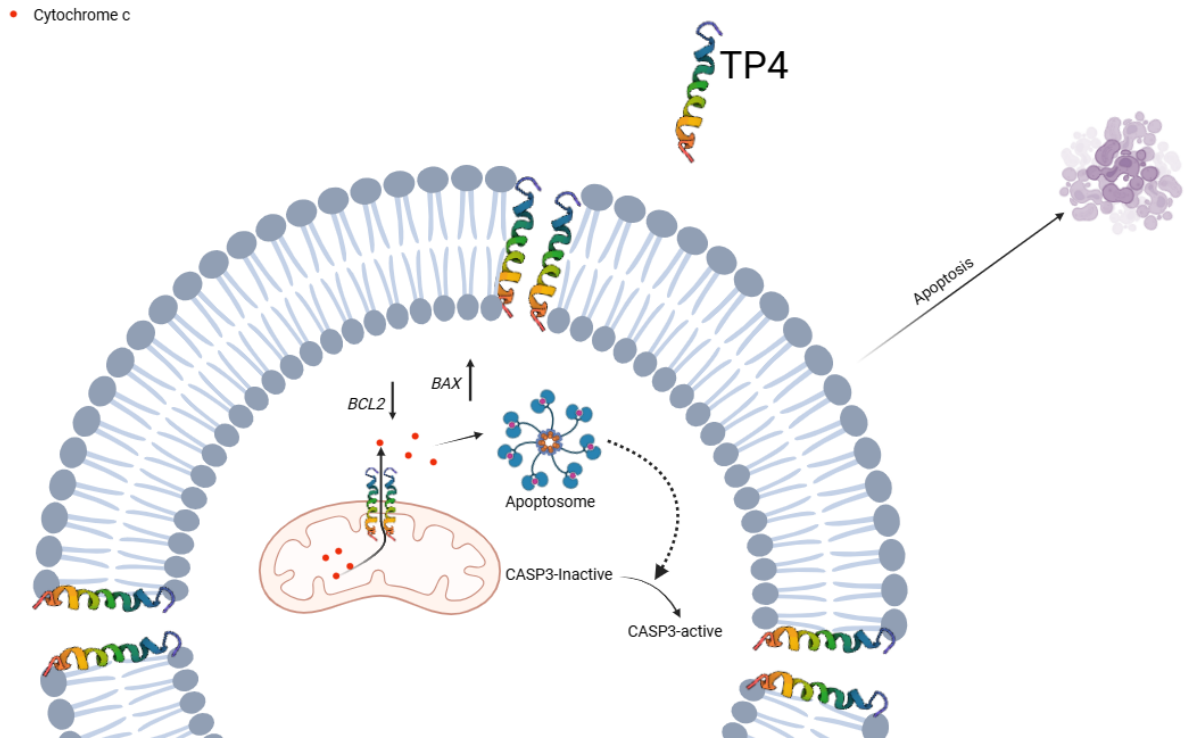

Figure 6. Apoptotic induction of TP4 on HUH-7 cells.

Kuo et al. (2018) applied TP4 stock solution (MSP-4) to MG63 osteosarcoma cells and observed that MSP-4 decreased anti-apoptotic protein BCL-2 levels and increased pro-apoptotic proteins BAX, CASP3 and BID in a dose-dependent manner. The study demonstrated that MSP-4 induced apoptosis via both the intrinsic (mitochondrial) and extrinsic (receptor-mediated) pathways [30, 31]. In this study, gene expression was analyzed using RT-PCR. The results were found to be consistent with the literature. Results showed that treatment with 4.5 µg/mL TP4 significantly upregulated *BAX* and *CASP3* genes expression on HUH-7 cells. Otherwise, gene expression levels of *BCL-2* downregulated in cells treated with 4.5 µg/mL TP4 (Figure 7). In cells treated with the same concentration, the ELISA findings of Bax and Casp-3 protein significantly increased compared to control, correlating with increased gene expression levels. The gene expression level of *BCL-2* was decreased in cells treated with 4.5 µg/mL TP4 (Figure 7). And ELISA result of Bcl-2 protein level was decreased and correlating with gene expression level. These findings provided important information about the pro-apoptotic effects of TP4 on HUH-7 cells. The dual effect of increased pro-apoptotic signals (*BAX* and *CASP3*) and decreased anti-apoptotic signals (*BCL-2*) demonstrated the strong apoptotic effect of TP4.

This study demonstrated that TP4 is particularly important for understanding its therapeutic potential. By promoting apoptosis in cancer cells, TP4 may serve as an effective anti-cancer agent, especially in cases where conventional treatments fail to trigger sufficient apoptotic response. Future research should focus on elucidating the detailed molecular mechanisms by which TP4 modulates these apoptotic pathways and evaluating its effects in various cell types and in vivo models (Figure 6).

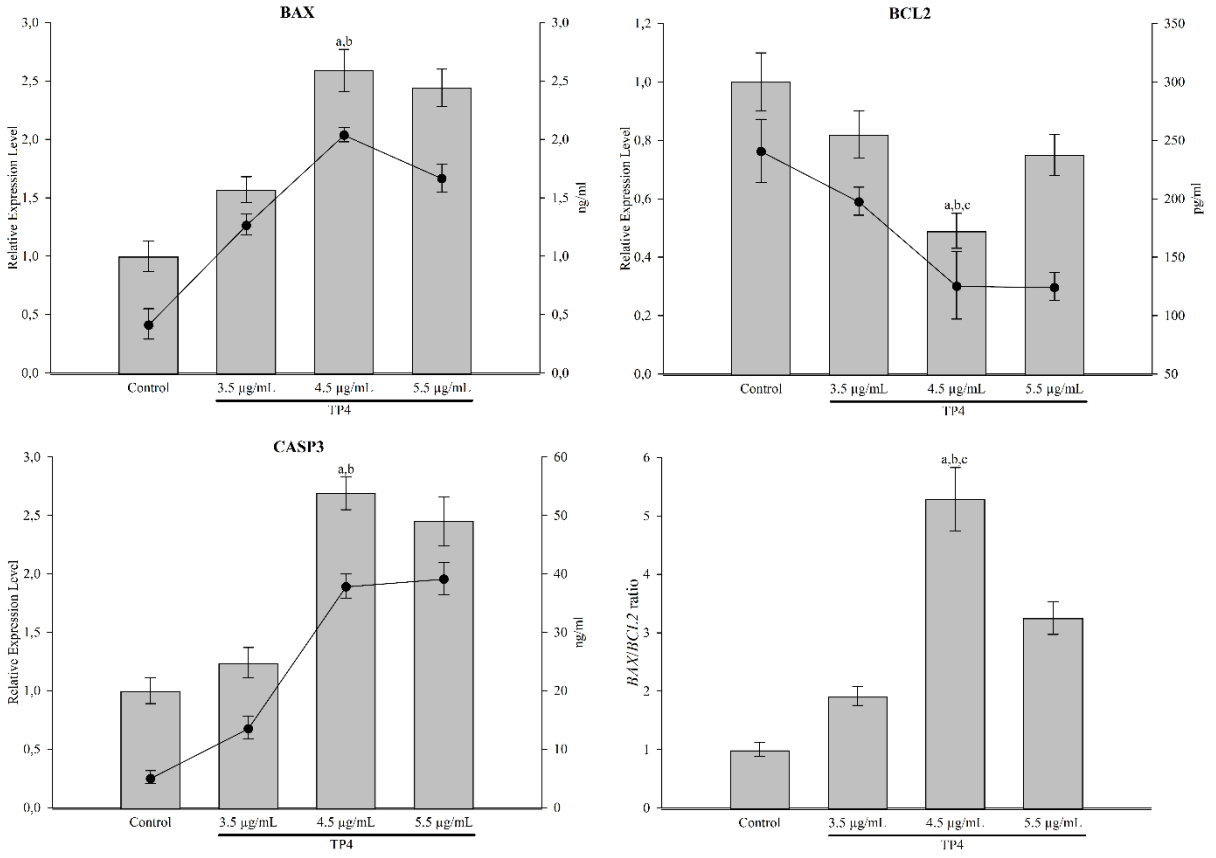

Figure 7. Expression levels of *BAX, BCL-2, CASP3* and *BAX*/*BCL-2* ratio in HUH-7 cells. Values are represented as mean ± standard error of mean. $^{a}p < 0.05$ versus control group, $^{b}p < 0.05$ versus 3.5 µg/mL TP4-treated group, $^{c}p < 0.05$ versus 5.5 µg/mL TP4-treated group.

TAS and TOS Status

Antioxidants play a crucial role in preventing oxidative damage caused by free radicals, whereas oxidants are reactive molecules that can harm cellular structures and contribute to disease. Su et al. (2021) demonstrated that TP4 initiates the production of reactive oxygen species (ROS) and induces necrotic cell death in Aska-SS and SW982 cell lines by reducing levels of antioxidant proteins, such as uncoupling protein-2 and superoxide dismutases SOD-1 and SOD-2. In this study, the total antioxidant status (TAS) and total oxidant status (TOS) in HUH-7 cells were measured using ELISA kits. The results are shown in Figure 8. These findings consistent with the literature, demonstrating that TP4 significantly affects the oxidative balance in HUH-7 cells. The decrease in TAS with increasing TP4 concentration indicates that TP4 induces oxidative stress, raising oxidant levels. The observed decrease in TOS suggests

that TP4 may deplete antioxidants or inhibit cellular antioxidant defenses. This highlights TP4's potential pro-oxidant role, which could cause cellular damage if oxidative stress is not managed. Additionally, the increase in oxidant levels implies that TP4 may stimulate ROS production or impair the cell's ability to neutralize them. Overall, this study shows that long-term oxidative stress can lead to apoptosis in cancer cells, suggesting TP4's potential for anticancer applications.

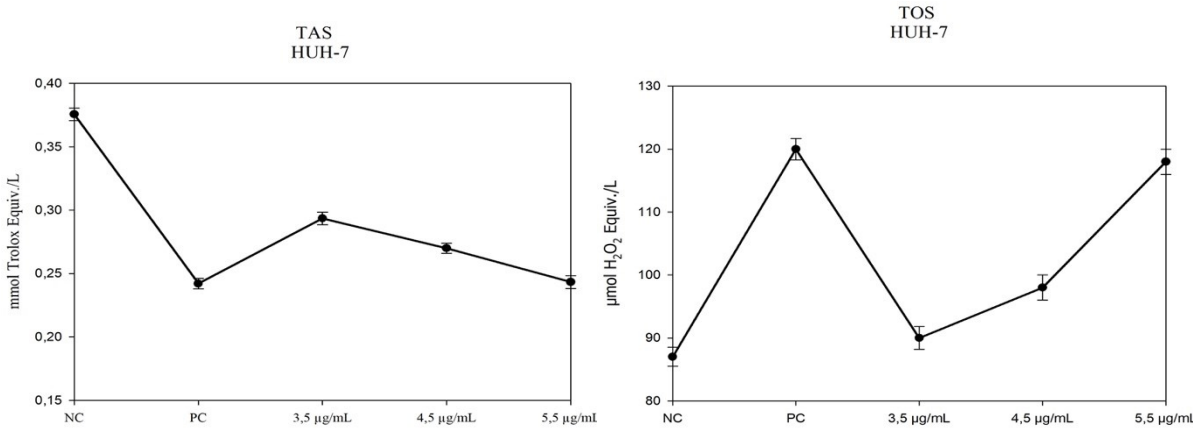

Figure 8. TAS and TOS status on HUH-7 cell line.

4. Conclusion

In this study, the recombinant production of TP4 was carried out in *P. pastoris,* and its effects on HUH-7 hepatocellular carcinoma cells were investigated. The results demonstrated that TP4 exhibits a strong anticancer effect, inducing apoptosis in HUH-7 cells through the induction of oxidative stress and the activation of pro-apoptotic genes. This study is the first to demonstrate that recombinant TP4 exhibits potent anticancer activity in the human HUH-7 cell line. The findings suggest that TP4, an antimicrobial peptide, may serve as a promising candidate for cancer treatment due to its significant anticancer effects. Additionally, investigating the potential synergistic effects of TP4 with other anti-cancer agents may provide valuable information for the development of combination therapies that increase efficacy while minimizing adverse effects.

Ethics in Publishing

There are no ethical issues regarding the publication of this study.

Author Contributions

The authors declare that they have contributed equally to the article.

Acknowledgements

This project was supported by Health Institutes of Türkiye (TUSEB) with project no. 31705.

References

[1]https://gco.iarc.fr/today/en/dataviz/pie?mode=cancer&group_populations=1&cancers=39&types=1, Ziyaret tarihi: 14.03.2024.

[2] Chen, Z., Xie, H. et al., (2020) Recent progress in treatment of hepatocellular carcinoma, Am J Cancer Res., 10 (9) 2993-3036.

[3] Lien, S. and Lowman, H.B. (2003) Therapeutic peptides, Trends Biotechnol., 21(12) 556-62.

[4] Boohaker, R.J., Lee, M.W., Vishnubhotla, P., Perez, J. M. and Khaled A. R. (2012) The Use of Therapeutic Peptides to Target and to Kill Cancer Cells, Current Medicinal Chemistry, 19 3794-3804.

[5] Chauhan, S., et al., (2021) Antimicrobial peptides against colorectal cancer-a focused review Pharmacological Research.

[6] Peng, K.C. et al., (2012) Five Different Piscidins from Nile Tilapia, *Oreochromis niloticus*: Analysis of Their Expressions and Biological Functions, plosone, 7, 11.

[7] Mahrous, K.F. et al., (2020) Piscidin 4: Genetic expression and comparative immunolocalization in Nile tilapia (*Oreochromis niloticus*) following challenge using different local bacterial strains, Developmental & Comparative Immunology.

[8] Dyshlovoy, S.A. and Honecker, F., (2020) Marine Compounds and Cancer: Updates 2020 Marine Drugs, 18, 643.

[9] Ting, C.H. and Chen, J. Y., (2018) Nile Tilapia Derived TP4 Shows Broad Cytotoxicity toward to Non-Small-Cell Lung Cancer Cells, Marine Drugs, 16, 506.

[10] Su, B.C. et al., (2019) Antimicrobial Peptide TP4 Induces ROS-Mediated Necrosis by Triggering Mitochondrial Dysfunction in Wild-Type and Mutant p53 Glioblastoma Cells, Cancers, 11, 171.

[11] Neshani, A. Et al., (2018) Extended-Spectrum Antimicrobial Activity of the Low Cost Produced Tilapia Piscidin 4 (TP4) Marine Antimicrobial Peptide, Journal of Research in Medical and Dental Science, 6 (5) 327-334.

[12] Zorko, M., Jerala, R. (2010) Production of recombinant antimicrobial peptides in bacteria, Methods Mol Biol., 618,61-6.

[13] Li, Y. (2011) Recombinant production of antimicrobial peptides in *Escherichia coli*: A review, Protein Expr Purif. 80:260-67.

[14] Hagenson, M.J. (1991) Production of recombinant proteins in the methylotrophic yeast *Pichia pastoris*, Bioprocess Technol., 12, 193-212.

[15] Cregg, J.M. (2007) Pichia Protocols Methods in Molecular Biology Series, Humana Press Inc., 389(2) 1-10.

[16] Huang, H.N. et al. (2020) Dietary supplementation of recombinant tilapia piscidin 4-expressing yeast enhances growth and immune response in Lates calcarifer, Aquaculture Reports, 16.

[17] Tai, H.M. et al. (2021) Scale-up production of and dietary supplementation with the recombinant antimicrobial peptide tilapia piscidin 4 to improve growth performance in *Gallus gallus domesticus*, PLOS ONE, 16(6).

[18] Su, B.C. (2021) Marine Antimicrobial Peptide TP4 Exerts Anticancer Effects on Human Synovial Sarcoma Cells via Calcium Overload, Reactive Oxygen Species Production and Mitochondrial Hyperpolarization, Mar. Drugs, 19, 93.

[19] Ting, C.H. (2016) Targeting FOSB with a cationic antimicrobial peptide, TP4, for treatment of triple-negative breast cancer, Oncotarget, 7, 26.

[20] Hazam, P.K. and Chen, J.Y. (2020) Therapeutic utility of the antimicrobial peptide Tilapia Piscidin 4 (TP4), Aquaculture Reports, 17.

[21] Karaoğlan, M., Erden-Karaoğlan, F. (2020) Effect of codon optimization and promoter choice on recombinant endo- polygalacturonase production in *Pichia pastoris*. Enzyme and Microbial Technology, 139, 109589.

[22] Wu, S. and Letchworth, G.J. (2004) High efficiency transformation by electroporation of *Pichia pastoris* pretreated with lithium acetate and dithiothreitol, Biotechniques, 36,152-154.

[23] Schägger, H. (2006) Tricine–SDS-PAGE, Nature Protocols, 1 (1) 16-22.

[24] Carson, M. et al. (2007) His-tag impact on structure. Acta Crystallographica Section D" Structural Biology, 63(3) 295-301.

[25] Spriestersbach, A. et al. (2015) "Purification of His-Tagged Proteins" Methods in Enzymology, 559, 1-15.

[26] Karaoğlan, M. and Erden-Karaoğlan, F. (2021) "Extracellular Production and Purification of the β-glucanase in *Pichia pastoris* Expression System". Erzincan University Journal of Science and Technology, 14 (2) 620-630.

[27] Günay, N., & Kuzucu, M. (2023). "Agonistic Effects of Deinoxanthin on Tamoxifen Antiproliferative Activity on HER2 Positive Breast Cancer: An In Vitro Study on MDA-MB-453". Erzincan University Journal of Science and Technology, 16(1), 138-154.

[28] Karaoğlan, M. (2012) "Pichia pastoris Alkol Oksidaz (AOX1 ve AOX2) Genlerinin İnaktif Edilmesi ve Elde Edilen Suşun Rekombinant Protein Üretiminde Kullanılması" (Yüksek Lisans), Akdeniz Üniversitesi Fen Bilimleri Enstitüsü, Antalya, 15-19.

[29] Li, Y. et. al. (2012) "Overview on the recent study of antimicrobial peptides: Origins, functions, relative mechanisms and application" Peptides, 37,207–215.

[30] Zhong, C. et. al. (2020) "A Review for Antimicrobial Peptides with Anticancer Properties: Re- purposing of Potential Anticancer Agents" BIO Integration, 1(4), 156-167.

[31] Deslouches, B. and Peter Di, Y. (2017) "Antimicrobial Peptides with Selective Antitumor Mechanisms: Prospect for Anticancer Applications." Oncotarget, 8(28), 46635-46651.

[32] Kuo, H.M. et al. (2018) "MSP-4, an Antimicrobial Peptide, Induces Apoptosis via Activation of Extrinsic Fas/FasL- and Intrinsic Mitochondria-Mediated Pathways in One Osteosarcoma Cell Line" marine drugs, 16,8.

[33] Portt, L. et. al. (2011) "Anti-apoptosis and cell survival: A review" Biochimica et Biophysica Acta 1813, 238–259.

[34] Kiraz, Y. ve ark (2016) "Major apoptotic mechanisms and genes involved in apoptosis" Tumor Biol., 37, 8471–8486