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Research Article

Evaluation of Anticancer and Antimicrobial Potentials of *Tarantula cubensis* Venom (Theranekron® D6)

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Abstract

Tarantula cubensis venom (Theranekron®D6) is widely used in veterinary medicine as a drug with anti-tumor, wound healing, anti-inflammatory, antiphlogistic properties. The purpose of the study was to explore Theranekron®D6 (TD6)'s antibacterial activity and its impact on apoptotic cell death in human colon and liver cancer cell lines. TD6 showed a dose and time dependent cytotoxic effect at 12h and 24h in HT-29 and HUH-7 cancer cell lines. The IC_{50} values of TD6 were calculated as 12.18 µg/mL and 25.10 µg/mL in HT-29 and HUH-7 cell lines at 24h, respectively. TD6 induced apoptosis in HT-29 and HUH-7 cell lines. In these cells exposed to TD6, while the *BAX/BCL-2* ratio and *CASP-3* mRNA level increased, the *HSP90* mRNA and protein level decreased. Also, TD6 exhibited antimicrobial activities against *Pseudomonas aeruginosa, Enterococcus faecalis, Klebsiella pneumonia, Staphylococcus aureus, Candida albicans* and *Candida utilis*. Obtained results demonstrated that TD6 has great potentials as alternative therapeutic for cancer and infectious diseases as apoptosis inducer and antimicrobial agent.

Keywords: Cancer, *Tarantula cubensis*, Apoptosis, Antibacterial, Antifungal

Tarantula cubensis Venomunun (Theranekron® D6) Antikanser ve Antimikrobiyal Potansiyellerinin Değerlendirilmesi

Öz

Tarantula cubensis venomu (Theranekron®D6), veteriner tıbbında anti-tümör, yara iyileştirme, anti-enflamatuar, antiflogistik özelliklere sahip bir ilaç olarak geniş bir şekilde kullanılmaktadır. Bu çalışmada, Theranekron®D6 (TD6)'nın antibakteriyel aktivitesi ve insan kolon ve karaciğer kanser hücre hatlarında apoptotik hücre ölümü üzerine etkisini araştırıldı. TD6, HT-29 ve HUH-7 kanser hücre hatlarında 12 saat ve 24 saatte doz ve zaman bağımlı sitotoksik etki gösterdi. TD6'nın HT-29 ve HUH-7 hücre hatlarında 24 saatte hesaplanan IC_{50} değerleri sırasıyla 12.18 µg/mL ve 25.10 µg/mL olarak bulundu. TD6, HT-29 ve HUH-7 hücre hatlarında apoptozu indükledi. TD6'ya maruz kalan bu hücrelerde, *BAX*/*BCL-2* oranı ve *CASP-3* mRNA seviyesi artarken, *HSP90* mRNA ve protein seviyesi azaldı. Ayrıca, *TD6, Pseudomonas aeruginosa, Enterococcus faecalis, Klebsiella pneumonia, Staphylococcus aureus, Candida albicans* ve *Candida utilis'e* karşı antimikrobiyal aktiviteler sergiledi. Elde edilen sonuçlar, TD6'nın kanser ve enfeksiyon hastalıkları için alternatif bir terapötik olarak büyük potansiyele sahip olduğunu, apoptoz indükleyici ve antimikrobiyal ajan olarak işlev gördüğünü gösterdi.

Anahtar Kelimeler: Kanser, *Tarantula cubensis*, Apoptoz, Antibakteriyel, Antifungal

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1. Introduction

Natural toxins are important pharmacological resources in the discovery of therapeutic biomolecules. Natural venoms derived from some animals such as snakes, scorpions and spiders have therapeutic potential against many diseases. Spider venoms are a complex mixture of nucleic acids, free amino acids, low molecular weight organic compounds, polypeptides, proteins, neurotoxins, and inorganic salts [1-4]. The biological activity of spider venom is quite impressive. Spider venom has been reported to exhibit potential therapeutic activity against a wide variety of human diseases such as microbial infections, cancer, malaria, and arrhythmia [3, 5, 6].

Tarantula cubensis is a large arachnid from the Theraphosidae spider family. Theranekron® D6 (TD6) is a product made from an alcoholic extract of the entire *tarantula cubensis.* It is a homeopathic drug commonly used in veterinary medicine. TD6 is effectively used in clinical veterinary in the treatment of foot and mouth lesions, pododermatitis, cutaneous papillomatosis and endometritis [7, 8]. A wide range of therapeutic effects of TD6 have been demonstrated in several clinical studies. It has been found to be effective in inhibiting the growth of canine tumors, uterine invasion in cattle, treating genital microbial diseases and oral lesions. According to these studies, TD6 shows antitumor, wound healing, anti-inflammatory, antiphlogistic and necrotizing effects Additionally, some reports have implied that TD6 has antimicrobial activity [9-18]. Although the antitumor effects of TD6 have been investigated in clinical veterinary medicine, more research is needed to reveal the antitumor action mechanism of TD6.

In this study, anticancer activity of TD6 was investigated in liver (HUH-7) and colon (HT-29) cancer cell lines. The gene expression level of *BAX*, *BCL-2*, *CASP-3* and *HSP90* in cancer cell lines exposed to TD6 was evaluated. *HSP90* protein level in these cells was determined using the ELISA kit. Also, antibacterial and antifungal activities of the TD6 were examined against *Enterococcus faecalis*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Klebsiella pneumonia*, *Candida albicans* and *Candida utilis*. Obtained results demonstrated that TD6 is a potent natural therapeutic in treatment of infectious diseases and cancer.

2. Material and Methods

2.1. Materials

TD6 was supplied from Richter Pharma AG (Wels, Austria). Dulbecco′s Modified Eagle′s medium (DMEM), Heat-inactivated fetal bovine serum (FBS), penicillin-streptomycin solution, RPMI-1640 medium, trypsin–EDTA, L-glutamine, phosphate buffer saline (PBS), and XTT cell proliferation kit were from Biological Industries. cDNA synthesis kit and SYBR Green master mix was obtained from Bioline. Total RNA isolation was from Favorgen Biotech Corp.. *BCL-2* (PPH00079B), *BAX* (PPH00078B) and *GAPDH* (PPH00150F), *HSP90* (PPH63391B) primers were purchased from Qiagen (Primer Assays; Qiagen, Valencia, CA, USA). *CASP-3* primer was synthesized by Macrogen (forward: 5’-ACATGGAAGCGAATCAATGGACTC-3’, reverse: 5’-

AAGGACTCAAATTCTGTTGCCACC-3'). *HSP90* ELISA Kit were purchased from Biont Shangai YL Biotech Co., Ltd. Bacterial strains (*P. aeruginosa ATCC 27853, E. faecalis ATCC 29212, K. pneumoniae ATCC 15380, S. aureus ATCC 25923*), fungal strains (C. utilis *ATCC 9950, C. albicans ATCC 90819*), and cancer cell lines (HUH-7 from the Japanese Collection of Research Bioresources (JCRB), HT-29 from the American Type Culture Collection (ATCC)) were obtained from Tokat Gaziosmanpasa University, Department of Bioengineering and Genetics, Turkey."

2.2. XTT Assay

HT-29 cells were cultured in RPMI-1640 medium, while HUH-7 cells were cultured in DMEM high glucose medium. Both cultures were supplemented with 10% fetal bovine serum, 1% L-glutamine, 100 IU/mL penicillin, and 10 mg/mL streptomycin. The cells were cultured under standard conditions at 37 °C in a humidified atmosphere containing 95% air and 5% CO_2.Colorimetric XTT cell proliferation kit was used to analyze the effects of TD6 on cell proliferation. The cells were seeded into 96-well plates at a density of 5×10^4 cells/mL. After 24 h, the cells were exposed to TD6 at concentrations ranging from 500 µg/mL to 3.9 µg/mL for 24 h. After the exposure period, 50 µL of the XTT reagent was added to each well and incubated for an additional 4 hours. Absorbance was then measured at 450 nm, and cell viability was expressed as a percentage relative to the untreated control group. IC_{50} values of TD6 were calculated with GraphPad Prism 8.0 software [17].

2.3. Gene Expression Analysis

Expression levels of *HSP90*, *BCL-2*, *BAX* and *CASP-3* genes in cells exposed to TD6 were determined using RT-PCR (Biorad CFX96™). Firstly, IC_{50} doses of TD6 were applied to HT-29 and HUH-7 cells for 24 hours. Then, the cells were trypsinized and total RNA was isolated from the cells. cDNA synthesis from total RNAs was performed according to the commercial kit protocol. The amplification process was conducted under the following conditions: 1 cycle at 95°C for 10 minutes, followed by 45 cycles at 95°C for 15 seconds and 60°C for 1 minute. The data of RT-PCR were analyzed using the $2^{-\Delta\Delta Ct}$ method and normalization of expression of genes was performed with *GAPDH* [19].

2.4. *HSP90* ELISA Assay

The alteration of *HSP90* protein level under IC_{50} doses of TD6 were measured in HT-29 and HUH-7 cell line by human ELISA kit following the manufacturer's instructions. The change in the *HSP90* protein level was calculated as a percentage relative to the control.

2.5. Antibacterial and Antifungal Activity Assays

Using the minimum inhibitory concentration (MIC) test, the antibacterial and antifungal properties of TD6 were assessed against *P. aeruginosa, E. faecalis, K. pneumonia, S. aureus, C. utilis, and C. albicans.* For this experiment, a 96-well plate was filled with 100 µL of the

nutritional soup in each well and TD6 was applied with two-fold dilutions from 500 µg/mL to 3.9 µg/mL. Then, the bacterial and fungal strains ($1x10^8$ CFU/mL) were added in nutrient broth and incubated overnight at 37°C. A microplate reader was used to detect absorbance at 600 nm at the conclusion of the incubation period and the percent inhibition was calculated compared to control group [20].

2.6. Statistical Analysis

Statistical analysis was conducted using GraphPad Prism 8.0 software, employing two-way ANOVA with Dunnett's and Sidak tests. A significance level of $p<0.05$ was used to determine statistical significance (****<0.0001).

3. Results and Discussion

3.1. XTT Assay

The XTT assay was applied to investigate the *in vitro* cytotoxic effect of TD6 on colon and liver cancer. The change in % cell viability of cells exposed to TD6 at different concentrations for 12h and 24h is shown in Figure 1. TD6 exhibited cytotoxic effect on HT-29 and HUH-7 cells in a time and dose dependent manner. IC_{50} values determined from % cell viability data of cells exposed to TD6 are given in Table 1. TD6 showed lower doses of cytotoxic effect in HUH-7 cells than HT-29 at 24h.

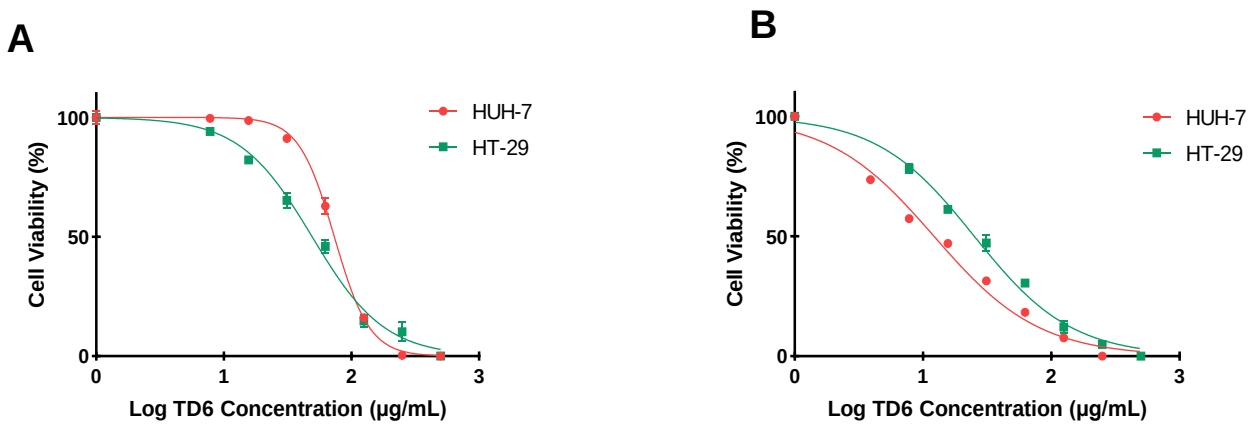

Figure 1 Cell viability of cancer cell lines exposed to TD6 in 12h (A) and 24h (B)

Table 1 IC_{50} values of TD6 on HT-29 and HUH-7 cancer cell lines

Cell Lines	**IC_{50} (µg/ml) 12 h**	**IC_{50} (µg/ml) 24 h**
HUH-7	74.47 ± 1.61	12.18 ± 4.23
HT-29	49.23 ± 3.94	25.10 ± 3.05

Colorectal cancer is an important type of cancer and approximately half of colorectal cancer patients die within 5 years [21]. The liver is the most common metastatic site for patients with colorectal cancer, and at least 25%-50% of patients develop colorectal liver metastasis [22]. Resistance to drugs and increase in relapse rate are an important problem in cancer treatment. Therefore, the need to discover new therapeutics in cancer is increasing day by day [23]. The use of natural anticancer agents is seen as powerful resources for overcoming cancer and getting promising results [24]. Poisonous animals play an important role in discovering new therapeutic candidates. Various poisons have been shown to inhibit the proliferation of cancer cells and promote cell death by decreasing or increasing protein expression in inducing apoptosis [25-27]. TD6, which is the venom of *Tarantula cubensis*, is a drug with known anticancer properties in veterinary medicine. In this study, *in vitro* antitumor effect of TD6 on human colon and liver cancer cells was investigated. TD6 caused a dramatic decrease in the viability of colon and liver cancer cells in a time and dose dependent manner.

3.2. Gene Expression Analysis

In order to investigate the apoptotic effects of TD6 on cancer cells, the expression of apoptotic genes was investigated by RT-PCR. Figure 2 shows the change in *BAX/BCL-2*, *CASP-3* and *HSP90* mRNA levels of HUH-7and HT-29 cells exposed to TD6 for 24 hours.

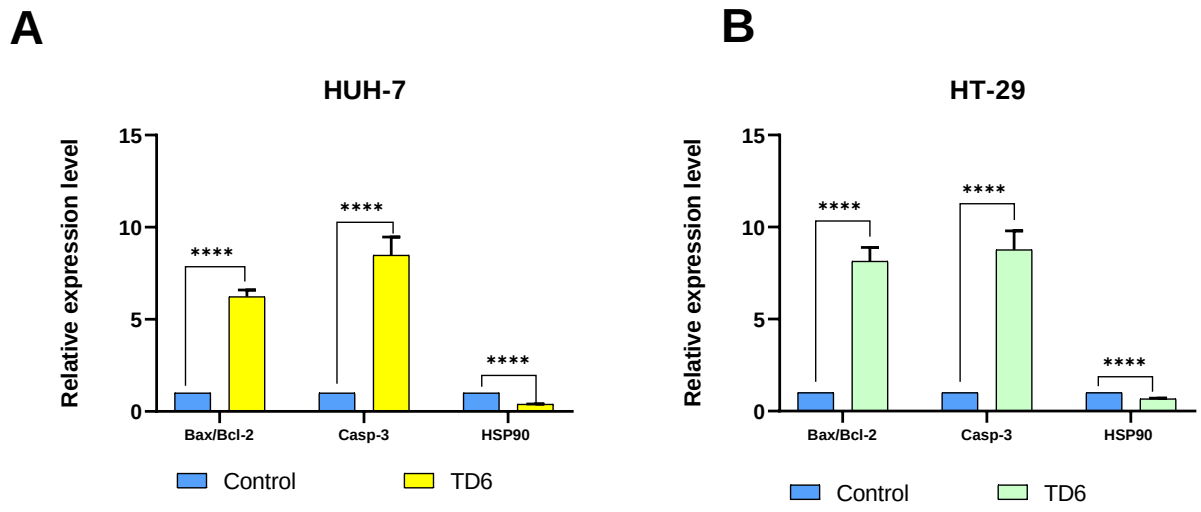

Figure 2 Illustrates the effect of TD6 on the mRNA levels of *HSP90* and apoptotic genes (*BAX*/*BCL-2*, *CASP-3*) in HT-29 and HUH-7 cell lines.

3.3. *HSP90* ELISA Assay

HSP90 protein level was investigated using *HSP90* ELISA kit in HT-29 and HUH-7 cells exposed to TD6. *HSP90* protein level decreased in TD6 treated HT-29 and HUH-7 cells (**Figure 3**). The decrease in *HSP90* protein level correlated with the decrease in mRNA level.

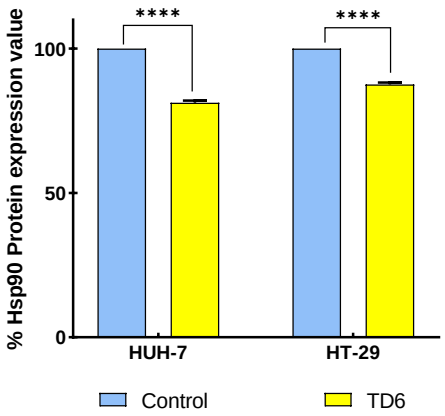

Figure 3 The effect of TD6 on *HSP90* protein level in HT-29 and HUH-7 cell lines

While *BAX/BCL-2* ratio and *CASP-3* mRNA level increased in cells exposed to TD6, *HSP90* mRNA and protein level decreased. *HSP90* plays a role in the metastasis, invasion, vascularization, and proliferation of tumors. In addition, it contributes greatly to the maintenance of oncoproteins, which are involved in the signal transduction pathway of apoptosis [28, 29]. *HSP90* has been found to be overexpressed in hepatocellular carcinoma [30]. In colorectal cancer, the high level of *HSP90* expression causes more aggressive phenotypes of

the tumor [31]. Therefore, *HSP90* represents an attractive therapeutic target for many cancers, including liver and colorectal cancer. In the literature, *HSP90* inhibitors have been shown to induce p53-mediated induction of *PUMA* and *BAX* and apoptotic response *in vitro* and *in vivo* by the mitochondrial pathway in colon cancer cells [32, 33]. In this study, an increase in the ratio of *BAX/BCL-2* and *CASP-3* mRNA level was observed in colon and liver cancer cells exposed to TD6. TD6 also caused a decrease in the *HSP90* mRNA and protein level in these cells. TD6 could potentially induce cellular apoptosis via the mitochondrial pathway, which emerges with the inhibition of *HSP90*. This inhibition results in the reduction of *BCL-2*, increase and stimulation of *BAX*, rendering mitochondrial membranes permeable, cytochrome c release, and caspase activation. Şumnulu et al. investigated the cytotoxic effects of TD6 on HepG2 (human liver cancer) and AML12 (mouse hepatocyte) cell lines. The researchers found an IC_{50} value of 143 µg/mL for HepG2 cells, resulting in a 31.04% increase in apoptotic and necrotic cells. In contrast, no substantial increase in cell mortality was detected in AML12 cells. Their investigation also found that apoptotic genes such as *BAX*, *CASP-3*, *APAF1*, and *p53* were significantly upregulated in HepG2 cells, but remained mostly unaltered in AML12 cells. Our study found comparable cytotoxic effects on liver cancer cells, with an IC_{50} value of 12.18 µg/mL in HUH-7 cells at 24 h. TD6 also strongly triggered apoptosis in HUH-7 and HT-29 cells, as demonstrated by increased *BAX/BCL-2* ratios, *CASP-3* expression, and reduced HSP90 levels. Our research and that of Şumnulu et al. reveal that TD6 may hold potential as a chemotherapeutic treatment for liver cancer, with significant variations in sensitivity between cancer and non-cancerous liver cells [34]. In another study on liver cancer, Vanli et al. investigated the effects of TD6 and sorafenib on hepatocellular carcinoma in rats. They found that TD6 reduced tumor incidence and size, improved biochemical markers of liver damage, and increased apoptosis markers such as *CASP-10* and *CASP-3*. Similar to Vanlı et al., we observed apoptosis induction in cancer cells through changes in the *BAX/BCL*-2 ratio and *CASP-3* levels [35]. Akçakavak and Özdemir investigated the effect of TD6 on colorectal cancer in rats. Their study found that TD6 treatment led to reduced cancer progression. In addition, TD6 decreased the expression of *KRAS* and *β-catenin*, while increasing the expression of *APC* and *p53* genes [36]. In another study, Taspinar investigated the combined antitumor effects of TD6 and cisplatin in neuroblastoma cells (SH-SY5Y). It was found that the co-administration of 100 µM TD6 with 40 µM cisplatin resulted in a significant cytotoxic effect of 60% and a 38% decrease in cell volume. The study also observed a 34.4% early apoptosis rate and an eight-fold decrease in mitochondrial membrane potential in SH-SY5Y cells compared to the control group. These results suggest that TD6 and cisplatin have a synergistic effect and enhance the efficacy of cisplatin. Similar to Taspinar et al., we observed significant apoptosis induction in cancer cells as indicated by changes in *BAX/BCL-2* ratio and *CASP-3* levels [37].

3.4. Antibacterial and Antifungal Activity Assays

Our results demonstrated a significant variation in the antimicrobial activity of TD6 depending on the different bacterial and fungal strains (Figure 4). TD6 exhibited antibacterial and antifungal activities against *E. faecalis, P. aeruginosa, S. aureus, K. pneumonia, C. albicans*

and *C. utilis* in dose dependent manner and the MIC values of TD6 were calculated ranged from 60.98 µg/mL to 89.89 µg/mL in these strains (Table 2).

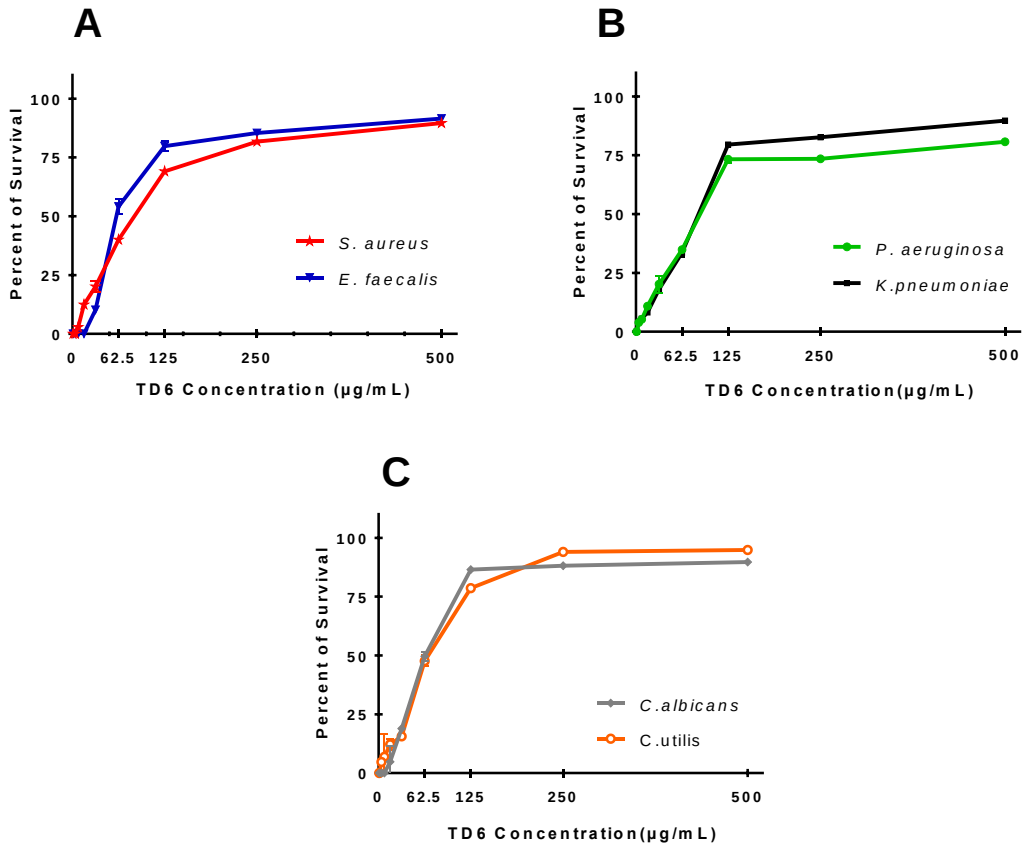

Figure 4 A) Growth curves of pathogen gram-positive bacterial strains, *S. aureus* and *E. faecalis*, with exposure to TD6 B) Growth curves of pathogen gram-negative bacterial strains, *K. pneumonia and P. aeruginosa*, with exposure to TD6 C) Growth curves of pathogen fungal strains, *C. utilis* and *C. albicans*, with exposure to TD6

Table 2 MIC values of TD6 against bacterial and fungal strains

Microorganism	MIC Value of TD6 (µg/mL)
E. faecalis	60.98 ± 4.87
P. aeruginosa	89.89 ± 6.11
S. aureus	85.21 ± 7.02
K. pneumonia	75.00 ± 6.55
C. albicans	64.12 ± 3.54

C. utilis	74.64 ± 4.31

Antimicrobial activities of venoms against bacterial and fungal strains have been extensively examined in the literature. Peptide and non-peptide-origin components of the animal venoms, including phospholipase A2s (PLA2s), L-aminoacid oxidases (LAAOs), hyaluronidases (HYAs), metalloproteinases, serotonin, histamine, citrate, nucleosides and inorganic ions, exhibit potent antimicrobial activities against different species of microbes [38-40]. In a nutshell, animal venoms have big potent for treatment of cancer and infectious diseases.

Current study is the first comprehensive report of the antimicrobial properties of TD6. TD6 demonstrated dose-dependent antimicrobial activity against four bacterial and two fungal strains. *E. faecalis* (MIC: 60.98 ± 4.87 µg/mL) and *S. aureus* (MIC: 85.21 ± 7.02 µg/mL) are gram-positive bacterial strains which displayed strong sensitivity to TD6. Also, TD6 showed significant antimicrobial activity against *P. aeruginosa* (MIC: 89.89 ± 6.11 µg/mL) and *K. pneumonia* (MIC: 75.00 ± 6.55 µg/mL). Hence, TD6 has a great antimicrobial potential against both gram-negative and gram-positive bacterial strains. Similarly to strains of bacteria tested in this study, fungal strains also exhibited strong sensitivity to TD6. The MIC values for *C. albicans* and *C. utilis* of TD6 were calculated as 64.12 ± 3.54 µg/mL and 74.64 ± 4.31 µg/mL, respectively. This extract displayed antimicrobial activity in *P. aeruginosa*, *E. faecalis, K. pneumonia, S. aureus C. utilis* and *C. albicans.*

4. Conclusion

Venoms of animals have been used as a therapeutic and healing agent in recent years. Especially, some cancer cell line and microbial strains have been treated with their alcoholic and aqueous extracts *in vitro* studies. In conclusion, we reported the results of antiproliferative activities of TD6 venom on colon cancer cell line and liver cancer cell line, and antimicrobial activity on two pathogenic bacteria and two fungal strains. The findings of this study suggest the therapeutic potentials of TD6 for cancer and infectious diseases. Further investigation of the biological activity of TD6 may contribute to its use as an alternative agent in the treatment of cancer and infectious diseases. However, this study has certain limitations. The in vitro nature of the experiments limits the direct applicability of the findings to the clinical practice. The methods by which TD6 exerts its effects, notably its interaction with specific biochemical pathways, have yet to be fully understood. Moreover, the study did not look at potential side effects or toxicity on noncancerous cells, which is crucial for determining the safety profile of TD6. In addition, broadening the study to include various cancer types and microbial strains might offer a more comprehensive understanding of TD6's biological activity. Furthermore, investigating the possible combination of TD6 with existing therapies could increase its efficacy and provide new avenues for treatment strategies.

Ethics in Publishing

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

Author Contributions

NGT conceptualized, design and coordinated the investigation. NGT and ÖK performed all the experiments. NGT, ÖK and AÖ analyzed the data. ÖK created the first draft of the manuscript which was edited by all the authors.

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