



In vitro cytotoxic activity of *Tarantula cubensis* alcoholic extract on different human cell lines

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Abstract

Spider venoms are known to have great potential for their antimicrobial, antifungal, and antitumor activities due to their rich peptide content. *Tarantula cubensis* alcoholic extract Theranekron[®] is a homeopathic remedy and it is frequently used in veterinary treatments. Specifically, the anti-inflammatory, antitumor and wound healing effects of Theranekron[®] provide a wide range of use in animal treatments. More importantly, it can reduce the growth rate of canine mammary tumors. This effect shows that Theranekron[®] can be a potential candidate for cancer treatment. In this study, the cytotoxic effect of Theranekron[®] was evaluated in four different human cancer cell lines and one non-cancerous human cell line. It was aimed to provide a foundation for further studies by conducting an *in vitro* screening for the cytotoxic effect of Theranekron[®]. Results showed that Theranekron[®] has a high cytotoxic effect on human breast cancer cell lines. On the other hand, the rate of cytotoxicity was found to be relatively lower on human small cell lung cancer, glioblastoma and human prostate cancer cell lines as compared to breast cancer cells. These results indicated that *T. cubensis* alcoholic extract might be utilized as an anticancer bio-therapeutic and should be further examined for its potential.

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1. Introduction

Venoms are described as poisonous secretions of animals and they contain a variety of chemicals, enzymes and distinctive bioactive peptides [1]. In general, venoms cause severe damage to cells and organs. However, they also hold great potential for novel pharmacological approaches and as medicine enhancements because of the bioactive peptides and chemicals [1,2]. Peptides that are obtained from various venoms are already being investigated as pain killers, against diabetes, cardiovascular diseases and cancer [3,4]. Spider venoms are rich in different peptides. In several studies, it was demonstrated that these peptides have antimicrobial, antifungal, cytolytic and antitumor activities [1,5].

Cancer is a global health problem [6], and it is crucial to find novel potential targets for cancer therapeutics. Cytotoxic effects of several venoms on cancer cells were investigated and provided promising results [4,7]. Theranekron[®] is the alcoholic extract of *Tarantula cubensis*. It is a homeopathic solution abstained from *T. cubensis* being processed and diluted in 60% alcohol [8]. *T. cubensis* belongs to the Theraphosidae family and its venom known to contain a variety of enzymes

[9]. Theranekron[®] is used as a veterinary remedy for the treatment of several indications as a commercial extract. It is used mostly for its anti-inflammatory effect. It is also reported to be effective on lesions, endometriosis and decrease the growth rate of canine tumors in veterinary clinical studies [10-12].

There is a number of maladies that Theranekron[®] was used for treatment on different animal groups. One of the most common applications is related to its wound healing effect. In one of these studies, 20×20 mm of full thickness wound composed on 7 months bulls and then treated with 6 mL Theranekron[®] subcutaneously. The results demonstrated that, the application of Theranekron[®] can quicken the epithelialization of the wounds [13]. In another study, the wound healing effect of Theranekron[®] was observed on rats with open wounds. Similarly, it was shown that Theranekron[®] was found to be stimulating on epithelialization of the wounds and fastens the wound healing [14]. *T. cubensis* alcoholic extract was shown to be effective for the treatment of oral lesions caused by Bluetongue disease in Holstein cattle. During an outbreak in Iran, Theranekron[®] was used in a controlled clinical trial, and it was found to be a potential application against the foot-and-mouth disease (FMD) of cattle. Finally,

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its effect was also observed against papillomatosis, which can be a widespread problem in cattle [15–17]. Along with these effects, it was shown that Theranekron® could be used for neutralizing the damaging impacts of aflatoxins [18]. Another beneficial use of Theranekron® was its peripheral nerve healing effects caused by nerve injury on rats. It was demonstrated that alcoholic extract of *T. cubensis* reduced the axon and myelin damage, which could be linked to its modifying effects on pro-inflammatory cytokines like TNF- α , IL-1 and IL-6 [19].

Along with many other applications, although the antitumor effect was demonstrated in clinical veterinary studies, the anticancer effect of Theranekron® on different cancer cell lines was not fully investigated. Only a few studies demonstrated that it holds a great potential [9,20], but expanded *in vitro* studies specifically on human cancer cell lines are still needed. The aim of this study is to investigate the cytotoxic effect of Theranekron® on different cancer and non-cancerous cell lines in order to provide a foundation for further studies.

2. Materials and Methods

2.1. Chemicals and reagents

Theranekron® (alcoholic extract of *T. cubensis*) purchased by Richter Pharma AG (Wels, Austria), was obtained from the local veterinary clinic with the permission of the veterinary physician. As described from Richter Pharma AG, it is prepared by processing the whole spider and diluted in alcohol. It is commercially sold in 50 mL bottles, each mL contains 1 mg alcoholic extract (1:100) from *T. cubensis* in an alcoholic solution. Other chemicals and reagents used for this study are given within the related sections.

2.2 Cell lines and cell culture conditions

For this study, five different cell lines consist of four different cancer, and one non-cancerous cell line were used to evaluate the cytotoxic effect of Theranekron®. Cell lines and culture conditions were summarized in Table 1.

Table 1. List of Cells and Cell Culture Conditions. ATCC, American Type Culture Collection; MEM, Eagle's Minimum Essential Medium; RPMI-1640, Roswell Park Memorial Institute medium 1640; FBS, fetal bovine serum

Cell line	Source	Medium	FBS (%)	L -glutamine (%)	penicillin-streptomycin
Human embryonic kidney cell line, HEK-293	ATCC CRL-1573	MEM	10	1	1
Multidrug resistant variant of the human small cell lung cancer cell line, H69AR	ATCC CRL-11351	RPMI-1640	20	1	1
Glioblastoma cell line, T98G	ATCC CRL-1690	MEM	10	1	1
Human prostate cancer cell line, PC3	ATCC CRL-1435	RPMI-1640	10	1	1
Human breast cancer cell line, MCF-7	ATCC HTB-22	RPMI-1640	10	1	1

All the cell lines were grown as monolayers in 75 cm² polystyrene flasks (Corning Life Sciences, UK), and maintained at 37°C in a humidified atmosphere with 5 % CO₂. Cell morphologies and growth rates of all the cell lines were monitored daily under the inverted microscope (Zeiss Primovert, Germany) and the cells were passaged when reached 90 % confluence.

2.3 MTT cell viability assay

Trypan blue dye exclusion test was used to determine cell viability and the cell numbers for all the cell lines. Countess Automated Cell Counter (Countess, Thermo

Fisher Scientific, Massachusetts, USA) was used for measuring the cell viability and cell numbers.

The cytotoxic effect of Theranekron® was evaluated via 3 - (4 , 5 - dimethylthiazol - 2 - yl) - 2 , 5 - diphenyltetrazolium bromide (MTT) assay [21]. Cells were seeded at 1×10⁴ cells/well, in a final volume of 200 μ L, in 96-well flat-bottomed plates. After 24h incubation, cells were treated with increasing concentrations (10-100 μ g/mL) of Theranekron®. Plates were then incubated at 37°C in a 5% CO₂ incubator for 24, 48 and 72 h.

At the end of the incubation, 20 μL (10% of the final volume) of MTT was added to each well. Plates were then incubated for 4 h at 37°C. Then, the medium was removed, and the formed formazan crystals were dissolved in 100 μL of dimethyl sulfoxide (DMSO). The absorbance of each well was measured at 570 nm against a reference wavelength at 690 nm using a microplate reader (TECAN PRO200, Männedorf, Switzerland). IC_{50} values were calculated from the mean of the triplicate experiments.

2.4 Statistical Analysis

Cytotoxicity experiments were carried out in triplicates, and GraphPad Prism 5.0 software (GraphPad Software, San Diego, USA) was used for data analysis. For significant values, a one-way analysis of variance test (ANOVA) followed by Dunnett's t-test. Statistically significant data were those with a p-value ≤ 0.05 . IC_{50} values were calculated via Biosoft CalcuSyn 2.1 software (Ferguson, MO, USA).

3. Results and Discussion

In this study, first the effect of Theranekron[®] on human embryonic kidney HEK-293 cells was determined. For this aim, HEK-293 cells were treated with the increasing concentrations (10-100 $\mu\text{g}/\text{mL}$) of

Theranekron[®] for 24, 48 and 72 h, and then MTT cell viability assay was performed. It was found that the Theranekron[®] treatment decreased the cell viability of HEK-293 cells specifically at 72h (Figure 1, 2). The IC_{50} value of Theranekron[®] was found as 88.3 $\mu\text{g}/\text{mL}$ at 72h for HEK-293 cell line. Acquired results in this study were consistent with two other studies that showed the cell viability levels of HEK-293 cell line treated with Theranekron[®] [9], [20].

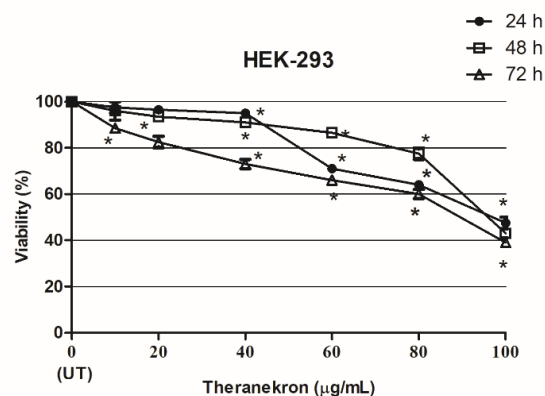


Figure 1. Time and concentration dependent effect of Theranekron[®] on human embryonic kidney HEK-293 cells (* $P < 0.05$ compared to the untreated control, UT: Untreated control)

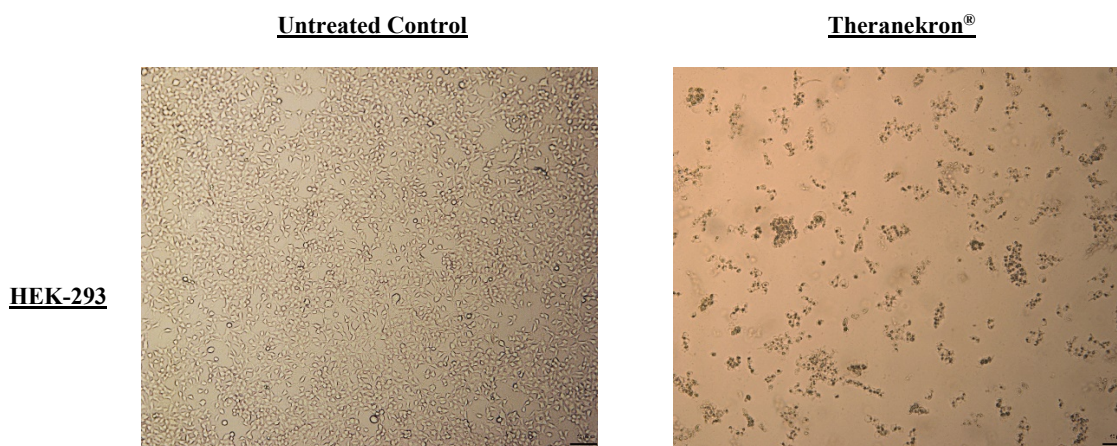


Figure 2. Images of HEK-293 cells treated with Theranekron[®] with the IC_{50} value of 88.3 $\mu\text{g}/\text{mL}$ for 72h. Reduced viability and aberrant adhesion morphology were observed on treated cells as compared to the untreated control group (Magnification 10X).

For the cancer cell lines, the effect of Theranekron[®] was evaluated on glioblastoma cell line T98G, human small cell lung cancer cell line H69AR, human prostate cancer cell line PC-3 and human breast cancer cell line MCF-7 with the increasing concentrations (10-100 $\mu\text{g}/\text{mL}$) of Theranekron[®] for 24, 48 and 72 h. Obtained MTT cell viability assay results indicated that Theranekron[®] is highly cytotoxic for breast cancer cell

line MCF-7 in a time- and concentration-dependent manner ($p \leq 0.05$). Its effect can be clearly observed after 48h (Figure 3, 4). The IC_{50} value of Theranekron[®] was found as 94.7 $\mu\text{g}/\text{mL}$ at 72h for MCF-7 cells.

While Theranekron[®] was found to be highly cytotoxic for breast cancer cell line MCF-7, its effect was found to be relatively lower for human small cell lung cancer

H69AR, glioblastoma T98G and human prostate cancer PC3 cell lines. IC₅₀ values were calculated as 278 µg/mL for H69AR, 295 µg/mL for PC3 and 118.9 µg/mL for T98G cell lines at 72h. As a difference,

decreased cell viability was observed after 24h for these three cell lines while it was observed after 48h for MCF-7 (Figure 3, 4).

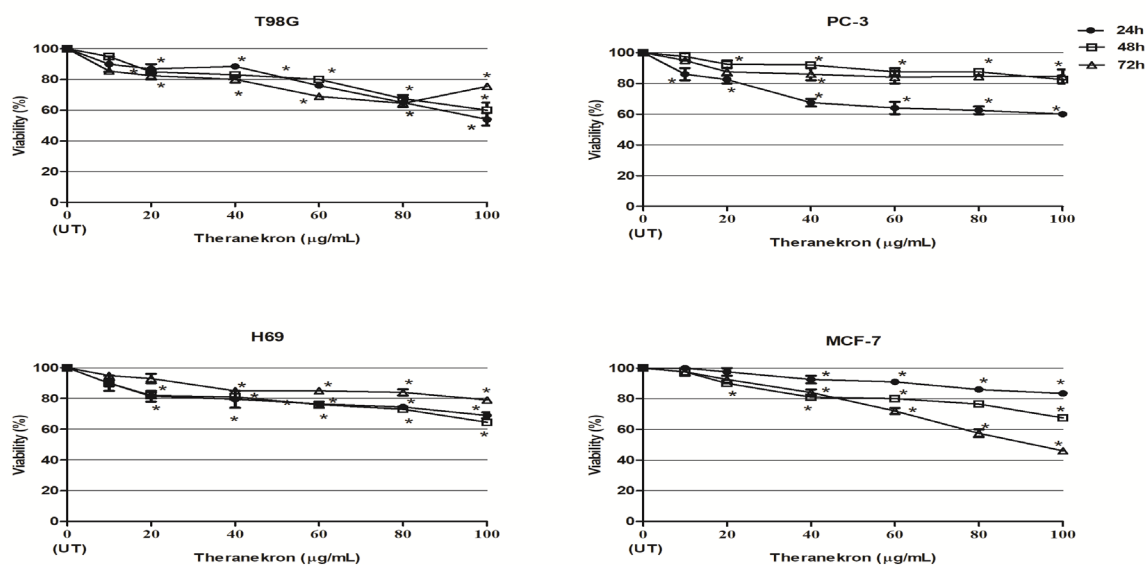


Figure 3. Time and concentration dependent effect of Theranekron® on glioblastoma T98G, human prostate cancer PC3, human small cell lung cancer H69AR and breast cancer cell line MCF-7 (*P < 0.05 compared to the untreated control, UT: Untreated control)

Spider venoms are known to have an anticancer perspective, via tempering the cell cycle, several cell death mechanisms, ion channels and receptors [22]. *T. cubensis* alcoholic extract, commercially named Theranekron®, is commonly used as a veterinary remedy [23]. It is known to have several therapeutic effects such as anti-inflammatory, antitumor and wound healing. These therapeutic effects make Theranekron® hold great potential for treatment [24–26]. More importantly, it is known to have a strong antitumor activity on canine mammary tumors. It was shown that *T. cubensis* extract supports the inhibition of tumor growth, thickening of tumor capsules and demarcation of the surrounding tissue. It was also demonstrated that it has an anti-inflammatory effect on lymph nodes [11]. In light of these, cytotoxic activity screening of Theranekron® is crucial to comprehend its activity and potential for also human cancers. The apoptotic activity of Theranekron® was investigated only in few other studies. In one study, it was shown that the *T. cubensis* alcoholic extract caused DNA fragmentation and remarkable cell death in MCF-7 and HN5 cell lines as a result of over activation of apoptosis [9]. It was explained that Theranekron® was affected the adhesion of the cells while decreasing the proliferation levels of the MCF-7 and HN5 cells. In another study, it was determined that alcoholic extract of *T. cubensis* induces apoptosis on MCF-7 cell line as

a result of increased tumor necrosis factor- α (TNF- α) and transforming growth factor- β (TGF- β) levels. Both of these factors are crucial for the proliferation, survival and cell death levels of the cells [20].

It was shown that although *T. cubensis* alcoholic extract has a cytotoxic effect on HEK-293, it is considerably more toxic for cancer cells, specifically for MCF-7 cells. Similar results were also shown in another study stating that although Theranekron® found to be slightly cytotoxic to HEK-293, its effect was higher on breast cancer cell line MCF-7 and human head and neck cancer cell line HN5 [9]. This phenomenon is explained as a result of the hyperactivation of apoptosis on these cell lines. To explain the reason for the different levels of cytotoxicity between the normal and cancer cells, it was stated that the rapid metabolism rate of the cancer cells causes increased vulnerability against the extract. When Theranekron® was tested on canine mammary tumors, immunohistochemical staining of these tumors revealed that Bcl-2 and Ki-67 expression levels were significantly affected after the Theranekron® treatment [27]. Bcl-2 expression levels are crucial for apoptosis regulation, specifically for human breast cancer [28]. Bcl-2 expression is known to be increased in human mammary tumors as well as in canine mammary tumors [29].

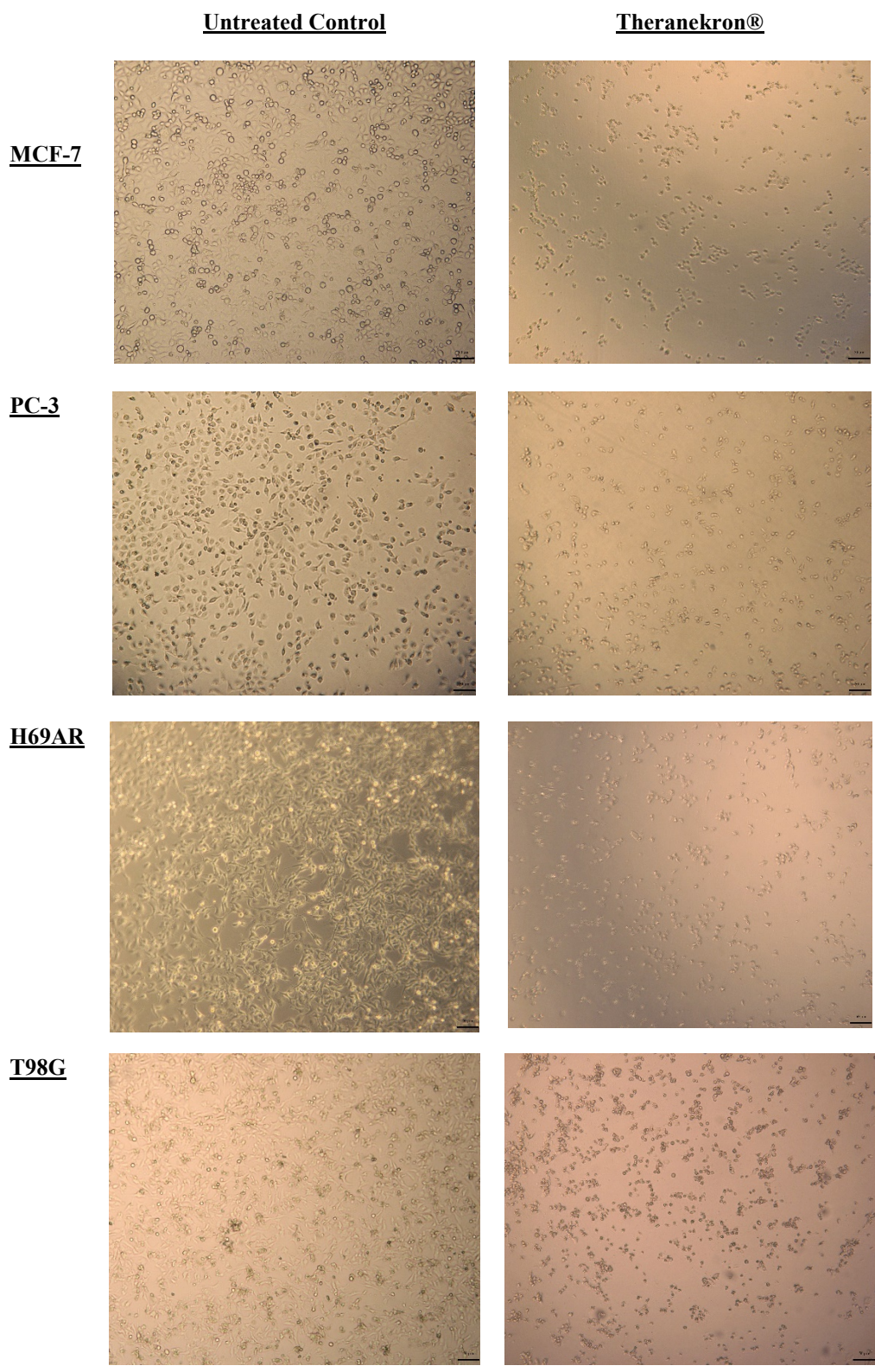


Figure 4. Images of cancer cell lines treated with Theranechron® with IC₅₀ values of each cell line for 72h. Reduced viability and cell number were observed highly on MCF-7 cells compared to other cell lines. While reduced viability was observed on all treated cells as compared to the untreated control groups (Magnification 10X).

For the canine tumors, it was found that Bcl-2 expression was significantly decreased in the Theranekron® treated tissues [27]. Considering the similarities between canine and human mammary tumors [29], the effect of Theranekron® on MCF-7 cells can be considered as expected.

When the comparison was made among the cancer cell lines, it was found that Theranekron® was more effective to MCF-7 cells, followed by T98G and finally H69AR and PC-3 cells. Since it was already demonstrated that *T. cubensis* alcoholic extract was effective on canine mammary adenocarcinomas [11] and was shown to have potential against endometriosis in a rat model [25], it can be hypothesized that Theranekron® has higher potential against estrogen-dysregulation related diseases. Endometriosis is known to be an estrogen-dependent disease [30,31] and MCF-7 cell line is also known as an estrogen receptor positive cell line [32]. Thus, higher levels of cytotoxicity of Theranekron® on MCF-7 cell line can be explained with this relation. Among the other screened human cancer cell lines, the IC₅₀ value of T98G cell line was 118.9 µg/mL at 72h for *T. cubensis* alcoholic extract. When looked at the commonalities, there are number of studies that explains that tamoxifen (estrogen receptor modulator used against ER positive breast cancers) can constrain the proliferation of malignant ER(-) glioma cells, and reports the expression and function of ER-related receptors in T98G cell line along with other cell lines [33,34]. On the other hand, the cytotoxicity levels were found relatively low for H69AR and PC-3 cells. Considering the complex molecular mechanisms of different cancer cell lines, further studies might be more enlightening on the effect of *T. cubensis* alcoholic extract on other cancer cells as well.

4. Conclusion

In this report, the cytotoxic effect of alcoholic extract of *T. cubensis* obtained from the local veterinary clinic was evaluated in five different cell lines. One non-cancerous human cell line and four different human cancer cell lines were used for *in vitro* screening for the cytotoxic effect of Theranekron® with the increasing concentrations (10-100 µg/mL). It was aimed to offer a basis for further studies on the anticancer effect of *T. cubensis* alcoholic extract.

Results showed that Theranekron® decreased the cell viability of non-cancerous cell line HEK-293 with an IC₅₀ value of 88.3 µg/mL at 72h. Among the cancer cell lines, the highest cytotoxic effect of *T. cubensis* extract was observed on human breast cancer cell line MCF-7.

The IC₅₀ value of Theranekron® was calculated as 94.7 µg/mL at 72h in MCF-7 cells. Its cytotoxic effect was apparent after 48h of treatment. However, the cytotoxicity rate of Theranekron® was noted relatively lower on the glioblastoma cell line T98G, human small cell lung cancer H69AR, and human prostate cancer cell line PC-3 measured up to MCF-7 cell line. Among these 3 cell lines, the highest effect was observed on T98G with the IC₅₀ value of 118.9 µg/mL at 72h. It is followed by H69AR and finally PC-3 cell lines with the IC₅₀ values of 278 µg/ml and 295 µg/mL respectively.

In conclusion, the anticancer effect of *T. cubensis* venom has a great prospect not only in canine tumors but also in human cancer cell lines. It can be also promising for other cancer cell lines, however, its effect should be further studied. Based on this conclusion, it can be suggested that by describing the molecular mechanisms and further understanding of the composition of *T. cubensis* extract, it can be considered as a novel bio-therapeutic for cancer in the future.

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Conflicts of interest

The author(s) declare that they have no conflict of interest.

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