Exp Biomed Res 2025; 8(1): 14-23 **https://doi.org/10.30714/j-ebr.2024.230**

Experimental Biomedical Research *Original article*

Investigation of the antitumoral activity of tarantula cubensis extract on human hepatocelular cancer cells

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ABSTRACT

Aim: To investigate the antitumor effects of tarantula cubensis alcoholic extract (TCE) on the human hepatocellular cell line (HepG2).

Methods: Various concentrations (1, 5, 10, 20, 50, 100, 250, and 500 µg/ml) of TCE were applied to HepG2 cells, which were then incubated for 24, 48, and 72 hours. Analysis was performed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays. Immunohistochemistry and apoptosis (Ki-67) analysis were conducted on cells treated with 1 and 5 µg/ml TCE for 24, 48, and 72 hours.

Results: The most appropriate doses of TCE for the HepG2 cell line were found to be 5 µg/ml at 24 hours and 1 µg/ml at 48 and 72 hours. Ki-67 H-SCOR in HepG2 cells treated with TCE significantly decreased compared to the control group (p<0.001).

Conclusion Our study suggests that TCE may alter normal cancer physiology by stimulating apoptosis in HepG2 cells via the Ki-67 pathway, leading to cell death. Therefore, TCE has the potential to provide a new perspective in the treatment of HCC.

Keywords: Apoptosis, HepG2, hepatocelluler cancer, Ki-67, tarantula cubensis alcholic extract, theranekron.

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Received: 2024-09-04 / Revisions: 2024-11-03

Accepted: 2024-11-24 / Published: 2025-01-01

1. Introduction

Hepatocellular cancer (HCC) constitutes 90% of liver cancers worldwide. Although knowledge about the pathophysiology and underlying causes of HCC has improved in the last decade, this has not yet been fully implemented into clinical practice. HCC is currently the fifth most common cancer and the third leading cause of cancer-related deaths, particularly in East Asia and sub-Saharan Africa [1,2].

A large number of cancer-associated genes have been identified in human cancers [3]. Ki-67 is one such gene involved in cell growth and proliferation. Ki-67 has been found to be an independent factor for overall and disease-free survival after resection of the primary tumor in HCC, and its presence may influence the decision to administer adjuvant therapy [4,5].

Peptides, which are the main compounds of spider venoms, have a wide range of bioactivities, including antimicrobial, antifungal, antiparasitic, antiarrhythmic, analgesic,

cytolytic, hemolytic, and antitumor effects [6]. Tarantula cubensis (T. cubensis), also known as the Cuban tarantula, is a large spider belonging to the Theraphosidae family [7]. T. cubensis venom (TCE) has been identified as a useful homeopathic remedy for a wide variety of conditions, including animal wounds and tumors. "Theranekron" is the commercially available alcoholic extract of T. cubensis and is used for its antitumor effects in veterinary medicine [8,9]. The antitumor effects of Theranekron® have also been demonstrated in vitro for human breast and head & neck cancers [10]. However, studies on the effect of Theranekron® on HCC, particularly those supported by immunohistochemistry, are lacking or limited in the literature. Therefore, the aim of this study was to investigate the anti-proliferative effect of Theranekron® on a hepatocellular cell line (HepG2) by measuring Ki-67 expression.

2. Materials and methods

2.1. Agents/Chemicals and Cell culture: In this research, HepG2 cells and TCE (Theranekron D6®, an alcoholic extract of T. cubensis, Enj. Sol. Richter Pharma, Austria) were used with the veterinarian's approval. According to information provided by Richter Pharma AG, TCE is made by digesting the entire spider and diluting it with alcohol. Each mL includes 1 mg of an alcoholic extract (1:100) from T. cubensis in an alcoholic solution, and it is commercially available in 50 mL bottles. The American Type Culture Collection (ATCC) provided the cell lines.

2.2. Cell Lines and Cell Culture Conditions: In this study, HepG2 (ATCC: HB-8065, Manassas, USA) cells were used as the human hepatocellular cancer cell line. Cells stored in the nitrogen tank were thawed under appropriate conditions, and cell density and viability were counted on a Thoma slide using trypan blue. The cells were then grown at a density of 2.0 x 10^5 - 3.0 x 10^5 in 25 cm^2 cell culture flasks with heat-inactivated 10% fetal bovine serum (FBS) (Sigma-Aldrich, Schnelldorf, Germany) and 1% penicillin-streptomycin (Capricorn Scientific, Ebsdorfergrund, Germany) supplemented with 5 mL DMEM (Pan-Biotech, Aidenbach, Germany) medium. Cell morphologies and growth rates of all the cell lines were monitored daily under an inverted microscope (CKX53, Olympus, Japan), and the cells were passaged when they reached 80% confluence. Cells with 80% confluences were passage into 75 cm^2 polystyrene flasks (Corning Life Sciences, UK) using 0.25% trypsin EDTA and maintained at 37°C in a humidified atmosphere with 5% CO_2.

2.3. MTT Test Assay: The trypan blue dye exclusion test was used to determine cell viability and count the cell numbers for all the cell lines. The TC20 Automated Cell Counter (Biorad Cell Counter, Hercules, CA, USA) was used for measuring cell viability and cell numbers. The cytotoxic activity of TCE was evaluated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide test (MTT) (Serva, Germany). The MTT assay is a colorimetric assay for assessing cell metabolic activity. NAD(P)H-dependent cellular oxidoreductase enzymes may, under defined conditions, reflect the number of viable cells present. HepG2 cells were seeded in 96-well culture flasks at a cell density of 5x10^4 cells/ml in each well with 100 µL of culture medium. The control group contained only pure HepG2 cells. After 24 hours of incubation, cells were treated with increasing concentrations (1, 5, 10, 20, 50, 100, 250, and 500 µg/ml) of TCE. Plates were then incubated at 37°C in a 5% CO_2 incubator for 24, 48, and 72 hours. After incubation, 10 µL of MTT solution was added to each well, and the plates were incubated for 4 hours at 37°C in 5%

CO_2. After incubation, 200 µL of DMSO (dimethyl sulfoxide) was added to dissolve the formazan salts. The absorbance value was measured at 570 nm using a nanodrop spectrophotometer (Epoch BioTek Instruments, Inc., Highland Park, USA). In the experimental sets, cells were seeded in triplicate for each drug dose, and the experiment was repeated three times independently. Cells without TCE extract and those with the solvent of TCE extract were used as controls. IC50 values were calculated from the mean of triplicate experiments.

2.4. Preparation of Cells for Immunocytochemical Methods: When the cells, which were allowed to proliferate under appropriate conditions, reached 80% confluence on the flask surface, they were treated with trypsin-EDTA and removed from the flask base. Cells stained with trypan blue dye were counted three times with the help of a Thoma slide and transferred to the nutrient environment. Cells were suspended in the previously prepared medium. Round 10 mm coverslips were placed in 24-well culture plates. The coverslips were sterilized by UV for at least 4 hours. To ensure cell adhesion to the glass coverslips, 50 µL of FBS was added to the wells and kept in an oven at 37°C for 30 minutes. FBS was withdrawn with a micropipette, and a mixture of 4x10^4 cell medium was added to each well until a total volume of 0.5 mL. Cell adhesion was achieved over 24-48 hours. The two most effective doses of TCE (1 and 5 µg/ml), determined by the MTT method, were applied to the cells. The cells, which were kept in the oven for 24, 48, and 72 hours in separate wells, were then discarded, and the contents were fixed with 4% paraformaldehyde for 30 minutes.

2.5. Investigation of Ki-67 Signaling Path Activity by Immunocytochemistry Method: The streptavidin-biotin complex (Strept-ABC) immunoperoxidase technique was used in immunocytochemical studies. This method ensures the specific binding of antibodies to antigens present in the cells, providing information about their amounts in the cells. After fixation, the cells were washed with PBS for 2x5 minutes. PBS solution containing 1% Triton X-100 was added to the cells, and permeabilization was performed by incubating the plates at +4°C for 5 minutes. After the permeabilization solution was removed, the cells were washed with PBS for 2x5 minutes. To suppress endogenous peroxidase activity, 4% H_2O_2 prepared in PBS was added, and the cells were kept at room temperature for 5 minutes. Non-specific binding was prevented by applying blocking solution for 1 hour at room temperature to coverslips washed with PBS for 2x5 minutes. After this process, the Ki-67 (anti-human monoclonal primary antibody) primary antibody was applied separately for all doses and extract applications without washing. The cells were incubated with the primary antibody, diluted 1/200 with PBS, at +4°C overnight. The cells were then washed with PBS for 3x3 minutes, and the appropriate biotinylated secondary antibody (rabbit polyclonal secondary antibody) was applied for 30 minutes to bind to the primary antibody. After washing the cells with PBS for 3x3 minutes, they were exposed to the streptavidin peroxidase enzyme complex for 30 minutes, allowing the enzyme to bind to biotin. After the coverslips were washed again with PBS for 3x3 minutes, DAB was applied to reveal the visible immune reaction. Mayers' hematoxylin was used for background (counter) staining. Coverslips were mounted on slides with mounting medium. Stained cell samples were examined with a Binocular Microscope (Carl Zeiss AX10) light microscope. Ki-67 positive cells were detected, and cells were examined in all preparations for 1 center, 5 periphery, a total of 6 regions. 100 cells were counted in terms of

immunoreactivity, and data for statistical analysis were created. H-Score Analysis was performed with "0 = None, 1 = Poor, 2 = Moderate, 3 Moderate to Intense, 4 = Intense" numerical data.

2.6. Statistical Analysis: Cytotoxicity experiments were carried out in triplicate. Log-transformed drug concentrations were plotted against concentration-response, and half-maximum inhibitory concentration (IC50) values were determined using GraphPad Prism 5.0 (GraphPad Software, San Diego, USA) response variable slope normalized with a non-linear regression log. Data with a *p*-value of ≤ 0.05 were considered statistically significant.

3. Results

3.1. Determination of Effective Anti-Proliferative Dose on HepG2 Cells: To determine the most effective anti-proliferative dose of TCE on HepG2 cells, eight different doses (1, 5, 10, 20, 50, 100, 250, and 500 µg/ml) of TCE were applied, and the cytotoxic effect was assessed using the MTT test at 24, 48, and 72 hours. The IC50 values for these time points were calculated (Table 1). It was found that the most appropriate dose of TCE for the HepG2 cell line was 5 µg/ml at 24 hours and 1 µg/ml at 48 and 72 hours. At 24 hours, the 5 µg/ml dose had an effective viability value of 95.2181%, while the 1 µg/ml dose had a viability value of 98.4539% at 48 hours and 96.0557% at 72 hours (Figure 1, Figure 2). TCE exhibited no activity and had a toxic effect at doses of 10 µg/ml and above on the HepG2 cell line (Table 1). The most effective doses of TCE for inhibiting HepG2 cell growth were 5 µg/ml after 24 hours, and 1 µg/ml after 48 and 72 hours. Ki-67 H-SCOR analysis showed a significant reduction in treated HepG2 cells compared to the control group ($p<0.001$)

3.2. Immunocytochemical Evaluation (ICC): To determine the intensity of Ki-67

Table 1. After application of TCE in HepG2 cell line, % cell viability at 24-48-72 hours.

Drug Concentration (µg/ml)	0/control	1	5	10	20	50	100	250	500
%Survival 24 hour	100	92,05	**95,21**	77,32	60,31	49,92	34,76	28,53	11,27
%Survival 48 hour	100	**98,45**	91,93	75,50	55,42	47,92	31,54	24,11	10,55
%Survival 72 hour	100	**96,05**	89,21	71,66	62,46	49,39	34,76	27,27	14,25
Std deviation (Stdv)	0,240	0,189	0,187	0,247	0,261	0,211	0,132	0,098	0,099
According to the percentage of vitality (Stdv)	17,59	13,87	13,72	18,16	19,13	15,51	9,721	7,212	7,31

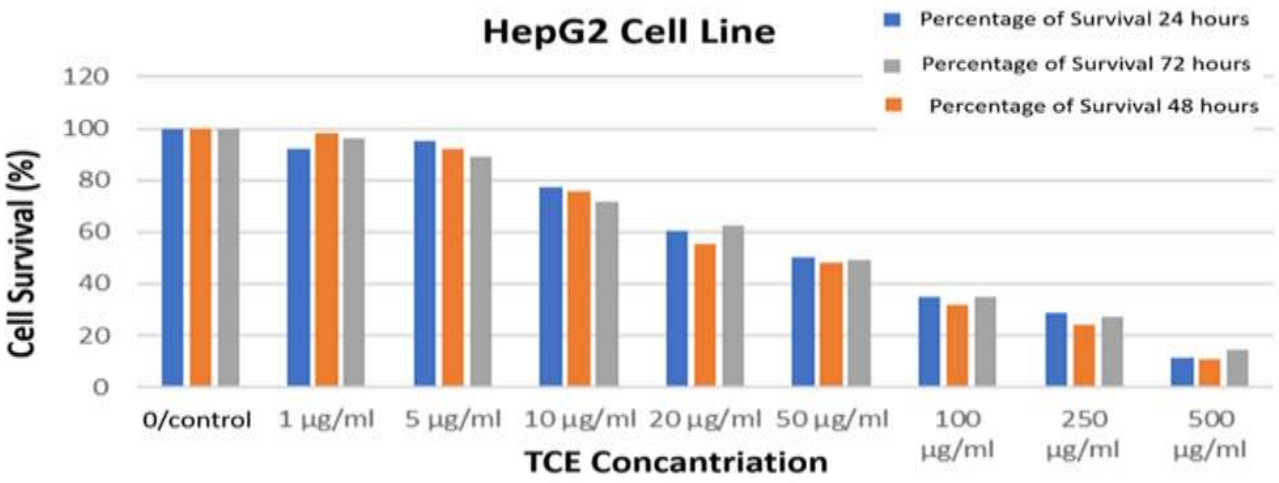

Figure 1. MTT result graph of TCE application on different doses and times for HepG2 cell.

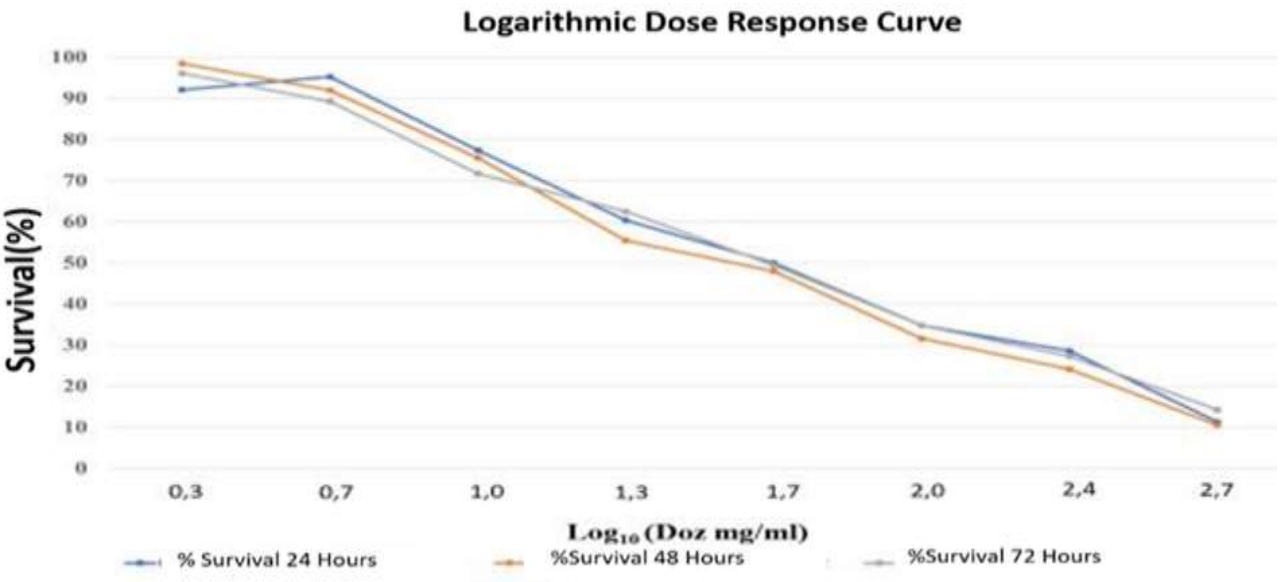

Figure 2. After determining the effective doses of TCE in HepG2 cell line as 1 and 5 µg/ml, logarithmic graph immunocytochemistry study of 24.-48-72 hours MTT viability-dose ratio with these doses.

Table 2. Ki-67 H-SCOR values in HepG2 cells incubated with 5 µg/ml TCE for 24 hours and 1 µg/ml

Variablaes	**24 hour (χ^2: 15.39) (p<0.001)**	**48 hour (χ^2: 64.53) (p<0.001)**	**72 hour (χ^2:56.54) (p<0.001)**
HepG2 +TCE ki-67	+	+/-	+
HepG2 ki-67	++	+++	+++

TCE for 48 and 72 hours ((+): weak, (++): middle, (+++): strong)

immunocytochemical staining in TCE-treated HepG2 cells, 100 cells were counted in five different areas, and immune positivity was categorized as strong, moderate, or weak. For HepG2 cells, Ki-67 immunocytochemistry staining was performed at doses of 5 µg/ml for TCE at 24 hours and 1 µg/ml for 48 and 72 hours. Ki-67 immunostaining in TCE-treated HepG2 cells was weak at all-time points. In contrast, the control group showed moderate staining at 24 hours and strong staining at 48 and 72 hours. Ki-67 H-SCOR in HepG2 cells treated with TCE was significantly decreased compared to the control group (p<0.001, Table 2).

In the study, 75 cells in the control group were immunostained with Ki-67, and 25 cells were not immunostained out of 100 cells examined at 24 hours. When TCE was administered at a dose of 5 µg/ml, the number of Ki-67 immunostained cells decreased to 48. A significant relationship was found between TCE application at 24 hours and the number of viable cells (immunostained with Ki-67) ($\chi2$ = 15.39, p<0.001) (Table 3). At 48 hours, 90 cells in the control group were immunostained with Ki-67 out of 100 cells examined. When TCE was administered at a dose of 1 µg/ml, the number of Ki-67 immunostained cells decreased to 35, while the number of non-viable cells increased to 65. A statistically significant relationship was found between TCE application at 48 hours and the number of viable cells ($\chi2$ = 64.53, p<0.001) (Table 3). At 72 hours, 92 cells in the control group were immunostained with Ki-67 out of 100 cells examined. When TCE was applied at a dose of 1 µg/ml, the number of immunostained cells

Table 3. Evaluation results of ki-67 immunocytochemistry staining data of HepG2 cell line which was applied 5 µg/ml dose at 24th hour and 1 µg/ml dose TCE at 48th hour and 72nd hour and control group.

24 Hour TCE HEPG2 Ki-67 ICC results	**Survival (n=100)**		**χ^2 value**	***p***
	(+)	**(-)**		
Control	75 (%75)	25 (%25)	15.39	*<0.001*
TCE 5 µg/ml dose	48 (%48)	52 (%52)		
48 Hour TCE HEPG2 Ki-67 ICC results	**Survival (n=100)**		**χ^2 value**	*p*
	(+)	(-)		
Control	90 (%90)	10 (%10)	64.53	*<0.001*
TCE 1 µg/ml dose	35 (%25)	65 (%75)		
72 Hour TCE HEPG2 Ki-67 ICC results	**Survival (n=100)**		**χ^2 value**	*p*
	(+)	(-)		
Control	92 (%92)	8 (%8)	56.54	*<0.001*
TCE 1 µg/ml dose	42 (%42)	58 (%58)		

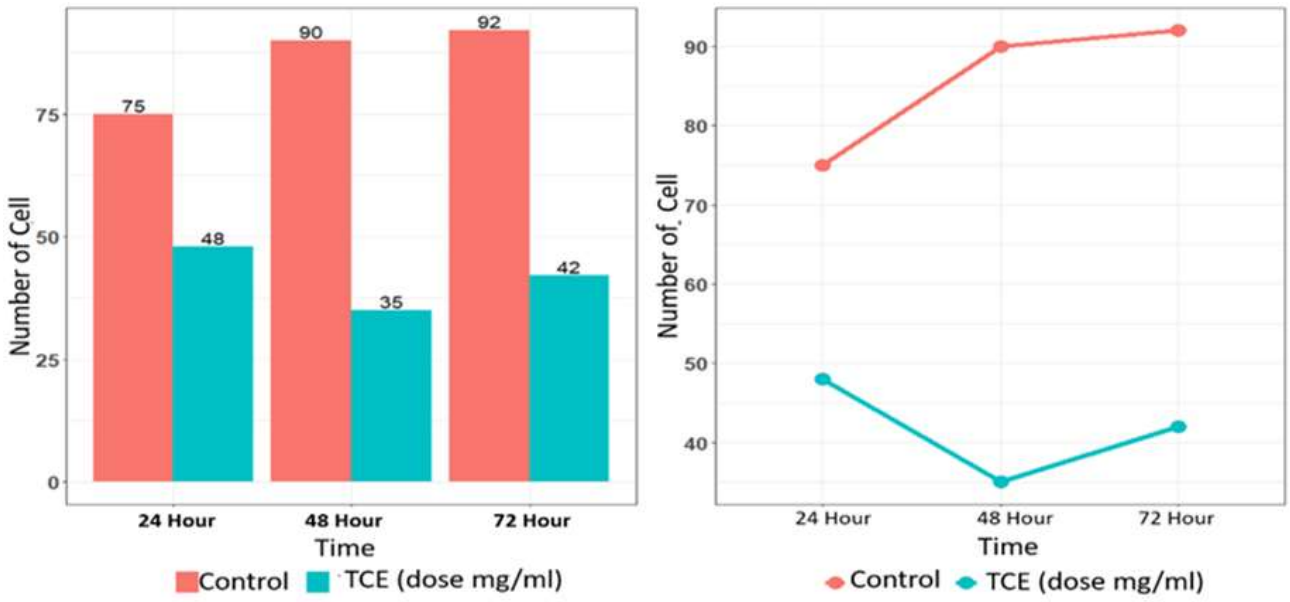

Figure 3. The number of cells stained with ki-67 at different times according to the drug groups given.

decreased to 42, and the number of non-viable cells increased to 58. A statistically significant relationship was found between TCE administration at 72 hours and the number of viable cells ($\chi 2 = 56.54$, $p<0.001$) (Table 3).

Figure 3 shows the number of cells stained with Ki-67 (viability) at different time intervals according to the groups at 24, 48, and 72 hours in column and line graphs. Ki-67 immunoreactivities in control and HepG2 cells incubated with 5 µg/ml for 24 hours and 1 µg/ml TCE for 48 and 72 hours under standard culture conditions are depicted in Figure 4 (A-F, Scale: 40 µm).

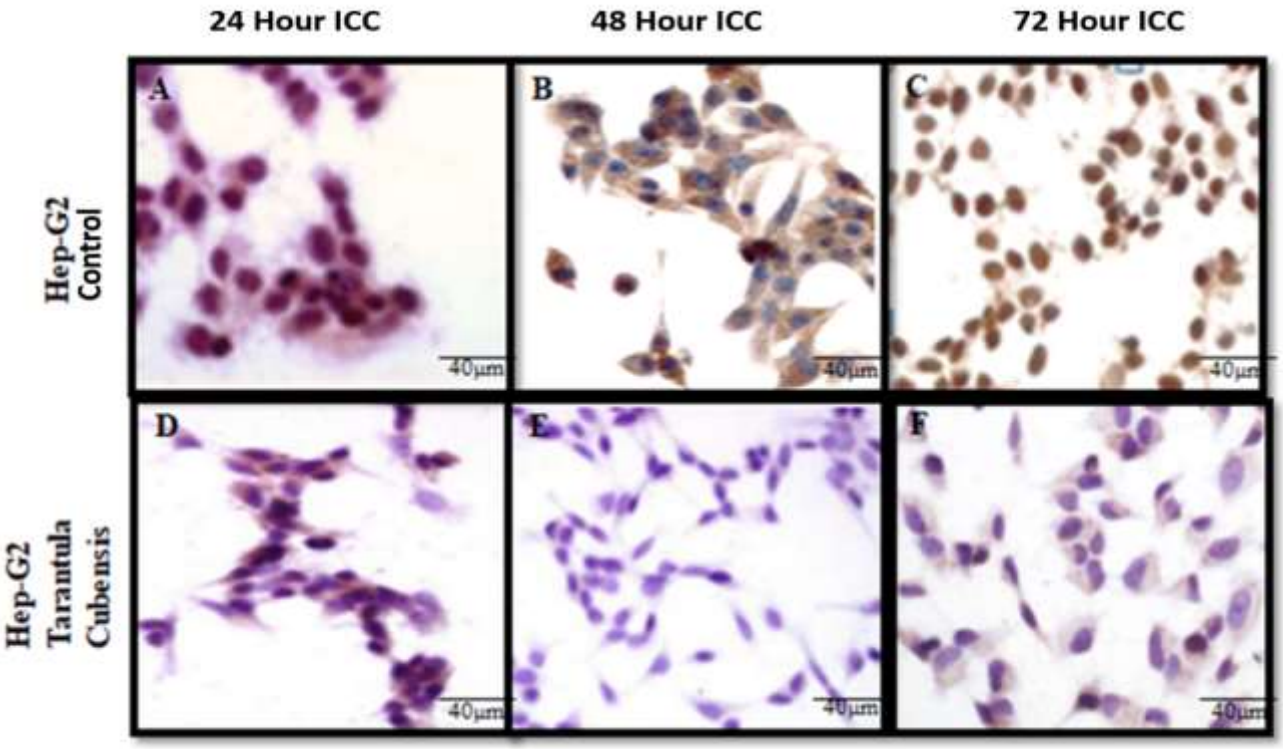

Figure 4. Ki-67 immunoreactivities (A-F, Scale: 40 µm) in control and HepG2 cells incubated with 5 µg/ml for 24 hours and 1 µg/ml TCE for 48 and 72 hours under standard culture conditions.

4. Discussion

Liver tumor resection and transplantation remain the cornerstone treatments for HCC. However, the majority of patients are unsuitable candidates for these interventions, and recurrence rates exceed 70% within five years [11,12]. While advancements in surgical methods, transarterial chemoembolization, and radiofrequency ablation have provided additional options, they have not significantly improved disease-free survival rates [13]. Moreover, these treatments are frequently associated with severe side effects, and chemotherapeutic agents are notorious for their high toxicity to both cancerous and normal cells. In advanced stages of the disease, current therapies merely slow progression, falling short of offering a cure. Furthermore, the effectiveness of adjuvant and neoadjuvant protocols in HCC management remains inconclusive. Consequently, there is a pressing need for innovative therapeutic approaches for HCC. To address this, our study focused on evaluating the effects of TCE extract on hepatocytes [14].

Despite advanced surgical methods, transarterial chemoembolization, and local ablation with radiofrequency, patients have low disease-free survival rates, necessitating new drugs to increase survival. Additionally, the treatments have a high incidence of side effects, and chemotherapeutic agents are known for their high toxicity to normal cells as well as cancer cells. In advanced-stage patients, the disease process can only be slowed down, and cure cannot be achieved with the available chemotherapeutic agents. There is no clear evidence about the efficacy of any of the adjuvant and neoadjuvant protocols used in HCC [15]. In this respect, new treatment agents are needed for HCC. Therefore, we aimed to investigate the effect of TCE extract on hepatocytes.

Our findings demonstrate that Tarantula cubensis extract (TCE) exhibits cytotoxic effects on liver cancer cells (HepG2). This effect appears to be mediated through the suppression of Ki-67 proliferation in HepG2 cells. To our knowledge, the anti-tumor potential of TCE on this specific cell line had not been explored prior to this study. Our results revealed that TCE effectively suppressed Ki-67 expression and exhibited anti-tumor activity at doses of 1 and 5 µg/ml doses.

Previous studies indicate that Ki-67 immunostaining in HCC lesions correlates with increased mitotic activity [16]. Elevated Ki-67 expression levels in HCC tissues are linked to more aggressive tumor grades and early recurrence. Furthermore, high Ki-67 levels have been significantly associated with advanced HCC, characterized by poor differentiation, larger tumor sizes, multiple nodules, metastasis, cirrhosis, and venous invasion [17,18].

Literature suggests that TCE has been predominantly utilized in veterinary medicine. Research on canine mammary adenocarcinomas revealed high pre-treatment levels of Bcl-2 and Ki-67, which significantly decreased following TCE administration. This indicates that TCE may effectively control tumor growth in canine mammary adenocarcinomas classified as T2N0M0 [9]. Additional studies have demonstrated that subcutaneous TCE injections reduce cell proliferation and promote apoptosis in cancer cells [19]. Moreover, TCE has shown anti-tumor activity in human breast cancer and head and neck cancer cell lines by inducing apoptosis through caspase-3 activation. Post-treatment analyses further confirmed significant reductions in Ki-67 and Bcl-2 expression levels [20].

The main limitation of this study is that only ki-67 was studied among the pathways involved in the regulation of apoptosis in tumor cells, and other pathways (such as bcl-2) were not examined. Another limitation of the study is that the effect of TCE on normal cells and its toxic dose are unknown. Since this study was conducted on diseased cells and healthy cells were not included in the experiment, the effect of TCE on healthy cells is unknown.

4.1. Conclucion**:** *In the present study, we observed that the suppression of Ki-67 expression in HepG2 cells suggests that TCE may offer promising therapeutic potential in the management of hepatocellular carcinoma (HCC). Notably, a key strength of our research lies in the evaluation of the anti-tumor effects of TCE using not only the MTT assay to assess cell viability but also immunocytochemistry to examine Ki-67 as a proliferation marker, providing a more comprehensive analysis compared to similar studies. Based on these findings, we propose that further investigation into the molecular mechanisms underlying the effects of TCE is warranted. Detailed studies focusing on its bioactive constituents and pathways of action are essential to facilitate the development of TCE as a novel biotherapeutic agent for HCC in the future.*

Acknowledgment

The experiments in this paper were performed at Bolu Abant İzzet Baysal University (Türkiye) – Faculty of Medicine, Department of Physiology Cell Culture Laboratory.

Funding: *Bolu Abant İzzet Baysal University Scientific Research Projects Coordination Unit number 2022.08.11.1539.*

Conflict of Interest: *The other authors declare that they have no conflicts of interest to report.*

Ethical Statement: *Since it was a cell culture study, ethical compliance was not required.*

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