International Journal of Secondary Metabolite

2023, Vol. 10, No. 3, 370–384

https://doi.org/10.21448/ijsm.1212761

Published at https://dergipark.org.tr/en/pub/ijsm **Research Article**

Effects of Grayanotoxin-III on different cell lines: in vitro ischemia model

Esin Akbay Çetin [1,*], **Çiğdem Özenirler** [1]

[1]Hacettepe University, Faculty of Science, Department of Biology, 06800, Ankara, Türkiye

Abstract: Grayanotoxins (GTXs) are natural products and are mostly found in plants of the *Ericaceae* family, especially in the *Rhododendron*. With their ability to bind to voltage-gated sodium channels, they keep these channels constantly active and cause tissue damage. However, despite this feature, the use of *Rhododendron* leaves or its secondary products as an alternative product is especially common in Turkey. This study aims to evaluate the possible dose-related effects of GTX-III in ischemia-induced *in vitro* cell models. Within the scope of the study, an ischemia model was established in two different cell lines (H9c2 and Cos-7) and treated with various concentrations of GTX-III. In this context, cell viability, cytotoxicity, apoptosis and necrosis were examined. In the results of MTT, a significant decrease ($^{+}p < 0.05$) in cell viability was observed in all GTX-III concentrations in H9c2 cells compared to the control, while a significant difference ($^{+}p < 0.05$) was observed in Cos-7 cells, especially at the 24th hour. LDH cytotoxicity was increased in a dose-dependent manner in both cell models. It was concluded that GTX-III caused apoptosis, and reduced cell viability in ischemia models; however, promoted cell proliferation in healthy cells. Based on the literature review, this study is the first to document the cytotoxic properties and apoptotic potential of GTX-III in an *in vitro* cell culture ischemia model. Our findings support the usage of GTX-III, however it should be remembered that the dose needs to be verified before being used medically.

ARTICLE HISTORY

Received: Nov. 30, 2022

Revised: Apr. 17, 2023

Accepted: June 20, 2023

KEYWORDS

Cardiomyocyte,

Cells culture,

Grayanotoxin-III,

Renal cell line,

Cytotoxicity.

1. INTRODUCTION

Rhododendron, comprising about 1215 taxa, is a cosmopolitan genus of the *Ericaceae* family (MacKay and Gardiner 2017). These plants are distributed mainly in Asia, with taxa also native to North America, Europe, Southeast Asia and Australia (Qiang *et al*., 2011; MacKay and Gardiner 2017). More than 25 Grayanotoxin (GTX) isoforms are known to be isolated from *Rhododendron* species (Qiang *et al*., 2011). These toxic agents can be found in the leaves, flowers, nectars, or secondary products (such as honey) of the aforementioned plants (Lim *et al*., 2016). Of these agents, GTX-III is the most toxic.

GTXs' cytotoxicity is caused by the blocking of sodium channels in the excitable cell membranes, preventing transmission. As a result of this blockage, the excitable nerve and muscle cells are kept in a state of continuous depolarization (Bilir *et al*., 2018). GTXs' also lead

*CONTACT: Esin AKBAY ÇETİN akbayesin@gmail.com Hacettepe University, Faculty of Science, Department of Biology, 06800, Ankara, Türkiye

e-ISSN: 2148-6905 / © **IJSM 2023**

to cardiac toxicity by increasing sodium channel permeability and activating the vagus nerve (Choo *et al*., 2008; Poon *et al*., 2008). Diterpenic GTXs prevent Na^+ channel inactivation (Mladěnka *et al*., 2018). In severe GTX-III intoxications, life-threatening cardiac complications such as complete atrio-ventricular block have been reported with an increase in resting sodium permeability as well as activation of voltage-sensitive sodium channels (Durdagi *et al*., 2014). All GTXs can cause different cardiac arrhythmias (Aliyev *et al*., 2009; Okuyan *et al*., 2010). As a result of studies conducted to elucidate this information, it has been shown that the mechanism underlying GTX-induced arrhythmias is the production of activity triggered by release after potentials (Brown *et al*., 1981).

Rhododendron species are used as traditional medicines for the treatment of inflammation, pain, skin ailments, colds, and gastrointestinal ailments (Popescu *et al*., 2013). Additionally, it is believed that *Rhododendron* honey reduces the risk of coronary heart disease and is also a sexual stimulant (Dubey *et al*., 2009). In addition, it is predicted that the misused dose of GTX-III causes oxidative stress in cells, and many diseases can be triggered by the initiation of this process (Incalza *et al*., 2018).

Apoptosis plays an essential role in cardiovascular and renal diseases, increasing oxidative stress, which is the primary cause of mitochondrial dysfunctions, such as the increase of ROS, saturation of antioxidant systems, and exhaustion of ATP. Programmed cell death is caused by mitochondrial dysfunction. There was a correlation between an increase in apoptosis and a decrease in cardiac functions in previous studies (Ritter and Neyses, 2003; Argun *et al.*, 2016). Apoptosis and oxidative stress are known to be associated with many cardiac events (Doğanyiğit *et al.*, 2020). Likewise, it is known that they are related with diseases of chronic kidney diseases (Small *et al*., 2012).

To the best of our knowledge, despite this strong evidence for anti-hypertensive effects, the effects and doses of GTX-III itself or the new GTX analogues on the heart muscle and renal cells in cardiovascular or renal diseases have not (yet) been fully investigated. This rationale led us to investigate whether GTX-III had potential dose-dependent effects on kidney cell and cardiomyocyte disease models in the present study.

2. MATERIAL and METHODS

2.1. Materials

GTX-III hemi (ethyl acetate) (CAS Number 4678-45-9) was obtained from Sigma Aldrich (Germany). All chemicals and solvents were obtained as cell culture grade. H9c2 and Cos-7 cell lines were purchased from American Type Culture Collection (ATCC, Rockville, MD).

2.2. Determination of Cell Type

The H9c2 cell line is widely used as a model in reperfusion-ischemia (RI) studies (Kuznetsov *et al*., 2015). The H9c2 cell line (derived from embryonic rat ventricular tissue and lost its pulsing characteristic) was used in this study due to its similarities (membrane morphology, electrophysiological properties) with primary cardiomyocytes in many ways. At the same time, it represents a homogeneous population of heart cells offering numerous experiments to simulate cardiac pathologies. Cos-7 cells most resemble human fibroblast cells and are thus often called Cos-7 monkey kidney fibroblast or Cos-7 fibroblast-like cells (Condreay *et al*., 1999).

2.3. Determination of Concentration of H_2O_2 and GTX-III

We selected the same H_2O_2 concentration used by Xu *et al.* (2016). 600 µM H_2O_2 was shown to regulate cellular stress responses, proliferation, survival, and differentiation within the scope of this study.

0.5 μM,1 μM, and 10 μM of the GTX-III used in cell culture were based on tissue concentration studies of its analogues. Similar concentrations chosen in this study were also used in other cell culture studies made with GTX-III (Brown *et al*., 1981). GTX-III was freshly dissolved in 0.1% DMSO as a stock solution and diluted with DMEM before use.

2.4. *in vitro* Cell Culture Studies and Experimental Groups

H9c2 and Cos-7 cells were grown in DMEM containing 10% FBS and 1% P/S in standard conditions (5% CO_2, 95% air at 37 °C). The medium change of cells was done every 2 to 3 days. After reaching adequate cell numbers, cells were inoculated in 96-well culture plates for 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT) test and in 24-well culture plates for Acridine orange/Propidium iodide (AO/PI) double staining (Türck *et al.,* 2022). After 24 h of incubation with and without H_2O_2, the media were removed from all groups. And, GTX-III prepared in three different concentrations (D1:0.5 μM, D2:1 μM, and D3:10 μM) were applied to the cell after both applications. The untreated cells were considered a "control (untreated group)". Cells treated only with H_2O_2 were named as a "treated group". Also, the cells were treated with only 0.1% DMSO which was regarded as a "solvent control group".

2.5. Cell Viability Assay

The cytotoxic effects of GTX-III on H9c2 and Cos-7 cells were determined using the MTT test. Briefly, H9c2 and Cos-7 cells were trypsinized, and $2x10^4$ cells/well were seeded into 96-well plates for each. At the 24th h following the cultivation, 600 μM H_2O_2 was applied for 120 minutes and then H_2O_2 was removed from the plates and GTX-III concentrations were added. GTX-III concentrations were applied directly to the groups that were not treated with H_2O_2. For the MTT test, GTX-III concentrations were removed from the cells after 24, 48, and 72 h incubation. Fresh culture medium containing 10% MTT solution was added to each well and incubated at 37 °C for 4 h. Isopropyl alcohol was added to the medium to dissolve the formazan crystals formed at the end of the incubation and the absorbance at 540 nm was recorded with a microplate reader (μQuantTM, BiotekW Instruments Inc, USA). The assay was repeated in three independent experiments (n=6). Cell viability percentage was calculated by the following equation 1:

$$\%Cellular\ Viability\ = \frac{Treated\ Group\ Absorbance}{Control\ Group\ Absorbance} x100 \quad (1)$$

The MTT results of the cells incubated with the standard medium (untreated group) were considered to be 100% viable and the viability percentages of the experimental groups were calculated by comparing them with the control group.

2.6. Apoptotic Staining and Scoring of Apoptotic Cells

Cell death occurs in many ways, from cell skeletal damage, cell contraction, plasma membrane condensation, membrane swelling, nuclear condensation, and DNA fragmentation, when cells enter the apoptosis pathway (Abdel *et al*., 2009). To investigate the potential of apoptosis and the type of death of the cells, the ratio of apoptosis was examined with AO/PI double staining. Staining was performed at 24, 48, and 72 h following the incubation. Briefly, cells were washed gently with PBS, after culture mediums were removed. Cells were stained with the equal volume combination of AO and PI for 20 seconds and washed twice with PBS. The stained sample was viewed under fluorescent microscopy (Olympus IX70, Japan). The apoptosis potential of cells was determined by counting according to the following criteria: viable cells; it has an organised structure and a uniform green core (1); early apoptotic cells, which have bright green areas due to the concentration of chromatin in the nucleus (2); late apoptotic cells; it has intense orange chromatin condensation areas (3); necrotic cells have a uniform orange

nucleus (4). Apoptosis potentials in groups were calculated according to the following equations 2 and 3:

$$\%Apoptotic\ Cells\ = \frac{Total\ number\ of\ apoptotic\ cells\ (early\ or\ late)}{Total\ count\ cell} x100 \quad (2)$$

$$\%Necrotic\ Cells\ = \frac{Total\ number\ of\ necrotic\ cells}{Total\ count\ cell} x100 \quad (3)$$

2.7. Lactate Dehydrogenase Assay

The lactate dehydrogenase (LDH) test is a widely used spectrophotometric test to determine cell membrane damage. The damage potential was determined using the LDH cytotoxicity test. LDH levels were determined in H9c2 cells seeded at $5x10^3$ cells/well and incubated for 24 h. Then, cells were treated with and without H_2O_2. Groups for LDH testing were designed as in MTT analysis. After incubation with experimental solutions, all wells were incubated for another 48 and 72 h. 10 µl of culture medium (cell supernatant) was used for the assay. LDH release was calculated by measuring according to the manufacturer's protocol (Thermo Scientific). Briefly, it was measured at an absorbance of 490 nm using a spectrophotometer (µQuantTM, Biotek® Instruments Inc, USA). In addition, the % cytotoxicity was calculated according to the manufacturer's protocols:

$$\%Cytotoxicity\ = \frac{Compound\ treated\ LDH\ activity - Spontaneous\ LDH\ activity}{Maximum\ LDH\ activity -\ Spontaneous\ LDH\ activity} x100 \quad (4)$$

2.8. Statistical Analysis

GraphPad Prism 5.01 (GraphPad Software Inc., San Diego, CA, USA) was used in statistical analysis. Differences between groups were analysed using analysis of variance (two-way ANOVA). Data were represented as mean ± standard deviation (SD) with a significance level of $p < 0.05$.

3. RESULTS

3.1. MTT Analysis

As shown in Figure 1, 600 µM H_2O_2 and GTX-III reduced H9c2 myocardial cell viability in the concentration range of 0.5 µM (D1), 1 µM (D2), and 10 µM (D3). A significant decrease was observed in all H_2O_2 application times compared to the control. Similarly, a significant decrease was observed in cell viability at 48 and 72 h when D1 concentration was applied from GTX-III groups following H_2O_2 application, while a significant decrease was observed only at 72 h after D2 and D3 concentration groups were applied ($^*p < 0.05$). Therefore, 48 and 72 h were selected for the LDH test based on these results for H9c2 cells.

There was no significant difference between H_2O_2 and dilutions of H_2O_2+GTX-III groups at all incubation times. In addition, significant increases were observed in D1, D2, and D3 of DMEM-GTX-III groups at 24 and 48 h compared to the control group ($^\#p < 0.01$). Also, cell viability was increased significantly within the DMEM-GTX-III concentrations at 72 h ($^+p < 0.05$) (Figure 1).

In cell viability experiments with the Cos-7 cell line, it was determined that H_2O_2 significantly reduced cell viability in all time periods ($^*p < 0.05$). This result showed that the ischemia pattern was formed in these cells as well. It was observed that different concentrations of GTX-III following H_2O_2 application also caused a significant decrease in the viability of

Cos-7 cells at 24 h ($^{*}p < 0.05$). However, despite a decrease in cell viability at 48 and 72 h, no significant difference was found between the GTX-III groups and the control. On the other hand, no significant difference was found in the DMEM+GTX-III groups when compared to the control. This result showed us that GTX-III had no effect on healthy Cos-7 cells unlike H9c2 cells (Figure 2).

Figure 1. Impact of H_2O_2 and post treatment of GTX on the % viability of H9c2 cells and GTX treatments on the H9c2 cells without any treatment, after incubation periods (24, 48, and 72 h) (n = 6). (D1: 0.5 μM; D2: 1 μM; D3: 10 μM) ($^{*}p < 0.05$, $^{\#}p < 0.01$, $^{+}p < 0.05$)

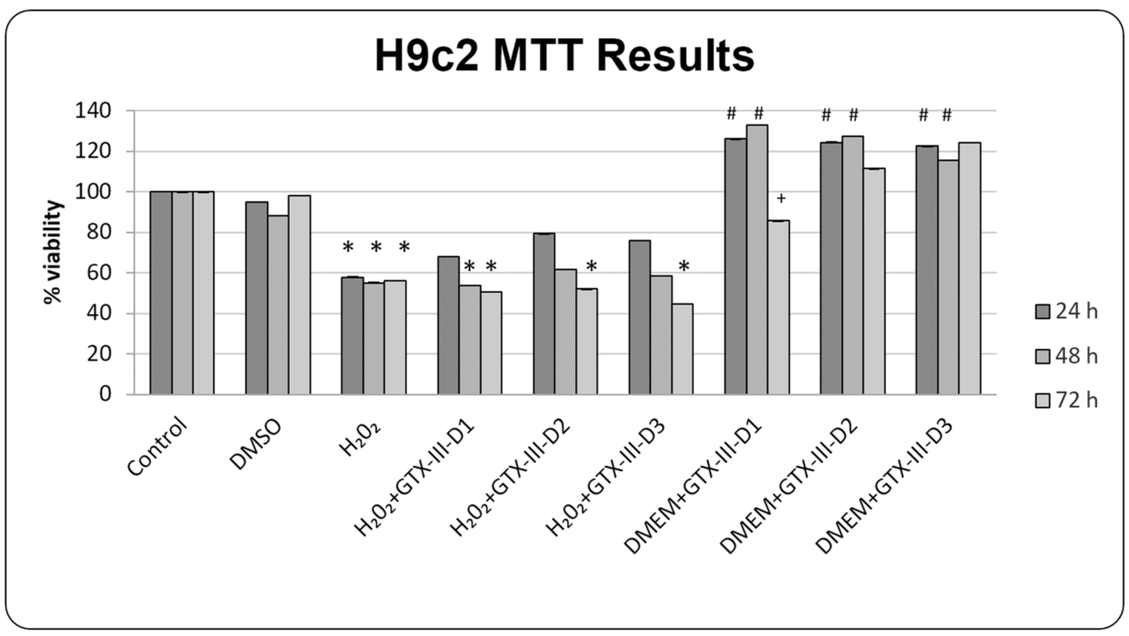
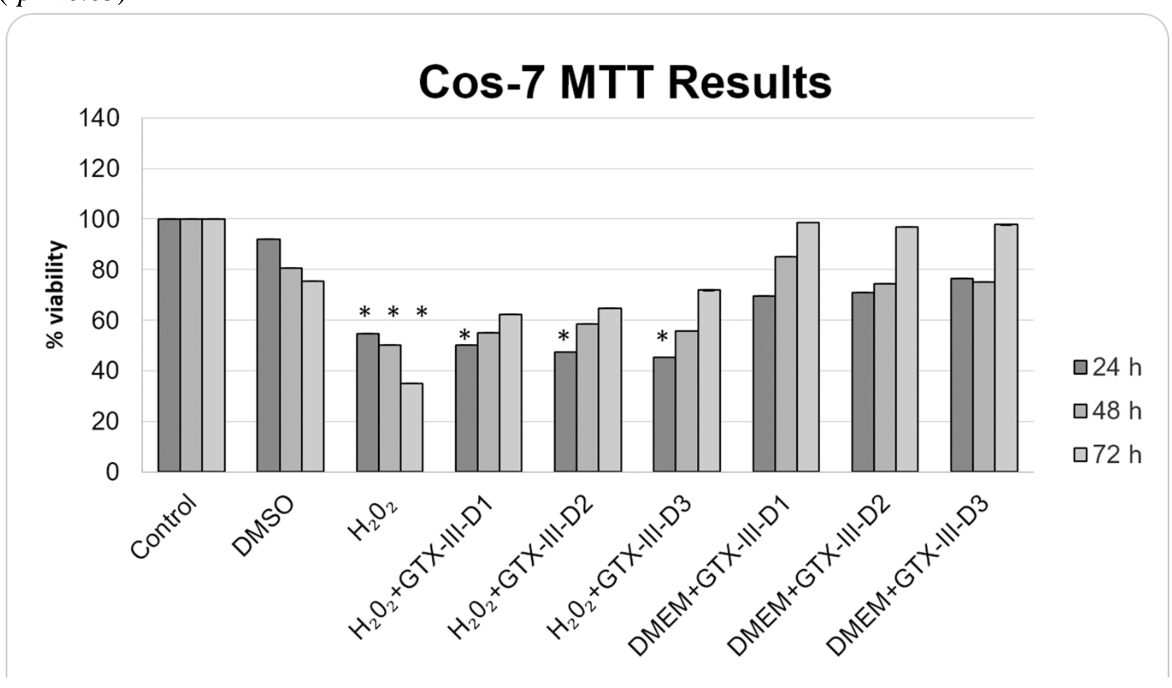


Figure 2. MTT results of Cos-7 cells after treatment with H_2O_2 and GTX's different concentrations for 24, 48, and 72 h. Results are derived from at least three replicates. (D1: 0.5 μM; D2: 1 μM; D3: 10 μM) ($^{*}p < 0.05$)



3.2. Apoptotic Staining Results

Results in H9c2 and Cos-7 cells showed an increase in apoptotic cell death in both cases after all incubation times at all concentrations of GTX-III used after H_2O_2. As shown in Figure 3-4, more apoptotic cells were observed in the H_2O_2 and H_2O_2-GTX-III groups than in the control and DMEM-GTX-III groups. According to these results, GTX-III has a protective effect against non-H_2O_2-induced cell apoptosis, while inducing apoptosis in H_2O_2-induced cardiomyocytes and renal cells.

When the percentages of apoptosis and necrosis in H9c2 cells were analysed, it was found that the H_2O_2 and H_2O_2+GTX-III groups showed a significantly higher percentage of both. The significant difference found was valid in all time periods. In the DMEM groups, there was no difference in all time periods in terms of both % apoptosis and % necrosis.

As a result of the analysis of Cos-7 cells, there was a significant increase in % apoptosis results of H_2O_2 and D2 and D3 concentrations of H_2O_2+GTX-III at 24th h compared to the control ($^*p < 0.05$). In addition, it was determined that there was a significant increase in the percentage of necrotic cells in the D2 concentration of DMEM+GTX-III ($^+p < 0.01$). A significant increase in both % apoptosis and % necrosis results was observed at 48 h for H_2O_2 and all concentrations of H_2O_2+GTX-III when compared to the control ($^*p < 0.05$, $^+ p < 0.01$). At the 72nd h, a significant increase was observed in % necrosis results in D3 concentration of H_2O_2+GTX-III and H_2O_2 when compared to the control ($^+p < 0.01$), while a significant difference in % apoptosis was observed only in D1 concentration of DMEM+GTX-III groups ($^*p < 0.05$) (Figure 5).

3.3. Lactate Dehydrogenase Assay Results

The LDH test revealed that cytotoxicity was significantly increased in H9c2 cells in H_2O_2 and in all GTX-III groups compared to the control group at 48 and 72 h ($P < 0.05$). LDH cytotoxicity was increased in a dose-dependent manner, representing an increase in the number of dead cells (Table 1). However, the small increase in all DMEM-GTX-III groups was not significant when compared with the control. Also, it was observed that increasing GTX-III doses showed a decrease in cytotoxicity effect over time. The maximum LDH value was observed in the 0.1 μM dose group of GTX-III at 48 and 72 h, whereas the viability percentage was observed to have the lowest percentage at 48 and 72 h (Figure 6).

Figure 3. AO/PI staining for apoptosis potential detection after H_2O_2 induce and non-induce H9c2 cardiomyocytes treated with different concentrations of GTX-III after 24, 48, and 72 h. The images of cells are x20 magnifications.

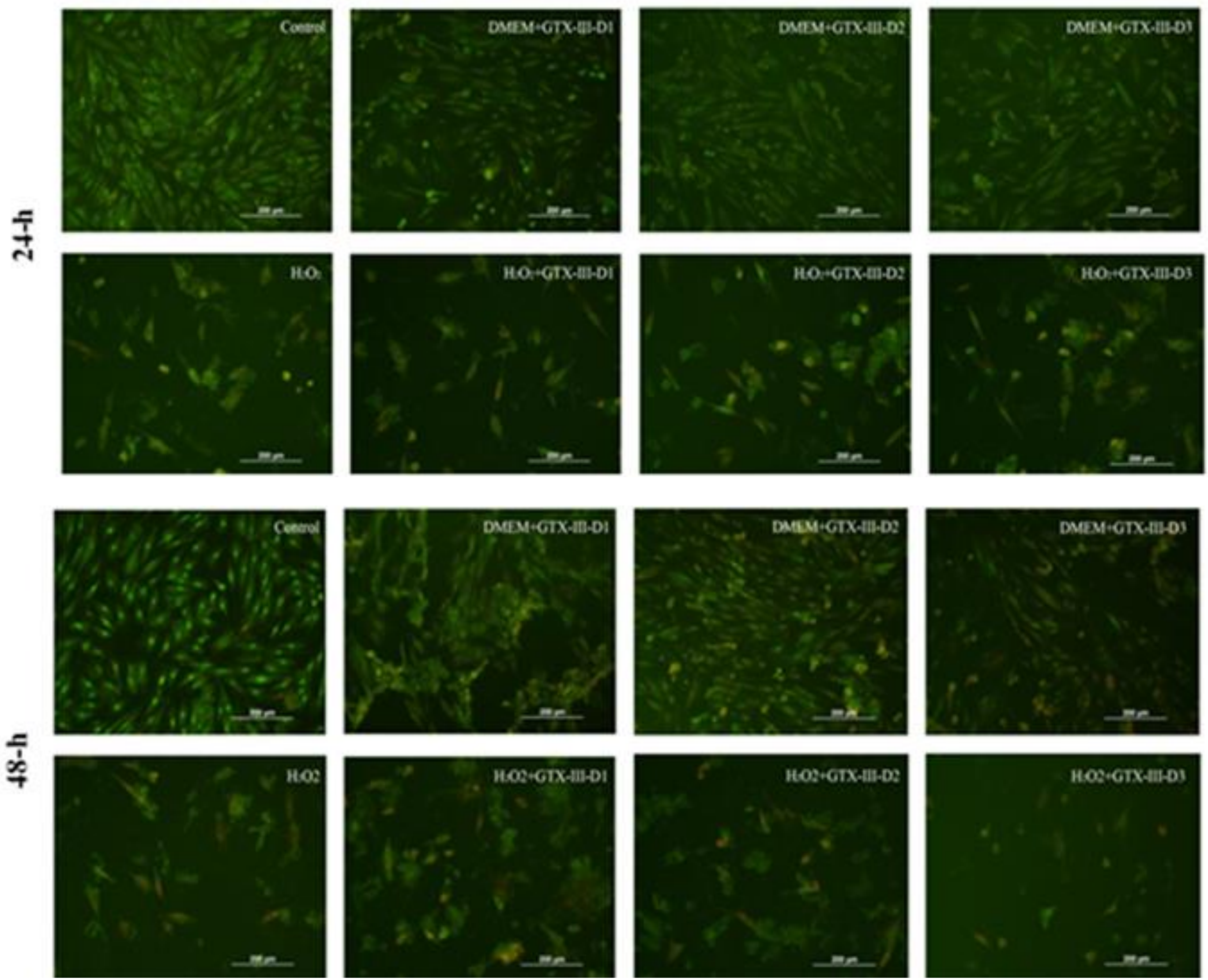


Figure 3. *Continues.*

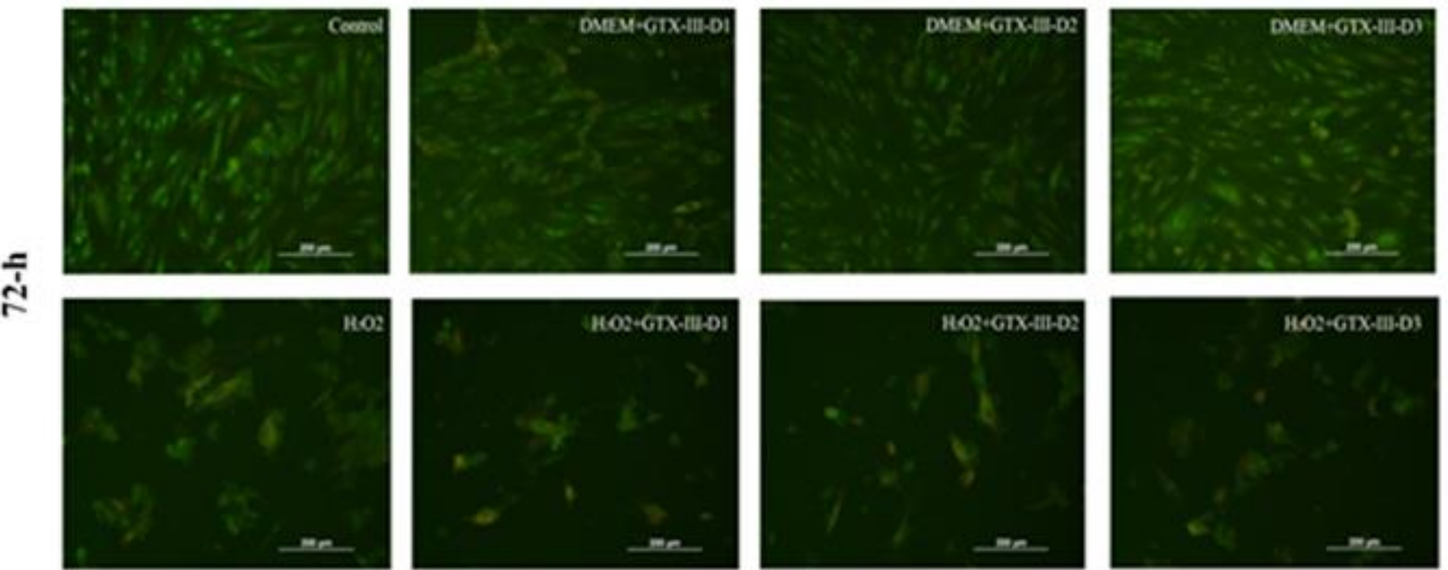


Figure 4. AO/PI staining for apoptosis potential detection after H_2O_2 induce and non-induce Cos-7 cells treated with different concentrations of GTX-III after 24, 48, and 72 h. The images of cells are x20 magnifications.

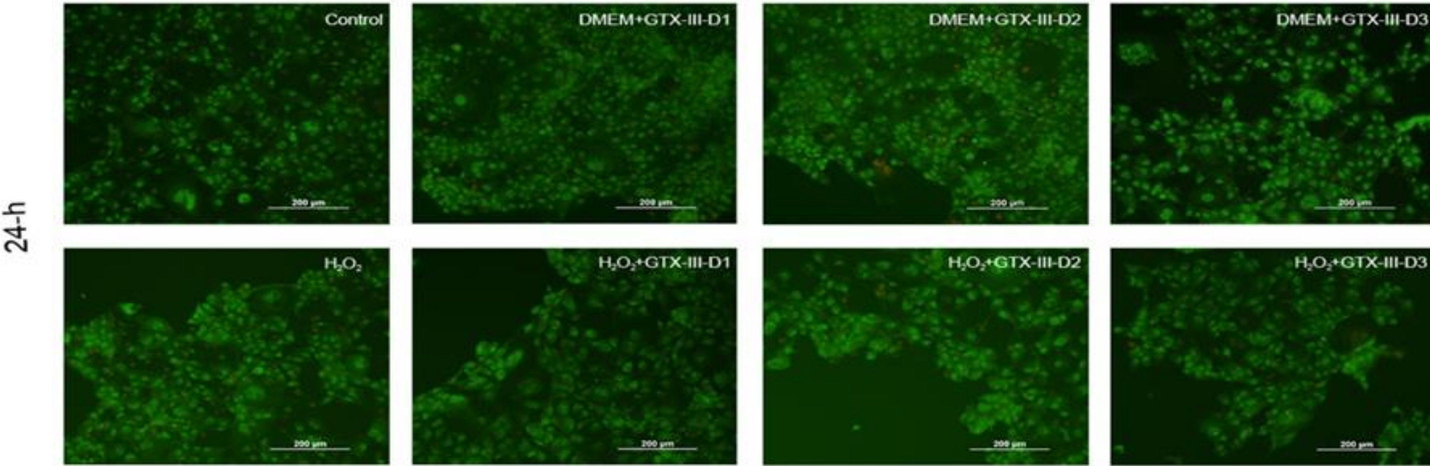


Figure 4. Continues.

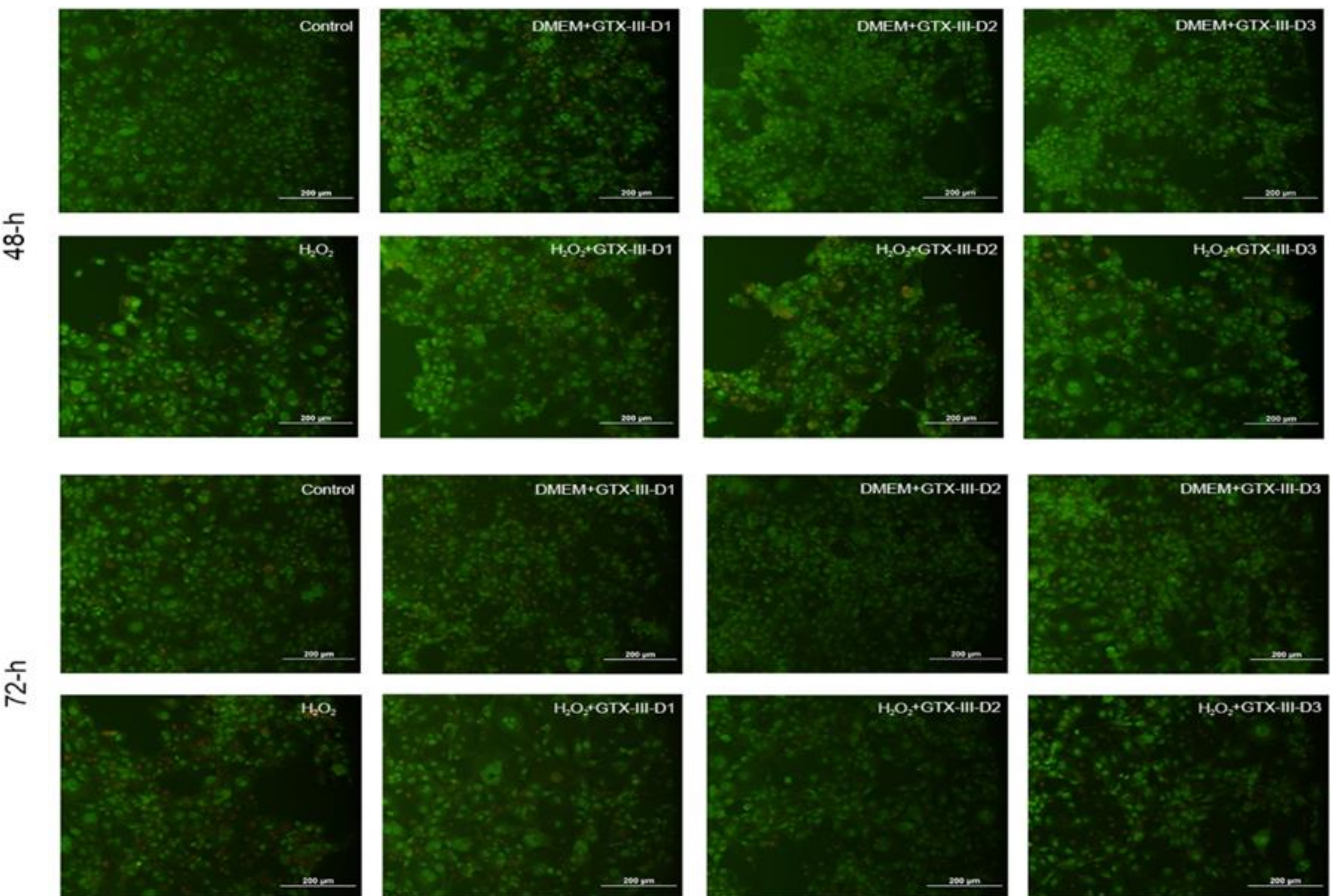


Figure 5. The % apoptotic and necrotic cells in H9c2 (A) and Cos-7 (B) treated with H_2O_2 and GTX's different concentration ($^{*}p < 0.05$, + $p < 0.01$).

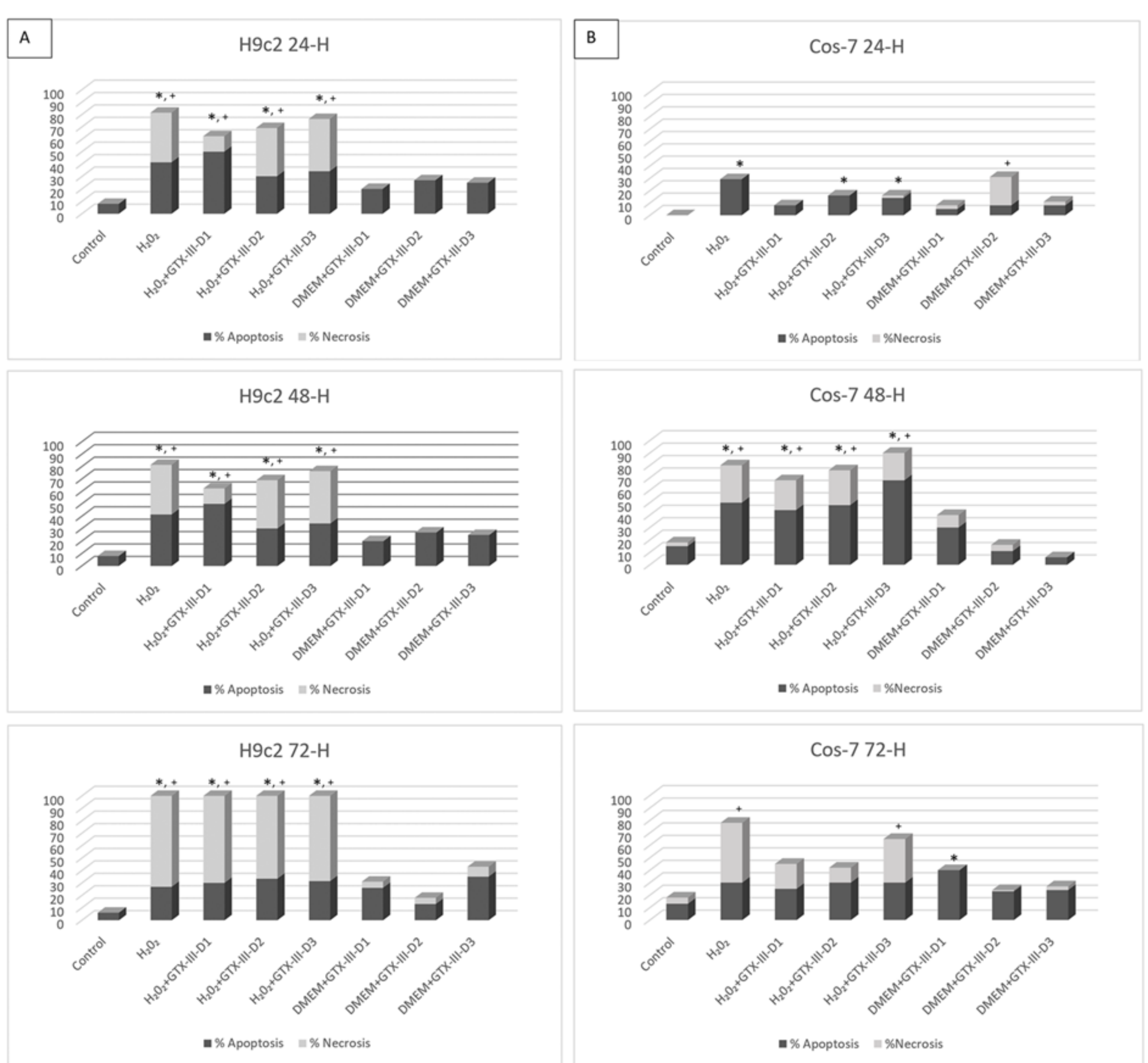


Table 1. Lactate dehydrogenase cytotoxicity assay results

	Control	H_2O_2	H_2O_2+ GTX-III-D1	H_2O_2+ GTX-III-D2	H_2O_2+ GTX-III -D3	DMEM+ GTX-III-D1	DMEM+ GTX-III-D2	DMEM+ GTX-III-D3
Cytotoxicity	Mean±SD	Mean±SD	Mean±SD	Mean±SD	Mean±SD	Mean±SD	Mean±SD	Mean±SD
48-hours	1.35±0.05	38.53±0.10	21.06±0.09	13.64±0.02	10.8±0.01	7.31±0.05	7.79±0.05	3.56±0.04
72-hours	2.38±0.02	37.22±0.12	13.40±0.00	8.37±0.00	8.63±0.02	5.76±0.02	5.00±0.09	3.37±0.02
p^*	0.026		0.027	0.028	0.035			

The results (Mean±SD) of three independent experiments are shown as percentages of the control.
D1: 0.5 µM; D2: 1 µM; D3: 10 µM; SD: Standard Deviation, *: Bonferroni post-tests

4. DISCUSSION and CONCLUSION

The current research aims to evaluate the potential dose-dependent effects of GTX-III on cardiomyocyte and renal cells in connection to the background of heart attack and renal failure. Within this frame, H9c2 and Cos-7 cells failed with an oxidative stress agent-H_2O_2.

Oxidative stress occurs when the balance between oxidants and antioxidants changes in favour of oxidants. The balance of reduction and oxidation events is critical for cell viability, activation, proliferation, and organ function. Oxidative stress formation causes the development of many pathological diseases (including cancer, neurological disorders, atherosclerosis, hypertension, ischemia/perfusion, diabetes, and asthma). Especially after coronary stenting, myocardial ischemia reperfusion injury (MI/RI) develops by triggering the pathways involved in oxidative stress damage and cell apoptosis. Myocardial or renal hypoxia and re-oxygenation cause an increase in many free radicals (superoxide radical (O_2-), hydrogen peroxide (H_2O_2), hydroxyl radical (-OH) and peroxynitrite (ONOO-)) in tissues (Abdel *et al.*, 2009; Elahi *et al.*, 2009). Apoptosis or death ultimately results from lipid peroxidation of the cell membrane, the main mechanism of RI (Incalza *et al.*, 2018). The change in oxygen free radicals triggered by MI/RI causes the initiation of a series of steps that induce cell death pathways associated with tissue damage. These damages are the observation of glucose and lipid peroxidation, enzyme inactivation, and protein denaturation, followed by DNA fragmentation. In short, oxidative stress-induced apoptosis is an important triggering factor in MI/RI development (Cakmak-Arslan *et al.*, 2020). Our results indicate that GTX-III has a cytotoxic effect on the ischemia cell models, possibly by reduced activity in mitochondria. It was observed that GTX-III concentrations decreased cell viability in H9c2 and Cos-7 cells below 60% compared to control cells. Degeneration mostly involved acute cell swelling. Acute cell swelling is known to be more related to reduce cellular oxygenation (Elahi *et al.*, 2009). When the oxygen used by the cell decreases with MI/RI, mitochondrial oxidative phosphorylation stops and the cells undergo anaerobic metabolism or die. If the cell fails to restore mitochondrial function, its entry into the path of death becomes irreversible and leads to cell necrosis (Wang *et al.*, 2018). One of the markers of apoptotic cell death, necrotic cell death, was also observed in high-dose groups of GTX-III. It was observed that the DMEM-GTX-III-D1 group had the lowest % necrotic rate in all incubation periods in both cells. According to these results, GTX-III induces apoptosis in ischemic cardiomyocytes. It appears to have a protective effect against apoptosis in healthy cells. However, we cannot say the same for kidney fibroblast cells. According to the cell viability results, it was observed that the viability rate approached the control in healthy fibroblasts only at 72 h. It was also observed that increasing doses of GTX-III showed an increase in cytotoxicity over time in ischemic cardiomyocytes but decreased over time in fibroblast cells.

Based on the fact that LDH results support the cell viability, results strengthen the confirmatory aspect of our hypothesis. LDH test was used in this study to determine the cell

membrane damage of the GTX-III dose effect on damaged cardiomyocytes. And results showed that the increase in the number of dead cells was dose-dependent.

All the tested doses of GTX-III induce cell proliferation in healthy H9c2 cells. This can be attributed to various reasons. Kühn *et al*. (2007) reported that PI3K/Akt pathway induces the re-entry of cardiomyocytes into the cell cycle. In another study, gap junctional coupling was reported to be an important factor in cardiomyocyte proliferation (Weeke-Klimp *et al*., 2010). The factors that trigger these have not been studied in detail. A commonly known feature of GTX-III is that it eliminates rapid sodium inactivation and channel activation causes a hyperpolarized shift in voltage dependence. It has also been reported to be a lipid-soluble toxin that exhibits a selective property on voltage-dependent sodium channels (Maejima *et al*., 2003). With these in mind, GTX-III may have a potential inductive effect on different membrane proteinsor may have a secondary effect on activating certain pathways in cardiomyocytes.

DMEM-GTX-III was observed to cause aggregation in healthy cardiomyocyte cells at 48 and 72 h, especially at D1 concentration. This cellular behaviour, specifically observed in these cells in particular, shows that the use of GTX-III at this dose promotes the aggregation movement of cardiomyocytes and may trigger pulse observation after some time.

Due to their use in folk medicine, these species have become the focus of interest for scientific studies. Medicinal use of this product without obtaining any information about the consumption dose of a natural product makes it a real risk factor. While the in vivo effects of GTX-III have been examined in almost all of the previous studies, the important feature of this study is that GTX-III is examined at the cell level *in vitro* model of the ischemia-reperfusion injury cell culture model (Maejima *et al*., 2003; Xu *et al*., 2016). Also, previous cell culture studies with plant extracts and GTXs were generally performed with different cancer cell lines (Doğanyiğit *et al*., 2019). However, there have not (yet) been any studies on heart muscle and kidney fibroblast cells. To the best of our knowledge, this is the first study to document the cytotoxic properties and apoptotic potential of GTX-III, the main substance of commonly consumed mad honey in the *in vitro* cell culture model.

This study's primary limitation was that it only included *in vitro* experiments and no animal or human research. Also, it was done with a single isoform of the GTX-III. It should give more valuable information if the other isoforms were also evaluated. But on the other hand, using a pure GTX-III is the main strength of our study.

In conclusion, it was revealed that GTX-III induces cell proliferation in healthy H9c2 cells whereas promotes apoptosis in H_2O_2–induced cell death H9c2 cardiomyocytes. In addition, it was observed that GTX-III supports proliferation at a lower rate in healthy Cos-7 cells than in cardiomyocyte cells. Our results may provide a theoretical basis for dose-dependent alternative use of GTX-III in humans without heart and kidney disease.

Acknowledgments

The authours thank Prof Dr Kadriye Sorkun for supporting the study from her project budget: F.Bİ.P65 (Hacettepe University). Also thank Prof Dr Mehmet Ali Onur for his valuable contributions to the methodology. The preliminary version of this study was presented in International Apicultural Research and Sustainable Regional Development Strategy Congress (2019).

Declaration of Conflicting Interests and Ethics

The authors declare no conflict of interest. This research study complies with research and publishing ethics. The scientific and legal responsibility for manuscripts published in IJSM belongs to the authors.

Authorship Contribution Statement

Esin Akbay Çetin: designed the study and supervised the experiments **Çiğdem Özenirler**: made contributed in the analysis of the results. All these authors have substantial contributions to the final manuscript and approved this submission.

Orcid

Esin Akbay Çetin https://orcid.org/0000-0002-0797-8322
Çiğdem Özenirler https://orcid.org/0000-0003-0390-2416

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