Exp Biomed Res 2021; 4(2): 89-98
Doi: 10.30714/j-ebr.2021267971

Experimental Biomedical Research **Original article**

Alcoholic extract of Tarantula cubensis (Theranekron®) induce autophagy on gastric cancer cells

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ABSTRACT

Aim: To evaluate the effects of theranekron in respect of autophagy on gastric cancer that is the fifth leading cancer type worldwide.

Metods: In the present study, metastatic AGS and non-metastatic MKN-45 human gastric cell lines were used together with HEK-293 non-cancer cells as controls. Cytotoxic effect of theranekron besides appropriate treatment time was investigated through cell proliferation by using Cell Proliferation assay Kit (MTT) using different concentrations of the drug. The autophagic effect of the drug was determined using the LC3-GFP translocation assay and western blot analysis. All experiments were performed also using the ethanol since Tarantula cubensis spider was processed and diluted in 60% alcohol to generate as a drug.

Results: MTT assay results demonstrated that the half maximal inhibitory concentration of theranekron was ~100 µM, its effect was found to be significant at 6 hrs, and theranekron decreased the cell viability in all cell lines without specificity in respect to the increasing concentrations. Additionally, a significantly increased GFP accumulation was detected in the autophagosomes of the cells treated with theranekron compared to non-treated cells, indicating the presence of autophagy.

Conclusion: These findings were confirmed by LC3-I to LC3-II conversion with the western blot analysis. The data of ethanol experiments; however, demonstrated that ethanol also induced a cytotoxic effect and autophagic cell death. Our results suggested that theranekron results in cell death and stimulate autophagy process, but it is not specific for cancer cells since it represented similar results on non-cancer control cells. Moreover, the effect of theranekron on cell death might mostly occur through alcohol in which it is extracted.

Keywords: Theranekron, tarantula cubensis, homeopathy, gastric cancer, autophagy.

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Received: 2020-11-12 Revised: 2021-01-13
Accepted: 2021-01-23 / Published online: 2021-04-01

Introduction

Gastric cancer is a type of cancer that originates at the mucosa epithelia of the stomach and expands rapidly to the lining of the stomach. Mostly, it is developed as ulcer. The cancer may spread from the stomach to other parts of the body, particularly to the liver, lungs, bones, lining of the abdomen and lymph nodes through blood [1]. Globally, stomach cancer is the fifth leading cancer type and the third leading cause of death from cancer according to 2012 cancer statistics [2]. It is also fifth leading cancer at

men and sixth leading cancer type at women in Turkey according to 2016 cancer statistics [3]. Gastric cancer occurs most commonly in East Asia especially Japan and South Korea and Eastern Europe. It is observed mostly at ages of 50 to 60 and occurs twice as often in males than females [2].

Theranekron® (Richter Pharma, Austria) is an alcoholic extract of the venom that is provided from a spider known as *tarantula cubensis*. *Tarantula cubensis* is the famous one among the venomous spiders and many therapeutic effects have been reported for its venom [4]. Theranekron is a homeopathic remedy that is used in cattle, horse, sheep, goat and dog. Homeopathy is a treatment method introduced by Dr. Samuel Hahnemann in the end of 18th century with the principle that ‘‘any substance causing symptoms of a disease in healthy people will cure similar symptoms in sick people’’. Mezger described the homeopathic effects of theranekron first time in 1977 [5]. Theranekron is used as a pharmaceutical compound serving in veterinary medicine with outstanding success for its antiphlogistic, necrotizing, and wound healing effects [4]. In the literature, the drug was studied in various fields such as many types of ulcer and abscess, peripheral nerve healing, as well as treating necrotic or proliferative cases in animals [6-9]. Koch and Stein reported first time that theranekron stimulate the demarcation of mammary gland tumors in dogs [10]. Later, Gultekin et al (2007) reported that theranekron application to the dogs resulted in regression and hardness of benign mammary tumors [11], and tarantula cubensis extract alters the degree of apoptosis and mitosis in canine mammary adenocarcinomas shown in 2015 [12].

Autophagy is a tightly-regulated process involving the degradation of a cell's own components through the lysosomal machinery [13]. Autophagy plays an important role in the homeostasis of the organelles and protein, and maintains a balance between synthesis and degradation in cells [14]. During the distribution of the homeostasis, autophagy leads to the cell death through activating the signaling pathways. Autophagic cell death is distinct from the apoptotic cell death, but the relationship between autophagy and apoptosis is more complex. It was shown that autophagy can delay apoptotic death following DNA damage [15] as well as can trigger a form of cell death in the absence of apoptosis [16]. The aim of the present study was to evaluate the effects of the alcoholic extract Theranekron on the gastric cancer cells in respect of autophagy.

Materials and Methods

Culture of cells

Metastatic gastric cancer cell line AGS, non-metastatic gastric cell line MKN-45, and non-cancerous cell line HEK-293 were purchased from American Type Culture Collection (ATCC, USA). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Sigma-Aldrich, Germany) containing 10% fetal bovine serum (FBS) (Sigma-Aldrich, Germany) and 1% penicillin-streptomycin (P/S) antibiotic solution (Sigma-Aldrich, Germany). Cells were grown in a 5% CO_2 incubator at 37˚C by replacing the medium every other day, and cells were passaged when they reached confluence.

The half maximal inhibitory concentration and cytotoxic effect of theranekron

AGS, MKN-45, and HEK-293 cells ($1x10^4$ cells/well) were treated with different concentrations of theranekron (Richter Pharma, Austria) by diluting the drug with culture medium. One group of cells was also treated

with same dilutions of alcohol as control since the drug was extracted in ethanol. Following different time points, the Cell Proliferation Kit I (MTT) experiment was performed. Culture medium was mixed with thiazolyl blue tetrazolium bromide powder (Sigma-Aldrich, Germany) at a ratio of 5 mg/ml, and 10 µl was added to each well. The cells were incubated with the solution for 3 h at 37˚C until the color turned into blue. Then, all the solution was taken away from the wells and the formazan salts were dissolved through DMSO about 20 min. The absorbance was measured at 540 nm.

LC3-GFP translocation assay

AGS, MKN-45, and HEK-293 cells were seeded into 6-well plate with 2 mL culture medium and transfected with plasmid coding LC3 fused with green fluorescence protein (GFP). For transfection, first plasmid was diluted in a tube with 125 µl serum free medium at a ratio of 1 µg/100 µl. At the same time, 6 µl Lipofectamine® 2000 (Invitrogen, USA) transfection reagent was diluted with 125 µl serum free medium in another tube. After 5 minutes of incubation time, both tubes were mixed and the mixture was then incubated at room temperature for 20 min to form lipid complexes. 250 µl of mixture was then added into each well. Transfected cells were incubated in CO_2 incubator for 24 h until the cells start to express LC3-GFP. Then, one group of transfected cells was treated with 100 µM theranekron and the other groups were treated with rapamycin as a positive control for autophagy and alcohol as negative control. Treated cells were incubated for 3, 6, and 12 hours for the best result. The cells were analyzed under Epi Fluorescence Microscope (Nikon, Japan).

LC3 protein analysis by western blot

AGS, MKN-45, and HEK-293 cells were treated with theranekron and alcohol for 6 hrs, then they were detached from plate, washed with PBS, and the pellets were lysed with 100 150 µl RIPA lysis buffer (Santa Cruz, USA). The protein concentration of the lysates was determined with Bradford protein assay reagent (Bio-Rad, USA) following the manufacturer's instructions. Each sample (20 µg) was loaded into a 10% separating acrylamide gel and electrophoresis was applied. After blotting, the membrane was incubated with LC3 (Cell Signaling Technology, USA) and β-actin (Santa Cruse Biotechnology, USA) primary antibodies at 4˚C. Images of the proteins were captured with a molecular imager (LI-COR Biosciences, USA) at a suitable time (change with respect to the primary antibody) after incubating the membranes with Western ECL Blotting Substrate (Bio-Rad, USA) for 5 min.

Results

Effect of theranekron on cell proliferation

The initial step for evaluating the effect of theranekron on gastric cancer cells was to determine the half maximal inhibitory concentration of the drug. The results of MTT assay showed that the IC_{50} value of theranekron on these cells was ~100 µM. Moreover, the significant effect of theranekron was found to be at 6 hrs. Therefore, 100 µM theranekron was applied to cells for 6 hrs in the subsequent experiments. After appropriate time and concentration were determined, the viability of cancer and non-cancer cells treated was analyzed for effectiveness and uniqueness. The results showed that theranekron affected similar in both AGS and MKN-45 gastric cells as it affected in control HEK-293 cells (Figure 1).

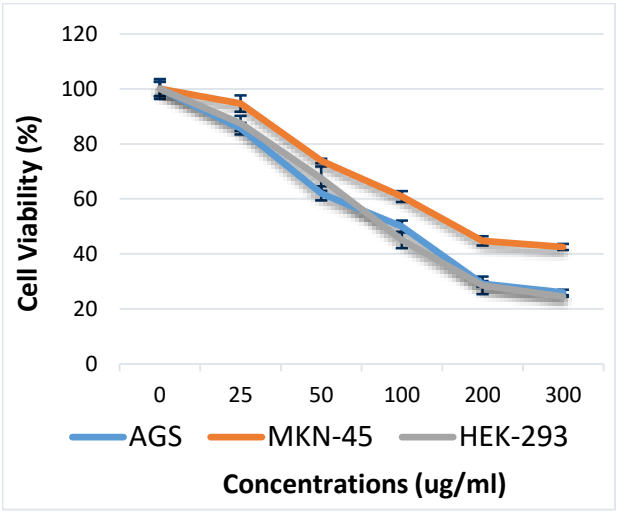

Figure 1. Comparison of theranekron's cytotoxic effects on AGS, MKN-45 gastric cancer and HEK-293 control cells at 6 hrs with different concentrations of the drug.

Moreover, theranekron was provided commercially and it was extracted in 60% ethanol. For this reason, theranekron and alcohol's cytotoxic effects were compared on gastric cancer and non-cancer cells to distinguish theranekron's effect from alcohol. All gastric cancer and control cells were treated with theranekron and ethanol at the same dilutions for 6 hrs. The results of proliferation assay demonstrated that alcohol showed similar results to theranekron (Figure 2).

Theranekron's autophagic effect in respect to the autophagy marker LC3

Since it was observed that theranekron decrease cell viability in all cells, additional experiments

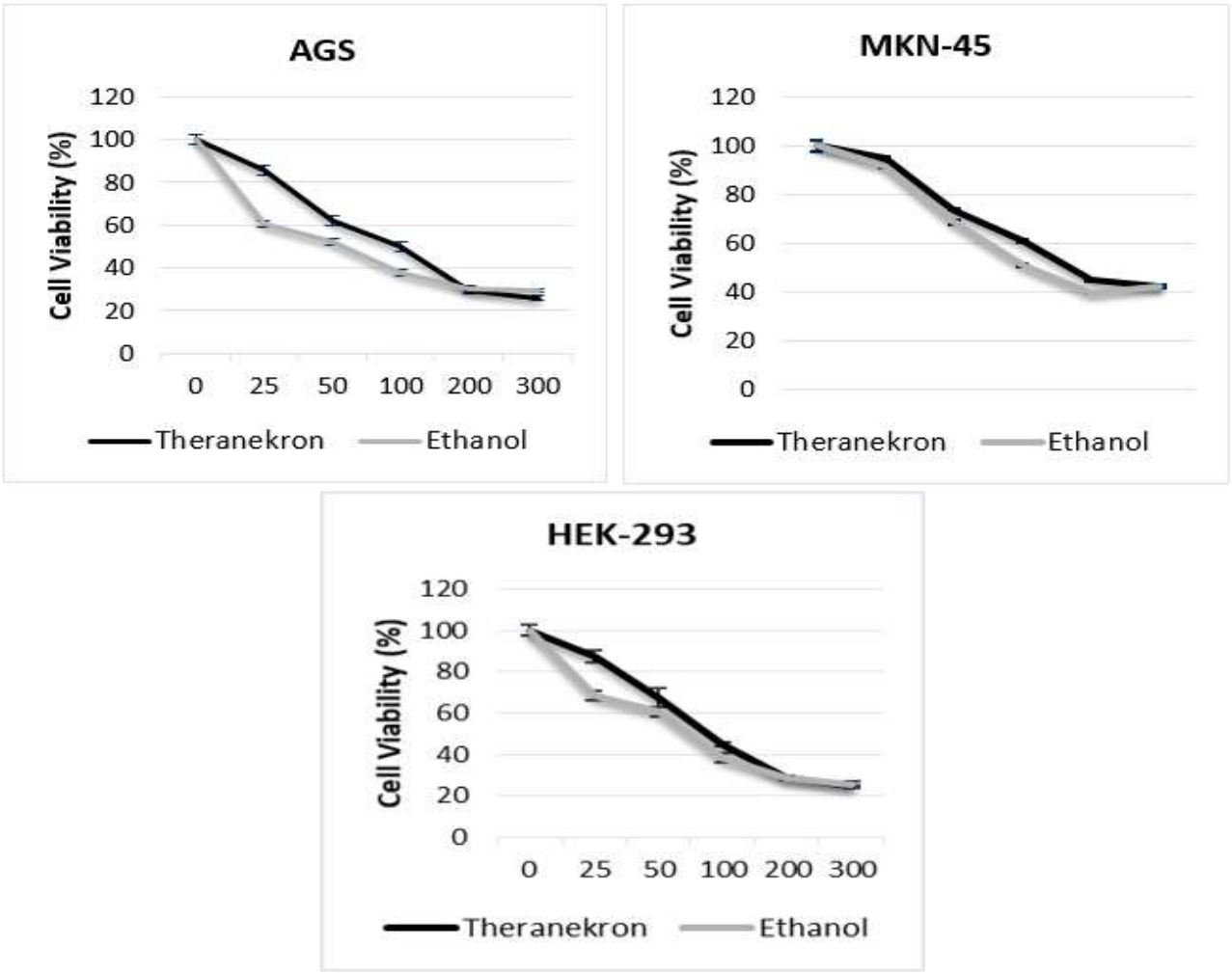

Figure 2. Comparison of theranekron and alcohol effects on AGS, MKN-45 and HEK-293 cells following treatment with theranekron and ethanol.

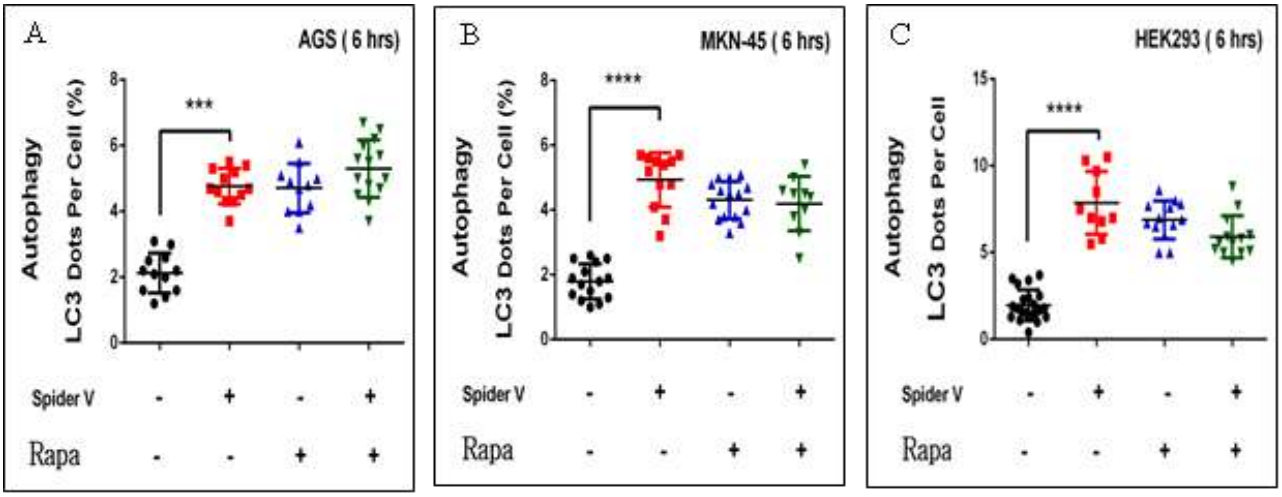

Figure 3. Autophagic effects of theranekron on (A) AGS, (B) MKN-45, and (C) HEK-293 cells displayed by LC3-GFP aggregation in the vacuoles of theranekron treated cells.

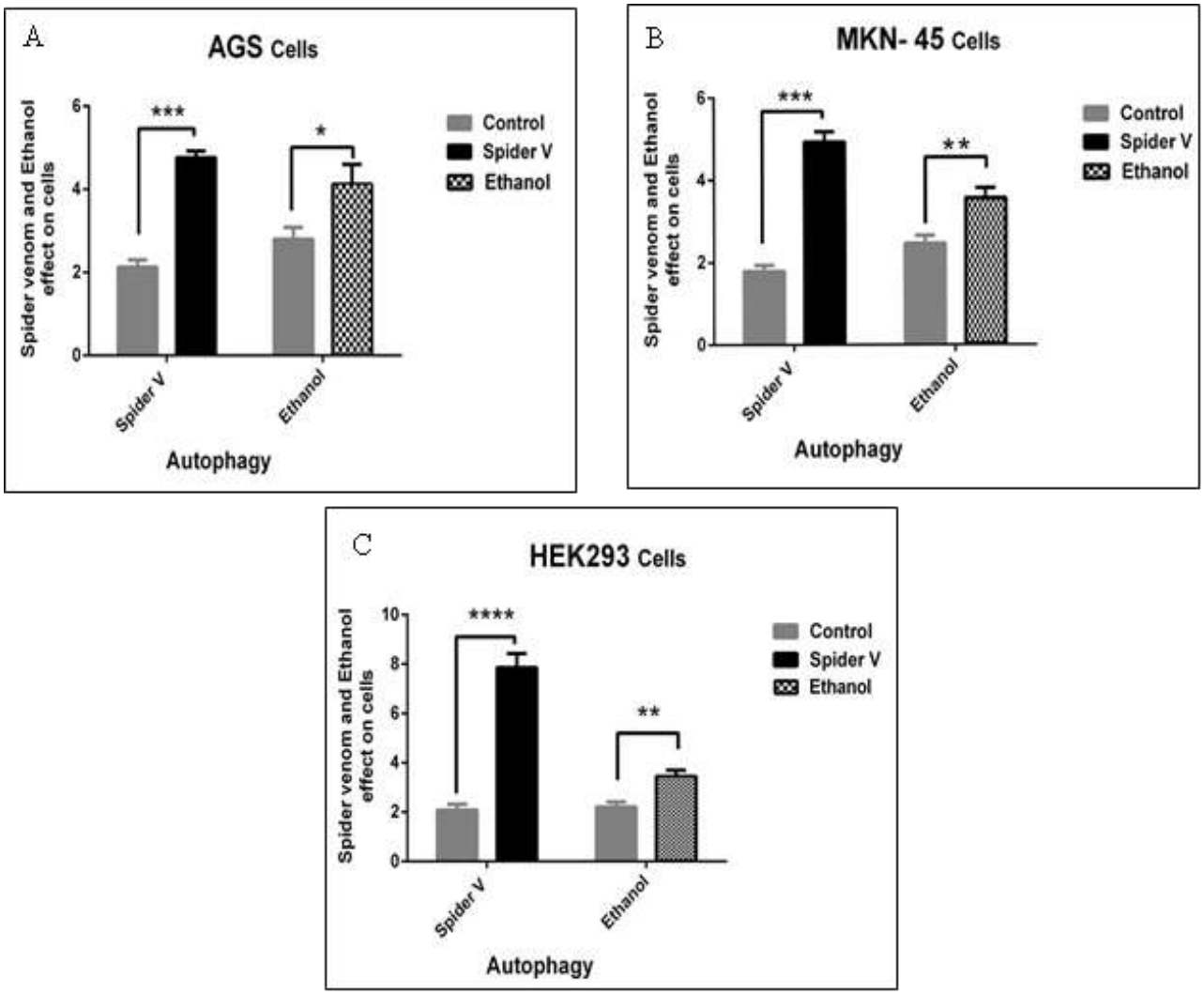

Figure 4. Comparison of autophagy effects of theranekron to ethanol on (A) AGS, (B) MKN-45, and (C) HEK-293 cells displayed by LC3-GFP aggregation in the vacuoles of theranekron and ethanol treated cells.

were carried out to investigate whether theranekron induce cell death through autophogic pathway. Initially, LC3 translocation assay was performed to analyze autophagy in treated cells. The results demonstrated that theranekron induced autophagy in gastric cancer cell lines AGS and MKN-45 when applied for 6 hrs at the similar level with rapamycin that is an autophagy inducer (Figure 3A and 3B). However, it was observed that autophagy was also induced in control HEK-293 cells, so the autophagy induction was not unique to cancer cells (Figure 3C).

In addition to that, alcohol's effect on autophagy was also tested on these cells since the drug was extracted in ethanol. According to the results, ethanol triggered authophagic cell death, but when compared to the effect of drug, there were less LC3 aggregated vacuoles in ethanol treated cells especially in HEK-293 cells comparing to gastric cancer cells (Figure 4A, 4B, 4C).

To confirm the LC3 translocation assay's results, theranekron treated gastric cancer and non-cancer control cells were analyzed for LC3-I to LC3-II conversion by western blotting, which is known as hallmark of autophagy. The results showed that theranekron induced autophagy in both AGS and MKN-45 gastric cancer cells at 6 h. LC3-I (18 kDa) to LC3-II (16 kDa) conversion was clearly visualized in the treated cells. As in LC3 translocation assay results, theranekron induced autophagy in gastric cancer cells similar to HEK-293 control cells. Moreover, the ethanol treatment resulted in the autophagy in all cell types, but its affect was less than theranekron's

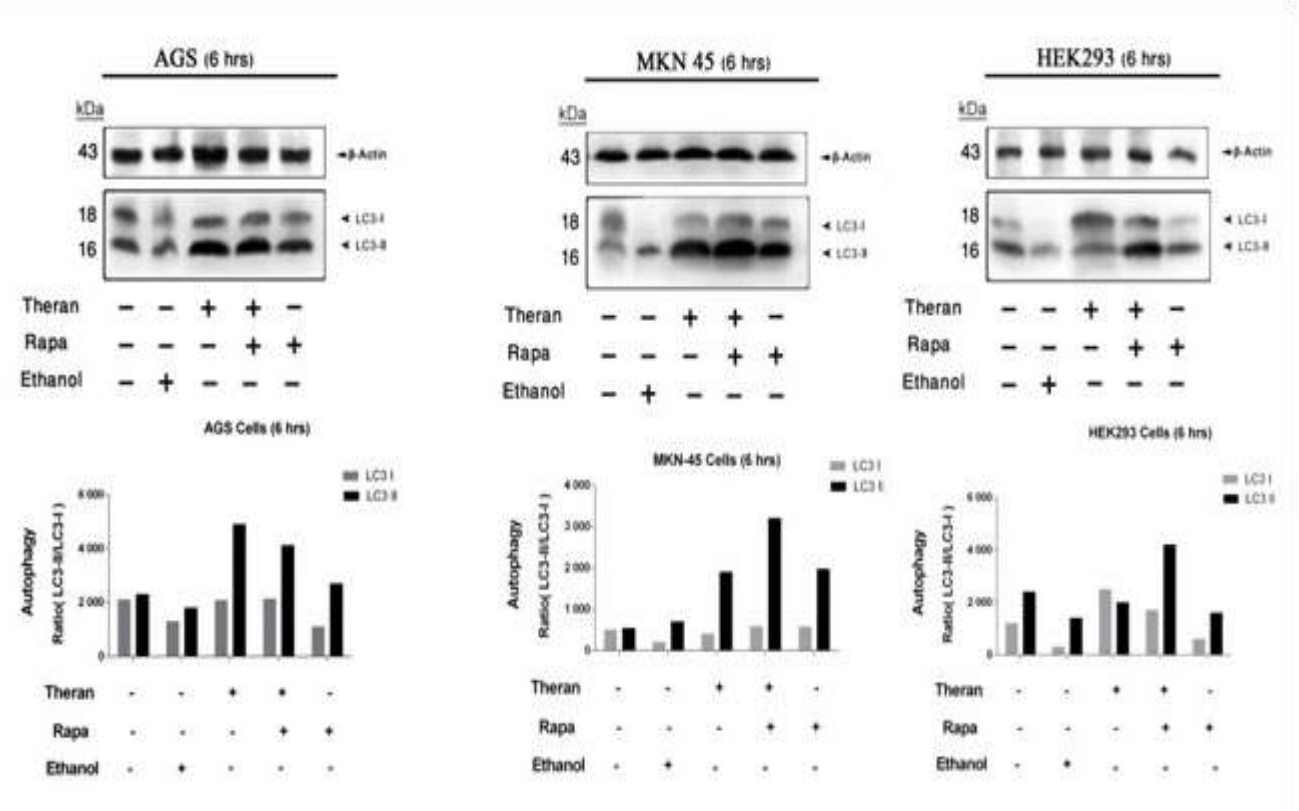

Figure 5. Autophagic effects of theranekron on AGS, MKN-45, and HEK-293 cells demonstrated by LC3-I (18 kDa) to LC3-II (16 kDa) conversion. All cell types were treated with theranekron, ethanol and rapamycin, separately, for comparison.

effect (Figure 5A, 5B, and 5C). This data also showed a consistency with LC3 translocation assay results. These results showed that theranekron induced more extensive autophagic cell death in both cell types when compared to the ethanol treated cells. However, theranekron did not show a significant difference in the gastric cancer cells for autophagic cell death when compared to the HEK-293 control cells.

Discussion

In this study, the effects of theranekron on gastric cancer cells were investigated focusing on autophagic cell death. Gastric cancer is one of the most common gastrointestinal tumors and fifth leading cancer type, globally [2]. Autophagy is a tightly-regulated homeostatic process that involves degradation of cells' own components through the lysosomal machinery. It is a major system to clear the defective or aging organelles and long-lived proteins in eukaryotic cells. It is reported that autophagy plays a dual role as tumor suppressor function at early stages while it plays an oncogenic function once the tumor is formed by providing cancer cells with survival contexts as nutrition [17]. Autophagy plays also tumor-suppressor and tumor-promoter role in gastric cancer [18]. Autophagy-related markers as Beclin1 might be considered as a potential marker of gastric carcinogenesis, aggressiveness and prognostic prediction, and as a target for gene therapy in gastric cancer [19] while autophagy inducers, such as rapamycin, show promise for gastric cancer treatment [20]. It was also reported that long-term *Helicobacter pylori* infection can disrupt autophagy process eventually promoting gastric cancer [21].

Theranekron is a homeopathic remedy that is mostly used in the veterinary medicine. It is an alcoholic extract produced by processing the whole spider known as Tarantula cubensis and diluting in 60% alcohol. There are many studies carried out with theranekron to present its beneficial effect in the veterinary medicine area such as wound healing [22], anti-inflammatory [23], and necrotizing action [4]. In addition to these studies, antibacterial effect of theranekron was evaluated, but they could not find significant antibacterial property of the venom [24]. Recently, theranekron's effect on peripheral nerve healing was also studied, and it was found that theranekron decreases axonal and myelin damage after sciatic nerve injury [9].

Beside of all these data, there is not much study investigated the theranekron's effect on cancer. For the first time, Koch and Stein published a paper in 1980 about theranekron's effect on dogs' mammary gland tumors [10]. They demonstrated that theranekron stops tumor growth in canine mammary tumors by forming demarcation from surrounding tissue when it is used preoperatively a week for three times with the dosage of 3-6 ml depending on the body weight. It was observed that tumors became smaller, no reoccurrence was observed for years. Then, Gultiken and Vural demonstrated in their 2007 paper that Tarantula cubensis extract applications resulted in regression and hardness of benign mammary tumors while only hardness was detected in malignant mammary tumors in the dogs [11]. The same group then published another study in 2015 about theranekron's apoptotic effect on canine mammary tumors. Pre- and post-treatment tumor tissues were immunohistochemically assessed and they showed that the expression of B-cell lymphoma 2 (Bcl-2) which is considered an important anti-apoptotic protein was found to be higher in pre-treatment compared to post-treatment tissues. They concluded that the apoptotic index was low before treatment and

increased during treatment, so apoptotic cell death increased through theranekron treatment [12]. Beside these results, Ghasemi-Dizgah et al (2017) and Ayse et al (2017) demonstrated in their *in vitro* studies that theranekron increases cell death through apoptosis [25, 26]. In the current study, it was shown that theranekron leaded to cell death, but also presented that the cell death induced by theranekron was also through autophagic pathway. Moreover, it was also shown that the cell death could be triggered by alcohol in which theranekron was diluted. Finally, this study also indicated that alcohol triggered autophagic pathway supporting previous studies in the literature about alcohol induces autophagy [27, 28].

The common ground of all studies performed before current study is that they had been carried out *in vivo*. *In vitro* cancer studies of theranekron is very limited in the literature. Moreover, autophagic effect of theranekron was not examined although its apoptotic effect was shown [25, 26]. In addition, ethanol, the solvent used to extract was not investigated in any study before to eliminate alcohol effect of theranekron. To our knowledge, the present study is the first to reveal the relationship between theranekron and autophagic cell death. Also, this is the first study to show theranekron may cause cell death through alcohol in which it is extracted.

Conclusions

In this study, it was demonstrated that theranekron result in cell death and stimulate the autophagy process, but it is not specific for cancer cells since it represented similar results on non-cancer cells. Moreover, the effect of theranekron on cell death might mostly occur through alcohol effect.

Acknowledgement

This manuscript was presented as oral presentation at 11th National Medical Genetic Conference on Septemper 24-27, 2014 at Istanbul, Turkey.

Further results were also presented as published abstract at European Human Genetics Conference 2015 on June 6 - 9, 2015 at Glasgow, Scotland, United Kingdom.

Funding: *There is no financial support and sponsorship.*

Conflict of Interest: *The authors declare that they have no conflict of interest.*

Ethical statement: *Since the study is a cell culture study, ethics committee approval was not obtained.*

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