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Evaluation of the Cytotoxic Efficacy of Thymoquinone and Capsaicin in the SH-SY5Y Neuroblastoma Cell Line

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Abstract

In our study, it was aimed to examine the effects of thymoquinone, the active ingredient of Nigella sativa, which has known anticancer activities, and capsaicin, which is an important part of the endocannabinoid system, on the neuroblastoma cell line SH-SY5Y cells. SH-SY5Y cells were grown in culture in conventional culture flasks in DMEM medium at 37 °C and 5% CO_2. When the cells were 70-80% confluent, morphological changes were examined under an inverted microscope. The cells were passaged into 96 microplates, and after passage, different concentrations of thymoquinone (2.5; 5; 10; 25; 50; 100; 200; 300 µM/ml) and capsaicin (0.675; 1.25; 2.5; 5; 10; 20; 50; 100 µM/ml) were applied to the cells. After administration, cytotoxic effect and proliferation rates/cell proliferation were analyzed by the MTT method. When compared to the control group, cultured cells treated with 200 and 300 µM thymoquinone and 100 µM capsaicin had reduced cell proliferation at statistically significant levels ($p<0.05$). Demonstration of antiproliferative activity of thymoquinone and capsaicin on neuroblastoma shows that phytotherapeutic approaches can be evaluated in cancer.

Key words: Cell culture techniques, Cytotoxicity test, MTT formazan, Thymoquinone, Capsaicin, Neuroblastoma

1. Introduction

Neuroblastoma is the most common extracranial solid tumor among childhood cancers that originates from the neural crest and develops anywhere in the sympathetic nervous system (Maris, 2010). An average of 700 children are diagnosed with neuroblastoma each year in the United States (Ward et al., 2014). In

neuroblastoma cases, about half of which are metastatic at the time of diagnosis (Tolbert and Matthay, 2018), the disease may regress spontaneously or may progress aggressively (Chung et al., 2020). As well as a fully cured treatment is still not available today, modern protocols including induction chemotherapy, surgical resection, high-dose chemotherapy combined with autologous stem cell transplantation, external beam radiotherapy, immunotherapy, and differentiating agents have produced results that increase the three-year survival rate to 60% (Park et al., 2016). Besides high drug resistance (Marengo et al., 2018), most patients with neuroblastoma, of whom 50% survive despite multimodality therapy, are severely affected by treatment complications. At this stage, patients with neuroblastoma urgently need new treatment targets with reduced toxicity and a high treatment rate (Park et al., 2020).

The SH-SY5Y neuroblastoma cell line is frequently used in neurodegenerative disease studies (Molina-Jimenez et al., 2003). Because it is unethical to obtain a primary human neuron, SH-SY5Y allows the study of human-specific proteins not found in rodent cultures, making the cell line valuable (Encinas et al., 2000). It is a unique experimental material for new molecule studies to be developed for neuroblastoma.

Phytochemicals are widely used in cancer as an antioxidative defense mechanism against DNA breaks caused by oxidative damage induced by oxidant radicals (Ames et al., 1993; Rajput and Mandal, 2011). Nigella sativa extract thymoquinone, which is frequently used in phytotherapy, is known to inhibit the proliferation, migration and invasion of cancer cells (Imran et al., 2018). Similarly, a different phytotherapeutic molecule, capsaicin (trans-8-methyl-N-vanillyl-6-nonenamide), is the primary and natural component of red pepper species (Cordell and Araujo, 1993). Capsaicinoids, which are included in a group of chemicals known as vanilloids, have many pharmacological and physiological activities such as anti-inflammatory, anti-apoptotic, antioxidant, anti-cancer, and neuroprotective properties (Chaudhary et al., 2019; Bujak et al., 2019; Zhang et al., 2019). Within the framework of these features, it is important to examine and compare thymoquinone and capsaicin in neuroblastoma cell lines to increase awareness of phytotherapeutic approaches to neurological cancers. Although they have been shown to be effective in many types of cancer: breast (Adinew et al., 2021; Dupoiron et al., 2022), prostate (Alshyarba et al., 2021; Sanchez et al., 2022), colorectal (Karim et al., 2022; Hou et al., 2019) and neuroblastoma (Firdaus et al., 2018; Baek et al., 2008), there is no study in the literature comparing the effectiveness of the two.

For this purpose, we aim to investigate the anti-proliferative effects of thymoquinone and capsaicin in SH-SY5Y neuroblastoma cells isolated from human tissue to develop treatment alternatives in neuroblastoma.

2. Material and Methods

Our research was carried out under in vitro conditions by using the Cell Culture Laboratory in Bolu Abant Izzet Baysal University Faculty of Medicine, Department of Physiology. Cell culture studies do not require ethics committee approval.

2.1. Drugs and reagents

Thymoquinone (Sigma-Aldrich Chemical Co., cat no: 490-91-5, St. Louis, Missouri, USA) and capsaicin (Tocris, cat no: 545395-94-6, UK) were acquired to be used in the study. Penicillin-streptomycin as antibiotic/antimycotic (Capricorn Scientific Ebsdorfergrund, lot no: CP21-4278, Germany), fetal bovine/bovine serum for cell culture experiment (Sigma-Aldrich, Schnelldorf, lot no: BCBT7617, Germany), dimethyl sulfoxide (DMSO, Pan-Biotek, Aidenbach, cat no: P60-36720100), trypsin/EDTA solution (Hyclone, Logan, UT, cat no: SH30042.01, USA), phosphate buffered water (PBS, Sigma-Aldrich Chemical Co., lot no: 6730915, St. Louis, Missouri, USA), and Dulbecco's Modified Eagle Medium/F-12 (DMEM/F-12) medium (Pan-Biotech, Aidenbach, cat no: P04-41250, Germany) were used. In order to obtain the final concentrations of thymoquinone and capsaicin in the in vitro experiment, thymoquinone was dissolved and capsaicin was diluted in ethanol at different concentrations: For thymoquonine 2.5, 5, 10, 25, 50, 100, 200, 300 µM /ml; for capsaicin (0.675, 1.25, 2.5, 5, 10, 20, 50, 100). We used our work control groups for the MTT assay. For the metabolic activity assays of control goups were untreated cells (Cel-) exposed to the maximum solvent concentrations with ethanol and DMSO. Both our working and control groups are presented as the mean ± SD of each with two replicates. Each treatment was compared to the 100% metabolic activity of the negative control (untreated cells).

2.2. Cell culture

The SH-SY5Y human neuroblastoma cell line purchased from ATCC (ATCC CRL-2266, Manassas, VA, USA) was used in the study. The cell culture protocol was completed with reference to previously published studies (Shipley et al., 2016). In brief, cells were quickly thawed in a 37 °C water bath and then centrifuged at 3000 rpm for 4 minutes. Next, all cells were cultured in 75 cm^2 flasks/flasks having fortified DMEM/F12 mixture/full medium supplemented with 10% heat-inactivated FBS and 1% penicillin-streptomycin at 37 °C in a humidified incubator/oven with 5% CO_2. One day later, the growth medium was rapidly changed to take away any DMSO that may be present in the freezing medium. Cells were maintained at the log phase and supplemented/replaced with fresh medium every 3-4 days. Cell morphologies and growth rates of the cell line were monitored daily under an inverted microscope (Olympus CKX41, Tokyo, Japan) and passaged by separation with 0.25% Trypsin-EDTA when cells reached 70-80% confluence.

2.3. Thymoquinone and capsaicin treatment

After reaching the appropriate confluence, the cells were passaged. Thymoquinone at concentrations of 2.5; 5; 10; 25; 50; 100; 200; 300 µM/ml and capsaicin at concentrations of 0.675; 1.25; 2.5; 5; 10; 20; 50; 100 µM/ml were prepared and given to the cells in equal volumes as 100 µl and incubated for the defined times (24 hours) used. For both drugs and each dose, 2 wells were inoculated and the cells were incubated at 37 ºC in a 5% CO_2 air mixture in a cell culture incubators, very high relative (around 90-95%) humidity environment. Humidity is homogeneous and created by evaporation from the water tank inside the incubator.

2.4. MTT assay

Trypan blue was used to establish cell viability and cell quantification. BioRad TC20 Automated Cell Counter was used to calculate cell viability and cell numbers. After cell counting in 96-well plates, nearly 1.5x104 cells/well were seeded in a total volume of 200 µL. Plates were then incubated at 37 °C for 24 hours for cell attachment.

When the cells in the flask reached 70- 80% confluence, thymoquinone (2.5; 5; 10; 25; 50; 100; 200; 300 µM/ml) and capsaicin (0.675; 1.25; 2.5; 5; 10; 20; 50; 100 µM/ml) in different dilutions were added to the 96-well plate(s) and kept in incubation for 24 and 48 hours. Cells incubated in 10% FBS were used as positive control and the viability of the cells was determined by MTT Method (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl- tetrazolium bromide) (Serva, Germany). The MTT test is a colorimetric method used to find out cell viability. It is based on the ability of NADPH-dependent cellular oxidoreductase enzymes to alter the tetrazolium MTT into insoluble formazan, which is purple. Briefly, 10 µl of MTT reagent was added to each well and the 96-well plate was incubated at 37 °C for 4 hours, then DMSO was added to the cells. Absorbance values/Change in Color were read at 570 nm with a colorimetric reader (spectrophotometer) (Epoch BioTek Instruments, Inc., Highland Park). The most suitable proliferative and inhibitory doses of thymoquinone and capsaicin were determined according to the cells. Thus, the effect of thymoquinone and capsaicin on the viability of cultured cells and the period of the effective dose were determined.

2.5. Statistical analysis

Statistical analyzes were made with SPSS 25.0 package statistics program. Cytotoxicity experiments were performed in duplicate and GraphPad Prism 5.0 software (GraphPad Software, San Diego, USA) was used for data analysis. Descriptive statistics were given as number of units (n), percentage (%), mean ± standard deviation (x ± SD) median (Q1-Q3) values. The normal distribution of the datasets were evaluated with the Kolmogorov-Smirnov (K-S) Test. Mann-Whitney U Test was used for nonparametric distributed variables as a multiple comparison test. $p < 0.05$ was considered statistically significant.

3. Results and Discussion

Thymoquinone (200 and 300 µM) decreased ($p<0.05$) the cell viability, while the other concentrations of thymoquinone did not alter the viability compared to medium-treated cells (Figure 1). Capsaicin (100 µM) decreased ($p<0.05$) the cell viability, while the other concentrations of capsaicin did not alter the viability compared to medium-treated cells (Figure 1).

The same doses of thymoquinone and capsaicin were compared. It was observed that capsaicin was found to be more cytotoxic when both drugs are administered at the same molarity in different wells: 5; 10; 100 µM on SH-SY5Y cells. Besides it was observed that thymoquinone was found to be more cytotoxic when both drugs are administered at the same molarity in different wells: 2,5; 25; 50 on SH-SY5Y cells (Figure 2).

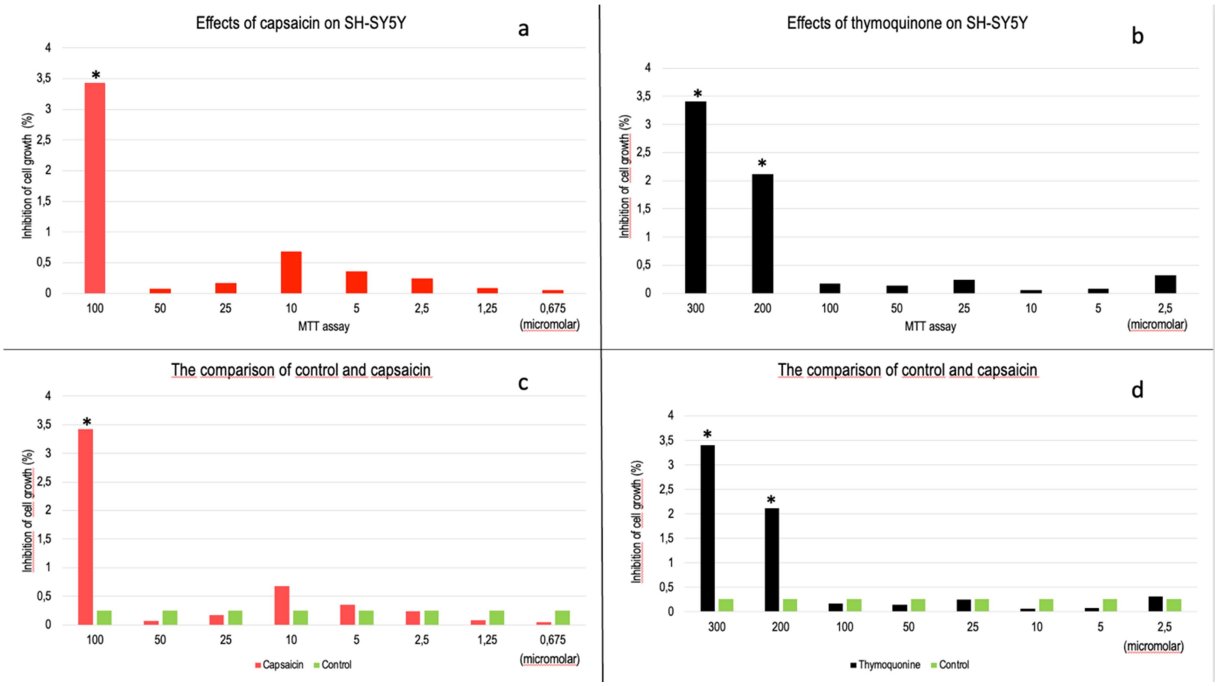

Figure 1. a. Effect of different doses of capsaicin on SH-SY5Y cell line. **b.** Effect of different doses of thymoquinone on SH-SY5Y cell line. **c.** Comparison of the control and capsaicin SH-SY5Y cell line. **d.** Comparison of the control and thymoquinone SH-SY5Y cell line.

*: p<0.05. There are significantly differences on 100 µM capsaicin-treated cells and 200 and 300 µM thymoquinone-treated cells versus non-treated control cells, each with the two replicates.

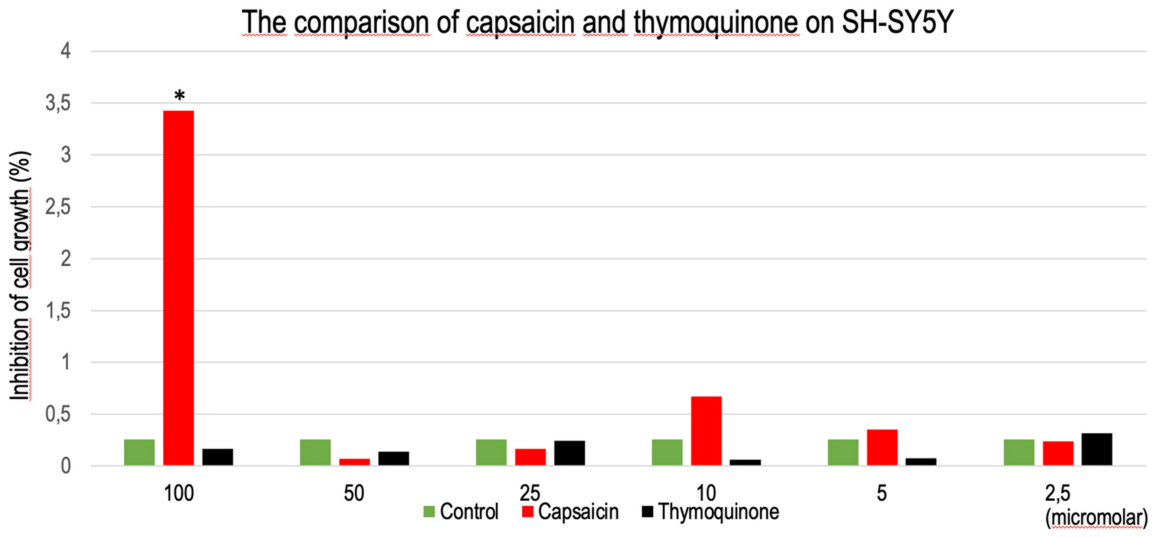

Figure 2. Comparison of the same doses of capsaicin and thymoquinone in SH-SY5Y cell line, each with the two replicates.

In the present study, the effectiveness of capsaicin and thymoquinone molecules on cell viability in the SH-SY5Y neuroblastoma cell line was evaluated, and it was observed that a 100 µM dose of capsaicin and 200 and 300 µM doses of thymoquinone decreased the number of neuroblastoma cells.

While phytotherapy was evaluated in the shadow of synthetic drugs in the 20th century (Kuete and Efferth, 2011) due to the recommendation of non-health professionals, it has come to the fore again in recent years due to the growing interest in medicinal plants (Falzon and Balobanova, 2017) so much so that today many drugs are obtained from plants. The fact that it has against cancer toxicity and improves health constitutes makes it a potential drug target. In this respect, intensive study of phytochemicals in medicine by researchers led to the award of the Nobel Physiology Prize to a phytotherapeutic product in 2015; a scientist named Youyou Tu introduced artemisinin in the plant *Artemisia annua*, known in Chinese medicine, to the literature by recommending it for the treatment of malaria (Efferth et al., 2015).

Due to surgical difficulties, chemotherapeutic side effects, and neurodegenerative processes, the demonstration of the effects of phytochemicals is important today to increase patient survival, especially in the field of neurological cancers. Thymoquinone, one of the active components of Nigella sativa, which has a strong traditional use among medicinal plants, is widely studied due to its pharmacological properties (Samarghandian et al., 2018). Although the molecular action pathways have not been fully revealed yet, thymoquinone has immunomodulatory, antioxidant, anticancer, antiapoptotic (Akter et al., 2021) and anti-inflammatory properties (Amin and Hossainzadeh, 2016) in healthy cells, and antiproliferative effects in cancerous cells (Aslan et al., 2021). It also has curative effects in neurological diseases such as epilepsy (Beyazçiçek et al., 2016), Alzheimer's (Elibol et al., 2020), depression (Safhi et al., 2019), and Parkinson Disease (Uddin et al., 2021). Thymoquinone has been shown to be effective in breast (Talib, 2017), prostate (Kolli-Bouhafs et al., 2012), blood (Hansen et al., 2003), bone (Thummuri et al., 2015), and colon (Lei et al., 2015) cancers. In the present study, 200 and 300 μM doses of thymoquinone in the neuroblastoma SH-SY5Y cell line effectively reduced cell viability and supported the anticancer property of thymoquinone.

Capsaicin is the primary and naturally occurring component found in red pepper species and is responsible for the pungency of peppers (Cordell and Araujo, 1993). It shows neuroprotective effects in neurodegenerative diseases as an analgesic in the treatment of neuropathic pain (Ilie et al., 2019) by reducing beta-amyloid accumulation in Alzheimer's Disease (Wang et al., 2020) and oxidative stress in Parkinson's Disease (Siddique et al., 2018). It has antiproliferative and cytotoxic effects on many peripheral cancer cells such as breast (Chen et al., 2021), prostate (Zhu et al., 2020), colon (Nisari and Eroz, 2020) and oral cancers (Chang et al., 2020). It is also known to exert apoptotic effects on brain-derived tumors such as glioblastoma (Xie et al., 2016). In our study, the use of capsaicin in neuroblastoma SH-SY5Y cell line with the cytotoxic effect of 100 μM dose in neurological cancers was also demonstrated.

Description of the action pathways of thymoquinone and capsaicin, which have been the potential drugs for the future, will allow to know both molecules better. Thymoquinone, has been shown to increase reactive oxygen products in brain cancer cell lines and trigger apoptosis by reducing the mitochondrial membrane potential (Firdaus et al., 2018). Similarly, it is known that capsaicin triggers the formation of superoxide radicals and initiates apoptosis by disrupting the intracellular mitochondrial membrane status in many cancer cell lines (Hail and Lotan et al., 2002). The similarity of the action mechanisms of both capsaicin and thymoquinone against

SH-SY5Y cells led us to compare the two molecules. Our study shows that the dose-response assessment of both drugs is not linear, as capsaicin at 100, 10, and 5 micromolar and thymoquinone at 2.5, 25 and 50 micromolar are more cytotoxic to SH-SY5Y cells when similar doses are compared. The limitation is that the signaling pathways of capsaicin and thymoquinone have not been evaluated by molecular, histopathologic and serologic techniques.

4. Conclusion

We anticipate that information from this research article would be capitalized for the future development of thymoquinone and capsaicin as potential therapeutic agents to be applied for neuroblastoma and the formulations of popular phytochemicals to promote optimum health and reduce cancers. In addition, it will be valuable for further studies to investigate the potential beneficial effects of thymoquinone and capsaicin in all aspects with animal experiments and clinical studies.

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Conflicts of Interests

There is no conflict of interest between authors.

Statement contribution of the authors

This study's experimentation, analysis and writing, etc. all steps were made by the authors.

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