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**RESEARCH ARTICLE**

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# Anti-Cancer Effects of *Trigonella foenum* in Neuroblastoma Cell Line

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**ABSTRACT**

**Objective:** Current approaches focus on the use of natural compounds in plants in the treatment of cancer. *Trigonella foenum* (Fenugreek) is mainly used in medicine, food, and cosmetics due to its bioactive and aromatic compounds. Many compounds with medicinal effects are found in Fenugreek seeds. These compounds have been demonstrated to be effective on numerous cancer cells. Neuroblastoma is one of the most common extracranial solid malignancies in children. In our study, the effects of Fenugreek on cell cytotoxicity, cell damage, migration, and sphere formation in neuroblastoma cells were investigated.

**Materials and Methods:** The powder extract was dissolved in DMEM and filtered to prepare a stock solution of Fenugreek. We used MTT assay for the detection of cytotoxic effects of Fenugreek on SH-SY5Y cells, crystal violet staining for the analysis of morphological change in cells, wound healing test for the cell migration analysis, and agarose gel electrophoresis for detecting DNA damage. We created a spheroid to test the effect we observed in 2D culture, in 3D culture as well.

**Results:** The findings of our study showed that Fenugreek treatments caused significant reductions in cell proliferation and migration capacities in 2D neuroblastoma cells at $IC_{50}$ (1500 µg/ml) doses, respectively. In addition Fenugreek changed cell morphology and increased DNA fragmentation. Furthermore, Fenugreek caused disruption of 3D spheroid formation of SHSY-5Y cells in a dose- and time-dependent manner.

**Conclusion:** The determination of the anti-cancer effect of Fenugreek on neuroblastoma cancer may be a useful and feasible intervention in neuroblastoma patients in the light of further studies and with the help of new nano drug delivery systems.

**Keywords:** Fenugreek, neuroblastom, proliferation, spheroid

## INTRODUCTION

An increasing number of research studies are now focusing on investigations of the anti-cancer potential of plants. Because bioactive chemicals in plants have been shown to have a variety of biological and pharmacological effects, natural products are regarded as powerful sources for new drug discovery and development. The various medicinal effects of natural compounds in traditional medicine have sparked interest in their use in cancer treatment (1,2).

The annual plant Fenugreek (*Trigonella foenum graecum*) belongs to the Leguminosae family. Fenugreek is one of the oldest medicinal herbs known as an aromatic legume plant native to many Asian, Middle Eastern and European countries due to its therapeutic and medicinal properties and is recognized by the FDA (American Food and Drug Administration) (3). The antioxidant and anti-inflammatory properties of Fenugreek that has rich content of Diosgenin, Apigenin, Luteolin, and Kaempherol have been reported. Although there are studies on

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the antiproliferative effects of Fenugreek on cancer cells such as breast and colon cancers, there is no comprehensive study that has reported its potential therapeutic efficacy against SH-SY5Y cells (4).

Neuroblastoma accounts for 10% of pediatric cancers and is characterized with a high risk of death due to its strong metastatic potential. Neuroblastoma is a very variable and difficult-to-treat tumor that can range from spontaneous remission to tumor development to aggressive malignancy. It is known to develop from embryonic neural crest cells, which play a critical role in the development of the sympathetic nervous system (5). The molecular mechanisms underlying the development and progression of neuroblastoma are unknown (6).

In this study, we aimed to detect the cytotoxic effect of Fenugreek, which is utilized in traditional medicine and widely used in Turkish cuisine, on SH-SY5Y cells. In addition its potential effects on cell migration and DNA damage were investigated. The effects of Fenugreek on SH-SY5Y cells generated as two-dimensional (2D) and three-dimensional (3D) spheroid models were examined.

## MATERIALS AND METHODS

### Preparation of Fenugreek Extract

Fenugreek extract was donated by INDUS BIOTECH, INDIA. Before use it was dissolved in Dulbecco's Modified Eagle Medium (DMEM) (Gibco, Waltham, MA, USA).

### Culture of The Neuroblastoma Cell Line 'SH-SY5Y'

The SH-SY5Y cell line from the American Type Culture Collection (ATCC) was cultured in DMEM (Gibco, Waltham, MA, USA) with 10% fetal bovine serum (FBS) (Serox GmbH, Mannheim, Germany), 2 mM L-glutamine (Sigma-Aldrich), 100 U/ml penicillin, and 100 g/ml streptomycin (Sigma-Aldrich). Cells were incubated in a 95% humidity and 5% $CO_2$ atmosphere at 37°C.

### The Cytotoxic Effect of Fenugreek in SH-SY5Y Cells: MTT Assay

The cytotoxic effect of the plant extract was assessed using the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltratrazolium bromide) (BioFroxx, Germany) assay. In a 96-well plate, SH-SY5Y cells were seeded at $5x10^3$ cells/well and allowed to attach to the surface for 24 h. The cells were exposed to 100 µg/ml, 500 µg/ml, 750 µg/ml, 1000 µg/ml, and 1500 µg/ml concentrations of plant extract for 24 h and incubated for 4 h by adding 5 mg/mL of MTT. To each well, 100 µl dimethyl sulfoxide (DMSO) (Merck) was added to reduce formazan crystals. Absorbance at 570 nm was measured by a multiplate reader (BioTekTM SynergyTM HTX). The mean absorbance of the control cells was accepted as 100% viable. The effective dose ($IC_{50}$) value was calculated.

### The Effect of Fenugreek on Morphological Changes in SH-SY5Y Cells: Crystal Violet Staining

SH-SY5Y cells were seeded at a density of $25x10^4$ cells per well in a 6-well plate and allowed to adhere to the surface for 24 h. After exposing the cells to the plant extract (750 µg/ml, 1500 µg/ml ($IC_{50}$), 3000 µg/ml for 24 h, the morphology of the cells was assessed using crystal violet staining. Phosphate buffered saline (PBS):methanol was added in a 1:1 ratio and maintained at +4°C for 3 h. After that, a solution of 0.1 % crystal violet and 5% methanol was added and incubated for 15 min at room temperature. The dye in the well was removed after the plate had been incubated. After waiting for the well to dry for 1-2 min, it was examined under a microscope (Olympus CKX53, 10X).

### The Effect of Fenugreek on Cell Migration in SH-SY5Y Cells: The *In Vitro* Wound Healing Model

SH-SY5Y cells were seeded at $5 \times 10^5$ cells/well in a 24-well plate and allowed to adhere to the surface for 24 h. A sterile plastic pipette tip was used to make a linear wound pattern on the sheet at once. Cells were exposed to Fenugreek at doses of 750 µg/ml, 1500 µg/ml ($IC_{50}$) and, 3000 µg/ml and compared to the control group. All of the samples were visualized using an inverted microscope (Olympus CKX53,10X) at $0^{th}$, $24^{th}$, $48^{th}$, $72^{th}$ h.

### The Effect of Fenugreek on DNA Damage in SH-SY5Y Cells: Electrophoretic Analysis

SH-SY5Y was seeded at $10^6$ cells/well in a 6-well plate. The cells were allowed to adhere to the 6-well plate overnight and exposed to the plant extract (750 µg/ml, $IC_{50}$, 3000 µg/ml) for 24 h. The cells were then scraped into a sterile tube, centrifuged for 5 min at 900 rpm, and resuspended in 1 ml of ice-cold PBS. DNA was extracted from the cell suspension using a commercial DNA isolation kit according to the manufacturer's instructions (Geneall, Korea). The concentrations and purity of the DNA samples were assessed in the Nanodrop. In the gel electrophoresis, DNA samples were run in 1% agarose at 140 V for 3 h. A UV Transilluminator was used for determination of the bands (Wealtec, USA).

### The Effect of Fenugreek on *In Vitro* 3D SH-SY5Y Cells: The Agarose Matrix Spheroid Model

A sterile 1.5 % agarose coating was applied to the 96-well plate. SH-SY5Y cells were seeded at $2x10^3$ cells/well in agarose matrix and cultured for 10 days to generate a spheroid model. SH-SY5Y cells were exposed to Fenugreek (750 µg/ml, 1500 µg/ml ($IC_{50}$) and, 3000 µg/ml) for 24 h after sphere formation. The results were evaluated by using a microscope (Olympus CKX53, 10X).

### Statistical Analysis

MTT assay results in order to determine the $IC_{50}$ value of Fenugreek were calculated with GraphPad Prism software v9. Non-linear regression analysis was performed for $IC_{50}$ calculations. $p<0.05$ was considered significant.

## RESULTS

### Fenugreek Induced Cytotoxicity in SH-SY5Y

The MTT assay was used to study the cytotoxic effect of different doses of Fenugreek (0-1000 µg/ml) on SH-SY5Y cells. After 24 h of treatment with various doses of plant extract, a dose-dependent decrease in cell viability was seen (Figure 1). After analyzing all of the concentrations with the GraphPad , the $IC_{50}$ dose was calculated to be 1500 µg/ml ($p<0.05$).

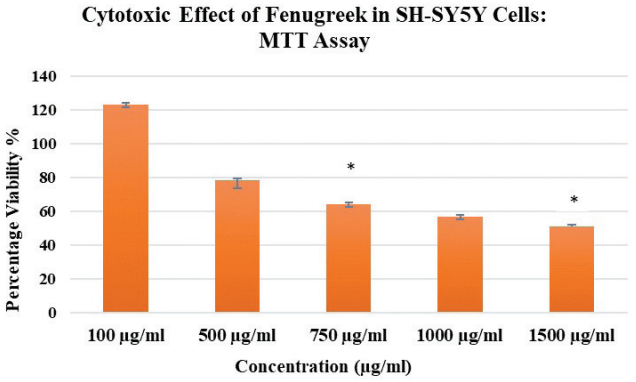


Figure 1. The cytotoxic effect of Fenugreek extract with different concentrations (100 µg/ml, 500 µg/ml, 750 µg/ml, 1000 µg/ml, 1500 µg/ml) on SH-SY5Y cells at 24th h. Experiments were carried out in triplicate. Data are expressed as mean±standard deviation (S.D.). *p<0.05.

**The Effects of Fenugreek on the Cell Morphology**

An inverted microscope was used to assess the effect of Fenugreek treatment on cell morphology. The results of the microscope examination revealed that cell viability had decreased, which was consistent with the MTT test results. Crystal violet dye was used to stain the cells after they had been morphologically examined (Figure 2). The increasing changes in the morphology of SH-SY5Y cells were observed in a dose-dependent manner. In comparison to the control group, cell viability was reduced in the wells given varying doses of Fenugreek in microscobic examination. More than half of the cells treated with 750 µg/ml Fenugreek survived, but around half of the cells treated with the $IC_{50}$ dose died. Almost all of the cells died at the toxic dose of 3000 µg/ml.

**Fenugreek Induced the Inhibition of Cell Migration**

In order to evaluate the metastatic feature of the tumor, it is important to demonstrate its invasion and migration capabilities at the first stage. To investigate the effect of Fenugreek on cell migration in SH-SY5Y, a wound healing model, which is an in vitro approach, was established. The cells of control groups were migrated to the wound area after 24 h. When compared to the control group, the cell migratory capacity of the groups exposed to Fenugreek at 750 µg/ml, 1500 µg/ml ($IC_{50}$) and 3000 µg/ml concentrations decreased (Figure 3).

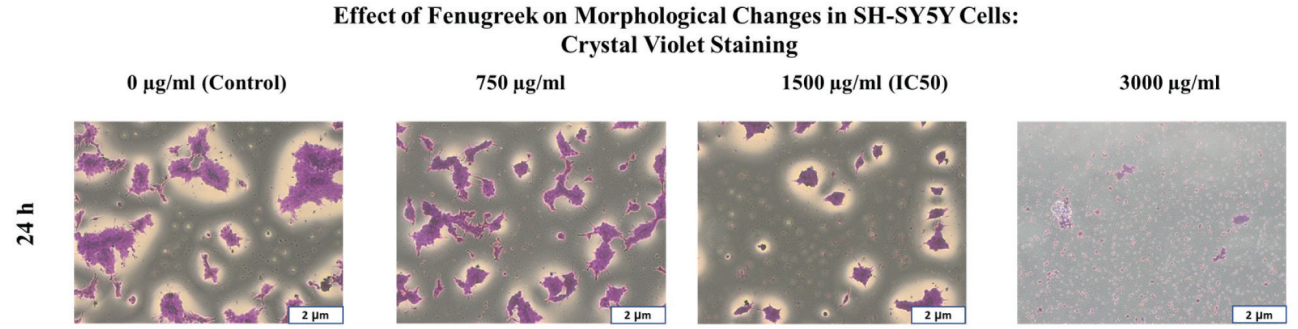


Figure 2. Crystal violet assay was performed in SH-SY5Y at 750 µg/ml, $IC_{50}$ (1500 µg/ml) and 3000 µg/ml doses.

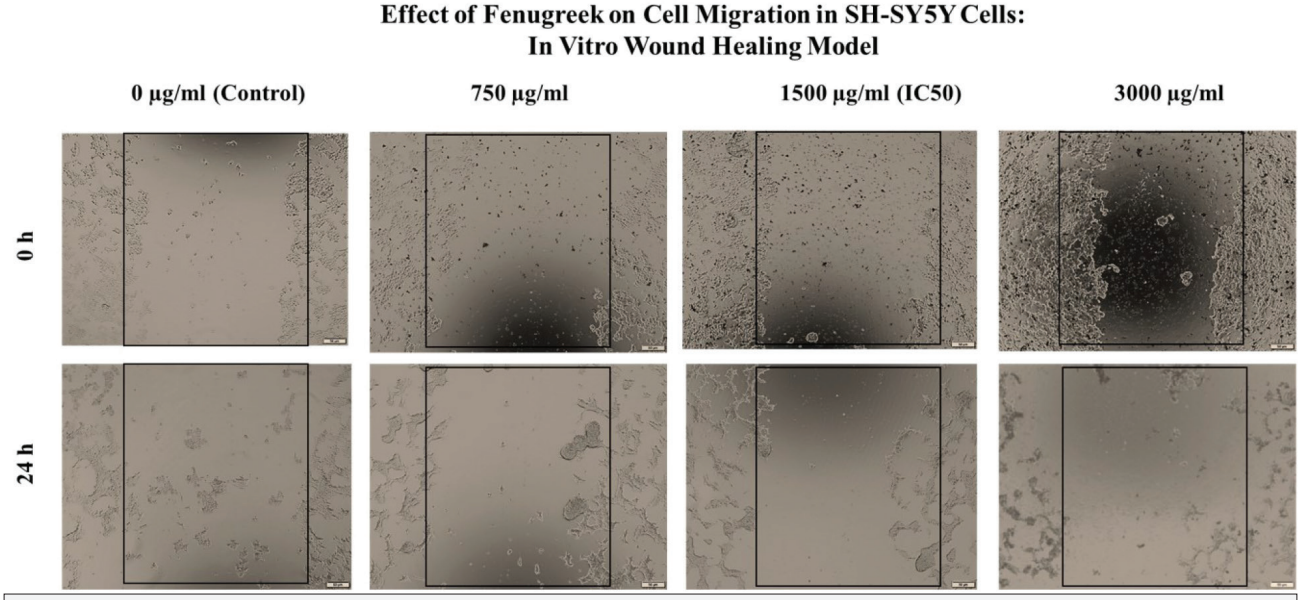


Figure 3. Cell migration was analyzed by wound-healing assay. Scale bar 50 µm.

**The Effects of Fenugreek on the DNA Fragmentation**

DNA fragmentation was studied using the traditional "DNA ladder" method, which involves extracting DNA from apoptotic cells and separating it on an agarose gel. In cells exposed to Fenugreek, chromosomal DNA was fragmented into short internucleosomal fragments, as illustrated in Figure 4, a biochemical hallmark of cells undergoing apoptosis. Longer DNA fragmentation was observed when higher Fenugreek concentrations were applied.

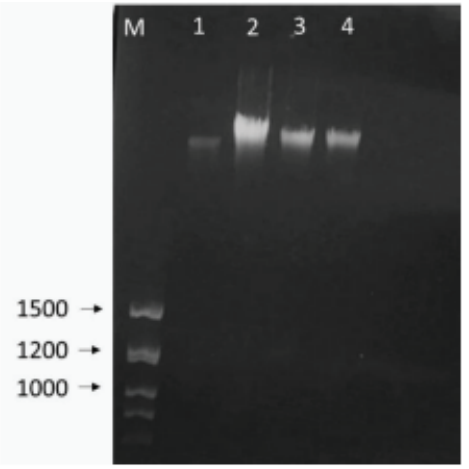


Figure 4. DNA Damage was examined with agarose gel electrophoresis in SH-SY5Y Cells. M:Marker, 1:Control (Untreated) 2: 3000 µg/ml, 3:1500 µg/ml, 4:750 µg/ml.

**The Effects of Fenugreek on Spheroid Forming Ability**

We investigated the effect of Fenugreek extract on spheroid formation of SH-SY5Y cells in 3D culture. In each well of a 96-well round bottom extra low attachment plate, 2000 cells were planted. When compared to the control group, the internal structure of the spheroid was more scattered in the $IC_{50}$ treated group after 24 h. In comparison to the control group, the spheroid structure in the 3000 µg/ml applied group began to dissolve dramatically after 24 h (Figure 5).

**DISCUSSION**

Natural components make up a portion of the chemotherapeutic and chemopreventive medications, which can be used alone or in combination with other cancer treatment methods (7). Medicinal plants containing natural components have been shown to be effective and versatile chemopreventive agents against cancer types such as lung, breast, and liver cancer (8). The Fenugreek plant from the Leguminosae family has antioxidant and immunostimulatory properties that make it interesting for its use in cancer treatment. In particular, Fenugreek seeds and leaves that contain flavonoids and alkaloids offer a wide range of therapeutic uses (9,10).

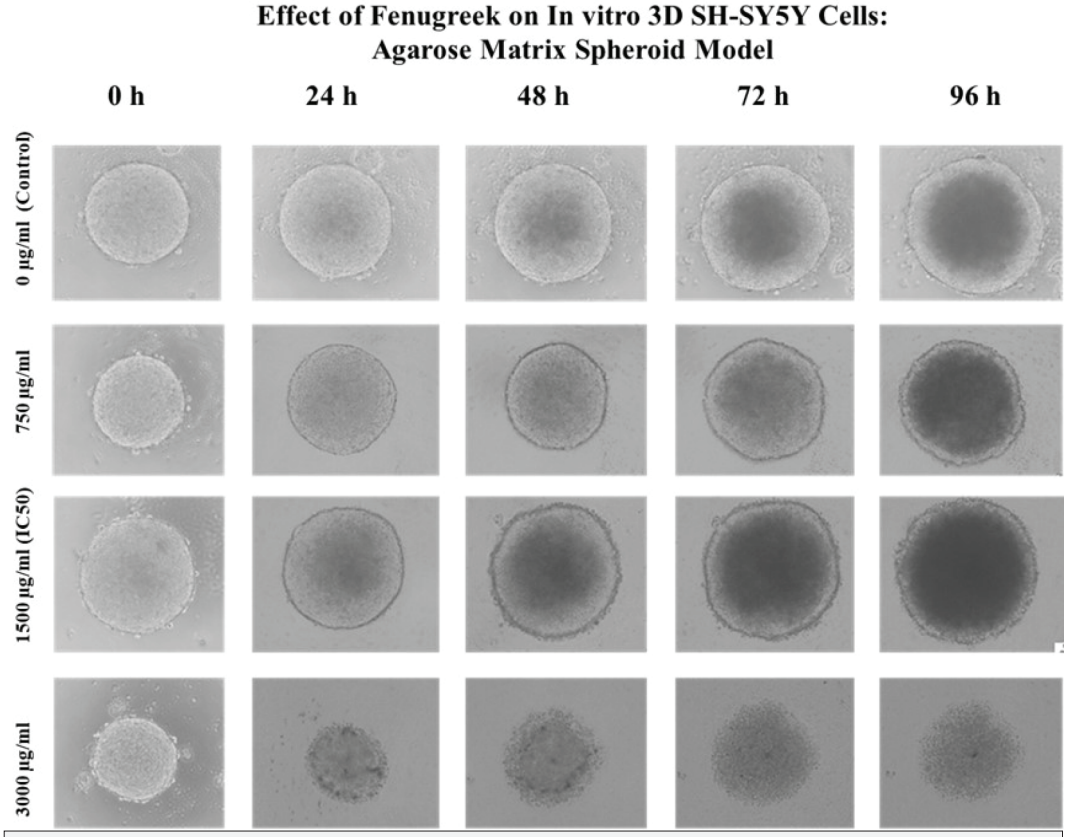


Figure 5. SH-SY5Y spheroids macroscopic aspect after Fenugreek extract treatment. Scale bar 50 µm.

Fenugreek seeds have been shown to have anti-oxidant, anti-diabetic and anti-nociceptive qualities, as well as hypocholesterolemic and anti-cancer capabilities (11). Furthermore Fenugreek constituents are beneficial to neurological health and its constituents have been shown to have hypolipidemic, hypoglycemic, antioxidant, and immunomodulatory effects, as well as a potential role in a variety of neurological disorders (12). With these known effects, we aimed to investigate the effects of Fenugreek extract on neuroblastoma cell viability in our study.

Several studies have focused on the cytotoxic effects of Fenugreek extract on cancer cells. Sebastian et al. showed that Fenugreek ethanolic extract reduced cell viability and triggered early apoptotic changes such as phosphatidylserine reversal and reduced mitochondrial membrane potential on the breast cancer cell line MCF-7 (13). In a different study, it was shown that both MeOH extracts from Fenugreek sprouts were more effective than those from seeds (14,15). Kaviarasan et al. found that Fenugreek had a cytoprotective effect against ethanol-induced cytotoxicity in human liver cells (16). Furthermore, Fenugreek whole plant extract has been also shown to be cytotoxic in vitro against a variety of cancer cell lines, including A549, HepG2, colon cells (502713, HT29), and IMR-32 (17).

Studies determining the effects of Fenugreek on neuroblastoma are few. Syed et al. used brain-derived fibroblast neuroblastoma cells in their study and they reported that IMR-32 neuroblastoma cell lines showed cytotoxic inhibition by Fenugreek extracts (18). We investigated its effect on the bone marrow-derived epithelial neuroblastoma cell line (SH-SY5Y) in our study. We discovered that cytotoxic activity of Fenugreek extract increased in the neuroblastoma cell line in a dose dependent manner.

Moreover, migration studies have been performed to detect the effect of Fenugreek on metastasis. Migration is one of the most important challenges in controlling the cancer process. Metastasis, or the migration of cells in cancer, has a negative impact on the treatment process in patients. As a result, it is critical to identify compounds that inhibit tumor cell migration. In the wound healing model created with SH-SY5Y cells, Fenugreek extract inhibited cell migration (19). In a study with breast cancer cell lines, they reported that the anti-metastatic effect of Fenugreek extract against breast cancer (20). In our study, which is similar to this publication, it was observed that applying Fenugreek extract to SH-SY5Y neuroblastoma cells had a dose-dependent anti-metastatic effect.

Plant extracts containing phenolic compounds can cause DNA damage. Several studies have shown that these compounds can cause mutagenic effects by intercalating with DNA (21). In the literature, it has been shown that Fenugreek extract may be associated with DNA damage in different cancer cells (3,22). Our results show that exposure to Fenugreek extract causes damage to the DNA of SH-SY5Y cells. We also showed that DNA fragmentation increased in a dose-dependent manner in Fenugreek-treated cells compared to the control.

In summary, the Fenugreek plant had an anti-cancer effect on neuroblastoma cells. Taking advantage of the current breakthroughs in 3D spheroid technology for disease research and drug development, we wanted to validate the results we obtained in 2D culture in 3D culture as it more closely reflects the in-vivo tumor microenvironment. We observed the effects of dose application depending on time in the microscopic observation we made by applying the cytotoxic effects obtained with the two-dimensional cell culture to the 3D culture. Our results showed that Fenugreek content caused the deterioration of the spheroid structures of the cells in a 3D structure and reduced cell viability in SH-SY5Y cells.

In order for Fenugreek to be included in neuroblastoma treatment strategies studies, it is very important to thoroughly examine the Fenugreek content and evaluate the activity of the individual compounds of this herb. Although there are numerous published studies on the chemistry and bioactivity of Fenugreek seeds, Fenugreek sprouts and components and their effects in mediating changes in cell viability and cell proliferation in various other cancer cells, this study is the first to investigate the anti-cancer effects of Fenugreek in SH-SY5Y cells.

## CONCLUSION

Fenugreek extract showed anti-cancer activity on the human neuroblastoma cancer SH-SY5Y cell line. Further studies are still needed to understand the cell viability inhibition mechanism. These positive results in the SH-SY5Y cell line indicate that Fenugreek extract may be a potential agent as an additive to widely used anticancer drugs for neuroblastoma cancer treatment.

**Peer Review:** Externally peer-reviewed.

**Author Contributions:** Conception/Design of Study- I.U., M.P.; Data Acquisition- I.U., M.P., H.I.K.C., A.E.; Data Analysis/Interpretation- I.U., M.P., H.I.K.C., A.E.; Drafting Manuscript- I.U.; Critical Revision of Manuscript- M.P.; Final Approval and Accountability- I.U., M.P., H.I.K.C., A.E.

**Conflict of Interest:** Authors declared no conflict of interest.

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