Cytotoxic and Apoptotic Effects of Teucrium Polium L Extract on Human Hepatocellular Carcinoma Cell Line SNU-449

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Research Article

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Dr. Massimo Gadina
Editor-in-Chief
Inflammation

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Dear Dr. Gadina,

I wish to submit the manuscript entitled “Cytotoxic and Apoptotic Effects of Teucrium Polium L Extract on Human Hepatocellular Carcinoma Cell Line SNU-449” by Slocum, A., B. Satilmis, T.T. Sahin, S. Yilmaz and M.M.M. Harputluoglu to be considered for publication as an original research article in “Inflammation”.

The article explores the possible cytotoxic and apoptotic effects of an extract obtained from Teucrium Polium L on SNU-449, which is a hepatocellular carcinoma (HCC) cell line. The cytotoxic and anti-cancer properties of Teucrium Polium have been evaluated extensively in recent years, using various cell lines and varying extraction methods. While numerous studies have reported anticancer and cytotoxic properties of Teucrium Polium on the cell lines of cervical cancer (HeLa), colon cancer (SW480), melanoma (Skmel-3), breast cancer (MCF-7), colon cancer (Cac0-2, HCT-116, LoVo, SW480), glioblastoma multiforme (GBM) (REYF-1), osteosarcoma (Saos-2), lung cancer (COR-123), prostate cancer (DU145, PC3), bladder cancer (EL), laryngeal cancer (HEP-2), epidermoid carcinoma (A431), hepatoblastoma (HepG2), and chronic myeloid leukemia (K562), the effects of Teucrium Polium extract on the human HCC cell line SNU-449 are yet to be investigated. The findings of our study are significant, as the treatment of HCC is very challenging. With surgery only being possible at early stages of HCC and limited systemic treatment options, research concerning the discovery of other possible anti-cancer agents is imperative.

Because our study primarily focuses on cytotoxicology and investigates inflammatory and apoptotic pathways triggered by the Teucrium Polium extract, we believe it may be suitable for publication in “Inflammation”. We believe our findings will be of interest to the readers of “Inflammation” and will inspire further similar research.

Our study article represents original research; it has not been previously published and is not currently being considered for publication elsewhere.

As the authors of this article, we declare that we have no conflicts of interest associated with this study. Also, as corresponding author, I confirm that the article has been read and approved for submission by all named authors.

We look forward to hearing back from you following your evaluation of our work.

Thank you kindly for your time and attention,

Sincerely,

Dr Ayshe Slocum

Cytotoxic and Apoptotic Effects of Teucrium Polium L Extract on Human Hepatocellular Carcinoma Cell Line SNU-449

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ABSTRACT

Purpose: The current study aims to evaluate the effects of T. polium extract on the human HCC cell line SNU-449 in vitro and to determine possible cytotoxic, apoptotic and anti-cancer properties.

Methods: The MTT assay was performed to determine the dose-dependent effects of T. polium on tumor cell proliferation and to calculate IC50. The colony formation assay was carried out using the IC50 dose of T. polium to evaluate its effects on colony formation. To study the effect of T. polium on in vitro migration of SNU-449 cells, the scratch assay was performed. Finally, Western blotting was performed for caspase-3 and cleaved caspase-3 to study possible apoptotic effects.

Results: MTT analysis results demonstrated a dose-dependent decrease in cell viability. IC50 was calculated as 90 µg/mL using measurements from the 48th hour results. The colony formation assay showed that colony formation efficiency was reduced by 50% among SNU-449 cells in the T. polium treated group. With the wound healing assay results, half-gap times for the T. polium treated group and the control group were determined as 50.5 and 28.5 hours, respectively. Comparison of the caspase-3 and cleaved caspase-3 levels measured in T. polium treated cells and control cells ~~by means of Western blotting~~ did not demonstrate a significant difference between the two groups.

Conclusion: Reducing proliferation and survival rates in SNU-449 cells, T. polium appears to be a potentially effective antineoplastic agent. However, the cytotoxic effect of T. polium on SNU-449 cells is not mediated by induction of apoptosis.

Key words: Hepatocellular carcinoma, Teucrium polium, SNU-449, MTT assay, colony formation assay, scratch assay.

~~***Competing Interests and Funding***: This study was funded by the Scientific Research Projects Coordination Unit of Inonu University (IUBAP) (Project number: TTU-2021-2701)~~

1. Introduction:

Medicinal plants and their extracts have been used to treat various illnesses and ailments for centuries. Teucrium polium L. (Lamiaceae) (T. polium), one such medicinal plant, has a history of use that dates back over two thousand years. Although T. polium, also widely known as Golden/Felty germander, is a shrub native to the Mediterranean region, the Middle East and southwestern Asia, it is quite ~~prevelant~~ prevalent and approximately 300 subspecies of T. polium have been identified worldwide. T. polium has been employed for its diuretic, antipyretic, sudorific, spasmolytic, anti-inflammatory, antihypertensive, analgesic, antibacterial and antidiabetic properties [1, 2]. Nevertheless, more recent studies have indicated that T. polium also has beneficial effects when utilized in the treatment of nonalcoholic steatohepatitis and cancer [3]. The findings of these studies suggest that the anticancer and cytotoxic properties of T. polium are mainly attributed to the diterpenoid, flavonoid, iridoid and sterol compounds it contains [4]; however, among these, the polyphenolic compounds and specifically flavonoids are acknowledged as the most effective inducers of apoptosis [5]. Studies also indicate that administering T. polium alongside chemotherapeutics, such as vincristine, vinblastine, and doxorubicin, can enhance their efficacy [6]. The anticancer and cytotoxic properties of T. polium have been evaluated using various cancer cell lines, including those cell lines of cervical cancer (HeLa), colon cancer (SW480), melanoma (Skmel-3), breast cancer (MCF-7) [7], colon cancer (Cac0-2, HCT-116, LoVo, SW480), glioblastoma multiforme (GBM) (REYF-1) [8], osteosarcoma (Saos-2), lung cancer (COR-123) [9], prostate cancer (DU145, PC3) [10], bladder cancer (EL), laryngeal cancer (HEP-2), epidermoid carcinoma (A431), hepatoblastoma (HepG2), and chronic myeloid leukemia (K562) [11]. According to the findings of these studies, T. polium may be effective in treating various types of malignancies. However, the effects of T. polium

extract on the human hepatocellular carcinoma (HCC) cell line SNU-449 have yet to be investigated.

HCC, an aggressive and often insidious type of cancer, usually develops as a primary liver tumor in the setting of cirrhosis and chronic hepatitis B virus (HBV) or hepatitis C virus (HCV) infection [12]. The sixth most common cancer and the second leading cause of cancer-related death worldwide, HCC is difficult to diagnose [13]. Due to its variable manifestations clinically in terms of signs and symptoms, in addition to its non-specific radiological presentation, HCC is often diagnosed late in its course and may require multiple specific imaging modalities to be identified. Median survival following diagnosis is approximately 6 to 20 months, and many patients are untreatable at the time of diagnosis [14].

Treatment of HCC, similar to its diagnosis, is challenging. Surgical resection remains the mainstay of treatment; however, it is only effective during the early stages of HCC. Unfortunately, by the time HCC is diagnosed, most patients are ineligible for surgery due to severe liver failure or large tumor size. Other treatment options include liver transplantation, percutaneous ethanol or acetic acid ablation, radiofrequency, microwave or cryoablation ~~ablation~~, transarterial chemoembolization (TACE), transarterial radioembolization (TARE), radiation therapy or systemic treatment options such as chemotherapy, molecular targeted therapies and immunotherapy [15]. Sorafenib [16], a molecularly targeted tyrosine kinase inhibitor that inhibits RAF (~~Radiply~~ Rapidly Accelerated Fibrosarcoma) kinase and vascular endothelial growth factor receptor (VEGFR), represents perhaps the most important component of systemic therapy for HCC. However, while sorafenib improves survival compared with best supportive care alone [17], it is generally not well tolerated by patients. The benefits of sorafenib and similar systemic therapies are often limited by the extent of liver dysfunction in patients.

Therefore, the development and discovery of alternate agents in the treatment of HCC is of vital importance. Accordingly, the present study aims to evaluate the effects of T. polium extract on the human HCC cell line SNU-449 and to determine its possible cytotoxic, apoptotic and anti-cancer properties.

2. Materials and Methods

~~1.~~ 2.1 Cell lines

The human HCC cell line SNU-449 utilized in this study was obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA). Southern blot hybridization examinations revealed the presence of HBV DNA in this cell line; however, HBV genomic RNA was not expressed. ATCC confirmed that this cell line contains HBV DNA sequences. Prior to initiation of the study, viable cells were cultured in 75 cm^2 tissue culture flasks in RPMI-1640 (Catalog No. 30-2001), which was formulated by ATCC for this specific cell line. They were maintained in an incubator at 37°C (Panasonic® MCO-18AC-PE incubator, Japan), with a humidified atmosphere of 5% CO2~~.~~ and 95 % air. The cells cultured in RPMI-1640 were routinely supplemented with 5% heat-inactivated fetal bovine serum (FBS) (Sigma-Aldrich® F5724, USA) and %1 Penicillin-Streptomycin-Neomycin (5,000 units of penicillin, 5 mg streptomycin and 10 mg neomycin/mL) (Sigma-Aldrich® P4083, USA) to achieve a final concentration of 10%, promoting cell growth and proliferation and allowing for a doubling time of approximately 36 hours [18]. Following cultivation, the cells adherent to the flasks were separated from the flask bottom using 1 mL ~~tripsin~~ trypsin (Sigma-Aldrich® T3924, USA). Finally, after being mixed with 10 µL of trypan blue solution (Sigma-Aldrich® T8154, UK), the cells were counted using Luna-II Automated Cell Counter (Logos Biosystems®, South Korea).

2.2. Plant materials and Extract preparation
~~2.~~

200 grams of T. polium plant material was obtained from the Inonu University, ~~School of Medicine Pharmacy,~~ Department of ~~Medical~~ Pharmacology in powder form. The ethanol extract of T. polium was prepared using the maceration method. The powdered plant material was soaked in 80% ethanol for 24 hours and then evaporated at 50°C with a rotary evaporator. The crude extract was subsequently fractionated using a solvent-solvent extraction method with petroleum ether [19, 20]. For the present study, 360 mg of T. polium extract was dissolved in 7.2 mL of dimethyl sulfoxide (DMSO) (Merck® 116743 Dimethyl Sulfoxide Emplura®, USA) to prepare a master stock solution at a concentration of 50 mg/mL. This master stock was then diluted with RPMI-1640 to create a second stock containing 500 µg/mL of T. polium extract. Before being applied to the cell cultures, further serial dilutions were performed using RPMI-1640 to obtain T. polium solutions with concentrations of 250 µg/mL, 100 µg/mL, 50 µg/mL, 40 µg/mL, 20 µg/mL, and 10 µg/mL, respectively.

~~3. Extract preparation~~

~~The ethanol extract of T. polium was prepared using the maceration method. The powdered plant material was soaked in 80% ethanol for 24 hours and then evaporated at 50°C with a rotary evaporator. The crude extract was subsequently fractionated using a solvent-solvent extraction method with petroleum ether [19, 20]. For the present study, 360 mg of T. polium extract was dissolved in 7.2 mL of dimethyl sulfoxide (DMSO) (Merck® 116743 Dimethyl Sulfoxide Emplura®, USA) to prepare a master stock solution at a concentration of 50 mg/mL. This master stock was then diluted with RPMI-1640 to create a second stock containing 500 µg/mL of T. polium extract. Before being applied to the cell cultures, further serial dilutions were performed using RPMI-1640 to obtain T. polium solutions with concentrations of 250 µg/mL, 100 µg/mL, 50 µg/mL, 40 µg/mL, 20 µg/mL, and 10 µg/mL, respectively.~~

~~4.~~ 2.3. Cell viability assay ~~ytotoxicity assays~~

Cells in the logarithmic growth phase were ~~previously~~ trypsinized ~~were~~ and resuspended in RPMI culture medium. For cytotoxicity assays, ~~10,000 (~~10^4~~)~~ viable cells in 100 microliters of RPMI-1640 were plated into each well of three 96-well plates, with one column of wells excluded in each plate. The plates were incubated overnight to allow for cell adhesion. The following day, the medium in each of the three plates for 24h, 48h, and 72h treatment was discarded and replaced by 100 microliters of T. polium at various concentrations (250 µg/mL, 100 µg/mL, 50 µg/mL, 40 µg/mL, 20 µg/mL, 10 µg/mL, 5 µg/mL, respectively). A total of 10 columns were used for each plate, including one control column containing 10^4 cells mixed with RPMI-1640, one DMSO column representing the highest DMSO concentration from the extract (1% DMSO in RPMI-1640), and one blank column that contained only RPMI-1640 without any cells. The first plate was incubated with T. polium for 24 hours, the second plate for 48 hours, and the third plate for 72 hours. ~~During the incubation of the cells with the T. polium-containing medium, MTT was prepared.~~ Methylthiazolyldiphenyl-tetrazolium bromide (MTT) analysis is a colorimetric technique used to assess cell viability. In this process, the yellow tetrazolium dye, MTT, is reduced to purple formazan by mitochondrial succinate dehydrogenase, an enzyme that is present only in viable cells. Therefore, this reaction serves as a reliable indicator of cellular metabolic function.The MTT (Sigma-Aldrich® M2128-5G, USA) used in this study was prepared by diluting it in PBS to a concentration of 5 mg/mL. After the incubation periods were completed, 10 µL of MTT solution was added to each well, including the blank wells. The plates were then incubated in the cell culture incubator for an additional 4 hours. At the end of 4 hours, the medium was aspirated and removed (BioTek® 50TM TS Microplate Washer 40-301, Germany). To solubilize the formazan, 100 µL of DMSO was added to each well. The plate was then incubated at 37 °C for 15 minutes. Following the incubation, spectrophotometric absorbance measurements were taken at 570 nm with a multimode microplate reader (BioTek® SynergyTM H1m Microplate Reader, USA). To determine the 50% inhibition

concentration (IC50) value for T. polium extract in SNU-449 cells, firstly a calibration graph was created using Microsoft Excel® (Microsoft, Redmond, WA, USA) based on the MTT absorbance measurements obtained at different concentrations of T. polium administration. The IC50 value, which corresponds to 50% cell viability, was then calculated using an equation derived from the graph.

5. Microculture Tetrazolium Technique (MTT) assay

MTT analysis is a colorimetric technique used to assess cell viability by measuring metabolic activity under different conditions. In this process, the yellow tetrazolium dye, MTT, is reduced to purple formazan by mitochondrial succinate dehydrogenase, an enzyme that is present only in viable cells. Therefore, this reaction serves as a reliable indicator of cellular metabolic function.

The MTT (Sigma-Aldrich® M2128-5G, USA) used in this study was prepared by diluting it in PBS to a concentration of 5 mg/mL. After the incubation periods were completed, 10 µL of MTT solution was added to each well, including the blank wells. The plates were then incubated in the cell culture incubator for an additional 4 hours. At the end of 4 hours, the medium was aspirated and removed (BioTek® 50TM TS Microplate Washer 40-301, Germany). To solubilize the formazan, 100 µL of DMSO was added to each well. The plate was then incubated at 37 °C for 15 minutes. Following the incubation, spectrophotometric absorbance measurements were taken at 570 nm with a multimode microplate reader (BioTek® SynergyTM H1m Microplate Reader, USA).To determine the 50% inhibition concentration (IC50) value for T. polium extract in SNU-449 cells, firstly a calibration graph was created using Microsoft Excel® (Microsoft, Redmond, WA, USA) based on the MTT absorbance measurements obtained at different concentrations of T. polium administration. The IC50 value, which corresponds to 50% cell viability, was then calculated using an equation derived from the graph.

Cytotoxicity and cell viability level were calculated using the following formulas:

Cytotoxicity % = 1 – (average absorbance of cells exposed to T. polium extract) / (average absorbance of control group) x 100

Viability % = 100 – cytotoxicity %

6. IC50 (Inhibition Concentration) Determination

To determine the 50% inhibition concentration (IC50) value for T. polium extract in SNU-449 cells, firstly a calibration graph was created using Microsoft Excel® (Microsoft, Redmond, WA, USA) based on the MTT absorbance measurements obtained at different concentrations of T. polium administration. The IC50 value, which corresponds to 50% cell viability, was then calculated using an equation derived from the graph.

7. 2.4. Colony Formation Assay

Before beginning the colony formation experiment, RPMI-1640, PBS, and trypsin were heated to 37 °C. Initially, 10^3 SNU-449 cells were seeded into each well of 6-well plates. The plates were then placed in an incubator at 37 °C overnight to allow for the adhesion of the cells to adhere to the surfaces. Following successful confirmation of cell seeding under the microscope, the existing medium was removed from the plates. RPMI-1640 was subsequently added to the control wells, while a sublethal dose of T. polium solution (IC50 = 90 µg/mL) diluted in RPMI-1640 was added to the T. polium wells. The cells were then incubated under standardized cell culture conditions. After 48 hours, both the

T. polium solution and the RPMI-1640 medium were removed from the cells. During the final stage of the experiment, RPMI-1640 was added to all wells, including both T. polium and control wells. The medium was changed every 2-3 days and the cells were monitored carefully for approximately 9-11 days until the cells in the control wells formed sufficiently large colonies. Once an adequate number of colonies were cultivated, the medium was removed from all the wells, and the cells were carefully washed with PBS (phosphate-buffered saline). Afterwards the PBS was aspirated from the wells and Methanol: Acetic acid (3:1) was added to the wells instead. The plates were incubated with Methanol: Acetic acid (3:1) for 5 minutes. Thus, cell fixation was achieved.

Following the removal of the fixation solution, a 0.5% crystal violet solution in methanol was added to the wells and left for 15 minutes to stain the cells. After the staining process, the plates were immersed in a container filled with water, and the excess crystal violet was rinsed away. Finally, the colonies were counted and measured using the calibrated eye-piece of an inverted microscope (Leica Dmi8, Germany). Plating efficiency (PE), is the ratio of the number of colonies that have formed to the number of cells seeded:

PE = (number of colonies formed) / (number of cells seeded) x 100%

The number of colonies formed after treatment is applied to the cells is called the survival fraction (SF) and is obtained with PE:

SF = (number of colonies formed after treatment) / (number of seeded cells x PE)

~~8. Colony Fixation and Staining~~

~~Once an adequate number of colonies were cultivated, the medium was removed from all the wells, and the cells were carefully washed with PBS (phosphate-buffered saline). Afterwards the PBS was aspirated from the wells and Methanol: Acetic acid (3:1) was added to the wells instead. The plates were incubated with Methanol: Acetic acid (3:1) for 5 minutes. Thus, cell fixation was achieved.~~

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~~9. Plating efficiency~~

~~Plating efficiency (PE), is the ratio of the number of colonies that have formed to the number of cells seeded:~~

~~PE = (number of colonies formed) / (number of cells seeded) x 100%~~

~~10. Surviving Fraction~~

~~The number of colonies formed after treatment is applied to the cells is called the survival fraction (SF) and is obtained with PE:~~

~~SF = (number of colonies formed after treatment) / (number of seeded cells x PE)~~

~~11.~~ 2.5. In vitro cell ~~wounding (“~~scratch~~”)~~ assay

10^6 SNU-449 cells were seeded in two 60 mm tissue culture petri dishes. They were then incubated at 37 °C overnight to ensure the adherence of the cells and formation of a confluent cell layer (monolayer). After the confluence of the cell layers was confirmed using an inverted microscope, the monolayers in both petri dishes were scratched in a straight line using a 100 µL sterile pipette tip. The cell dishes were then washed once with 1 ml of PBS to correct the scratch edges and to remove the debris formed by the lysed cells.
5 mL of RPMI was added to the control petri dish, while 90 µg/mL of T. polium dissolved in 5 mL of RPMI was added to the other petri dish. Both cell layers were later placed in the incubator and incubated at 37 °C for 48 hours. Photos were taken of both cell layers every 4 hours using the Paula Smart Cell Imager (Leica Microsystems, Germany), in order to evaluate the cells' ability to repopulate the wound and study their migratory patterns.

12.2.6 Western Blotting for Caspase-3 and Cleaved Caspase-3

SDS-PAGE electrophoresis was performed using a 4–20% Mini-PROTEAN® TGX™ Precast Protein Gel (Bio-Rad, USA) on the Bio-Rad Mini-Protean electrophoresis system (Bio-Rad, USA). Equal amounts of protein samples were loaded into the wells, and electrophoresis was carried out for 30 minutes in an electrophoresis buffer containing 25 mM Tris, 192 mM glycine, and 0.1% SDS, pH 8.3 (Bio-Rad, USA), at a constant voltage of 200 V. At the end of the run, stain-free gel imaging was conducted using a gel imaging device (Bio-Rad ChemiDoc, USA) to visualize the proteins separated by gel electrophoresis between the cassettes. Proteins that were separated using SDS-PAGE electrophoresis were transferred to a membrane using the Trans-Blot® Turbo™ RTA Mini 0.2 µm PVDF Transfer Kit (BioRad 1704272, USA) and the Trans-Blot® Turbo™ Transfer System (BioRad 1704150, USA). At the end of the blotting process, stain-free blot imaging and stain-free gel imaging were performed on the imaging device to visualize the proteins that migrated from the gel to the PVDF membrane. The PVDF blotting membrane was blocked using the EveryBlot blocking solution (BioRad 12010020, USA) prior to the incubation with the primary antibodies. The blocked membrane was then incubated overnight at +4 °C with Caspase-3 Rabbit monoclonal antibody (mAb) (Cell Signaling 14220, USA) and Cleaved Caspase-3 Rabbit mAb (Cell Signaling 9664, USA) in EveryBlot solution at the recommended dilutions provided by the manufacturer. After incubation, the membrane was washed five times for five minutes each with Tris Buffered Saline (TBS) (BioRad 1706435, USA) containing Tween 20 (BioRad 1610781, USA) on a horizontal shaker (IKATM KS130). The washed membrane was subsequently incubated for one hour at room temperature with Anti-rabbit IgG, HRP-linked Antibody (Cell Signaling 7404, USA) in EveryBlot solution at the manufacturer’s recommended dilutions. Finally, the membrane was washed six times for five minutes each with Tris Buffered Saline containing Tween 20 on a horizontal shaker. The PVDF membrane, which had been incubated with primary and secondary antibodies, was treated with Clarity Max Western ECL Substrate (BioRad 1705062, USA) at the volume and for the duration recommended by the manufacturer. After the incubation period, chemiluminescence imaging of the blot was performed using a gel imaging device. Analysis was conducted with chemiluminescence blot images using Image Lab Software (BioRad, USA).

MATERYAL -METOD KISMINDA BÖYLE BİR BİLGİYE GEREK YOK TARTIŞMADA KULLANABİLİRSİN Caspase (Cysteine-requiring Aspartate Protease) (Caspase) is a family of enzymes that play a crucial role in the execution phase of apoptosis. Among these, caspase-3 acts as an "executioner" caspase. Its zymogen form is activated in apoptotic cells through both extrinsic (death receptor-mediated) and intrinsic (mitochondria-mediated) pathways. Once activated, caspase-3 is cleaved and processed to form cleaved caspase-3, which is the active form of the enzyme in vitro. Caspase-3-mediated protein cleavage is a crucial step in the molecular mechanism of apoptosis. It also plays a significant role in nuclear apoptosis, which includes processes such as chromatin condensation, DNA fragmentation, and cell blebbing. When apoptosis begins,

activated caspase-3 cleaves its associated substrates in the endochilema and cytoplasm, ultimately leading to cell death.

In this study, the possibility of apoptosis after T. polium treatment in SNU-449 cells was investigated using Western blotting and specific validated primary and secondary antibodies for caspase-3 and cleaved caspase-3.

13. SDS-PAGE Electrophoresis

SDS-PAGE electrophoresis was performed using a 4–20% Mini-PROTEAN® TGX™ Precast Protein Gel (Bio-Rad, USA) on the Bio-Rad Mini-Protean electrophoresis system (Bio-Rad, USA). Equal amounts of protein samples were loaded into the wells, and electrophoresis was carried out for 30 minutes in an electrophoresis buffer containing 25 mM Tris, 192 mM glycine, and 0.1% SDS, pH 8.3 (Bio-Rad, USA), at a constant voltage of 200 V. At the end of the run, stain-free gel imaging was conducted using a gel imaging device (Bio-Rad ChemiDoc, USA) to visualize the proteins separated by gel electrophoresis between the cassettes.

14. Blotting

Proteins that were separated using SDS-PAGE electrophoresis were transferred to a membrane using the Trans-Blot® Turbo™ RTA Mini 0.2 µm PVDF Transfer Kit (BioRad 1704272, USA) and the Trans-Blot® Turbo™ Transfer System (BioRad 1704150, USA). At the end of the blotting process, stain-free blot imaging and stain-free gel imaging were performed on the imaging device to visualize the proteins that migrated from the gel to the PVDF membrane.

15. Incubation with Primary and Secondary Antibodies

The PVDF blotting membrane was blocked using the EveryBlot blocking solution (BioRad 12010020, USA) prior to the incubation with the primary antibodies. The blocked membrane was then incubated overnight at +4 °C with Caspase-3 Rabbit monoclonal antibody (mAb) (Cell Signaling 14220, USA) and Cleaved Caspase-3 Rabbit mAb (Cell Signaling 9664, USA) in EveryBlot solution at the recommended dilutions provided by the manufacturer. After incubation, the membrane was washed five times for five minutes each with Tris Buffered Saline (TBS) (BioRad 1706435, USA) containing Tween 20 (BioRad 1610781, USA) on a horizontal shaker (IKATM KS130). The washed membrane was subsequently incubated for one hour at room temperature with Anti-rabbit IgG, HRP-linked Antibody (Cell Signaling 7404, USA) in EveryBlot solution at the manufacturer's recommended dilutions. Finally, the membrane was washed six times for five minutes each with Tris Buffered Saline containing Tween 20 on a horizontal shaker.

16. Incubation and Imaging with Chemiluminescence Reagent

The PVDF membrane, which had been incubated with primary and secondary antibodies, was treated with Clarity Max Western ECL Substrate (BioRad 1705062, USA) at the volume and for the duration recommended by the manufacturer. After the incubation period, chemiluminescence imaging of the blot was performed using a gel imaging device.

17. Analysis

Analysis was conducted with chemiluminescence blot images using Image Lab Software (BioRad, USA).

3. Results

~~1.~~ 3.1. Assessment of the Cytotoxic Effects of T. polium Extract on the SNU-449 Cell Line

Absorbance measurement results from MTT assays, obtained spectrophotometrically using varying concentrations of T. polium, were converted into percentages indicating cell viability ~~(Table 1-3)~~. Ultimately, each measurement demonstrated a dose-dependent decrease in cell viability ~~(Graph 1-3)~~ (Figure 1).

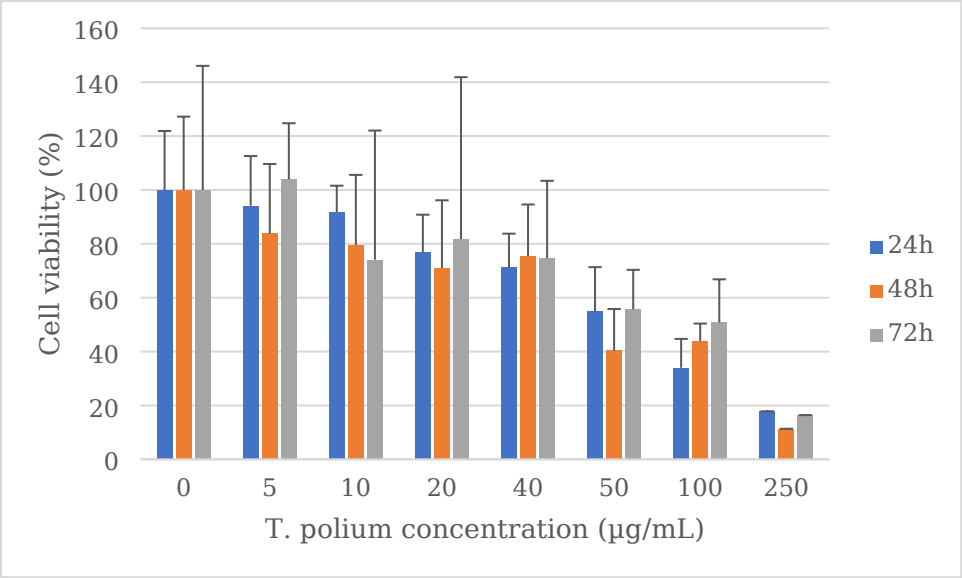


Figure 1. *Cell viability of the SNU-499 cells which were treated with varying concentration of T.Polium during 24, 48, and 72 hours.*

~~***Table 1**: 24th hour data from MTT analysis using T. polium extract at various concentrations*~~

	~~***Control***~~	~~***DMSO***~~	~~***5 µg/mL***~~	~~***10 µg/mL***~~	~~***20 µg/mL***~~	~~***40 µg/mL***~~	~~***50 µg/mL***~~	~~***100 µg/mL***~~	~~***250 µg/mL***~~
~~**Average absorbance**~~	~~0,489875~~	~~0,5275~~	~~0,46125~~	~~0,449625~~	~~0,377125~~	~~0,3495~~	~~0,26875~~	~~0,1665~~	~~0,087375~~
~~**Cell viability %**~~	~~100,00~~		~~94,15667~~	~~91,78362~~	~~76,98392~~	~~71,34473~~	~~54,86093~~	~~33,98826~~	~~17,83618~~

~~***Table 2**: 48th hour data from MTT analysis using T. polium extract at various concentrations*~~

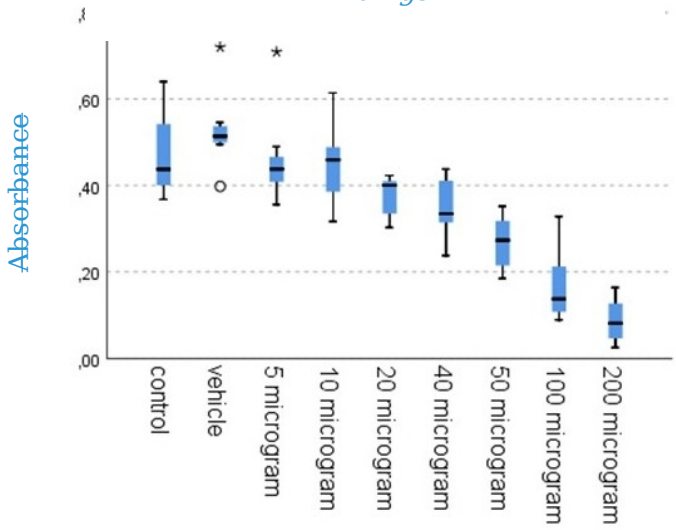
~~**Control**~~	~~**DMSO**~~	~~**5 µg/mL**~~	~~**10 µg/mL**~~	~~**20 µg/mL**~~	~~**40 µg/mL**~~	~~**50 µg/mL**~~	~~**100 µg/mL**~~	~~**250 µg/mL**~~

Average absorbance	0,586375	0,470714	0,491714	0,466286	0,416429	0,442571	0,236667	0,257375	0,0665
Cell viability %	100,00		83,85663	79,52005	71,01745	75,47584	40,36097	43,89256	11,34087

***Table 3**: 72nd hour data from MTT analysis using T. polium extract at various concentrations*

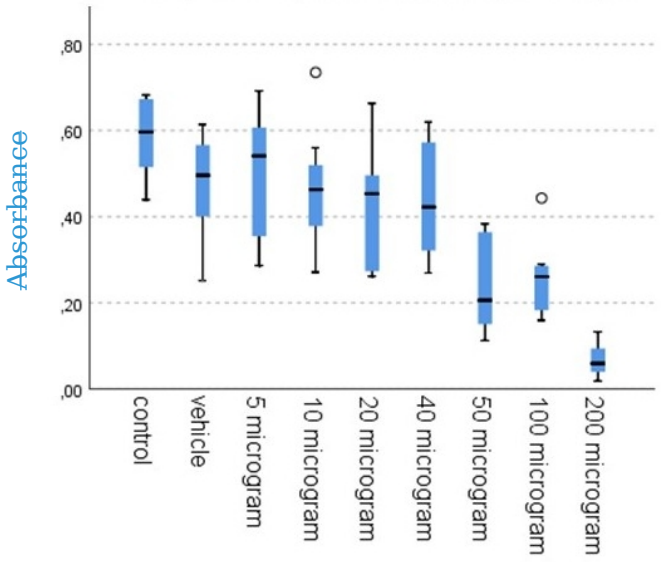
	Control	**DMSO**	**5 µg/mL**	**10 µg/mL**	**20 µg/mL**	**40 µg/mL**	**50 µg/mL**	**100 µg/mL**	**250 µg/mL**
Average absorbance	0,488	0,461625	0,507125	0,361625	0,399125	0,364143	0,2718	0,247875	0,080143
Cell viability %	100,00		103,9191	74,10348	81,78791	74,61944	55,69672	50,79406	16,42272

***Graph 1:** MTT Analysis 24th hour findings*

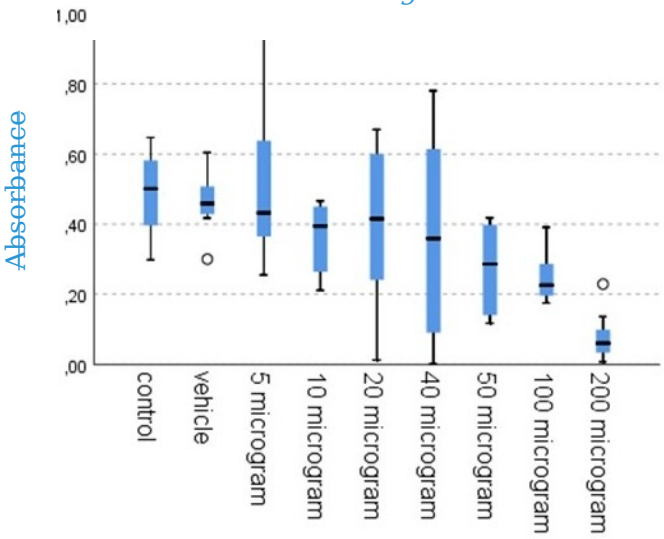


Concentrations of T. polium extract solution

***Graph 2:** MTT Analysis 48th hour findings*

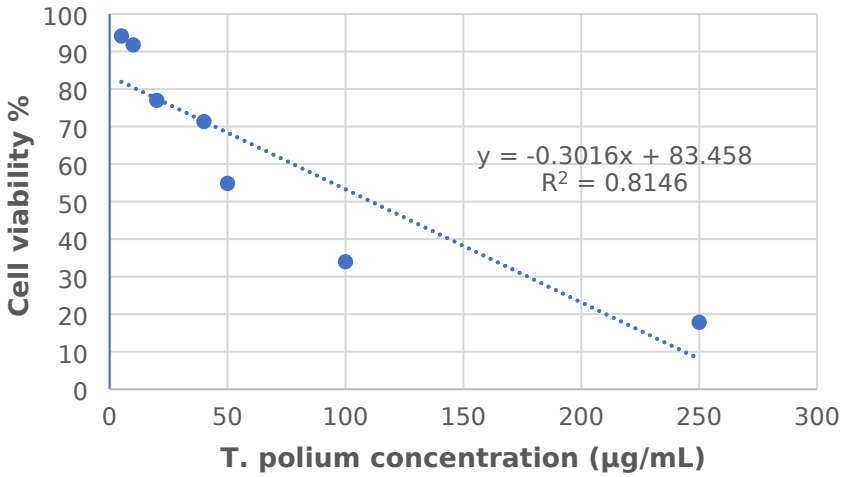


Graph 3: *MTT Analysis 72nd hour findings*

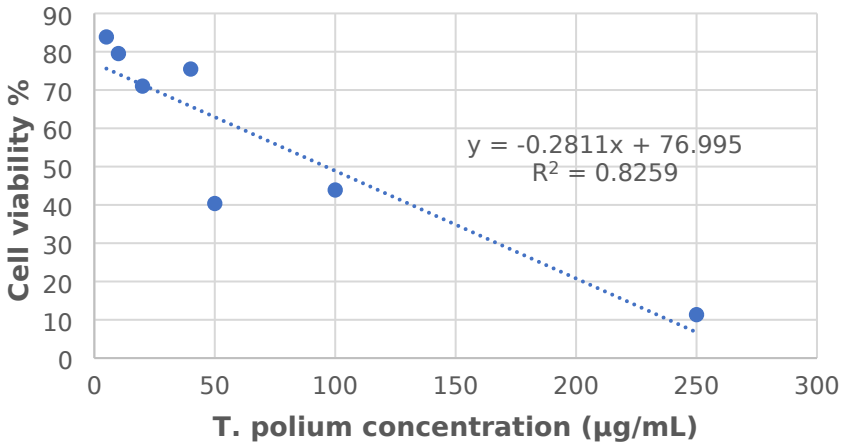


Calibration curves were created using Microsoft Excel based on MTT absorbance measurements obtained from different concentrations of T. polium applications. Using these values, line equations were created to represent cell viability corresponding to T. polium concentrations. The IC50 values were determined to be 110.935 µg/mL, 96.033 µg/mL, and 120.057 µg/mL at 24, 48, and 72 hours, respectively. For the colony formation and wound healing tests, the results at the 48-hour mark were selected, and the IC50 value of 90 µg/mL, which corresponds to 50% cell viability at this time point, was utilized (Graph 4-6).

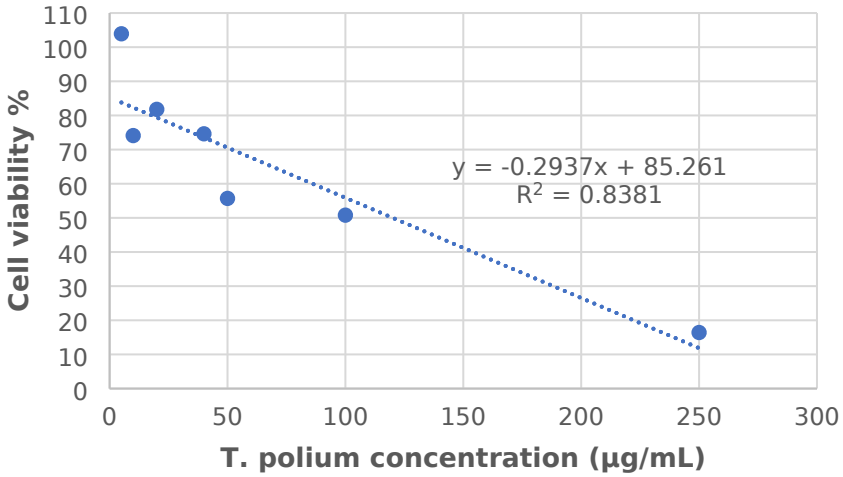
~~Graph 4: Cell viability at 24 hours~~



~~Graph 5: Cell viability at 48 hours~~



~~Graph 6: Cell viability at 72 hours~~



~~2.~~ 3.2. ~~the~~ Effect of T.Polium treatment on Colony Formation ~~Assay Findings~~

The ~~proliferative~~ effect of T. polium extract on the proliferation of the SNU-449 cell line was assessed using a colony formation assay. We examined the number of colonies formed after applying a sublethal dose of T. polium extract solution (IC50 = 90 µg/mL) diluted in RPMI-1640 at the 48-hour mark. Notable differences were observed between the treatment and control groups when comparing the number of colonies formed to the number of cells seeded. To evaluate these differences, we first calculated the plating efficiency (PE) for both control cells and those treated with the T. polium extract by dividing the number of colonies formed by the number of cells seeded. The surviving fraction (SF) was then calculated by dividing the PE values of the treated cell group by the PE values of the control cell group, which represents the proportion of colonies formed after treatment with the T. polium extract.

Plating Efficiency (PE)= (Number of colonies formed / Number of cells seeded) × 100
Surviving Fraction (SF) = (PE of cells treated with 90 µg/mL T. polium / PE of control cells) × 100

Calculations yielded the following results:

PE of control cells = ((62 + 14) / 2000) × 100 = 3.8%
PE of cells treated with T. polium (90 µg/mL) = ((19 + 19) / 2000) × 100 = 1.9%
Survival rate of cells treated with T. polium = (1.9 / 3.8) × 100 = 50%

These results indicate that T. polium extract reduced colony formation efficiency by 50% compared to the control group (RPMI-1640) (Figure ~~1-~~2~~, Graph 7~~).

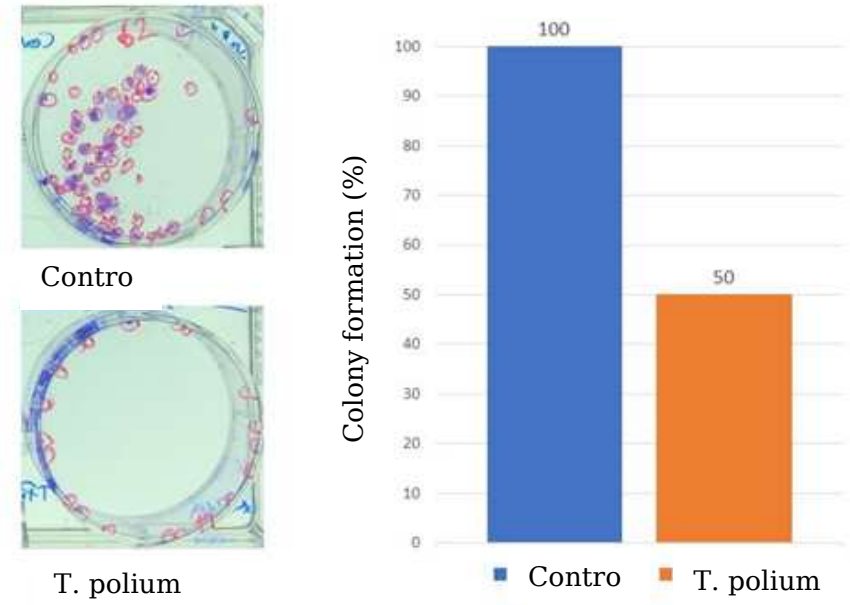


Figure ~~1-~~2~~., Graph 7:~~ *Colonies formed in the SNU-449 cell line control group and following the application of T. polium extract solution, a quantitative analysis of the number of colonies formed as a result of the colony formation experiment expressed as a percentage compared to the control cell group*

~~3.~~ 3.3. The ffect of T.Polium on In Vitro Scratch Assay ~~Cell Migration~~ Analysis ~~(Scratch Assay) Findings~~

The scratch test conducted to assess the effects of T. polium on the in vitro migration capabilities of SNU-449 cells, as well as on in vitro wound healing, revealed that the half-wound healing times were 50.5 hours for the group treated with T. polium and 28.5 hours for the control group that did not receive treatment, respectively. At the end of the test, it was observed that the scratch area in the group treated with 90 µg/mL of T. polium closed by 47.5%. For this T. polium-treated group, the average cell migration rate, representing the speed at which cells collectively migrated into the gap, was calculated to be 2.2 µm/hour, with a migration rate of 1% per hour. Additionally, the time required for 50% of the original surface area of the wound to close, referred to as the wound half-healing time (t1/2 gap), was determined to be approximately 50 hours and 31 minutes (or around 50.5 hours) (see Figure 3-4).

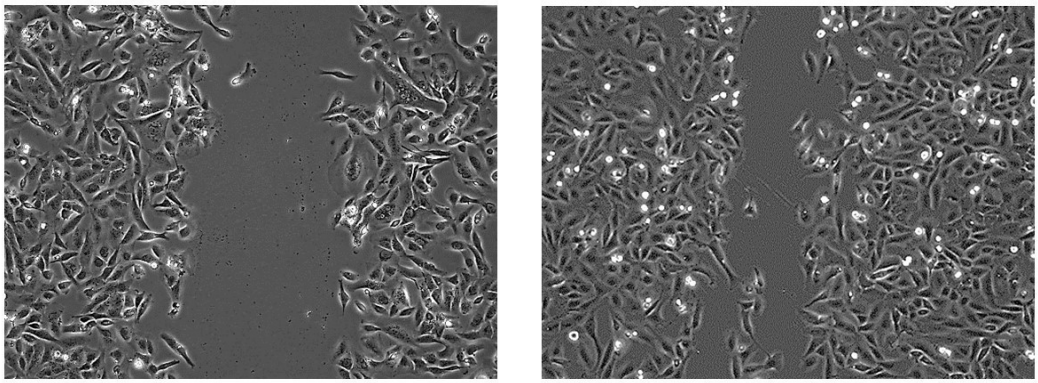


Figure 3-4: *Wound healing at the end of the 48th hour in the T. polium administered group versus the control group*

In contrast to the T. polium group, in the control group, by the end of the test the scratch area closed by 96%. The average cell migration rate, or $v^{migration}$, in the control group was measured at 2.6 µm/hour, resulting in a migration rate of 1.9% per hour. Additionally, the half-healing time for the wound, also known as t1/2 gap, was determined to be approximately 28 hours and 35 minutes (approximately 28.5 hours).

In the independent samples t-test comparing the test group treated with T. polium to the control group, statistically significant differences were found in the cell density rates within the confluence region of interest in the scratched area. The gap fill percentages for the two groups also showed significant differences, with p-values of 0.004 and 0.001, respectively (with $p \leq 0.05$ considered significant). However, no significant differences were noted in the cell migration rate ($p = 0.928$) or the gap fill area ($p = 0.146$) between the two groups. When comparing the hourly cell migration percentages (migration percentage per hour) of the T. polium test group and the control group using an independent samples t-test, a notable difference was observed, although it was not statistically significant ($p = 0.058$) (Table 4).

Table 4: *Independent t-test results performed using SPSS vs 26.0 with data obtained from in vitro cell migration analysis*

4.3.4 Western Blotting Findings for The effect of T. Polium treatment on Caspase-3 and Cleaved Caspase-3 expression

In order to investigate how T. polium induces apoptosis in SNU-449 cells, Western blotting was conducted to measure the levels of caspase-3 and cleaved caspase-3. The results of the Western blotting showed that there was no statistically significant difference in caspase-3 and cleaved caspase-3 levels between the T. polium-treated cells and the control cells. The Mann-Whitney U test, performed using SPSS version 26.0,

yielded significance levels of p = 0.827 for caspase-3 and p = 0.513 for cleaved caspase-3. Therefore, it was concluded that the cytotoxic effect of T. polium on SNU-449 cells was not mediated by the induction of caspase-dependent apoptosis (Tables 5).

Table 5: *Mann-Whitney U test results using the levels of caspase-3 and cleaved caspase-3 measured in T. polium-treated cells and control cells*

Ranks				
	Group	**N**	**Mean Rank**	**Sum of Ranks**
Caspase-3	Control	3	3,67	11,00
	T. polium	3	3,33	10,00
	Total	6		
Cleaved caspase-3	Control	3	4,00	12,00
	T. polium	3	3,00	9,00
	Total	6		

Test Statistics		
	Caspase-3	**Cleaved caspase-3**
Mann-Whitney U	4,000	3,000
Wilcoxon W	10,000	9,000
Z	-,218	-,655
Asymp. Sig. (2-tailed)	,827	,513
Exact Sig. [2*(1-tailed Sig.)]	1,000	,700

4. Discussion

This study was designed to investigate the potential cytotoxic and antiproliferative apoptotic effects of T. polium extract on the human HCC cell line SNU-449. The findings revealed that T. polium extract exhibits a dose-dependent cytotoxic effect on SNU-449 cells, leading to a reduction in both proliferation and survival rates. Consequently, due to these observed cytotoxic and anti-proliferative effects, T. polium may serve as a potentially effective antineoplastic agent. However, it is important to note that the cytotoxic effect of T. polium on SNU-449 cells was not mediated by the induction of caspase-dependent apoptosis.

Supporting the findings of the current study, numerous studies utilizing various human cancer cell lines have demonstrated that T. polium exhibits cytotoxic effects. For example, a study published by Nematollahi-Mahani et al. in 2007, which is similar to the present research, investigated the potential cytotoxic effects of an ethanol extract from T. polium in vitro. The results of tThis study, which used various cancer cell lines, including A549 (human lung adenocarcinoma), BT20 (human breast ductal carcinoma), MCF-7 (human breast adenocarcinoma), and PC12 (mouse pheochromocytoma). Cytotoxicity assessment was conducted using the WST-1 assay, which, like MTT, is a tetrazolium salt reduced to formazan by mitochondrial dehydrogenases present in living cells. The results indicated that the T. polium extract exhibited cytotoxic effects on all the cell lines included in the study. Based on the WST-1 analysis, the IC50 value for the human lung adenocarcinoma cell line A549 was calculated to be 90 µg/mL. This finding is consistent with the value determined in the present study for SNU-449. Additionally, the IC50 values measured for the BT20, MCF-7, and PC-12 cell lines were reported to be 106 µg/mL, 140 µg/mL, and 120 µg/mL, respectively [11]. In a study conducted by Eskandary et al. in 2007, the cytotoxic effects of water-based and methanolic extracts of T. polium were evaluated on a new GBM cell line, REYF-1, using in vitro methods. The study found that the IC50 values were 95 µg/ml for the methanolic extract and 1400

µg/ml for the water-based extract (the methanolic extract was found to be more effective, likely due to the better solubility of its active compounds in methanol). These findings, obtained through MTT analysis, are consistent with the results of the current study. Ultimately, it was concluded that T. polium exhibited dose-dependent cytotoxic effects on the REYF-1 cell line [21]. A study conducted by Nematollahi-Mahani et al., published in 2012, examined the effects of various concentrations of a water-based crude extract of T. polium, as well as its petroleum ether and diethyl ether fractions. The researchers investigated the antitumor and inhibitory effects of T. polium on the U87 malignant glioblastoma (GBM) cell line, as well as on non-malignant embryonic stem cells derived from human umbilical cord matrix mesenchymal cells (hUCM), which possess similar properties and a high proliferative capacity in vitro. The results indicated that T. polium produces a dose-dependent cytotoxic effect. As a result of the WST-1 analysis conducted in this study, it was found that all forms of T. polium led to significant decreases in cell viability when applied at increasing concentrations (ranging from 0 to 750 µg/ml) over 24 and 48 hours. After 48 hours, the compound with the highest cytotoxic activity against U87 cells was identified as the petroleum ether fraction of T. polium, with an IC50 of 64.47 µg/ml. This was followed by the diethyl fraction, which had an IC50 of 134.45 µg/ml, and lastly, the crude extract, with an IC50 of 176.10 µg/ml [20]. Similarly, in a 2016 study conducted by Nikodijević et al., the cytotoxic and pro-apoptotic effects of methanolic extracts from T. polium and T. montanum were investigated on breast cancer (MDA-MB-231) and colorectal carcinoma (SW-480) cell lines. The study reported that both plant extracts led to a dose- and time-dependent decrease in the viability of MDA-MB-231 cells, indicating cytotoxic effects. Specifically, for T. polium, the 24-hour IC50 was 429.37 µg/ml, while the 72-hour IC50 decreased to 118.26 µg/ml. The cytotoxic effect on SW-480 cells was observed at a lower level, becoming evident 24 hours after applying the extracts, with the 24-hour IC50 for T. polium at 242.93 µg/ml and the 72-hour IC50 at 292.13 µg/ml [22]. In their study published in 2020, Noumi et al. investigated the cytotoxic and potentially anticancer effects of T. polium methanolic extract on breast ductal carcinoma (Walker 256/B) and prostate cancer (MatLyLu) cells using MTT analysis. In their 2020 study, Noumi et al. investigated the cytotoxic and potential anticancer effects of T. polium methanolic extract on breast ductal carcinoma (Walker 256/B) and prostate cancer (MatLyLu) cells using MTT analysis. The extract was applied to both cell lines, which are known for their high metastatic potential, at concentrations of 0, 50, 100, and 200 µg/ml for either 24 or 48 hours. The results showed that T. polium methanolic extract exhibited cytotoxic and antiproliferative effects against both Walker 256/B and MatLyLu cells, which are known for their high metastatic potential. The lowest cell viability was observed with T. polium extract at a concentration of 200 µg/mL for both cell lines. It was noted that the cytotoxic effect was dose-dependent, becoming more pronounced after 48 hours of application [5]. In a study published by Hashem-Dabaghian et al. in 2020, the cytotoxic and anti-mutagenic effects of T. polium were investigated on the HT29 (human colon adenocarcinoma) cell line. The HT29 cells were exposed to T. polium essential oil at concentrations of 0, 10, 25, 50, 75, 100, and 150 µg/mL for a duration of 48 hours. Using the MTT assay, the IC50 value calculated to be 66.867 ± 1.37 µg/mL at the end of this 48-hour period. The cytotoxic effect of T. polium essential oil on the HT29 cell line showed a concentration-dependent increase, aligning with the findings of the present study [23]. In a study conducted by Menichini et al. in 2008, the researchers investigated the essential oils from four different species of Teucrium: Teucrium flavum, Teucrium montbretii ssp. heliotropiifolium, Teucrium polium ssp. capitatum, and Teucrium brevifolium. They evaluated the cytotoxic and antiproliferative effects of these essential oils on three cancer cell lines: amelanotic melanoma (C32), colon adenocarcinoma (CACO-2), and lung cancer (COR-L23). The analysis revealed that the essential oil from T. polium exhibited the highest cytotoxic effect on the CACO-2 cell line, with an IC50 value of 52.7 µg/mL as determined by the MMT assay [24].

Several cytotoxicity studies have examined how T. polium affects proliferation in various human cancer cell lines and its potential antiproliferative effects, revealing results that align with those of the present study. For example, the study conducted by Eskandary et al. in 2007 investigated the effects of T. polium on the REYF-1 GBM cell line. The results of the colony formation experiment indicated that after applying T. polium

methanolic extract at concentrations of 30, 60, 100, and 200 µg/mL, colony formation in REYF-1 cells decreased to 91%, 65%, 35%, and 0.006%, respectively, compared to the control group. The IC50 value was found to be 69 µg/mL [21]. In a 2010 study by Kandouz et al., researchers investigated the effects of T. polium extract on PC3 and DU145 human prostate cancer cell lines. They applied a concentration of 100 µg/mL of the extract to these cells over a period of 1 to 4 days as part of a colony formation test. The results showed that the proliferation of the treated PC3 and DU145 cells was inhibited when compared to the untreated cells. This aligns with the present study's findings, indicating that T. polium has antiproliferative effects [10]. In a study conducted by Haïdara et al. in 2011, researchers used two human lung cancer cell lines, referred to as H322 and A549, for a colony formation experiment. Likewise, the results indicated that, compared to the control group, the proliferation of both H322 and A549 cells was inhibited following the application of T. polium [9].

As discussed earlier, numerous publications illustrate similarities with the results of this study, supporting the cytotoxic and antiproliferative effects of T. polium. However, there are also some studies that dispute T. polium's anticancer properties. For example, in a study published by Ljubuncic et al. in 2005, the researchers investigated the antioxidant and cytotoxic effects of T. polium and seven different extracts on rat pheochromocytoma (PC12) cells and human hepatoblastoma (HepG2) cells. They conducted cytotoxicity tests and assessed cell membrane integrity using MTT analysis and LDH analysis, respectively. The presence of the cytosolic enzyme LDH in the cell culture medium serves as an indicator of cell membrane damage. The findings indicated that T. polium did not exhibit cytotoxic or inhibitory effects on either PC12 or HepG2 cells [25]. Khader et al. reported in their 2007 study that water-based crude T. polium extract did not exhibit antimutagenic and cytoprotective activity [26].

Upon examining the reasons for the differences in the results of the aforementioned studies, it is believed that these discrepancies primarily stem from the different methods used to obtain the T. polium extracts applied in the studies [21]. Some studies used ethanol or methanolic extracts of T. polium, while others utilized water-based crude extracts. Khader et al. reported in their 2007 study that the water-based crude extract of T. polium exhibited neither antimutagenic nor cytoprotective activity [26]. On the other hand, in their 2010 study, the authors reported that the ethanol extract of T. polium inhibited the mutagenicity of N-methyl-N'-nitro-N-nitrosoguanidine [27]. The variations observed among results from different studies can also be attributed to the use of different parts of the plant for extract preparation and geographical variations in the plant. Also, there are over 134 active components in various parts of T. polium subspecies, including neoklerodane diterpenoids, monoterpenes, sesquiterpenes, polyphenols, flavonoids, and fatty acids. Some studies suggest that terpenoids and flavonoids contribute to the anticancer effects of T. polium. However, further research is needed, particularly after fractionation, to identify which of these components are the active ingredients. It is also possible to argue that there may be synergistic or additive effects among the active components of T. polium, leading to strong cytotoxic effects and enhancing its overall anticancer properties [21].

In this study, the possibility of apoptosis after T. polium treatment in SNU-449 cells was investigated using Western blotting and specific validated primary and secondary antibodies for caspase-3 and cleaved caspase-3.
Cysteine-requiring Aspartate Protease (Caspase) is a family of enzymes that play a crucial role in the execution phase of apoptosis. Among these, caspase-3 acts as an "executioner" caspase. Its zymogen form is activated in apoptotic cells through both extrinsic (death receptor-mediated) and intrinsic (mitochondria-mediated) pathways. Once activated, caspase-3 is cleaved and processed to form cleaved caspase-3, which is the active form of the
enzyme in vitro. Caspase-3-mediated protein cleavage is a crucial step in the molecular mechanism of apoptosis. It also plays a significant role in nuclear apoptosis, which includes processes such as chromatin condensation, DNA fragmentation, and cell blebbing. When apoptosis begins, activated caspase-3 cleaves its associated substrates in the endochilema and cytoplasm, ultimately leading to cell death.

Western blotting analysis conducted in this study for caspase-3 and cleaved caspase-3 revealed that the cytotoxic and anti-proliferative effects of T. polium on the SNU-449 cell line were not mediated by caspase-induced apoptosis. In comparison to these results, in their 2010 study, Kandouz et al. reported a limited induction of apoptosis in both cell lines after application of 100 µg/mL T. polium extract to PC3 and DU145 human prostate cancer cells for 1 to 4 days [10]. Similarly, Iin a study conducted by Hashem-Dabaghian et al. and published in 2020, the where the HT29 human colon adenocarcinoma cell line which was treated with T. polium and later assessed was analyzed for sub-G1 apoptosis and assessed using Annexin V flow cytometry, . The results indicated that treatment of HT29 cells with T. polium resulted in morphological changes consistent with apoptosis [23]. In a study conducted by Nematollahi-Mahani et al. in 2012, the effects of T. polium on the U87 malignant glioblastoma (GBM) cell line were investigated. The researchers aimed to assess cell viability in 200 cells and identify the patterns of cell death (pre-apoptotic, apoptotic, and necrotic). To achieve this, they performed differential staining using the trypan blue staining test. This test works by allowing trypan blue to penetrate damaged cell membranes, staining the cell cytoplasm and nucleus; membrane damage is one of the final events in the cell death cascade that is triggered during apoptosis. Additionally, they used propidium iodide (20 mg/mL) and bisbenzamide (Hoechst, 1 µM) after applying T. polium for 48 hours. Both staining techniques revealed the presence of pre-apoptotic and apoptotic cell death patterns, as well as necrotic cell death in the cells treated with T. polium [20].

Similar to the present study's findings, there are also other studies that have indicated that T. polium does not induce apoptosis. n a 2010 study, Khader et al. reported that the ethanolic extract of T. polium exhibits anti-mutagenic effects by directly interacting with mutagens. This interaction enhances recovery from DNA damage and induces detoxifying enzymes, while the extract does not have an impact on necrosis or apoptosis [27]. In a the study conducted by Nikodijević et al. in 2016, researchers analyzinged the levels of caspase 8 and 9 in human breast cancer (MDA-MB-231) and colorectal carcinoma (SW 480) cell lines to investigate the presence of apoptosis after the application of T. polium,. The results indicated that apoptosis in MDA-MB-231 cells occurred through the external pathway, activated by the internal apoptotic pathways mediated by caspase 8 and 9. In contrast, in SW 480 cells, apoptosis occurred by means of was observed through a caspase-independent pathway [22].

Analysis of the mechanisms through which T. polium extract induces cytotoxic and anti-proliferative effects on the SNU-449 cell line in the present study indicates that these effects primarily result from the extract's impact on the migration of SNU-449 cells. Our in vitro cell migration analysis (scratch assay) revealed that T. polium significantly reduced the migration and motility of SNU-449 cells. In their in vitro analysis of cell migration, Kandouz et al. applied a 100 µl/ml of T. polium extract to PC3 cells and DU145 human prostate cancer cells for 24 and 48 hours. Their findings, consistent with the results of our study, indicated that T. polium extract inhibited the invasion and migration abilities of cells in both cell lines examined [10]. It is possible to suggest that the anticancer effects of T. polium on SNU-449 cells may result from various mechanisms. These could include cell cycle arrest, induction of a conversion to an epithelial phenotype in malignant SNU-449 cells through a process known as "mesenchymal-epithelial transition," or caspase-independent apoptosis, or the re-localization of proteins such as E-cadherin and catenin. These proteins play a crucial role in providing intercellular adhesion at the cell surface and within the intracellular membrane. The In a study conducted by Kandouz et al. in 2010 , it was demonstrated that T. polium extract induced cell cycle arrest in the S phase of the PC3 and DU145 prostate cancer cell lines. The extract also and led to a reduction in the duration of the G0–G1 phase, thereby inhibiting cell proliferation. Furthermore, it was determined that T. polium extract prompted differentiation into the epithelial phenotype through a process known as "mesenchymal-epithelial transition." This transition, which is crucial in the context of cell invasion and metastasis, resulted in a significant decrease in the invasion and motility abilities of PC3 and DU145 prostate cancer cells compared to untreated cells. In addition to these changes, it was reported that T. polium extract

causes re-localization of the expression patterns of E-cadherin and catenin. . It inhibits the phosphorylation of β-catenin through Src dephosphorylation, which in turn shifts its role from being a transcription regulator to functioning as an intercellular adhesion molecule. According to the results of the study, T. polium extract inhibits the signaling pathways regulating the E-cadherin/catenin complex and other intercellular adhesion genes through modification of β-catenin [10].
Nonetheless, further research and investigations are required to determine these effect mechanisms of T. polium in SNU-449 cells.

5. Conclusion

This study demonstrates that T. polium exhibits significant antiproliferative and cytotoxic effects; however, it does not trigger caspase-mediated apoptosis in the SNU-449 human HCC cell line. Further in vitro and in vivo studies are necessary to investigate the mechanisms of action of T. polium as a potential anticancer agent against HCC and to identify its active components.

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Conflict of Interests: The authors declare no conflict of interest.

REFERRENCES

1. Khazaei, M., S.N. Nematollahi-Mahani, T. Mokhtari and F. Sheikhbahaei. 2018. Review on Teucrium polium biological activities and medical characteristics against different pathologic situations*. J Contemp Med Sci* 4:1-6
2. Tariq, M., A.M. Ageel, M.A. Al-Yahya, J.S. Mossa, and M.S. Al-Said. 1989. Anti-inflammatory activity of Teucrium polium. *Int J Tissue React* 11(4):185-188
3. Nosrati, N., S. Aghazadeh, and R. Yazdanparast. 2010. Effects of Teucrium polium on insulin
resistance in nonalcoholic steatohepatitis. *J Acupunct Meridian Stud* 3:104-110. https://doi.org/10.1016/S2005-2901(10)60019-2
4. Movahedi, A., R. Basir, A. Rahmat, M. Charaffedine, and F. Othman F. 2014. Remarkable anticancer activity of Teucrium polium on hepatocellular carcinogenic rats. *Evid Based Complement Alternat Med* 2014:726724. https://doi.org/10.1155/2014/726724
5. Noumi, E., M. Snoussi, E.H. Anouar, M. Alreshidi, V.N. Veettil, S. Elkahoui, M. Adnan, M. Patel, A. Kadri, K. Aouadi, and V. De Feo. 2020. HR-LCMS-based metabolite profiling, antioxidant, and anticancer properties of Teucrium polium L. methanolic extract: Computational and in vitro study. *Antioxidants* 9(11):1089. https://doi.org/10.3390/antiox9111089
6. Rajabalian, S. 2008. Methanolic extract of Teucrium polium L potentiates the cytotoxic and apoptotic effects of anticancer drugs of vincristine, vinblastine and doxorubicin against a panel of cancerous cell lines. *Exp Oncol* 30(2):133-138
7. Abu-Dahab, R., and F. Afifi. 2007. Antiproliferative activity of selected medicinal plants of Jordan against a breast adenocarcinoma cell line (MCF7). *Sci Pharm* 75:121-146. https://doi.org/10.3797/scipharm.2007.75.121
8. Line, M.C. 2007. Evaluation of cytotoxic effect of Teuerium polium on a new glioblastoma multiforme cell line (REYF-1) using MTT and soft agar clonogenic assays. *Int J Pharmacol* 3:435-437. https://doi.org/10.3923/ijp.2007.435.437
9. Haïdara, K., A. Alachkar, and A.E. Al Moustafa. 2011. Teucrium polium plant extract provokes significant cell death in human lung cancer cells. *Health* 3(6):366-369. https://doi.org/10.4236/health.2011.36062
10. Kandouz, M., A. Alachkar, L. Zhang, H. Dekhil, F. Chehna, A. Yasmeen, and A.E. Al Moustafa. 2010. Teucrium polium plant extract inhibits cell invasion and motility of human prostate cancer cells via the restoration of the E-cadherin/catenin complex. *J Ethnopharmacol* 129:410-415. https://doi.org/10.1016/j.jep.2009.10.035
11. Nematollahi-Mahani, S.N., M. Rezazadeh-Kermani, M. Mehrabani, and N. Nakhaee. 2007. Cytotoxic effects of Teucrium Polium on some established cell lines. *Pharm Biol* 45:295-298
12. Singal, A.G., P. Lampertico, and P. Nahon. 2020. Epidemiology and surveillance for hepatocellular carcinoma: New trends. *J Hepatol* 72(2):250-261. https://doi.org/10.1016/j.jhep.2019.08.025
13. Sung, H., J. Ferlay, R.L. Siegel, M. Laversanne, I. Soerjomataram, A. Jemal, and F. Bray. 2022. *Global Cancer Observatory: Cancer Today 2020*. http://gco.iarc.fr/today/data/factsheets/cancers/11-Liver-fact-sheet.pdf. Accessed 24 May 2022
14. Cancer of the Liver Italian Program (CLIP) Investigators. A new prognostic system
for hepatocellular carcinoma: a retrospective study of 435 patients. 1998. *Hepatology* 28:751-755. https://doi.org/10.1002/hep.510280322

15. Forner, A., M. Reig, and J. Bruix. 2018. Hepatocellular carcinoma. *Lancet* 391:1301-1314. https://doi.org/10.1016/S0140-6736(18)30010-2
16. Liu, L., Y. Cao, C. Chen, X. Zhang, A. McNabola, D. Wilkie, S. Wilhelm, M. Lynch, and C. Carter. 2006. Sorafenib blocks the RAF/MEK/ERK pathway, inhibits tumor-angiogenesis, and induces tumor cell apoptosis in hepatocellular carcinoma model PLC/PRF/5. *Cancer Res* 66:11851-11858. https://doi.org/10.1158/0008-5472.CAN-06-1377
17. Llovet, J.M., S. Ricci, V. Mazzaferro, P. Hilgard, E. Gane, J.F. Blanc, A.C. De Oliveira, A. Santoro, J.L. Raoul, A. Forner, and M. Schwartz M. 2008. Sorafenib in advanced hepatocellular carcinoma. *N Engl J Med* 359:378-390. https://doi.org/10.1056/NEJMoa0708857
18. American Tissue Culture Collection. 2022. https://www.atcc.org/products/crl-2234. Accessed 26 May 2022
19. Sheikhbahaei, F., M. Khazaei, and S.N.Nematollahi-Mahani. 2018. Teucrium polium extract enhances the anti-angiogenesis effect of tranilast on human umbilical vein endothelial cells. *Adv Pharm Bull* 8(1):131–139. https://doi.org/10.15171/apb.2018.016
20. Nematollahi-Mahani, S.N., Z. Mahdinia, R. Eftekharvaghefi, M. Mehrabani, V. Hemayatkhah-Jahromi, and F. Nabipour F. 2012. In vitro inhibition of the growth of glioblastoma by teucrium polium crude extract and fractions. *Int J Phytomed* 4(4):582-588
21. Eskandary, H., S. Rajabalian, T. Yazdi, M. Eskandari, K. Fatehi, and N.A. Ganjooei NA. 2007. Evaluation of the cytotoxic effects of Teucrium Polium on a new glioblastoma multiforme cell line (REYF-1) using MTT and soft agar clonogenic assays. *Int J Pharmacol*. 3(5):435-437. https://doi.org/10.3923/ijp.2007.435.437
22. Nikodijević, D., M. Milutinović, D. Cvetković, M. Stanković, M.N. Živanović, and S. Marković. 2016. Effects of Teucrium polium L. and Teucrium montanum L.: Extracts on mechanisms of apoptosis in breast and colon cancer cells. *Kragujevac J Sci* 38:147-159. https://doi.org/10.5937/KgJSci1638147N
23. Hashem-Dabaghian, F., A. Shojaii, J. Asgarpanah, and M. Entezari. 2020. Anti-Mutagenicity and Apoptotic Effects of Teucrium polium L. Essential Oil in HT29 Cell Line. *Jundishapur J Nat Pharm Prod* 15(3)79559. https://doi.org/10.5812/jjnpp.79559
24. Menichini, F., F. Conforti, D. Rigano, C. Formisano, F. Piozzi, and F. Senatore F. 2009. Phytochemical composition, anti-inflammatory and antitumour activities of four Teucrium essential oils from Greece. *Food Chem* 115(2):679-686. https://doi.org/10.1016/j.foodchem.2008.12.067
25. Ljubuncic, P., H. Azaizeh, I. Portnaya, U. Cogan, O. Said, K.A. Saleh, and A. Bomzon. 2005. Antioxidant activity and cytotoxicity of eight plants used in traditional Arab medicine in Israel. *J Ethnopharmacol* 99(1):43-47. https://doi.org/10.1016/j.jep.2005.01.060
26. Khader, M., P.M. Eckl, and N. Bresgen N. 2007. Effects of aqueous extracts of medicinal plants on MNNG-treated rat hepatocytes in primary cultures. *J Ethnopharmacol* 112(1):199-202. https://doi.org/10.1016/j.jep.2007.01.027
27. Khader, M., N. Bresgen, and P.M. Eckl PM. 2010. Antimutagenic effects of ethanolic extracts from selected Palestinian medicinal plants. *J Ethnopharmacol* 127(2):319-24. https://doi.org/10.1016/j.jep.2009.11.001

Figures

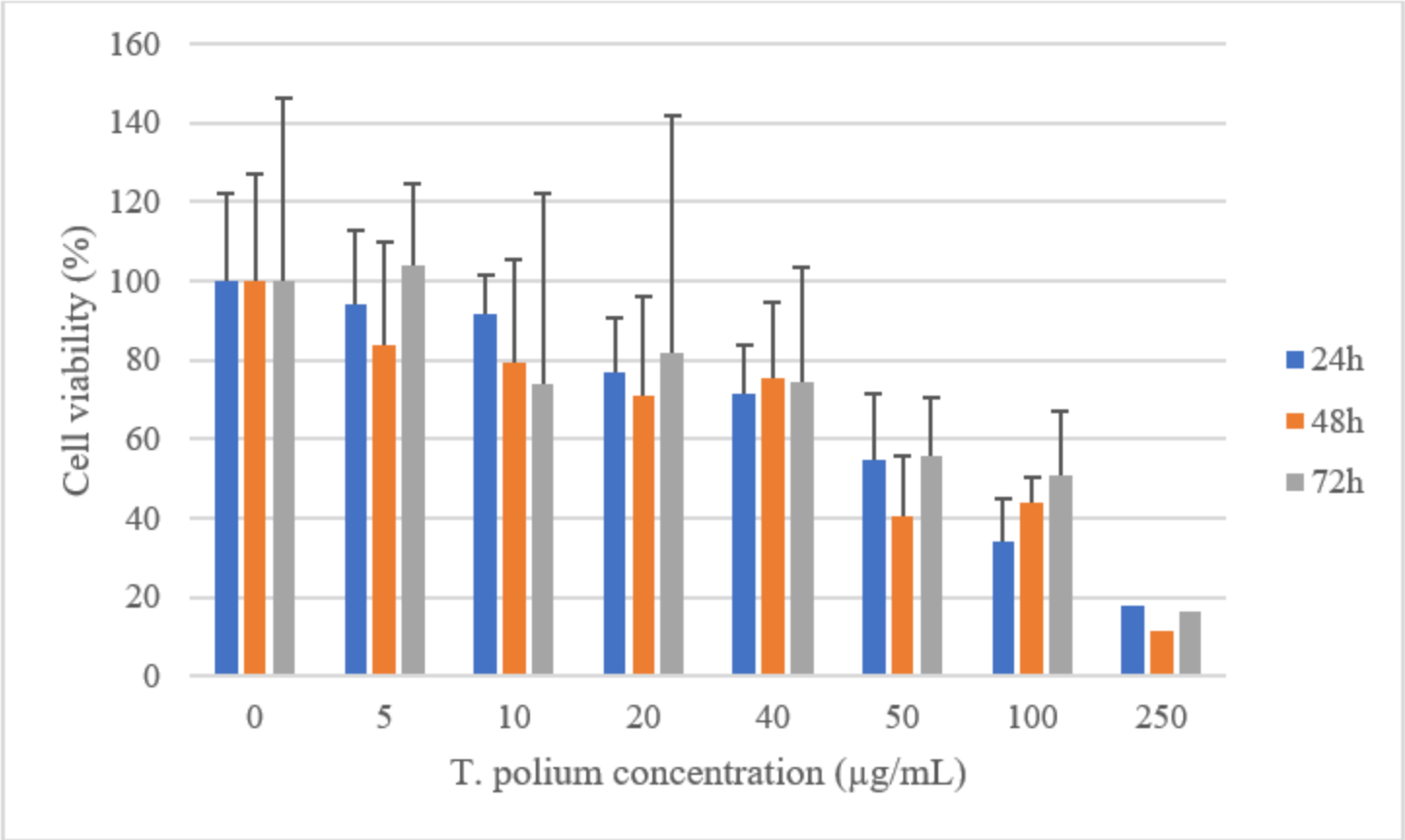

Figure 1

Cell viability of the SNU-499 cells which were treated with varying concentration of T.Polium during 24, 48, and 72 hours.

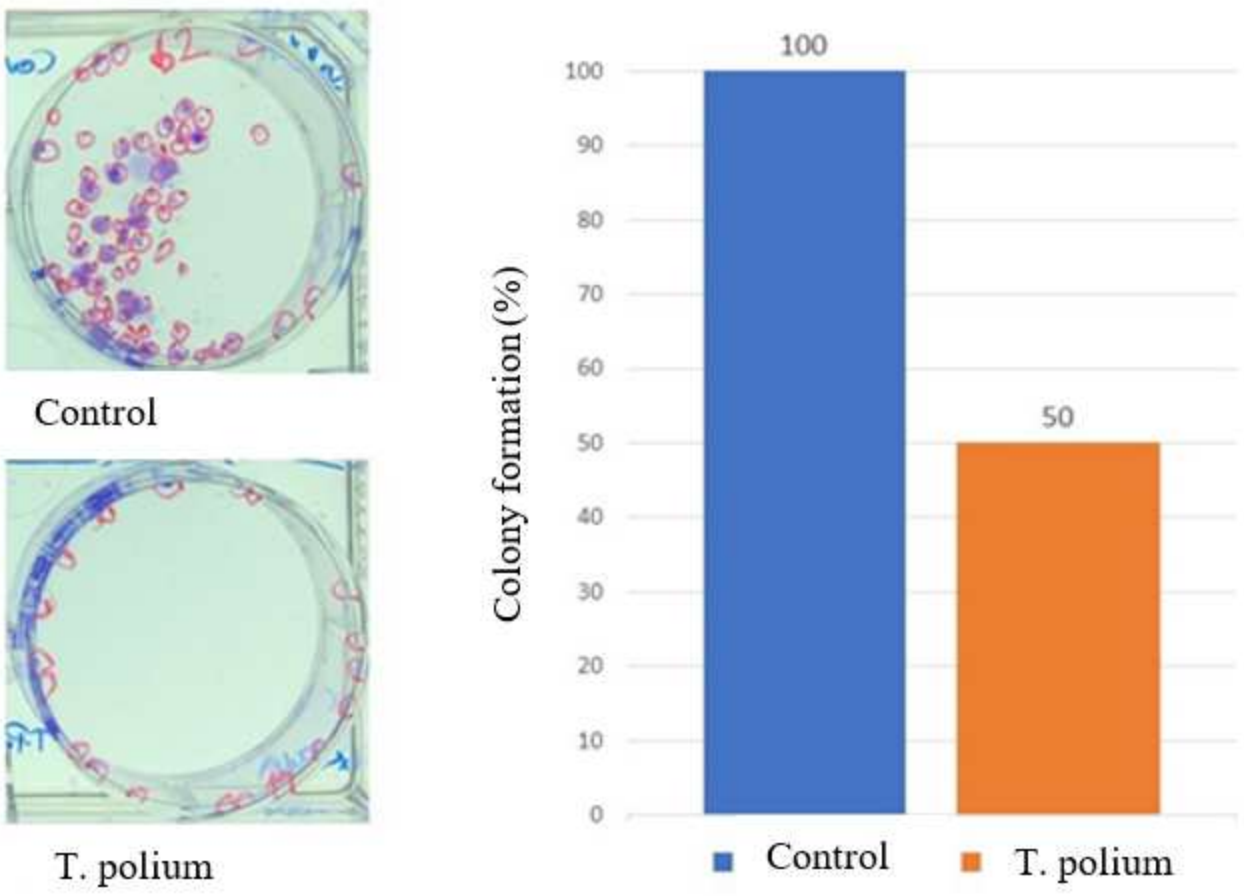

Figure 2

Colonies formed in the SNU-449 cell line control group and following the application of T. polium extract solution, a quantitative analysis of the number of colonies formed as a result of the colony formation experiment expressed as a percentage compared to the control cell group

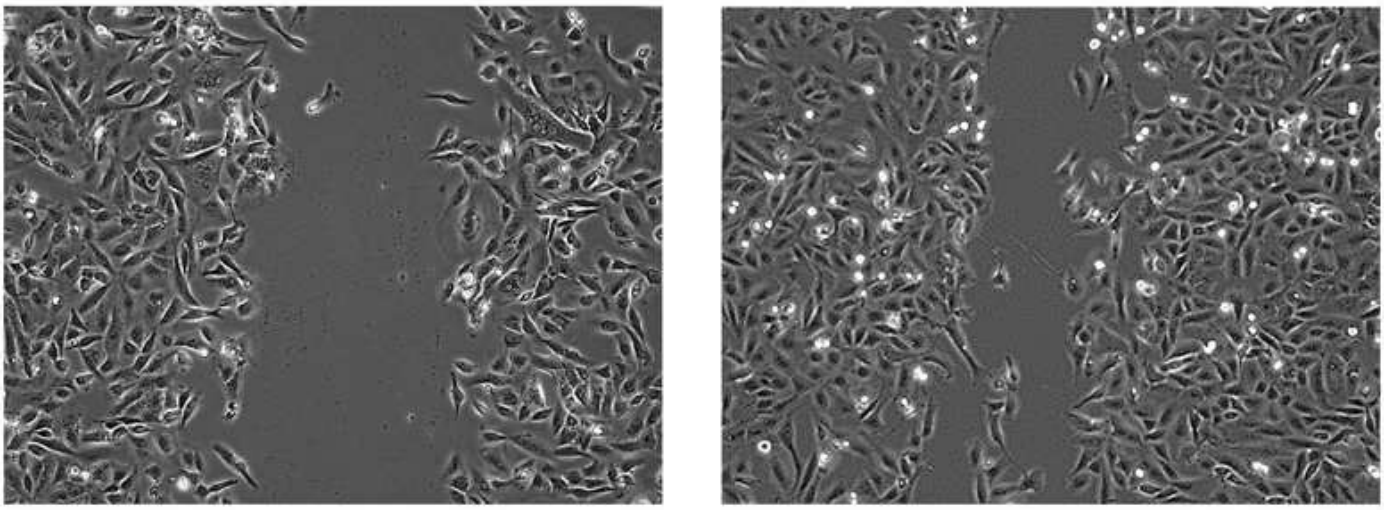

Figure 3

Wound healing at the end of the 48th hour in the T. polium administered group versus the control group

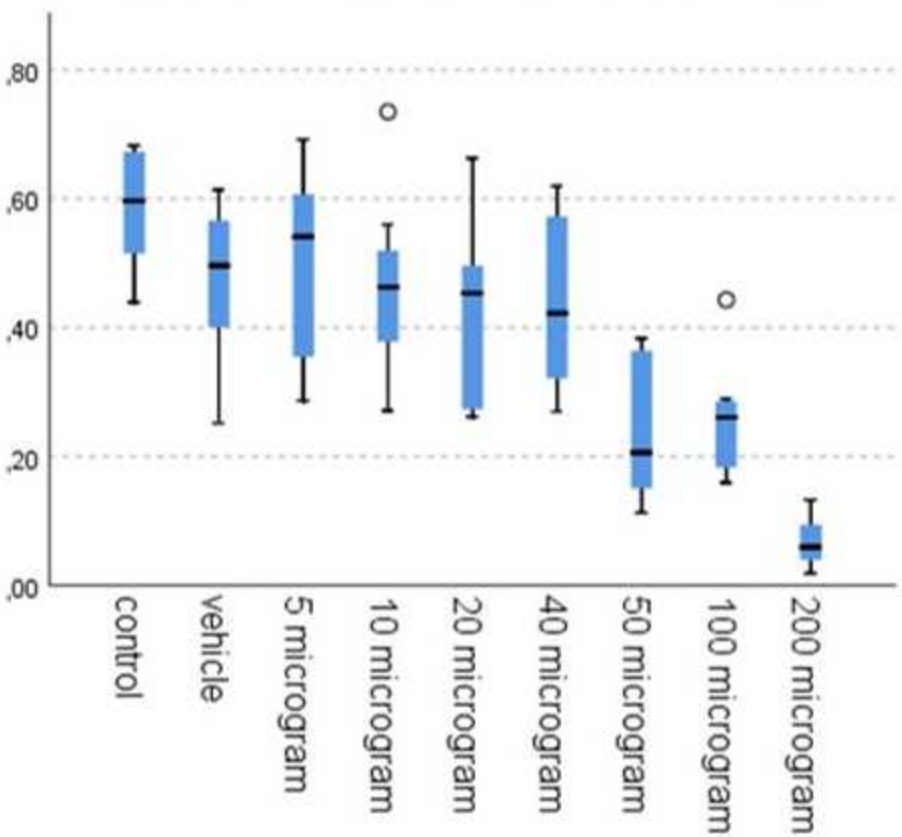

Figure 4

Unnumbered image in the Result section.