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Research Article

Cytotoxic effect of *Trigonella coerulescens* subsp. *ayvalikensis* Erdoğan, Selvi & Tümen in prostate and colon cancer cell lines

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Abstract: *Trigonella coerulescens* subsp. *ayvalikensis* is an annual local endemic taxon distributed only in Ayvalık/Balıkesir district and evaluated in the Vulnerable Category (VU). *Trigonella* L. is an important genus with medicinal and economic value in the Fabaceae family. Seeds of the genus *Trigonella* are known to contain several groups of secondary metabolites, the most abundant compounds being steroidal saponins, as well as flavones, isoflavones, and polysaccharides. In our study, the cytotoxic effect on two different cancer cell lines namely, PC-3 (prostate) and SW480 (colon), was investigated by extracting *Trigonella coerulescens* subsp. *ayvalikensis* with different solvents. The cytotoxic effects of extracts obtained from plant seeds with different solvents (hexane, methanol, ethanol, acetone) were investigated. The MTT test was used to examine the cytotoxic effect, which was studied with PC-3 and SW480 cancer cell lines. The different concentrations (23.45 µg/µL, 46.875 µg/µL, 93.75 µg/µL, 187.5 µg/µL, 375 µg/µL) of seed extracts were applied to the cells at different times points (24h, 48h and 72h) and absorbance was taken at 550 nm in the spectrophotometer. As a result of cytotoxic studies, it was observed that hexane extract had the most reducing effect on PC-3 compared to the control groups. In the SW480 cell line, a proliferative effect was observed in extracts prepared with methanol, hexane, and acetone in the early period of 24 hours. In the later period (72 hours), the extract prepared with hexane and acetone showed the most cytotoxic effect on SW480 cells.

1. INTRODUCTION

Trigonella L. is an important genus with medicinal and economic value in the Fabaceae family. Members of the genus *Trigonella* are distributed in areas such as the Eastern Mediterranean, Western Asia, Southern Europe, North and South Africa, Near East, India, and Ethiopia (Akan *et al*., 2020; Koç, 2002). This genus, known by vernacular names such as Çemenotu, Boyotu, Poyotu, Pıtlan, Pıltan, or Hulbe in Turkey, is represented by 106 species in the world and 32 species (34 taxa), 11 of which are endemic in Turkey (Güner *et al*., 2012; POWO, 2024). The seeds of species belonging to the genus *Trigonella* (fenugreek) are popularly used as a breast

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softener, expectorant, and laxative. They have also been used since ancient times as a tonic and sexual stimulant (Baytop, 1999).

Seeds of the genus *Trigonella* are known to contain several groups of secondary metabolites, the most abundant compounds being steroidal saponins, as well as flavones, isoflavones, and polysaccharides (Krol-Kogus *et al*., 2020). In recent years, herbal foods and plant-derived supplements have become more preferred in traditional medicine and health services. Some herbal products with rich bioactive and nutritional content have been used in traditional medicine to prevent various human diseases. The effectiveness of *Trigonella* species on different cancer cells has been demonstrated in several studies. The methanol and ethyl acetate extracts of *Trigonella foenum-graecum* L. seeds inhibited neurodegeneration in the hippocampus and cortex regions of the brain compared to the control group (Ahmed *et al*., 2017). The extracts exhibited anticancer activity against Hep2 and MCF-7 cells, low cytotoxicity against HCEC, and protected healthy cells in vitro. The hydroalcoholic extract of *T. foenum-graecum* seed had cytotoxic and anti-angiogenic effects *in vitro* (HUVEC and 3T3) and *in vivo* chick chorioallantoic membrane (CAM) (Iranmanesh *et al*., 2018).

The cytotoxicity, antitumor, antimetastatic, and antiangiogenic effects of the steroidal compound, ethyl iso-allocholate, isolated from *T. foenum-graecum* seeds were studied on A549 lung cancer cells in vitro and in vivo. The steroidal derivative isolated from *T. foenum-graecum* seeds induces caspase-dependent apoptosis in cancer cells, reduces tumor growth, metastasis, and angiogenesis in vivo, and is safe on normal tissues (Thakur & Ahirwar, 2019). The two protein hydrolysates of *T. foenum-graecum* (Purafect and Esperase) were studied in treating and progressing colorectal cancer. It has been suggested that hydrolysates of *T. foenum-graecum* protein could be used as nutraceutical molecules in treating colorectal cancer (Allaoui *et al*., 2019). In another study, in which two different aqueous extracts from the dry seed and germinated seed of *T. foenum-graecum* were used, evaluated the anticancer activity, its growth inhibitory effect on MCF7 human breast and pancreas (AsPC-1) cells was detected Abas & Naguib (2019); Mahapatra *et al*. (2020) also reported that the Fe (II) Schiff base complex MCF-7 in *T. foenum-graecum* induces cytotoxicity and DNA fragmentation through intracellular ROS production. They found that Fe-complex treatment also inhibited tumor growth in the solid tumor model without any side effects.

Stefanowicz-Hajduk *et al*. (2021) evaluated the cytotoxic activities of SKOV-3, HeLa and MOLT-4 cancer cell lines by obtaining extracts from the compounds (sapogenins, flavone C-glycosides, alkaloid trigonelline) in the seeds of the *T. foenum-graecum*. The extract showed a strong cytotoxic effect on cancer cell lines and was observed to significantly increase ROS production and caspase activity in the studied cells. *T. coerulescens* (M. Bieb.) Halácsy. subsp. *ayvalikensis* Erdoğan, Selvi & Tümen was introduced to the scientific world in 2017 with an international article by Erdoğan *et al*. (2017). This taxon has been evaluated in the VU (Vulnerable) category because its population is only found on the Badavut coast of Ayvalık district and the coastal edges of Küçükköy and carries the risk of being exposed to anthropogenic pressures (Erdoğan *et al*., 2017).

This study investigated for the first time the cytotoxic effect of the seeds of *T. coerulescens* subsp. *ayvalikensis*, which is locally endemic in Türkiye. The cytotoxic effect was evaluated in prostate cancer (PC3) and colon cancer (SW480) cells by MTT test.

2. MATERIAL and METHODS

2.1. Plant Collection and Extract Preparation Process

Mature seeds of the *T. coerulescens* subsp. *ayvalikensis* were collected in May-2022 from the following locality. The collection point is also shown in Figure 1. Türkiye, B1 Balıkesir: Ayvalik, Küçükköy, west of Badavut beach, Sarımsaklı beach, sandy coast, 39°16′23″ N, 26°37′43″ E, 1 m, 7.04.2013, (GT 3102).

Figure 1. Collection point of the taxon (indicated with an asterisk).

The seeds were left to dry for two days in a cool and airy room without light. Then, they were thoroughly grounded into powder in a metal mortar. It was then thoroughly ground into powder in a metal mortar. 0.75 grams were weighed on a precision scale and transferred to 4 centrifuge tubes of 50 mL for each solvent (Methanol, Ethanol, Acetone Hexane), and 10 mL of each solvent was added as a solvent and kept in a shaking incubator at +4 °C for one night. At the end of the process, the upper liquid part was transferred to a separate tube, and 10 mL of each solvent was added to the solid part at the bottom and kept at room temperature until it settled to the bottom. The liquid that rose to the top was transferred back to the centrifuge tube. The solvent was added again to the solid part at the bottom and centrifuged at 4000 RCF for 10 minutes. The liquid parts were transferred back to the centrifuge tube. The resulting extract was placed in a 100 mL bottle, the solvents were removed in a rotary evaporator and the extract was collected. The extract taken from the evaporator was dissolved in DMSO to create a stock solution (Habib-Martin *et al.*, 2017).

2.2. Culturing of Cells

Dulbecco's Modified Eagle's Medium (DMEM) high glucose (EuroClone) medium was used to grow the SW480 (Human Colon Carcinoma) cells used in the cell culture study, and DMEMF12 Mix (EuroClone) medium was used for PC-3 (Human Prostate Carcinoma) cells. By adding 10 % FBS (Fetal Bovine Serum) to these media, the cells were grown by passage 2-3 times a week at 37 °C in a humid atmosphere containing 5 % CO_2 (Cömert *et al*., 2016).

2.3. Identification of Live Cells and Cell Counting

One of the staining methods, using a solution called Trypan Blue, was applied to detect and count cells that remained viable. Thoma (hemocytometer) Slide was used to determine the number of cells in one milliliter of the total cell suspension. Cells separated from the surface by trypsinization process were centrifuged at 1000 rpm for 5 minutes and dissolved in DMEM with 10 % FBS content. To determine live cells, 10 µL of cell suspension and 10 µL of Trypan blue were mixed in a 1:1 dilution ratio and incubated at room temperature for 3-5 minutes, and then homogenization was achieved by pipetting. 10 µL of this prepared mixture was placed on a Thoma slide and the cells were counted under the microscope.

2.4. MTT Assay

In the MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) method, when the mitochondria of living cells create a reaction, the tetrazolium ring in the MTT solution is broken down by the dehydrogenase enzymes present in the cell mitochondria and formazan crystals appear. MTT is a yellow formazan salt. Proliferating cells convert MTT into purple-colored water-insoluble formazan crystals with increased mitochondrial dehydrogenase enzyme activity (Cömert *et al*., 2016).

Cells counted the previous day were seeded into 96-well plates. PC-3 cells were plated at a density of $1x10^4$ cells per well and SW480 cells at $5x10^3$ cells per well. Extracts prepared in different solvents (Methanol, Ethanol, Acetone, and Hexane) were administered to the cells at different doses: 23.45 µg/µL, 46.875 µg/µL, 93.75 µg/µL, 187.5 µg/µL, 375 µg/µL. It was

applied a day later. These doses were applied to each cell line separately in triplicate. After 24, 48, and 72 hours of application periods, stock MTT solution was added to each well with a final concentration of 0.5 mg/mL and kept in an incubator device containing 5% CO_2 for 4 hours at 37 °C. At the end of 4 hours, the medium was removed, the crystals were dissolved with isopropanol containing 0.004 M HCl, and the absorbance was measured at a wavelength of 550 nanometers (nm) in a spectrophotometer. The results were graphed using the GraphPad Prism 8 program. The results were evaluated statistically with One Way Anova in the program ($p<0.05$ * was considered significant). The experiment was carried out in 3 repetitions (Cömert *et al*., 2016).

3. RESULTS

The effect of *T. coerulescens* subsp. *ayvalikensis* extract on the viability of human colon cells, SW480, and human prostate cancer cells, PC-3 was evaluated in vitro. One of the important processes in the pharmaceutical and nutritional supplement industries is the extraction of active ingredients from herbal products. In our study, different solvents, such as methanol, ethanol, hexane, and acetone, were used in seed extraction. All of the extracts obtained were dissolved in DMSO and evaluated for their effect on cell proliferation in selected cancer cell lines. This study used the MTT assay, which measures cell growth in vitro using human cell-mediated tetrazolium reduction. The assay is commonly used to determine the cytotoxicity of potential anticancer agents. The quantity of viable cells produced directly correlates with the formazan concentration. Figure 2 show the effect of 5 different concentrations (23.45–345 µg/µL) of each extraction of the PC-3 cell line for 24, 48, and 72 h. In the PC3 cell line, an increasing effect was observed in *T. coerulescens* subsp. *ayvalikensis* methanol extract at the dose of 23.24 µg/µL applied at the 24th hour compared to the control groups, while a decreasing effect was observed at all doses applied at the 48th and 72nd hours (Figure 2A). subsp. *ayvalikensis* ethanol extract at doses of 187.5 µg/µL and 375 µg/µL applied at the 24th hour; A reducing effect was observed compared to the control group at all doses administered at the 48th and 72nd hours (Figure 2B). In the PC3 cell line, an increasing effect was observed in subsp. *ayvalikensis* acetone extract compared to the control group at doses of 23.45 µg/µL and 93.75 µg/µL applied at the 24th hour, while a decreasing effect was observed compared to the control group at all doses applied at the 48th and 72nd hours (Figure 2C). A reducing effect was observed at all doses administered in the hexane extract compared to the control group. It was observed that among the extracts applied to the PC-3 cell line, hexane extract had the most reducing effect compared to the control group (Figure 2D).

On the other hand, extracts show the differential effects on SW480 cells (Figure 3). In the methanol extract applied to SW480 cells, at doses of 46,875 µg/µL and 375 µg/µL applied at the 48 hours; While a reducing effect was observed in all doses at the 72 hours compared to the control group, an increasing effect was observed in other applied doses compared to the control group (Figure 3A). In the ethanol extract applied to SW480 cells, doses of 46.875 µg/µL, 187.5 µg/µL, and 375 µg/µL were applied at the 24th hour; While an enhancing effect was observed compared to the control group at the dose of 187.5 µg/µL applied at the 48th hour, at the doses of 23.45 µg/µL and 93.75 µg/µL applied at the 24th hour; At doses of 23.45 µg/µL, 46.875 µg/µL and 375 µg/µL applied at the 48th hour; A reducing effect was observed compared to the control groups at doses of 23.45 µg/µL, 46.875 µg/µL, 187.5 µg/µL and 375 µg/µL applied over 72 hours (Figure 3B). An increasing effect was observed in the acetone extract applied to SW480 cells compared to the control group at all doses applied at 24 hours, while a decreasing effect was observed compared to the control group at all doses applied at 48 and 72 hours (Figure 3C). In the hexane extract applied to SW480 cells, an increasing effect was observed compared to the control group at the doses of 23.45 µg/µL, 46.875 µg/µL, 187.5 µg/µL and 375 µg/µL applied at the 24th hour, while a decreasing effect was observed compared to the control groups at all other doses. It was observed that among the extracts applied to the SW480

cell line, hexane extract had the most reducing effect compared to the control group (Figure 3D).

In summary, *T. coerulescens* subsp. *ayvalikensis* extracts have different effects in different concentrations and at different time points in colon cancer cells (SW480) and prostate cancer cells (PC-3 cells). It is clear that the anti-cancer effect of the *T. coerulescens* subsp. *ayvalikensis* extracts must be detailed. The anti-tumoral effect should be tested cell-specifically and the underlying molecular mechanism should be explained.

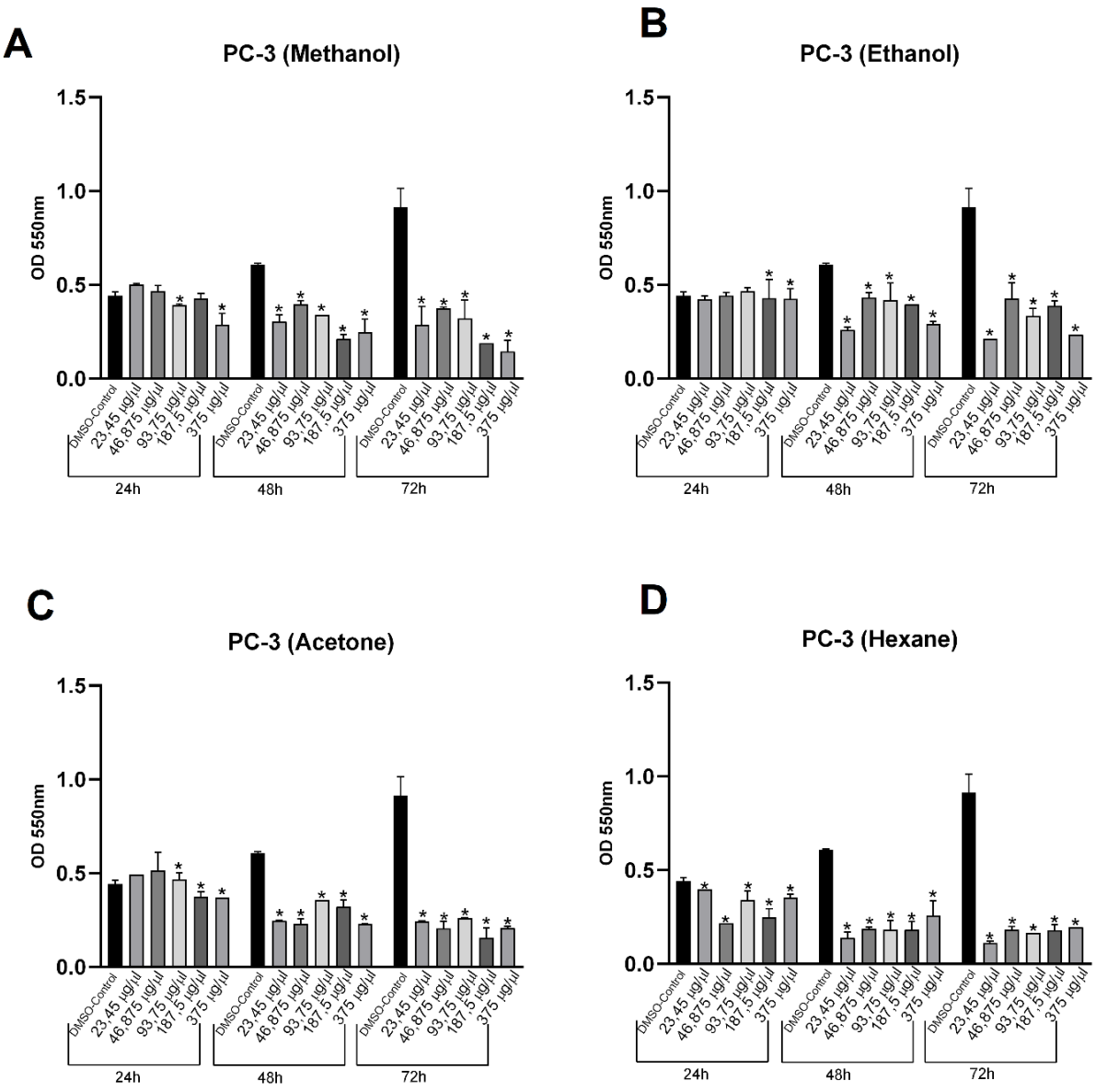

Figure 2. *In vitro* cytotoxicity *T. coerulescens* subsp. *ayvalikensis* extracts methanol (A), ethanol (B), acetone (C), and hexane (D) against malignant human prostate cells (PC-3). The cells were treated with DMSO vehicle or the indicated concentrations of *T. coerulescens* subsp. *ayvalikensis*. Cell viability was determined using MTT assay and p-value was obtained with ANOVA *$p < 0.05$ and was considered statistically significant.

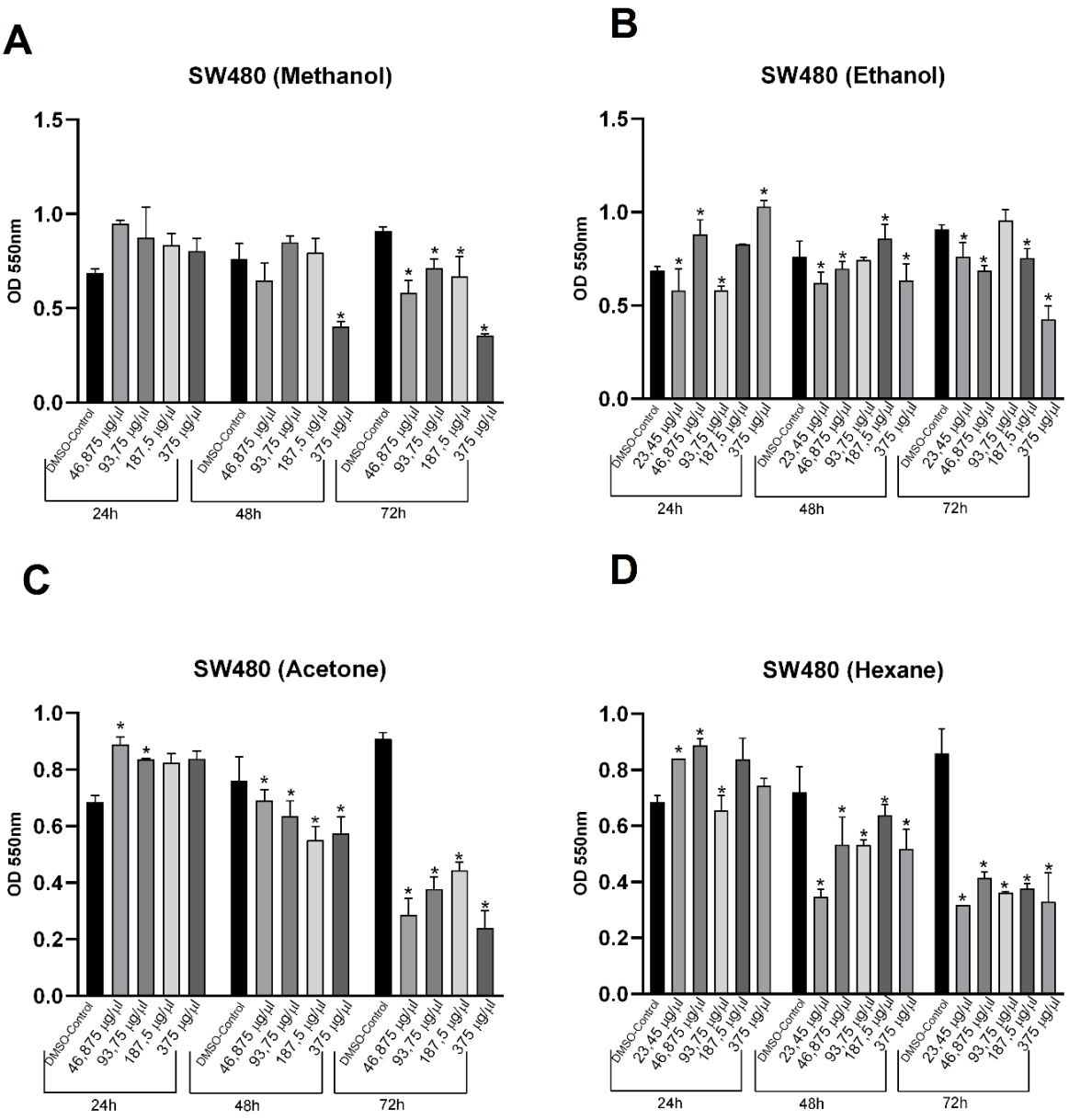

Figure 3. *In vitro* cytotoxicity *T. coerulescens* subsp. *ayvalikensis* extracts methanol (A), ethanol (B), acetone (C), and hexane (D) against malignant human colon cells (SW480). The cells were treated with DMSO vehicle or the indicated concentrations of *T. coerulescens* subsp. *ayvalikensis*. Cell viability was determined using MTT assay and *p*-value was obtained with ANOVA *$p < 0.05$ and was considered statistically significant.

4. DISCUSSION and CONCLUSION

Cytotoxicity refers to the rate of toxic effects on living cells. Cells may die due to autophagy, apoptosis, and necrosis. Cytotoxicity studies aim to determine the viability rates (amount of live/dead cells) of cells exposed to cytotoxic substances, depending on the dose and duration of effect(Tokur & Aksoy, 2017).

One of the methods used in cytotoxicity studies is the MTT method. MTT method; cytotoxic analysis is one of the preferred quantitative colorimetric methods for cell viability and proliferation. In this method, the tetrazolium ring in the MTT solution is broken down by the dehydrogenase enzymes in the cell mitochondria in the early stage of apoptosis or the reaction carried out by the mitochondria of living cells, creating colored formazan crystals. The MTT method is known as the method by which the mitochondria of healthy cells break down the tetrazolium ring of the MTT dye.

In this study, information on cytotoxicity studies of *T. coerulescens* subsp. *ayvalikensis* was presented for the first time. One of the important processes in the pharmaceutical and nutritional supplement industries is the extraction of active ingredients from herbal products. The solvent used in plant extraction can change the content of the products (Akinmoladun *et al*., 2022; Nawaz *et al*., 2019). Product content is affected by the time of plant collection, ambient temperature, extraction time, and solvent content (Dzah *et al*., 2020). In studies conducted with different plants, it has been reported that total phenol, flavonoid, and antioxidant activity values vary depending on the solvents, and it is necessary to use different solvents for each plant and the content to be obtained (Çoklar *et al*., 2016; Ribeiro *et al*., 2020; Salem *et al*., 2011). It is also clear that changing extract content depending on the solvent will affect biological activity. In our study, different solvents such as methanol, ethanol, hexane, and acetone were used in the extraction process from seeds. All of the extracts obtained were dissolved in DMSO and evaluated for their effect on cell proliferation in selected cancer cell lines.

Generally, when the phytochemical analysis of the seed content of the *Trigonella* genus is examined, it has been determined that flavonoid (vitexin, orientin, isoorientin, vicenin-1, vicenin-2, vicenin-3), saponin (yamogenin, tigogenin, diosgenin) and alkaloid (trigonelline) compounds are found in high amounts (Krol-Kogus *et al*., 2020; Krol-Kogus *et al*., 2021; Stefanowicz-Hajduk *et al*., 2021).

In the literature, in cytotoxicity studies conducted with extracts obtained from the seeds of the *Trigonella* genus, Stefanowicz-Hajduk et al. (2021), in SKOV-3, HeLa and MOLT-4 cell lines; Thakur and Ahirwar (2019) in the A549 (lung cancer) cell line; Abas and Naguib (2019), in MCF-7 and AsPC-1 cell lines; Goyal *et al*. (2018) in A431 (skin cancer) cell line; Al-Dabbagh *et al*. (2018) observed a cytotoxic effect on cancer cells in the HepG2 (human hepacellular cancer) cell line.

According to the cytotoxicity effect of *T. coerulescens* subsp. *ayvalikensis* hexane extract had the most reducing effect on PC-3 cells compared to the control groups. In the SW480 cell line, a proliferative effect was observed in extracts prepared with methanol, hexane, and acetone in the early period of 24 hours. In the later period of 72 hours, the extract prepared with hexane and acetone showed the most cytotoxic effect on SW480 cells. In this study, *T. coerulescens* subsp. *ayvalikensis* dose, time, and cell type-dependent effect was detected. Cell proliferation and antiproliferation occurred simultaneously, allowing the determination of the antitumoral and toxic effects for high doses of the extracts only at later periods (48, 72h). Based on the literature, at least two mechanisms can be put forth to explain this result. The first mechanism suggests that the drug's protective effects may be due to an anti-apoptotic mechanism; the second mechanism involves an antioxidant mechanism that increases the intracellular antioxidant enzymatic system's activity. Consequently, we propose that different solvents vad different extract concentrations of *T. coerulescens* subsp. *ayvalikensis* may impact the survival of human colon cancer cells through comparable anti-apoptotic and antioxidant mechanisms at early time points but apoptotic mechanisms may activated at late time points. More research is required to determine the precise cutoff value and concentration limits of *T. coerulescens* subsp. *ayvalikensis* extracts before their use as an anti-neoplastic agent, as well as to explain the mechanism(s) underlying this possible dual role of *T. coerulescens* subsp. *ayvalikensis* on the survival and growth of cancer cells.

As a result, we believe that the data we obtained will be an important resource for systematic studies to be conducted with similar genera or taxa in the future and for studies showing the effects of the extracts obtained for use in cytotoxicity experiments on different cancer cell lines. We think that the mechanism by which its effect on cancer cells occurs should be investigated in future studies.

Declaration of Conflicting Interests and Ethics

The authors declare no conflict of interest. This research study complies with research and publishing ethics. The scientific and legal responsibility for manuscripts published in IJSM belongs to the author(s). Ethics Committee Approval and its number should be given by stating the institution name which gave the ethical approval.

Authorship Contribution Statement

Sümeyye Aydoğan Türkoğlu: Investigation, Resources, Visualization, Writing -original draft, Supervision, and Validation. **Selin Koç**: Investigation, Resources, Methodology. **Fatma Poyrazlı**: Investigation, Resources, Methodology. **Selami Selvi**: Plant collection and identification, Supervision, and Validation.

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