In vitro evaluation of selective cytotoxic activity of Chaerophyllum macropodum Boiss. on cultured human neuroblastoma SH-SY5Y cells

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Abstract

Neuroblastoma is the most common solid tumor in children. New treatment approaches are needed because of the harmful side effects and costs of the methods used in the treatment of neuroblastoma. Medicinal and aromatic plants are important for new treatment approaches due to their minimal side effects and economic advantages. Therefore, the present study was carried out to examine the cytotoxic effect of *Chaerophyllum macropodum* extract on human neuroblastoma (SH-SY5Y) and fibroblast (HDFa) cell lines. 3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide (MTT) and Lactate dehydrogenase release (LDH) assays were used to determine the cytotoxic effect of *Chaerophyllum macropodum*. The results revealed that *Chaerophyllum macropodum* had a significant cytotoxic effect on neuroblast cells at all concentrations used (p < 0.05). But it did not show any effect on human fibroblasts. As a result, the obtained data clearly showed that *Chaerophyllum macropodum* exerts a selective effect on neuroblastoma cells for the first time.

Introduction

Cancer is an illness caused by the abnormal growth of cells that occurs in certain parts of the metabolism and can spread to other organs. According to the data of the World Health Organization, cancer-related deaths in the world were 9.6 million in 2018 ([WHO 2018](#)), while this number increased to 10 million in 2020 ([WHO 2020](#)). There are some childhood cancers that are of medical concern, although this illness rarely occurs before the age of 20 ([Steliarova-Foucher et al. 2017](#)). Neuroblastoma is a solid neuroendocrine tumor responsible for approximately 15% of childhood cancer-related deaths ([Louis and Shohet 2015](#); [Fusco et al. 2018](#)). Since this tumor occurs during the development of the sympathetic nervous system, prevention and screening are not possible ([Cheung and Dyer 2013](#)).

Despite some advances in clinical practice over the past 30 years, neuroblastoma remains a formidable challenge for scientists ([Luo et al. 2018](#)). The standard method used in the treatment of neuroblastoma is a combination of drugs consisting of cyclophosphamide, vincristine, cisplatin and doxorubicin this treatment causes chemoresistance, making the tumor very aggressive and metastatic ([Tibullo et al. 2018](#)). It has also been reported that the survival rate is low in patients with malignant metastatic tumors, despite the use of chemotherapeutic agents, radiation and immunotherapy, as well as multimodal therapeutic applications. ([Smith and Foster 2018](#)). Therefore, formulation of new agents is necessary to obtain good results from treatments applied in severe malignant groups. Specifically, natural products are of interest in recent approaches to cancer treatment ([Li et al. 2020](#)).

Today, about 80% of the world’s population uses herbal medicines in the treatment of diseases, which is reflected in the reports of the world health organization ([De Pasquale 1984](#); [Fabricant and Farnsworth 2001](#)). It is known that many medicinal plants show pharmacological activity because they have active ingredients with similar targets to pharmaceutical drugs ([Ahn 2017](#)). In addition, herbal medicines can be very effective in slowing the progression of cancer, as they induce apoptosis, inhibit enzymes and hormones and regulate the DNA repair process that support tumor development ([Lee et al. 2002](#);
Gehanege et al. 2017; Russo et al. 2019). In addition, it has been shown that medicinal plants may be effective in reducing the systemic toxicity caused by chemotherapy in some cancer types (Rao et al. 2008).

Apiaceae is one of the largest aromatic plant families and has approximately 450 genera and 3700 species distributed in temperate regions (Lee et al. 2019). In Turkey, 451 species belonging to the Apiaceae family and approximately 101 genera have been identified. Plants of this family are widely used as food and medicine (Özhatay et al. 2009). Chaerophyllum species belonging to this family are widely used in the kitchen with their aromas (Tarakçı et al. 2006). These species are used in cheese production and is also consumed as an edible vegetable in Turkey and Iran (Demirci et al. 2007; Çoruh et al. 2007; Durmaz et al. 2006). It has also been reported that plants of these species are used as herbal tea to relieve sore throat, cough and allergies (Prokopiou et al. 2021). Chaerophyllum macropodum (C. macropodum) is traditionally used in the production of herbed cheese, a famous cheese type in Turkey (Durmaz et al. 2006). Moreover, it has been reported that C. macropodum has antioxidant (Abdolrasoul et al. 2010), antimicrobial (Köse and Ocak 2018), antifungal (Khajehie et al. 2017), antiparasitic (Jabari et al. 2015). In addition, it has been concluded that C. macropodum has glutathione-S-transferase (Çoruh et al. 2007) and angiotensin converting enzyme inhibitory effects (Çelikezen et al. 2021). Although C. macropodum is a widely used plant, studies on its bioactivity are extremely limited. As much as we know, there is no report that indicates anti-cancer activity of C. macropodum. Therefore, the present work was planned for the first time to investigate the cytotoxic effects of C. macropodum on human neuroblastoma and fibroblast cell lines.

Material And Methods

Plant Material

Chaerophyllum macropodum Boiss. was collected in Diz Stream of Hakkari Cilo mountain at 1730 m in May. Scientific diagnoses of the plants were made by Mehmet FIRAT from University of Van YYU, Faculty of Education, Department of Biology.

Plant Extract

C. macropodum samples were frozen at -80°C for a week and grounded by using a tissue lyser (Qiagen®, Germany) into powder at maximum speed for 5 minutes. Samples were incubated in ultra-pure distilled water at room temperature for 24 hours and supernatant were obtained by using centrifugation at 10.000 RPM for 3 minutes. The aqueous extracts were passed through a 0.40 µm membrane filter and lyophilized to obtain dried solutes. Extract quantities were determined via the use of an analytical balance (Shimadzu®, Japan).

Cell Cultures
The human fibroblast cell line (HDFa, ATCC® PCS-201-012™) was grown in DMEM medium with a 1% penicillin/streptomycin antibiotic mixture, 10% FBS, 5% CO₂, and 37°C temperature until it reached 80% confluency.

Neuroblastoma cell line (SH-SY5Y, ATCC® CRL-2266™) was grown by using 1% penicillin/streptomycin, 10% fetal bovine serum in DMEM/F12 culture media. After the prepared medium was brought to 37°C, 5 ml was taken and transferred into a T25 flask and SH-SY5Y cells were cultivated in 37°C and 5% CO₂ incubator. Cells were incubated until they reached 80% density in the flask.

**Cytotoxicity and Cell Viability Analyses**

Lactate dehydrogenase (LDH, Cayman Chemical Company®, USA) assay was used to analyze cell death ratios for the cell cultures in respect to manufacturer's instruction. Briefly, cell cultures were seeded to 96-well plate and compounds were applied to the cultures in various concentrations (1.65 µg/m to 100 µg/m) for 24 hours. After the incubation period ends, 100 µL of culture supernatant were discarded into a fresh 96-well plate and 100 µL of the reaction mixture was added and immediately color intensity was investigated at 490 nm absorbance. Samples were incubated at room temperature for 30 minutes and again absorbance was monitored by using a microplate reader at 490 nm.

Cell viabilities for the fibroblast and the neuroblastoma cell cultures were investigated by using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. For the assay, compounds were applied to the cell cultures at wide dose intervals (1.65 µg/m to 100 µg/m) and incubated for 24 hours. After the application period, 10 µL of MTT solution was added to each well and incubated for 3 hours at 37°C. Then, cell mediums were discarded and 100 µL of DMSO was added to each well to dissolve formazan crystals to yield blue color. Color intensities were measured by using a microplate reader at 570 nm wavelength.

**Hoechst 33258 Fluorescent Staining and Nuclear Abnormality Analysis**

Hoechst 33258 staining was used to detect abnormal nuclear structures. Compounds were applied to the fibroblast cell culture at 100 µg/ml concentrations and cell cultures were incubated for 24 hours to monitor the nuclear morphology. Then, cells were fixed by using phosphate-buffered saline with 4% paraformaldehyde at 4°C for 30 minutes. Cell cultures were washed two times with PBS and samples were incubated with 1 µM Hoechst 33258 fluorescent dye at room temperature for 5 minutes. Cells were observed and photographed under a fluorescent microscope (Leica® DM IL LED).

**Statistical Analyses**

Statistical calculations were performed by using the GraphPad Prism (GraphPad Software®, USA, San Diego) statistical program and analyses were gathered via the use of one-way ANOVA, Dunnett comparison test. The statistical significance level was accepted as P <0.05.
Results

Cell viability and cytotoxicity analyses on the fibroblast cell line put forth that *C. macropodum* extract did not show significant toxicological properties up to 100 µg/ml concentration. Moreover, anticarcinogenic properties of *C. macropodum* extract were investigated on the neuroblastoma cell line by using LDH cytotoxicity and MTT cell viability assays. According to the analyses, *C. macropodum* extract showed anticarcinogenic properties up to 1.65 µg/ml concentration.

Figure 1.

Figure 2:

Figure 3.

Figure 4.

Figure 5.

Table 1

<table>
<thead>
<tr>
<th>Nuclear abnormalities (NA)</th>
<th>Treatment</th>
<th>Total MN</th>
<th>Total lobbed</th>
<th>Total notched</th>
<th>Mean NA/1000 cells ± SD</th>
</tr>
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<td>7</td>
<td>8</td>
<td>6</td>
<td></td>
<td>0.022 ± 0.005(^a)</td>
</tr>
<tr>
<td>Plant Extract</td>
<td>10</td>
<td>6</td>
<td>5</td>
<td></td>
<td>0.021 ± 0.002(^a)</td>
</tr>
</tbody>
</table>

Discussion

Despite significant advances in the development of new drugs for use in cancer treatment, deaths from cancer still rank second only to cardiovascular disease in the world. (Siegel et al. 2015). Although beneficial results are obtained in treatment with chemotherapy, it is not selective against cancer cells and has serious side effects. This creates an urgent need for drugs with greater efficacy and target specificity (Bagnyukova et al. 2010). Plants and plant-based products are very important source material for the discovery of effective anticancer agents. Since these herbs show less systemic toxicity than chemotherapy, they have been reported to be effective in preventing or slowing down oncogenesis (Akindele et al. 2015; Al-Rimawi et al. 2016; Wong et al. 2013). Researchers are investigating medicinal
plants and plant-based drugs for the development of effective anticancer agents because of their low cost and minimal side effects (Lichota and Gwozdzinski 2018; Tilaoui et al. 2018). Moreover, it has been reported that examining natural compounds to detect anti-proliferative and antitumor activities may be important in identifying new therapeutics for neuroblastoma and other cancer types (Busch et al. 2015). Therefore, the present study was designed to investigate cytotoxic effects of *C. macropodum* in human SH-SY5Y and human HDFα cell lines for the first time.

Cultured human SH-SY5Y samples were treated with different concentrations of *C. macropodum* extract. The results obtained showed that the *C. macropodum* extract exhibited statistically significant cytotoxic activity at all concentrations tested (from 1.65 µg ml⁻¹ to 100 µg ml⁻¹), as shown in Fig. 1. (p < 0.05). In addition, LDH assay was also used to determine cytotoxic effects of *C. macropodum*. The obtained data confirmed that the extract of *C. macropodum* led to statistically significant decreases at all tested concentrations as shown in Fig. 2. (p < 0.05). On the other hand, the generated data indicated that *C. macropodum* did not show a cytotoxic effect in HDFα cell line at used concentrations Fig. 3 and Fig. 4. Also, nuclear abnormalities were monitored via the use of the Hoechst 33258 fluorescent nuclear staining technique in HDFα cell cultures. Results put forth that *C. macropodum* extract did not exert any mutagenic property on the healthy human cell line (Fig. 5 and Table 1).

It has been observed that there are few studies on the cytotoxic activities of *Chaerophyllum* species on cancer cells during data mining. To our best knowledge, there is no information about the *in vivo* and *in vitro* anti-cancer features of *C. macropodum*. However, from other *Chaerophyllum* species, it has been reported that *Chaerophyllum hirsutum* has cytotoxic effects against the human intestinal adenocarcinoma cell line (Dall’Acqua et al. 2004) and *Chaerophyllum macrospermum* against HepG2 and S17 cell lines (Zengin et al. 2020). Furthermore, it has been reported that anti-cancer drugs should act on cancer cells while protecting healthy cells (Blagosklonny, 2004). At this point, *C. macropodum* showed that it could be an important candidate for cancer therapy by acting selectively against the neuroblastoma cell line.

On the other hand, although little information is available about the cytotoxic activities of the Apiaceae species used in our study, similar properties have been reported for different Apiaceae species. Nishumura et al. (2007) reported that chalcones obtained from *A. keiskei* had toxic effects on human neuroblastoma cells. In the study, it was shown that the isobavacalcone and xanthoangelol compounds in the content of *A. keiskei* induced apoptosis against neuroblastoma cells, not normal cells. Kimura and Baba (2003) and Kimura et al. (2004) reported the tumor-selective properties of xanthoangelol and 4-hydroxyderricin compounds isolated from *A. keiskei* in a DNA synthesis experiment using human HUVEC cell lines. Aydoğanuş-Öztürk et al. (2019) indicated that visnagin isolated from *Ammi visnaga*, has a cytotoxic effect on HT144 (human malignant melanoma) cell lines in a dose-dependent manner but did not show a cytotoxic effect on 3T3 cell (normal mouse fibroblast) lines. In another study, Pakfetrat et al. (2015) demonstrated that the ethanolic extract of *Ammi visnaga* had an inhibition potential on the growth of HeLa and MCF-7 cell lines. Aydin et al. (2013) showed that carvone a component of *Carum carvi* L. (Apiaceae) administration reduced the cell viability ratios in a significant manner in rat neuron
and N2a cell lines. Yang et al. (2018) reported that Angelica sinensis polysaccharide (AP) inhibited tumor formation in SH-SY5Y cells. Caputo et al. (2016) demonstrated that essential oils of Coriandrum sativum has a cytotoxic effect on SH-SY5Y cell line. In the study, researchers detected that Coriandrum sativum essential oil caused %78 cell death and they found IC$_{50}$ value as 591.8 µg/mL. Tabata et al. (2005) also investigated the cytotoxic activity of xanthoangelol, a major component of A. keiskei. In the study, they have been confirmed that xanthoangelol has antitumor activity at the concentration of 10$^{-4}$ M. Jeong and Kang (2011) evaluated that ethanol extract of A. keiskei induced apoptosis in breast cancer cell lines. Akihisa et al. (2011) stated that ethylacetate-soluble fraction of the methanol extract of A. keiskei roots has been reported to exert cytotoxic activity against human CRL1579 (melanoma), A549 (lung), AZ521 (stomach) and HL60 (leukemia) cell lines. Cheng and Ying (2021) investigated the anti-human neuroblastoma activity of saikosaponin A (SSA) isolated from the roots of Radix Bupleuri. They used the MTT method to detect the activity of SSA in inhibiting proliferation in the human SK-N-AS cell line and reported that SSA can inhibit proliferation, migration and invasion of human SK-N-AS. Rahman et al. (2016) showed that Angelica polymorpha Maxim root extract (APRE) may be a novel therapy. In the study, researchers examined APRE cytotoxic action on target neuroblastoma cells (SH-SY5Y) using cell viability assays. At the end of the study, authors found that APRE reduced the cell viability on the human SH-SY5Y and rat B103 neuroblastoma cells after 24 h of treatment with the value of IC$_{50}$ as 7.85 µg/ml.

**Conclusion**

As far as we know, the present study is the first to show that C.macropodum has a selective effect on human SH-SY5Y without affecting human HDFa cell lines. Our results suggest that further investigation of C. macropodum as an important candidate agent for neuroblastoma therapy will be important.

**Declarations**

**Author Contribution**

FCC and HT designed the study and wrote the paper. MF picked up plant samples and made scientific identification. HT, MEA and SÖ conducted the experiments.

**Conflict of interest**

The authors declare that there is no conflict of interest.

**References**


Figures
Figure 1

MTT analyses of neuroblastoma cell culture (SH-SY5Y) against *C. macropodum* extract application for 24 hours. (*) symbols represent a statistically significant decrease compared to the negative control (p < 0.05).

Cell viability and cytotoxicity analyses on the fibroblast cell line put forth that *C. macropodum* extract did not show significant toxicological properties up to 100 µg/ml concentration. Moreover, anticarcinogenic properties of *C. macropodum* extract were investigated on the neuroblastoma cell line by using LDH cytotoxicity and MTT cell viability assays. According to the analyses, *C. macropodum* extract showed anticarcinogenic properties up to 1.65 µg/ml concentration.
Figure 2

LDH analyses of neuroblastoma cell culture (SH-SY5Y) against *C. macropodum* extract application for 24 hours. (*) symbols represent a statistically significant decrease compared to the negative control (*p* < 0.05).
Figure 3

MTT analyses of the fibroblast cell culture (HDFa) against *C. macropodum* extract application for 24 hours.
Figure 4

LDH analyses of the fibroblast cell culture (HDFa) against *C. macropodum* extract application for 24 hours.
Hoechst 33258 fluorescent staining of fibroblast cell culture. a) Negative control (no application), b) 100 µg/ml of *C. macropodum* extract application to the fibroblast cell cultures.

Hoechst 33258 fluorescent staining was used to investigate nuclear abnormalities against *C. macropodum* extract application. Results showed that there is no significant difference in nuclear morphologies and cell surface distributions (Figure 5). Also, nuclear abnormality assessment counts exhibited that there were no significant differences in nuclear morphologies in respect to MN: micronucleus, L: lobbed, and N: notched nuclear abnormalities (Table 1).