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Cytotoxic activities of some Turkish medicinal plants against HeLa cells *in vitro*

Cagla Bozkurt-Guzel[1]*, Tuba Serbetci[2] & Sukran Kultur[3]

[1]Department of Pharmaceutical Microbiology, Faculty of Pharmacy, Istanbul University, Beyazit, Istanbul, 34116, Turkey;
[2]Herbasphere Biotechnology R&D Company, Inc., Istanbul University Technocity, Avcılar, Istanbul, 34310, Turkey;
[3]Department of Pharmaceutical Botany, Faculty of Pharmacy, Istanbul University, Beyazit, Istanbul, 34116, Turkey
E-mail: caglabozkurt@hotmail.com

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The aim of this study was to characterize the biological activities of traditionally used medicinal plants generally collected from Kırklareli, Turkey against cancer. We evaluated the cytotoxic activities of different extracts prepared from *Urtica dioica* L. **1**, *Achillea millefolium* L. ssp. *pannonica* **2**, *Malva sylvestris* L. **3**, *Stachys cretica* L. ssp. *mersinaea* (Boiss.) Rechf.f. (endemic) **4**, *Melissa officinalis* L. **5**, *Cotinus coggyria* Scop. **6**, *Sorbus aucuparia* L. **7** , and *Plantago major* L. ssp. *major* **8** species. Ethanol **(a)**, petroleum ether **(b)**, dichloromethane **(c)** and ethyl acetate **(d)** fractions of each plant material were obtained. Cytotoxicity in HeLa cells was evaluated using the MTT assay. Among the extracts, 7c, 2b, 6d, 7d, 7a, 2c, and 3b showed potent cytotoxic activity with IC_{50} values of less than 50 µg/mL, in descending order. Dichloromethane extract of *S. aucuparia* is considered highly active, with IC_{50} of 15±03 (mean ± SD) µg/mL. The extracts 8a and 8b also showed good cytotoxic activity. According to the results, dichloromethane extracts of *S. aucuparia* had the most potent anticancer activity in HeLa cells and should be considered as a potential clinical agent. The results support the ethnomedical claims for these species and suggest further *in vitro* and/or *in vivo* studies of the active extracts.

Keywords: Medicinal plants, Cancer treatment, Ethnomedicine, HeLa cells, MTT
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Cancer is a broad group of diseases involving unregulated cell growth. In cancer, cells divide and grow uncontrollably, forming malignant tumors, which may invade nearby parts of the body. Cancer may also spread to more distant parts of the body through the lymphatic system or bloodstream. There are over 200 different known cancer cells that affect humans. The major characteristic is the lack of control of cell proliferation, differentiation and death, invading organs and tissues. Many treatment options for cancer exist with the primary ones including anticancer agents, surgery, radiation therapy, and palliative care. However, there are many difficulties in the prevention and treatment but the most frequently faced ones are drug resistance, toxicity, and low specificity. Therefore, in cancer research, traditional medicine has aroused renewed interest in the search for safe, potent and selective anticancer compounds[1]. Natural products derived from plants continue to be investigated as a source of novel medicinal agents, with a focus on the discovery of novel anticancer drugs. Semi-synthetic and synthetic derivatives of plant molecules are important sources of anticancer drugs. Over 50 % of the drugs in clinical trials for anticancer activity were isolated from natural sources or are related to them. Plant-derived agents, such as vinblastine and vincristine, etoposide, paclitaxel (Taxol), docetaxel, topotecan, and irinotecan, are among the most effective cancer chemotherapeutics currently available[2].

Turkey has one of the richest flora in the temperate zone, with approximately 10,000 species of vascular plants, one-third of which are endemic to the country[3-6]. Since ancient times, they have been used in traditional Turkish medicine to treat numerous diseases, including cancer. The search for new drugs exhibiting activity against several types of cancer is one of the most interesting subjects in the field of natural products research. Recently, ethnobotanical studies have been conducted to survey local residents regarding the regional usage of plants[7-17]. Previously, Kultur *et al.*[7,8] had done ethnobotanical studies and the locals interview subjects reported that some of the local plants are effective for treating various types of cancer. Starting from this point of view, in the present study we aimed to examine the cytotoxic effects on cancer cells by plant species with rich ethnomedicinal history. We investigated the *in vitro* cytotoxic activities against HeLa cells (human tumor-derived cell lines from cervical

*Corresponding author

cancer) of ethanol, petroleum ether, dichloromethane and ethyl acetate extracts from *U. dioica* L., *A. millefolium* L. ssp. *pannonica* (Schelek), *M. sylvestris* L., *S. cretica* L. ssp. *mersinaea*, *M. officinalis* L., *C. coggyria* Scop., *S. aucuparia* L. and *P. major* L. ssp. *major* species. Some of these plants are used in the treatment of cancer in the Western part of Turkey[12,14,16,17]. To our knowledge, this is the first study that focused on the investigation of cytotoxic activity of these Turkish folklore plants against HeLa cells.

Methodology

Plant material

In this study, *U. dioica* (ISTE 81041), *A. millefolium* ssp. *pannonica* (ISTE 80989), *M. sylvestris* (ISTE 81044), *M. officinalis* (ISTE 81042), *C. coggyria* (ISTE 80926), *S. aucuparia* (ISTE 80916) and *P. major* ssp. *major* (ISTE 81039) species were collected from different districts of Kirklareli Province. Endemic *Stachys cretica* ssp. *mersinaea* (ISTE 98210) was collected from Mersin Province. The recorded species were collected by Prof. Sukran Kultur, Department of Pharmaceutical Botany, Faculty of Pharmacy, Istanbul University, and were taxonomically identified according to "Flora of Turkey and the East Aegean Islands"[18-20] and compared to the specimens kept in the Herbarium of Faculty of Pharmacy, University of Istanbul (ISTE). Voucher herbarium specimens were prepared and deposited in the ISTE Herbarium.

Preparation of crude extracts

All crude air-dried plant materials were weighed, pulverized, and macerated/homogenized in absolute ethanol for 3 h in an ultrasonic bath. The ethanol extract (1a, 2a, 3a…8a) was then dried under reduced pressure and the residue was dissolved in water and filtered. The filtrate was extracted with petroleum ether (1b, 2b, 3b…8b), dichloromethane (1c, 2c, 3c…8c) and ethyl acetate (1d, 2d, 3d…8d). In total, 32 different extracts were prepared.

Cell line, growth conditions and culturing

The HeLa cell is the oldest and most commonly used immortalized cell line in medical research. The cell line was derived from cervical cancer cells taken from Henrietta Lacks, who died from cancer in 1951 (Fig. 1). Initially, the cell line was said to be named after a "Helen Lane," to preserve Lack's anonymity[21].

The HeLa cell line used in this study was obtained from Dr. Cemalettin Bekpen at the Bogazici University, Istanbul. The cells were grown in Dulbecco's Minimal Essential Medium (DMEM) (Gibco Life Technologies, UK) supplemented with 10 % fetal bovine serum (FBS) (Biochrom, Germany) and 100 µg/mL streptomycin and 100 IU/mL penicillin, as a monolayer in tissue culture flasks in humidified atmosphere of 5 % CO_2 at 37 °C in the CO_2 incubator (Heraus, Germany). The trypan blue dye exclusion method was used to assess cell viability and counting using a Neubauer chamber. Cells were harvested using Trypsin-EDTA (Biochrom, Germany) (0.05 %:0.02 %) solution.

Cytotoxicity evaluation by MTT assay

The samples of each plant extract were prepared as stock solutions in DMSO. Working solutions at the final concentrations (1µg/mL, 10 µg/mL, or 100 µg/mL) were prepared in the medium prior to testing. Cytotoxic effects were determined by MTT colorimetric assay[22]. Briefly, exponentially growing cells were harvested and $1.5x10^4$ cells were plated per well on 96-well cell culture plates (Greiner Bio-one, Germany). After the cells were incubated overnight in the 37 °C CO_2 incubator to attach to the wells, they were treated with different concentrations of plant extracts. After every 24 h of incubation until 72 h, fresh medium with 5 mg/mL of MTT was added and incubated for 3 h at 37 °C. Upon media removal, water insoluble, purple, MTT-formazan crystals formed inside the living cells were dissolved in DMSO and the absorbance at 570 nm proportional to the number of living cells was measured on an ELISA Microplate Reader (Bio-Rad, Novo Path, CA, USA). Control groups received the same amount of DMSO. All experiments were performed three times in triplicate. The percentage of growth inhibition was calculated using the formula below:

% cell inhibition= 100- [(At-Ab)/(Ac-Ab)] x100
where,
At= Absorbance value of test compound
Ab=Absorbance value of blank
Ac=Absorbance value of control

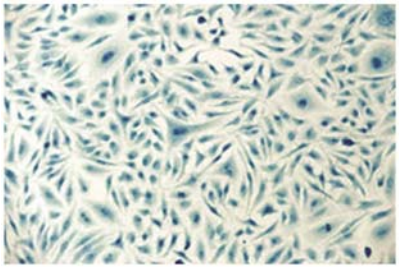

Fig. 1 — HeLa cell line

A crude extract is considered active if it exerts an IC_{50} values lower than 30 µg/mL in carcinoma cells, following incubation between 48 h and 72 h, according to the American National Cancer Institute[23].

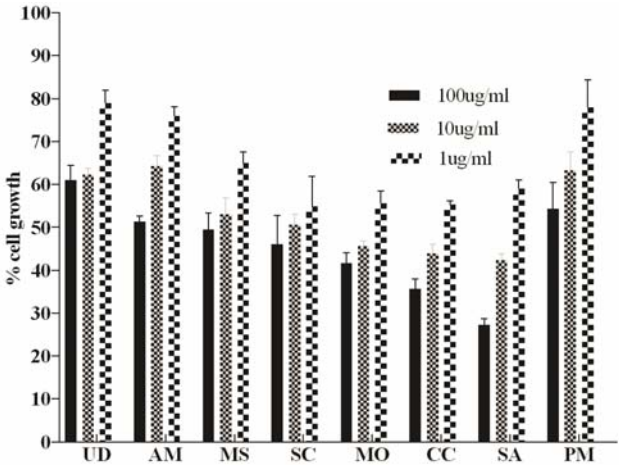

Fig. 2 — Cytotoxic effects of ethanol extracts from 8 plants on HeLa cell line growth after 72 h of incubation at 1 µg/mL, 10µg/mL, 100 µg/mL of the plant finale concentration range evaluated by MTT colorimetric technique. Data represent mean value of three independent experiments done in triplicate. (UD=*Urtica dioica*, AM=*Achillea millefolium* ssp. *pannonica*, MS=*Malva sylvestris*, SC=*Stachys cretica* ssp. *mersinaea,* MO =*Melissa officinalis,* CC =*Cotinus coggyria,* SA =*Sorbus aucuparia,* PP= *Plantago major* ssp. *major*)

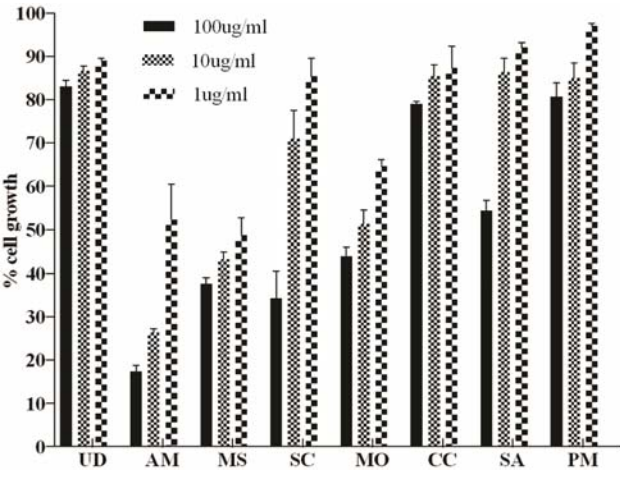

Fig. 3 — Cytotoxic effects of petroleum ether extracts from 8 plants on HeLa cell line growth after 72 h of incubation at 1µg/mL, 10 µg/mL, 100 µg/mL of the plant finale concentration range evaluated by MTT colorimetric technique. Data represent mean value of three independent experiments done in triplicate. (UD=*Urtica dioica*, AM=*Achillea millefolium* ssp. *pannonica*, MS=*Malva sylvestris*, SC=*Stachys creticas*sp. *mersinaea,* MO=*Melissa officinalis,* CC=*Cotinus coggyria,* SA=*Sorbus aucuparia,* PP= *Plantago major* ssp. *major*)

Statistical Analysis

All experiments were performed in triplicate and statistical analysis was performed by SPSS software. The means and standard errors were recorded. Cytotoxicity was expressed as the 50 % inhibitory concentration (IC_{50}), which is the concentration needed to reduce the cell number of treated cells by 50 %, compared to the control (untreated cells). IC_{50} values were calculated from the Prism dose-response curve.

Results

The results depicted in figures summarize the cytotoxic effects on HeLa cell line of the 32 extracts from eight different plant species from various families. The results are presented as a percentage of cell growth compared to control (Figs 2-5). The results are also expressed as IC_{50}. The results from the initial screening showed that the extracts from eight extracts exhibited promising activity against HeLa cells, with cell survival of less than 50 % at the concentration of 50 µg/mL. Among studied extracts, 2b, 2c, 3b, 6d, 7a, 7c, 7d showed potent cytotoxic activity with IC_{50} values of less than 50 µg/mL. The potency of the extracts in descending order was as follows: 7c, 2b, 6d, 7d, 7a, 2c, 3b.

A crude extract is considered active if it exerts an IC_{50} value ≤ 30 µg/mL in carcinoma cells, following

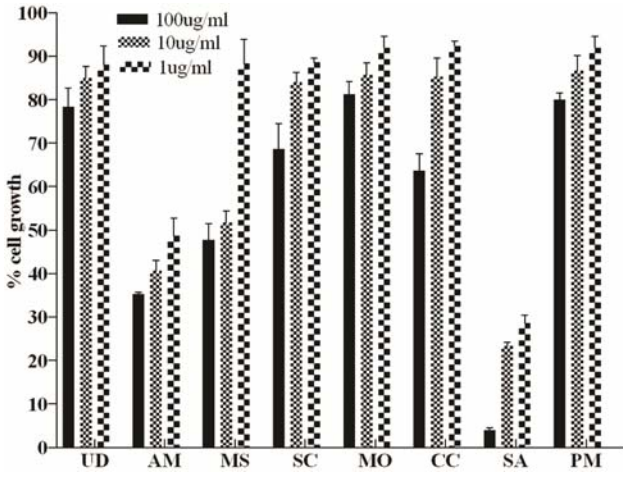

Fig. 4 — Cytotoxic effects of dichlormethane extracts from 8 plants on HeLa cell line growth after 72 h of incubation at 1µg/mL, 10 µg/mL, 100 µg/mL of the plant finale concentration range evaluated by MTT colorimetric technique. Data represent mean value of three independent experiments done in triplicate. (UD=*Urtica dioica*, AM=*Achillea millefolium* ssp. *pannonica*, MS=*Malva sylvestris*, SC=*Stachys cretica* ssp. *mersinaea,* MO=*Melissa officinalis,* CC=*Cotinus coggyria,* SA=*Sorbus aucuparia,* PP= *Plantago major* ssp. *major*)

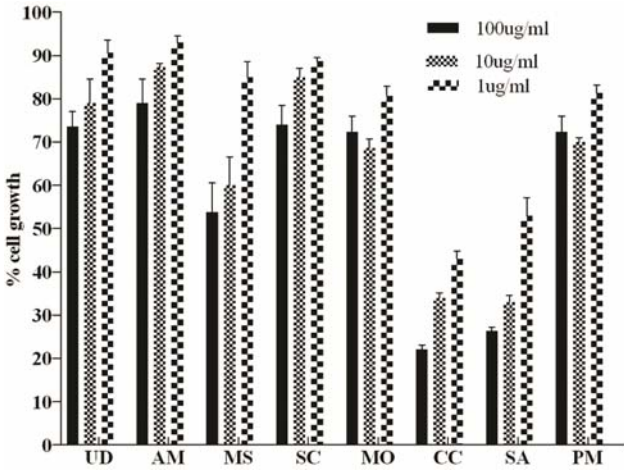

Fig. 5 — Cytotoxic effects of ethyl acetate extracts from 8 plants on HeLa cell line growth after 72 h of incubation at 1µg/mL, 10 µg/mL, 100 µg/mL of the plant finale concentration range evaluated by MTT colorimetric technique. Data represent mean value of three independent experiments done in triplicate. (UD=*Urtica dioica*, AM=*Achillea millefolium* ssp. *pannonica*, MS=*Malva sylvestris*, SC=*Stachys cretica*ssp. *mersinaea*, MO=*Melissa officinalis,* CC=*Cotinus coggyria,* SA=*Sorbus aucuparia,* PP=*Plantago major* ssp. *major*)

incubation between 48 h and 72 h, according to the National Cancer Institute[23]. Based on this criterion, the extract from dichloromethane of *S. aucuparia* (7c)is considered highly active with IC_{50} of 15±03 (mean ± SD) µg/ml. Plant extracts 7a and 7b also showed good cytotoxic activity. 2b, 6d, 7d, 7a, 2c and 3b have IC_{50} values 19.5±6, 21±5, 24±55, 25±34, 29±55 and 29±8, respectively.

Discussion

Recently, natural products have been shown to play a very important role in new drug discovery and development. Naturally occurring substances that block or suppress the proliferation of tumor cells are potentially potent antitumor agents[24,25].

In the present study, we have carried out a preliminary investigation of the cytotoxic effects of ethanol, petroleum ether, dichloromethane and ethyl acetate fractions (extracts) of eight plants (*U. dioica*, *A. millefolium* ssp. *pannonica*, *M. sylvestris*, *S. cretica* ssp. *mersinaea*, *M. officinalis, C. coggyria, S. aucuparia,* and *P. major* ssp. *major*). The extracts were evaluated in cells from a human cervical cancer-derived cell line, using the MTT assay. This *in vitro* method is used for the detection of cytotoxic and other negative effects on cell viability, following exposure to test materials. Cells were treated with increasing concentrations of the plant extracts (1, 10, 100 µg/mL) for 72 h. The MTT results were used to determine the IC_{50} values. Notably, we found that all extracts reduced cell viability in a dose-dependent manner. The cytotoxicity of all extracts was found to be concentration-dependent (Figs. 2-5).

***U. dioica* (Urticaceae)** leaves are a rich source of essential amino acids, ascorbic acid, several mineral elements and vitamins. This plant is believed to be anticarcinogenic, antiulcer, antioxidant, anti-inflammatory, immune suppressive, and antirheumatoid[26]. Nahata *et al*.[27] screened *U. dioica* for its cytotoxicity against various human cancer cell lines, namely lung (A549), prostate (PC-3 and DU-145), colon (Colo-205), neuroblastoma (IMR-32), and breast cancer (MCF-7), but they found that *in vitro* cytotoxicity induced by *U. dioica* was not significant. So, keeping these results in mind, in this study, we aimed to investigate cytotoxic activity against a different cell line –namely, HeLa. In accordance with the results of other researchers we could not find any significant results.

In European and American countries, ***Achillea* L. (Compositae)** species (commonly known as *yarrows*) have been used in various preparations (juice, ointment, oil, etc.) as traditional herbal medicine to treat cancer of the breast and liver and to treat hardness of the uterus. Experimentally, the anticancer activity of *Achillea* species has been shown in only a few instances. The cytotoxic effects of *A. alexandri-regis*, *A. clavennae*, *A. ageratum* and *A. millefolium* extracts have been demonstrated in various malignant tumor cell lines. In previous studies, the chloroform-soluble extract of *A. millefolium* was shown to inhibit cell proliferation in HeLa and MCF-7 cells, with a moderate effect on A431 cells. That study also demonstrated that centaureidin was the most effective constituent of the aerial parts of yarrow, and high cell growth inhibitory activities were observed especially in HeLa (IC_{50}: 0.0819 µm) and MCF-7 (IC_{50}: 0.1250 µm) cells[28]. In keeping with these observations, we found that petroleum ether and dichloromethane extracts of *A. millefolium* ssp. *pannonica* showed significant cytotoxic activity.

It is well known that ***M. sylvestris* (Malvaceae)** can be utilized as an anti-inflammatory substance for the respiratory tract, GI tract, and the skin[29]. The plant can be used topically or in a bath to treat abscesses, bruises, burns, dermatitis, swellings, and various ulcers[30,31]. Razavi *et al*.[32] reported that methanol extracts of *M. sylvestris* flower and leaves reduce the viability of McCoy cells, with IC50 value of 265.3 and 311.0 g/mL, respectively. Therefore, components of this plant could

be antiproliferative agents. Decoctions of *M. sylvestris* that are prepared from the leaves and flowers have been reported to be used against cancer in Turkey[11]. In our study, we had relatively high cytotoxic activity against HeLa cell line with the IC_{50} value of 29.8 µg/mL. There are no other cytotoxicity studies in the literature with this plant effects on HeLa cells.

The genus ***Stachys* L. (Lamiaceae)** contains approximately 70 species that are spread in the northern hemisphere and tropical Australasia, with a center of biodiversity in the Mediterranean and Middle East, including South and East Anatolia, Caucasia, North-West Iran, Iraq and the Balkan Peninsula. With 83 recorded species and a level of 48 % endemism, Turkey has some of the richest levels of *Stachys* diversity[33]. In previous studies, cytotoxic activity of *Stachys* plants against MCF-7, HeLa, and A431 cells has been reported. Treatment of HeLa cells with *S. recta* and *S. palustris* stem extract was shown to result in greater than 25 % inhibition of proliferation[34]. In our previous study, we tested the ability of the ethanolic extracts of the *Stachys* species to inhibit the growth of two human tumor cell lines. The IC_{50} values of *S. cretica* L. ssp. *lesbiaca* Rech. fil. and *S. cretica* L. ssp. *trapezuntica* Rech. fil. were determined as 100 µg/mL for the HL-60 cell line and 200 µg/mL for the Ishikawa cell line[33]. In our study, 100 µg/mL of *S. cretica* L. ssp. *mersinaea* (Boiss.) reduced cell growth to 34 %, relative to control.

The aromatic herb ***M. officinalis* L. (Lamiaceae)** can be used as an easily accessible source of natural antioxidants and is used as a food supplement and as a phytochemical. In a previous study, chloroform extract of *M. officinalis* showed a strong antiproliferative effect, with 50 % inhibitory concentration values of 0.09 mg/mL and 0.10 mg/mL for HeLa and MCF-7 cell lines, respectively[35]. It has been reported that decoction prepared from the leaves of *M. officinalis* has been used against gastric and lung cancers in Babaeski (Kırklareli), Turkey[12]. Here, we demonstrated that ethanol and petroleum ether extracts of *M. officinalis* showed similar cytotoxic activities.

***C. coggyria* L. (Anacardiaceae)**, commonly known as smoke tree, is native to a large area, from southern Europe, East and Central Asia to the Himalayas and northern China[36]. In a previous study, the methanol extracts of *C. coggyria* leaves and flowers have been shown to possess cytotoxic activity towards HeLa and LS174 human cancer cell lines *in vitro*, with stronger inhibition against growth of HeLa cell growth than against LS174 cell growth[37]. Decoctions of *C. coggyria* that are prepared from the leaves[12] and woods have been reported to be used against cancer in Antakya: A multicultural district in Hatay province on Turkey[13]. In our study, ethyl acetate extract of *C. coggyria* exhibited good cytotoxic activity, with less than 50 % of cells surviving at 50 µg/mL.

A recent study from the Estonian Folklore Archives showed potential anticancer properties elicited by different plants. Among these was ***S. aucuparia* L. (Rosaceae)**, whose anticancer activities have not been previously described in the scientific literature[38]. According to our results, the dichloromethane extract of *S. aucuparia* showed the highest anticancer activity.

Different species of ***Plantago* L. (Plantaginaceae)** have been utilized as remedies against cancer in folk medicine. However, there is no scientific validation for this use. One goal of the present study is to understand better its use.

P. major ssp. *major* fresh leaves are consumed against cancer in Turkish Folk medicine[14]. Similarly other studies showed decoction of this plant that is prepared from the leaves has been reported to be used against cancer[15,16]. In another local study, entire plants decoction is used to treat throat cancer in the South part of Izmir Gulf, in Turkey[17].

It has been demonstrated that *Plantago* extracts have growth inhibitory and cytotoxic effects on MCF-7 and UACC-62 (melanoma) cell lines recommended by National Cancer Institute[39]. In contrast to this claim, we found that cells showed more than 50 % cell growth with all concentrations of the four extracts of ***P. major* ssp. *major***.

Although many cytotoxic effects of these plants have been reported[33,40,41], such effects against HeLa cell line have not been previously described for ***U. dioica*, *M. sylvestris*, *S. aucuparia*** and ***P. major* ssp. *major*** plants, with respect to their anticancer activities. Our findings suggest the existence of potential anticancer compounds in herbal materials. This is the first report of the cytotoxic activities of the aforementioned plants in HeLa cells. Thus, this report provides promising results for the use of some of these Turkish medicinal plants against HeLa cells *in vitro*.

The results obtained from this study indicate that 7 out of a total of 32 plants used in Turkish folklore medicine exhibited promising cytotoxic activity against HeLa cells. Among those, dichloromethane extracts of *S. aucuparia* had the most potent

anticancer activity in HeLa cells, and should be considered as a potential clinical agent. The results of this preliminary screen justify further purification of the crude extracts and isolation of active compounds, with the goal to explore their potential as anticancer drugs. This study also justifies and reinforces the use of these plants as traditional medicines. Although all *in vitro* experiments are limited with respect to possible *in vivo* efficacy, these results are very promising and will serve as a resource for researchers studying the treatment of specific medical conditions with folkloric medicine. Furthermore, it is still necessary to investigate *in vivo* bioactivity and cytotoxicity of these plants, to explore in more depth the possible use of these plants in treating other diseases, such as microbial infection.

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