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Research article

Screening revealed the strong cytotoxic activity of *Alchemilla smirnovii* and *Hypericum alpestre* ethanol extracts on different cancer cell lines

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Abstract: Compounds of plant origin are considered promising alternative approaches in the development of medicines for the prevention and treatment of cancer. The large diversity of herbal species still requires careful exploration as a source for new anticancer compounds. The goal of the study was to screen different herbal extracts traditionally used in Armenian folk medicine for their cytotoxic effect against some cancer cell lines, and to find the prospective plant species among them. The cytotoxicity of the plant ethanol extracts was evaluated with MTT test against HeLa (human cervical carcinoma) and A549 (human lung adenocarcinoma) cells. Antioxidant properties were assessed with DPPH free radical scavenging assay. Five of the tested ten herbal extracts exhibited significant growth-inhibiting activity on HeLa cells. Moreover, *Alchemilla smirnovii* and *Hypericum alpestre* extracts also showed potent cytotoxicity on human lung adenocarcinoma cells. These two plants possessed high antiradical activity as well. Their DPPH stoichiometric values were 0.4234 and 0.14437 respectively, meaning that 1 µg of plant extract brought the reduction of DPPH equal to the respective stoichiometric values in µg. Thus, *A. smirnovii* and *H. alpestre* extracts expressed themselves as potent cytotoxic and antioxidant agents and could have promising anticancer potential. Further evaluation of their *in vivo* anticancer properties has much interest.

Keywords: herbal extract; Armenian flora; cytotoxicity; antiradical activity; *Inula helenium*

Abbreviations: DMEM: Dulbecco's modified Eagle medium; DPPH: 1,1-diphenyl-2-picrylhydrazyl; DW: Dry weight; FBS: Fetal bovine serum; GAE: Gallic acid equivalent; MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PBS: Phosphate-buffered saline; ASI: Accumulated survival index; ROS: Reactive oxygen species; NF-κB: Nuclear factor kappa B; MAPK: Mitogen-activated protein kinase

1. Introduction

Compounds of plant origin have been used for the treatment and prevention of various diseases since time immemorial, and now they remain extremely important [1]. Our previous investigations suggest numerous plant extracts and essential oils as preventive and treating agents for medicine and veterinary [2–5].

Scientists around the world are trying to reveal whether plant-derived antioxidants are cancer inhibitors or enhancers, their potential role in cancer treatment, and possible action mechanisms and targets in cancer cells. A huge number of investigations confirming the cytotoxic and potential anticancer activity of such plants are existing. Recent review articles cite hundreds of research data that state the positive effects of different plants in cancer treatment [6,7]. In other investigations it was shown that plant-derived metabolites can influence as enhancers and modulators of the activity of chemotherapeutic drugs in some types of cancers, sometimes expressing synergistic effects [8]. These could be possible due to different mechanisms such as blocking and/or reversing the drug-resistance mechanisms [9,10] reducing the chemoresistance by decreasing efflux proteins, and promoting apoptotic cell death [11]. There are also described other possible mechanisms of anticancer influence of the metabolites, extracted from different plant species including inhibition of carcinogen activity, tumorigenesis, cell proliferation, angiogenesis, and induction of cell death. These can be associated with the possible modulation of reactive oxygen species (ROS) production, inhibition of nuclear factor kappa B (NF-κB), down/upregulation of mitogen-activated protein kinase (MAPK) activation, and, eventually, the regulation of epigenetic change [12].

Armenian flora is very rich in plants with high biological activity, which could be considered for the development of different preparations with therapeutic values [13]. Many of these plants are possibly capable of being applied in the treatment and prevention of cancer, but there is an extremely low quantity of scientific information about this.

Therefore, this open field for research as well as the high rate of cancer evidence in Armenia [14,15] forced us to investigate the antiproliferative and cytotoxic effects of extracts, obtained from plants represented in the Armenian flora in order to find prospective herbal species.

2. Materials and methods

2.1. Chemicals and reagents

All applied chemicals and reagents were purchased from Sigma-Aldrich Co. Ltd.

2.2. Plant material

The plant materials were harvested from the Tavush region of Armenia (1400–2400 m above sea level) according to already established protocol [13]. Identification of plant materials was done at the Department of Botany and Mycology, Yerevan State University (YSU) (Armenia). Plant voucher specimens were deposited to the Herbarium of YSU and added to the collection with assigned voucher numbers (For details see Table 1).

Plant crude extracts were prepared by maceration technique using pure ethanol (96%) [13]. Ground plant materials (100 mg) were soaked with ethanol at 10: 1 solvent-to-sample ratio (v/w). The mixture was vortexed for one minute and left in an ultrasonic bath for 15 min and centrifuged at 1000 rpm for 5 min. The supernatant was transferred into a new tube and further, fresh solvent was added to precipitate at the same ratio, vortexed for one minute again and left in the ultrasonic bath for the second round. After the second centrifugation the supernatants were combined. Thus, 50 mg DW/mL crude ethanol extracts were prepared. The concentration of dissolved compounds/yield in 50 mg DW/mL extract was determined by drying 500 µL of extract and weighing each sample in three independent replicates (See Table 2).

2.3. Cell lines and culture

HeLa (human cervical carcinoma) and A549 (human lung adenocarcinoma) cells have been maintained in Dulbecco's Modified Essential Medium (DMEM) supplemented with 10% Human serum, 1x Pen/Strep. Cells have been seeded in tissue culture-treated multi-well plates at a maximum density of 2×10^5 cells/cm^2. The cells have been propagated at 37 ℃ in an atmosphere of 5% CO_2 in a CO_2 incubator (Biosan S-Bt Smart Biotherm).

2.4. MTT assay

The MTT test [16] was performed to assess the inhibition of growth of HeLa and A549 cells exposed for 4, 24, or 72 h to different concentrations (0.5, 0.25, and 0.125 mg DW/mL) of the test-plant extracts. Treatments were performed as three technical replicates. Three independent replicates of each treatment were also performed. Cytotoxicity was expressed as percent growth inhibition of cells exposed to tested plant extract compared to control cells treated with the appropriate volume of solvent only (1% ethanol in the final mixture), whose growth was regarded as 100%. Results were expressed as accumulated survival index (ASI), calculated as the sum of areas under survival curves for each exposure time for the same concentration range (0.125, 0.250 and 0.5 mg DW/mL).

2.5. DPPH free radical scavenging assay

The antioxidant potential of tested extracts was evaluated spectrophotometrically, using DPPH (1-diphenyl-2-picrylhydrazyl) radicals according to the procedure described before [16]. The stock solution of DPPH was prepared prior to measurements; DPPH with ethanol until the absorbance reached 0.9 ± 0.02 at $\lambda = 515$ nm. Measurements of absorbance were carried out in 48-well plates using SPECTRO star Nano microplate reader (BMG Labtech, Germany). The stoichiometry values (n_{10}) represent the number of oxidant molecules reduced by one molecule of

antioxidant after 10 min of reaction were determined at room temperature. Regression coefficient, which was defined as the tangent of the line representing the relationship between the amount of scavenged DPPH (μg) of a radical scavenger and the quantity of the tested antioxidant extract present (μg) in the mixture after 10 min of reaction (n_{10}) [17].

2.6. Determination of total phenolic content

The total phenolic content of plant extracts was measured exploiting the Folin & Ciocalteu's phenol reagent (FC) employing a calibration curve of gallic acid (GA) (0–250 μg/mL) using a UV-Vis spectrophotometer (Genesys 10S, Thermo Scientific, USA) [4].

2.7. Statistical analysis

All statistical analyses were performed using GraphPad Prism 8 software (GraphPad Software, Inc., USA). A *p*-value of less than 0.05 was considered significant. All results are presented as means ± SD (standard deviation). An unpaired Student's t-test ($p \leq 0.05$) was used to evaluate antioxidant activity in the chemical (DPPH) test.

3. Results and discussion

There is a high biodiversity of flora in the area of the Republic of Armenia which includes herbal species widely used in folk medicine [13,18–21]. This diversity can also be a source of plant products with potential anticancer properties. During this investigation, we screened alcoholic extracts of 10 herbal species for their cytotoxic properties on cancer cell lines of different origin in order to select promising plant samples for further more targeted and comprehensive studies (See Table 1). The initial selection of herbal species was based on their uses in folk medicine which could indicate the possible anticancer activities according to traditional medical handbooks [19,20].

At the initial stage of the study the effect of ethanol extracts of 10 herbal species on the growth of HeLa cells, which is one of the most common cancer cell lines, was evaluated. It is important to point out that only low concentrations of the extracts were used (ranging from 0.125–0.5 mg DW/mL) in order to find herbal species with strong cytotoxic properties. The extraction yield and concentration of dissolved compounds in 50 mg DW/mL extract are presented in Table 2.

Based on obtained data, five of the tested herbal extracts showed strong growth-inhibiting activity on HeLa cells (Figure 1). These plants are *A. smirnovii, H. alpestre, I. helenium, C. pallasii* (flowers with leaves) and *R. canescens*. For estimation of the overall cytotoxic properties of different herbal extracts accumulated survival index (ASI) parameters were calculated, which are defined as the sum of areas under survival curves determined for individual treatments for the same concentration range. ASI values are allowing to compare cell growth inhibiting effect of different samples. Obtained ASI parameters clearly indicated the high cytotoxic properties of these five extracts compared to the reminded samples (See Figure 1).

Table 1. The list of the tested plant species names with common names, family names, tested parts and traditional uses in folk medicine.

Plant name[a]	Common name	Family	Part tested	Traditional uses[b]
Alchemilla smirnovii Juz.	lady's mantle	*Rosaceae*	aerial part	Purulent wounds, eyelid inflammations
Bunias orientalis L.	hill mustard	*Brassicaceae*	aerial part	Wounds, hypertension, infertility, lung oedema, diarrhoea, tumours, *etc*.
Crataegus pallasii Griseb	woodland hawthorn	*Rosaceae*	fruits; flowers with leaves	cardiovascular diseases, cancer, diabetes
Inula helenium L.	horse-heal	*Compositae*	flowers with leaves	Gastrointestinal inflammation, whooping cough, yellow fever
Gentiana cruciata L.	star gentian	*Gentianaceae*	aerial part	Mucosal inflammation, hepatitis, splenomegaly, tumours
Hypericum alpestre subsp. *polygonifolium* (Rupr.) Avet. & Takht.	hypericum	*Hypericaceae*	aerial part	Pneumonia, tumours, wounds, hepatitis, cholecystitis gastrointestinal inflammation, nephritis, skin diseases
Rubus canescens DC.	Woolly blackberry	*Rosaceae*	flowers with leaves	Tumours, anaemia, diarrhoea, wounds, diabetes, diuretic, hemorrhoids, burns, flu, immunotonic
Vaccinium· myrtillus L.	European blueberry	*Ericaceae*	fruits	diarrhea, inflammation of the mouth, urinary problems, diabetes, tumours, *etc.*
Carum carvi L.	caraway	*Apiaceae*	whole plant	Dyspepsia, indigestion, pneumonia, bloating, diarrhea, flatulent colic, *etc*.

[a] The plants names has been checked with http://www.theplantlist.org/, [b] Traditional uses in accordance with Armenian traditional medicinal handbooks [19,20].

Table 2. Yield and concentration of dissolved compounds in tested 50 mg DW/mL crude plant ethanol extracts.

Plant sample	Tested organs	Concentration of dissolved compounds (mg/mL)	Yield (%)
A. smirnovii	aerial part	15.33 ± 1.15	30.67 ± 2.31****
B. orientalis	aerial part	5.67 ± 0.58	11.33 ± 1.15
C. pallasii	fruits	15.33 ± 1.15	30.67 ± 2.31****
C. pallasii	flowers with leaves	9.33 ± 1.15	18.67 ± 2.31**
I. helenium	flowers with leaves	10.00 ± 0.00	20.00 ± 0.00***
G. cruciata	aerial part	11.33 ± 1.15	22.67 ± 2.31****
H. alpestre	aerial part	8.5 ± 1.15	17.33 ± 2.31*
R. canescens	flowers with leaves	10.67 ± 1.15	21.33 ± 2.31***
V. myrtillus	fruits	27.00 ± 1.00	54.00 ± 2.00****
C. carvi	whole plant	7.67 ± 0.58	15.33 ± 1.15ns

*The results represent the means of three independent experiments with SD. Tukey's multiple comparison test was used to compare the significance of yield differences within different plant extracts compared to *B. orientalis* extract (the plant extract with lowest yield). The number of asterisks refer to: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$, ns: not significant.

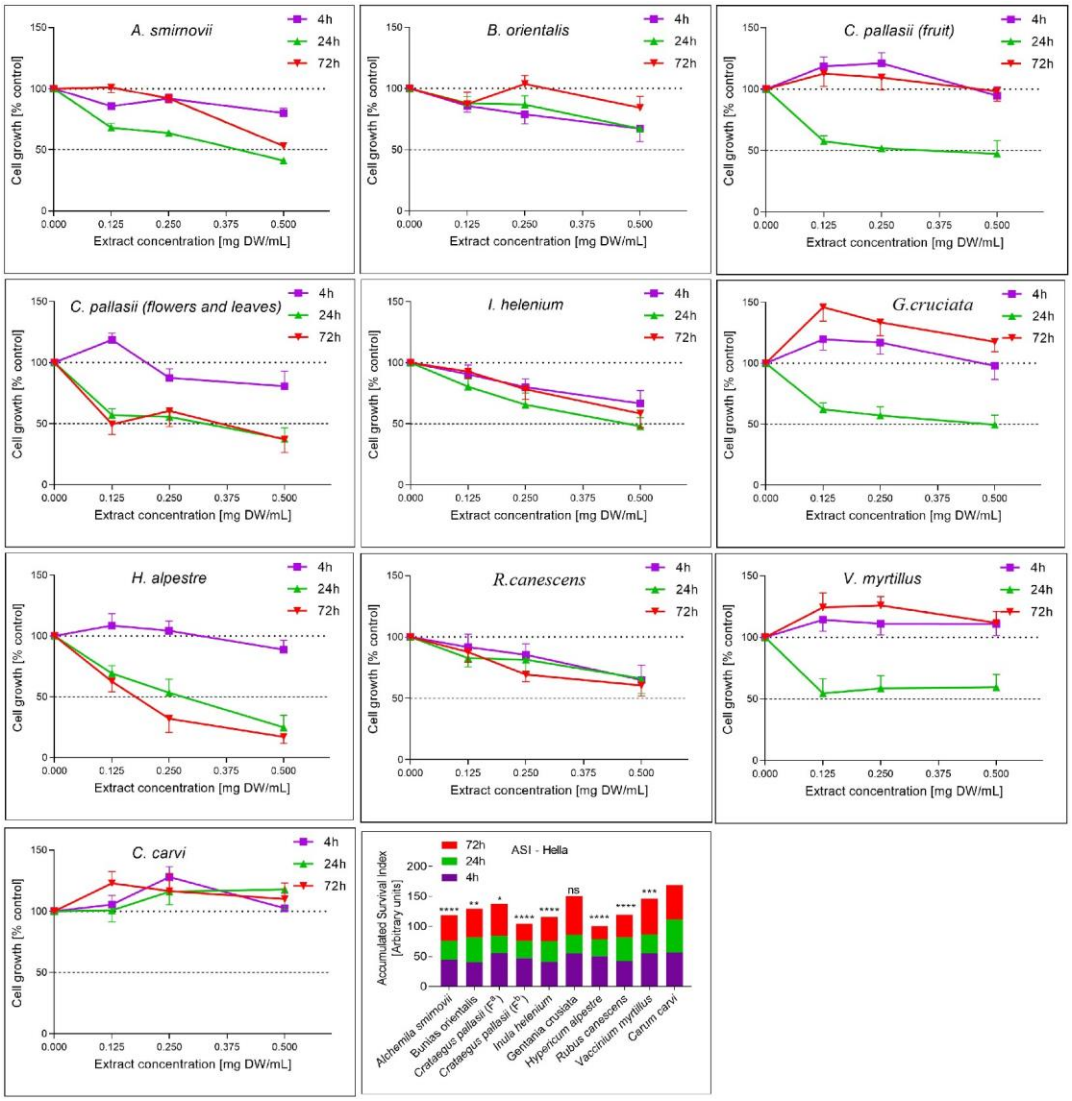

Figure 1. Growth inhibition of HeLa cells treated with test plant ethanol extracts for 4, 24 and 72 h (MTT test). Accumulated survival index (ASI) is defined as the sum of areas under survival curves determined for individual treatments for the same concentration range (0.125–0.5 mg DW/mL). Tukey's multiple comparison test was used to compare the significance of differences within different plant extracts compared to *C. carvi* extract ASI (the plant extract with lowest cytotoxic activity). The number of asterisks refer to: *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001, ns: not significant. [a]FL—flowers with leaves, [b]F—Fruits. The results represent the means of three independent experiments; SD values did not exceed 15%.

Further comparisons of the growth-inhibiting effectiveness of mentioned five samples based on higher cytotoxic effects at lower concentrations and short exposure times allowed the selection most promising three samples for more comprehensive analysis. These plant samples are *A. smirnovii*, *H. alpestre*, and *I. helenium*. The extracts from these plants or their relative species within the same genus are reported to possess considerable cytotoxic properties according to literature data as well. For instance, the strong cytotoxic activity of *H. alpestre* alcoholic extracts was revealed in our

previous research work [5]. During the evaluation of its neuroprotective properties on microglial BV-2 cells we revealed the strong cytotoxic properties of this plant extract. This led to further evaluation of this plant extract as a source of cytotoxic agents. There was a lack of other literature reports about the cytotoxic influence of *H. alpestre* extract. However many of the species from the *Hypericum* genus were known to possess high cytotoxicity on different cancer cell lines [22]. For instance, considerable cytotoxicity of *H. retusum* flower methanol extracts on HeLa and NRK-52E cell lines was shown [23]. Literature reports about cytotoxic properties of *I. helenium* extracts mainly refer to its root extracts [24,25]. For example, a highly selective cytotoxicity of *I. helenium* root extract on different tumor cell lines (HT29, MCF-7, Capan-2 and G1) was reported by Dorn et al. [26]. However, some data is also available about the cytotoxic properties of aerial parts of this plant as well. Aqueous extracts of *I. helenium* aerial parts were reported to exhibiting considerable cytotoxicity in a human U-87 MG glioblastoma cell line [27]. There was a lack of literature data about cytotoxic properties of *A. smirnovii*, however, high cytotoxic activity of the other species within a genus was reported (*A. mollis*, *A. vulgaris*, *etc.*) against different cancer cell lines, including breast, ovarian and cervical carcinoma [28–30].

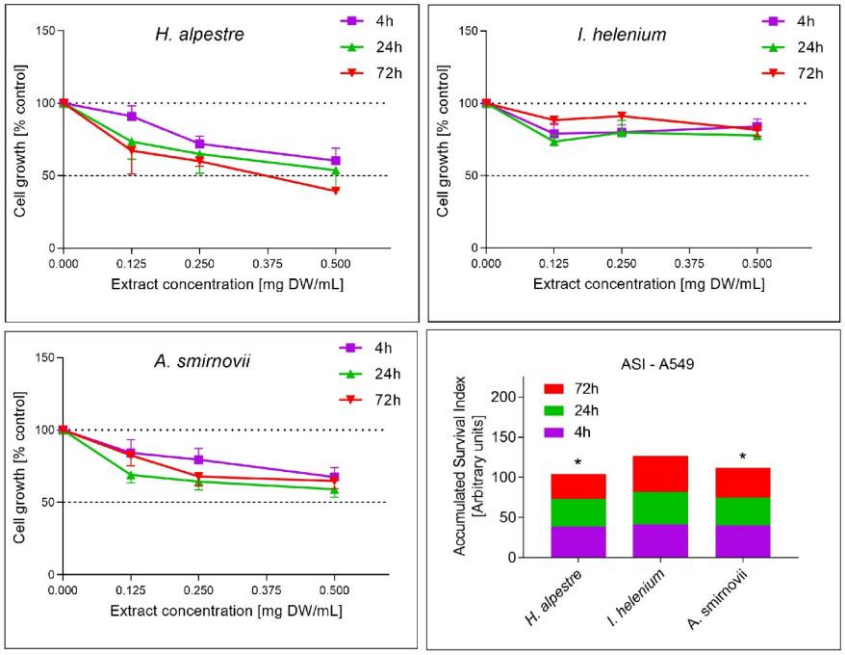

Figure 2. Growth inhibition of A549 (a) cells treated with selected test plant ethanol extracts for 4, 24 and 72 h (MTT test). Accumulated survival index (ASI) is defined as the sum of areas under survival curves determined for individual treatments for the same concentration range (0.125–0.5 mg DW/mL). Tukey's multiple comparison test was used to compare the significance of differences within different plant extracts compared to *I. helenium* extract ASI. The number of asterisks refer to: $*p < 0.05$. The results represent the means of three independent experiments; SD values did not exceed 15%.

Further, growth-inhibiting properties of selected three herbal extracts on human lung adenocarcinoma (A549) cells were explored. According to obtained data, only *A. smirnovii* and *H. alpestre* ethanol extracts showed strong cytotoxicity on A549 cells, whereas *I. helenium* extracted

showed only slight growth inhibiting properties (Figure 2). ASI parameters also clearly point out the higher cytotoxic properties of *A. smirnovii* and *H. alpestre* extracts compared to extract of *I. helenium* (See Figure 2). It was important to mention, that exposure time had no significant impact on the growth-inhibiting the effectiveness of extracts of *A. smirnovii* and *H. alpestre*. Moreover, the *A. smirnovii* and *H. alpestre* extracts exhibit strong cytotoxicity even at the lowest tested concentration (0.125 mg DW/mL).

Antioxidant/pro-oxidant properties of the plant extracts can play an important role in the consent of possible anticancer properties. Therefore, for the assessment and selection of the most potent plants with possible anticancer properties their antioxidant properties were explored through DPPH colorimetric method.

In particular, the DPPH stoichiometry values of the test extracts were determined, which describe the number of oxidant molecules reduced by one molecule of antioxidant after 10 min of reaction (n10) (Figure 3a,b). According to obtained data, *A. smirnovii* ethanol extract possessed the highest radical scavenging activity followed by *H. alpestre*. *I. helenium* possessed low radical-scavenging activity compared to other plant samples. In our previous research work metal chelating, hydrogen peroxide reducing and DPPH reducing the activity of methanol extract of *H. alpestre* was shown [31]. High DPPH scavenging activity of *A. smirnovii* was reported for the first time. Though literature data confirms high radical scavenging activity of species within the genus *Alchemilla*, such as; *Alchemilla vulgaris* [28], *Alchemilla. mollis* [29], *Alchemilla jumrukczalica* [32].

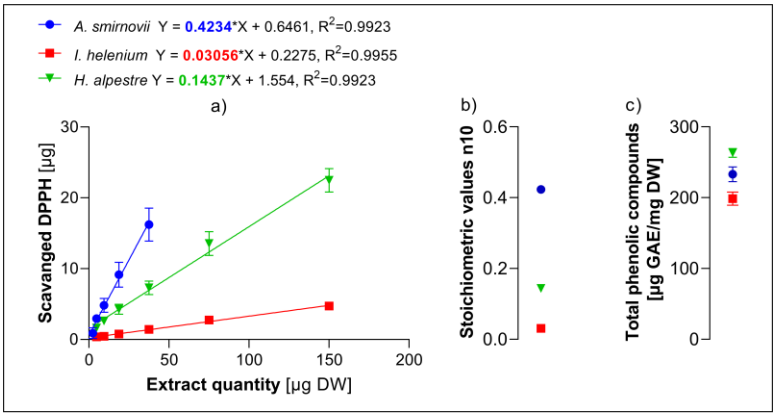

Figure 3. Antioxidant activity of selected plant ethanol extracts determined by spectrophotometric (DPPH) analyses and their total phenolic content. (a) The linear relationship between the quantity of reduced radicals (in µg) and quantity of plant crude extracts (in µg DW); (b) comparison of stoichiometric values n10 calculated based on DPPH method; (c) total phenolic content. The results are means ± SD from three independent determinations, $p < 0.05$.

Total content of phenolic compounds was determined in the selected plant extracts taking into account the importance of phenolics on biological activities of the extracts including antioxidant and cytotoxic properties [3,4]. The highest total phenolic content was present in the extract of *H. alpestre* followed by *A. smirnovii* and *I. helenium* (Figure 3c). Generally total phenolic content of the plant

extracts is in correlation with their radical scavenging activity [31,33], however, *A. smirnovii* extract exhibited higher scavenging activity compared to *H. alpestre* extract. This phenomenon can be due to the high content of non-phenolic antioxidant compounds in *A. smirnovii* extract.

4. Conclusions

Based on the screening of cytotoxicity of crude ethanol extracts of 10 herbal species on different cancer cell lines: two herbs with strong cytotoxicity were selected, which are *A. smirnovii* and *H. alpestre*. These herbs possessed high radical scavenging activity as well. Strong cytotoxic and antiradical activity of *A. smirnovii* was reported for the first time. We assumed that *A. smirnovii* and *H. alpestre* extracts could have promising anticancer potential and need further evaluation including elucidation of the metabolome, *in vivo* anticancer studies, etc. Although there is a lack of literature data about the cytotoxic properties of *I. helenium* areal part extracts, and it showed strong cytotoxic activity only on the HeLa cell line, we suggest that it can have great potential as a source of antitumor compounds and requires further investigation.

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Conflict of interest

The authors declare no conflict of interest.

Author contributions

All authors contributed to the study's conception and design. GS, MG, NA, HJ, SH, NZ and NS carried out the investigations and analyzed the outcomes. NZ identified plant samples. MG and NS wrote the manuscript. MG, NA, ZK and NS directed the experiments, corrected, and edited the manuscript. All authors revised and accepted the final version of the manuscript.

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