

Anticancer, Antiproliferative and Lactate Dehydrogenase Enzyme Activities of *Astragalus elongatus* subsp. *nucleiferus* on Human Cancer Cells

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ABSTRACT

In this study, potential anticancer and antiproliferative activities of ethanol and water extracts from aerial parts and roots of *Astragalus elongatus* subsp. *nucleiferus* were evaluated. MTT, trypan blue, and LDH enzyme activity assays were performed to determine cytotoxicity and cell proliferation potentials of the plant extracts against human A549, H1299, C6 cancer cells, and non-tumorous HUVECs. As results of MTT and trypan blue assays, dose-dependent anticancer and antiproliferative effects were observed on tested human lung and brain carcinoma cells. The water extracts obtained from the root exerted higher anticancer activity with IC₅₀ values ranging from 5.81±0.46 to 18.24±0.12µg/mL than the extracts of aerial parts. In contrary anticancer and cytotoxicity results, the ethanol extract of the root was also caused to the highest apoptosis level in a dose dependently. Regarding LDH activity, the plant extracts obtained from aerial parts and roots were demonstrated the highest LDH activity towards H1299 cells. The obtained results showed that the roots of the plant are able to inhibit cell growth in cancer cells in a time and concentration dependently.

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İnsan Kanseri Hücrelerinde *Astragalus elongatus* subsp. *nucleiferus*' un Antikanser, Antiproliferatif ve Laktat Dehidrojenaz Enzim Aktiviteleri

ÖZET

Bu araştırmada, *Astragalus elongatus* subsp. *nucleiferus*' un toprak üstü ve kök kısımlarından elde edilen etanol ve su özütlerinin potansiyel antikanser ve antiproliferatif aktivitelerinin araştırılması amaçlanmıştır. Bitki özütlerinin A549, H1299, C6 insan kanser hücreleri ve kanserli olmayan HUVEC hücrelerine karşı sitotoksikite ve hücre proliferasyon potansiyellerini araştırmak için; MTT, tripan mavisi ve LDH enzim aktivite analizleri yapılmıştır. MTT ve tripan mavisi deneylerinin bir sonucu olarak, test edilen insan akciğer ve beyin karsinoma hücrelerinde doza bağlı antikanser ve antiproliferatif etkiler gözlenmiştir. Bitkinin kökünden elde edilen su özütleri, 5.81±0.46 ile 18.24±0.12µg / mL arasında değişen IC₅₀ değerleriyle, toprak üstü özütlerinden daha yüksek bir antikanser aktivite göstermiştir. Antikanser ve sitotoksikite sonuçlarının aksine, kök etanol özütleri doza bağlı olarak en yüksek apoptoz seviyesine neden olmuştur. LDH aktivitesi ile ilgili olarak; toprak üstü kısımdan ve köklerden elde edilen bitki özütlerinin H1299 hücrelerine karşı en yüksek LDH aktivitesi göstermiştir. Elde edilen sonuçlar; bitki köklerinin, kanser hücrelerinde hücre büyümesini konsantrasyon ve zamana bağlı olarak önleyebildiğini göstermiştir.

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Anahtar Kelimeler

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Apoptoz,
Astragalus sp.,
Sitotoksikite,
Hücre proliferasyonu

INTRODUCTION

In recent years, there has been a widespread interest in herbal medicines due to their therapeutic and prevention potentials in cancer cases. Boosting the immune system is the most effective remedy strategy in most cancers (Newman and Cragg, 2016; Roleira et al., 2018; Gezici and Sekeroglu, 2019a). From this point of view, immune stimulating plants have critically importance for cancer patients. The genus *Astragalus* L., the largest in the Fabaceae family, are of important medicinal plants that known as immunostimulant, hepatoprotective, antiviral, anti-depressive, and anti-inflammatory agents (Block and Mead, 2003; Ionkova et al., 2014).

The plants of *Astragalus* genus are rich of bioactive secondary metabolites such as polysaccharides, saponins, and cycloartanes that can deeply affect to immune system (Shao et al., 2004; Yalçın et al., 2012; Ionkova et al., 2014). Their wide range of bioactive metabolites are responsible for their diverse pharmacological effects, leading us to investigate biological activities of wild grown *Astragalus* species in Turkey, particularly under mild-climate areas in sandy soils. In our previous study, aerial parts and root extracts of *A. elongatus* subsp. *nucleiferus* were analyzed for their potential neuroprotective effects and antioxidant capacities along with polyphenolic contents (Gezici et al., 2018). Taking our previous results, we aimed to screen potential anticancer and antiproliferative activities of extracts from aerial parts and roots of *A. elongatus* subsp. *nucleiferus* in the presented research.

Although there is scientific evidence about therapeutic properties of *Astragalus* species, there has been no report yet determining anticancer potentials of aerial parts and root extracts from *A. elongatus* subsp. *nucleiferus* against human lung carcinoma (A549), non-small lung cancer (H1299), and glioma (C6) cancer cells. According to literature survey, our findings in this paper ought to be the first report, which was carried out for investigation of *in vitro* anticancer, antiproliferative, and lactate dehydrogenase releasing activities of water and ethanol extracts obtained from aerial part and root of *A. elongatus* subsp. *nucleiferus*.

MATERIALS AND METHODS

Plant Material

The plant samples were collected during the months of April-May 2017 from Gaziantep, Turkey. Taxonomic identification of the plant was performed by a senior taxonomist Fatih Yayla, from Department of Biology, Gaziantep University (Gaziantep, Turkey). The voucher specimen was deposited at the Herbarium of Department of Biology, Gaziantep University, Turkey (GAUN1706). The plant materials were dried in shade and stored at 25°C in dark until extraction.

Preparation of Crude Extracts

The air-dried and powdered plant material (50 g) obtained from aerial part and root of *A. elongatus* subsp. *nucleiferus* were extracted with distilled water (dH₂O) and ethanol (EtOH) by maceration extraction procedure as described in previous study (Gezici and Sekeroglu, 2019b). Extract yields of the EtOH and dH₂O of the aerial parts and roots were 10.606%, 7.198%, 8.926%, and 5.312% (w/w), respectively.

Cell Lines and Culture Conditions

Human lung and human brain cancer cell lines: A549 (lung carcinoma), H1299 (non-small cell lung cancer), C6 (glioma) and non-tumorous HUVECs (human umbilical vein endothelial cells), purchased from the American Type Culture Collection (ATCC, USA) for analysis. A549 and H1299 lung cancer cells were cultured in Roswell Park Memorial Institute Medium (RPMI, ThermoFisher Scientific), and the other cell lines were grown in Dulbecco's modified Eagle medium (DMEM): Ham's F12 nutrient medium (1:1) (ThermoFisher Scientific) in the flasks at 37°C in a humidified CO₂ (5%) incubator. All the media and laboratory conditions were the same as given in our previous studies (Gezici et al., 2017; Gezici, 2018; Gezici, 2019a). All the experiments were repeated minimum of three times.

Anticancer Activity Determination by MTT Assay

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was used to determine cytotoxic properties of the various extracts according to the method of Mosmann (1983) with small changes. Briefly, densities of 5×10^3 cells were seeded in 200 µl medium into 96-well plates for 24 h, and *A. elongatus* subsp. *nucleiferus* extracts with different concentrations ranged from 10 to 200 µg/mL were added to wells, and then all the wells incubated at 37°C for 48 h. The medium was discarded and 50 µL/well of MTT solution (5 mg/mL) was added into each well after incubation. And then, the absorbance was measured at 570 nm with a Thermo Lab systems 408 Multiskan multiplate spectrophotometer, and the dose response curve was used to generate the IC₅₀ (µg/mL) values for each cell line.

Antiproliferative Activity Determination by Trypan Blue Assay

Extracts' antiproliferative activities were evaluated with Trypan Blue exclusion method described by Strober (2001) for A549, H1299, C6, and HUVEC cell lines. Chemicals and conditions used in the all analysis were the same as in our former publications (Gezici et al., 2017; Gezici, 2019b). Stock concentration as 1 mg/mL for all the *A. elongatus* subsp. *nucleiferus* extracts were prepared in DMSO and used at different

concentrations (10, 50, 100, and 200 µg/mL) from each extract, and they were incubated at 37°C for 24, 48, and 72 hours. The cell viability was determined by microscopically (Nikon, Japan), and the viable cells were counted by Cedex XS Analyzer, an automated cell counter.

Lactate Dehydrogenase (LDH) Release Activity Determination

Release of the Enzyme Lactate Dehydrogenase (LDH) Activity assays were achieved by detection of necrosis in the cells according to method of Al-Qubaisi et al. (2011). Chemicals and conditions used for all the analysis were the same as given our former papers (Gezici, 2018; Gezici, 2019a). LDH percentage release in medium was calculated by comparing to total LDH in the same well. In order to generate the IC₅₀ (µg/mL) values, dose response curve was used.

Statistics

The data obtained from analysis were expressed as mean value and standard deviation of the mean (mean±SD) from at least triplicate analyses. A linear regression analysis was performed to calculate IC₅₀ values. P<0.05 and p<0.01 values were considered as statistically significant, and very significant, respectively.

RESULTS AND DISCUSSION

Anticancer Activity Results

MTT assay was assessed to evaluate the cytotoxic activities of the *A. elongatus* subsp. *nucleiferus* extracts on three human cancer cell lines (A549, H1299, and C6), compared to cytotoxic activity of non-tumorous HUVECs. Anticancer activity results are given in Table 1 regarding of IC₅₀ values after 48h treatment period.

MTT assay results showed that the extracts of *A. elongatus* subsp. *nucleiferus* exhibited remarkable cytotoxicity towards the tested human lung and brain carcinoma cells even at lower dose and minimum exposure time. As summarized in the Table 1, the water extracts obtained from root was exerted higher anticancer activity on A549, H1299, and C6 calls, with the IC₅₀ values 6.14±0.02, 5.81±0.46, and 10.68±0.19µg/mL (at 200µg/mL concentration p<0.01), respectively, than the extracts obtained from the aerial parts of the plant. Furthermore, the ethanol extract obtained from aerial part of the plant was exerted lower cytotoxicity towards the cancer cells, compared to that of the water extract as dose and time dependently. In terms of the cancer cell types, all the extracts were found to be highly cytotoxic with IC₅₀ values in range of 5.81±0.46 – 10.75±1.16µg/mL on H1299 cells at the highest concentration for 48h, which

was followed by A549 and C6 cells, respectively (Table 1). Based on the findings on cytotoxicity, *A. elongatus* subsp. *nucleiferus* with aerial part and root demonstrated anticancer activity that against the cancer cells in a dose dependent manner, thus the plant could have significant potential in the prevention of cancer development.

Antiproliferative Activity Results

To screen antiproliferative activity of the ethanol and water extracts of *A. elongatus* subsp. *nucleiferus*, trypan blue assay was used, and the cells were counted according to their viabilities after treated with different doses (10, 50, 100 and 200 µg/mL) for 24, 48, 72h. Results of cell viability (%) percentage of A549, H1299, and C6 cancer cells by comparing with non-tumorous HUVECs were determined after 48h treatment at the highest concentration of the extracts (Figure 1).

In consistent with MTT assay, results of trypan blue assay demonstrated that cell growth and viability in the cancer cells were inhibited by the extracts from *A. elongatus* subsp. *nucleiferus*, in a time and dose dependent manner. As can be seen in the Figure 1, antiproliferative activity assay was also resulted in the superiority of the water extracts obtained from the root of the plant against H1299 lung carcinoma cells. On the other hand, water extracts of root showed significant reduction in cell growth of A549, H1299, and C6 cancer cells with survival percentage of 28%, 20%, and 35% against the cells, respectively. However, ethanol extract obtained from aerial part of the plant demonstrated lower reduction in cell growth with 46%, 40%, and 60% viability percentage to the A549, H1299, and C6 cancer cells, respectively, even at the lowest concentration (Fig 1.). The results revealed that *A. elongatus* subsp. *nucleiferus* extracts had quite inhibitory effects against to the cell growth in all the cancer cells.

According to the results we obtained from this research, the *A. elongatus* subsp. *nucleiferus* extracts inhibited the cell growth in lung carcinoma and brain glioma cancer cells in a dose-dependent manner. It was clear that increasing concentration of the extracts resulted in decreasing the number of live cells. Moreover, increasing exposure time was resulted in a significant inhibition of cell growth in the cancer cells. It is noteworthy that *A. elongatus* subsp. *nucleiferus* could have significant potential as an anticancer agent in the management of decreasing the amount of cancer cells. The anticancer and growth inhibitory effects of the extracts may be due to the fact that rich secondary metabolites components of the plant as described previously (Block and Mead, 2003; Shao et al., 2004; Ionkova et al., 2014; Ranjbar and Mahmoudian, 2015).

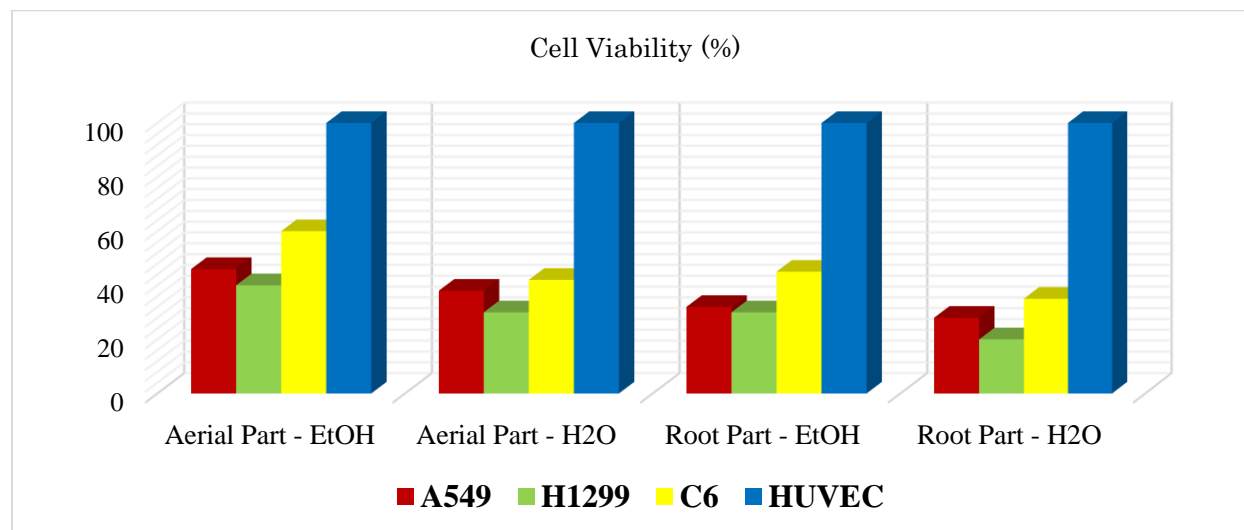


Figure 1. Cell viability (%) of A549, H1299 and C6 cancer cells

* Cells were treated with 200µg/mL concentration of the extracts for 48h.

** HUVEC cells were used as control and set as 100% cell viability

Table 1. Anticancer effects of *A. elongatus* subsp. *nucleiferus* extracts against A549, H1299 and C6 cells for 48h

Cell Lines	Plant Part-Extract Type	Concentrations of the Extracts (µg/mL) ^a				
		10µg/mL	50µg/mL	100µg/mL	200µg/mL	
A549	Aerial Part	Ethanol	58.67 ± 1.02**	46.36 ± 0.38**	43.85 ± 0.70*	13.07 ± 0.64**
		Water	51.45 ± 0.96*	40.91 ± 1.06*	32.03 ± 0.08**	10.08 ± 1.14**
	Root Part	Ethanol	46.09 ± 0.01**	36.04 ± 1.54**	24.79 ± 0.68**	8.15 ± 0.18*
		Water	48.01 ± 0.18**	30.96 ± 0.15**	22.25 ± 1.02*	6.14 ± 0.02**
H1299	Aerial Part	Ethanol	55.46 ± 1.14**	42.44 ± 1.60*	24.22 ± 1.09**	10.75 ± 1.16
		Water	47.16 ± 1.26	35.07 ± 0.05**	26.64 ± 0.93*	7.86 ± 0.14**
	Root Part	Ethanol	44.68 ± 0.16**	29.18 ± 0.98*	18.72 ± 0.42**	7.16 ± 1.01**
		Water	41.72 ± 0.49**	25.45 ± 0.02**	14.01 ± 0.08**	5.81 ± 0.46**
C6	Aerial Part	Ethanol	74.04 ± 0.61**	54.09 ± 0.74**	49.80 ± 0.17**	18.24 ± 0.12**
		Water	60.02 ± 0.08*	45.88 ± 0.68*	28.04 ± 0.96**	12.69 ± 0.46**
	Root Part	Ethanol	42.98 ± 0.14**	38.30 ± 0.47**	30.84 ± 0.46*	16.01 ± 1.15**
		Water	46.19 ± 0.82**	32.69 ± 0.03**	24.15 ± 0.45**	10.68 ± 0.19**
Doxorubicin ^b			7.48 ± 0.06	3.20 ± 1.01	2.78 ± 0.05	2.06 ± 0.12
DMSO (dimethyl sulfoxide) ^c			0	0	0	0

^a Values are expressed as IC₅₀ ± SD (n=3).

^b Doxorubicin is used as a positive control.

^c DMSO; dimethyl sulfoxide, negative control.

**P value of <0.01 and *P value of <0.05.

Lactate Dehydrogenase (LDH) Activity Results

In this assay, the release of LDH from the A549, H1299 and C6 human cancer cells was measured after treatment with *A. elongatus* subsp. *nucleiferus* extracts. The leakage of LDH was measured in regards of IC₅₀ values in a time dependent manner for 24, 48, and 72h treatment period. (Table 2). LDH release results of the extracts were presented in Table 2 for each cancer cells in a time dependently.

As presented in the Table 2, the IC₅₀ values for A549, H1299, and C6 cancer cells were calculated as comparing with non-tumor HUVECs and doxorubicin as positive control (IC₅₀= 2.48±0.02 – 3.02±0.46 µg/mL). The extracts obtained from aerial parts and roots of the

plant were demonstrated the highest LDH activity towards H1299 cells, which was followed by A549 cells, and the results were found to have in accordance with anticancer activity results.

In another word, a significant increasing was observed in the IC₅₀ value from LDH by depending the time for all the tested extracts, which was supported by IC₅₀ values obtained from MTT assay. Among the plant part, root part was found to have high LDH activity (IC₅₀ values ranging from 12.64±1.01 to 38.11±0.64 µg/mL, for 24h), whilst, aerial part was found to have moderate LDH activity towards the cancer cells with IC₅₀ values in range of 27.82±1.02 – 51.83±1.21 µg/mL, after 24h treatment (Table 2).

Table 2. LDH release results of *A. elongatus* subsp. *nucleiferus* extracts for A549, H1299, and C6 cells

Plant Part	Extract Type	Treatment Hours	IC ₅₀ (µg/mL) values ^a		
			A549	H1299	C6
Aerial Part	Ethanol	24 h	30.16 ± 0.18**	27.82 ± 1.02**	51.83 ± 1.21*
		48 h	49.85 ± 0.83*	35.06 ± 0.68**	76.05 ± 0.03**
		72 h	67.42 ± 0.12**	65.16 ± 0.62**	78.46 ± 1.01**
	Water	24 h	32.04 ± 1.03**	28.32 ± 0.41*	34.04 ± 1.06**
		48 h	35.02 ± 0.25**	41.24 ± 0.22**	45.24 ± 0.04**
		72 h	44.15 ± 0.08*	35.12 ± 1.40*	58.07 ± 1.05**
Root Part	Ethanol	24 h	29.16 ± 0.58**	21.78 ± 1.04*	38.11 ± 0.64**
		48 h	33.08 ± 1.06**	40.12 ± 0.16**	40.05 ± 1.02**
		72 h	42.03 ± 1.02**	48.20 ± 1.06**	54.10 ± 1.04**
	Water	24 h	21.38 ± 0.78**	12.64 ± 1.01**	29.06 ± 0.80*
		48 h	26.48 ± 0.12**	18.06 ± 0.09**	36.07 ± 1.64**
		72 h	35.10 ± 0.26**	24.06 ± 0.34**	42.01 ± 1.04**
Doxorubicin ^b			2.48 ± 0.02	2.54 ± 0.12	3.02 ± 0.46

^a The values are expressed as IC₅₀ ± SD (n=3) at 200µg/mL concentration.

^b Doxorubicin, positive control.

**P value of <0.01 and *P value of <0.05.

Releasing of LDH enzymes from the cells indicates damaging in the cells, since this enzyme releases from the necrotic cell membrane (Chan et. al., 2013). In view of this point, measuring the activity of LDH enzyme gives information about the percentage of dead and necrotic cells, as observed in this study

According to previous reports, there has been no study so far examining anticancer activity of *A. elongatus* subsp. *nucleiferus* extracts. On the other hand, this is the first evaluation of *in vitro* anticancer and antiproliferative activities of the extracts obtained aerial and root part of the plant by combining with cytotoxic activities of the cells and LDH enzyme activities that releases from the necrotic cells.

CONCLUSION

In current study, potential anticancer and antiproliferative activities of aerial part and root extracts obtained from *A. elongatus* subsp. *nucleiferus* were analyzed in detail by using various *in vitro* methods. Overall, the results revealed that the root extracts of the plant could have remarkable anticancer and antiproliferative activities through enhancement of apoptosis and necrosis, even at lower concentration and with a minimum exposure time. The data obtained from this studies could be considered as the first report for the literature, and the results should be validated by using further *in vitro* and *in vivo* techniques to assure of the molecular mechanisms underlying these wide ranges of anticancer effects of the plant.

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Conflict of interests

No conflict of interest.

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