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Evaluation of *In Vitro* Anticancer Effect of *Plantago major* L. and *Plantago lanceolata* L. Leaf Extracts from Sivas

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Abstract: Recently, there have been studies on the use of some synthetic and semi-synthetic compounds as pharmaceuticals instead of herbal medicines, although drugs produced from plant extracts, are widely used all over the world. In addition, it is known that the various parts of the plant, passed through processes directly simple as ethno pharmacological way, widely used for the treatment among the people. Although the interest is increasing every day, the scientific data about the biological effects and activities of plant-derived extracts are still insufficient. Among the various known therapeutic effects of *Plantago* plants, a few recent studies have shown that preparations of the crude extracts of *Plantago* leaves could prevent or regress the growth of some kind of tumours. In this study, showing the distribution in our country and known to be used for medicinal purposes in the treatment of various diseases, *Plantago major* and *Plantago lanceolata* extracts' the cytotoxic effects were investigated using human breast adenocarcinoma cell line (MCF-7).
Key words: Antitumoral activity, Plant extract, *Plantago lanceolata*, *Plantago major*

Sivas İli *Plantago major* L. (Sinirotu) ve *Plantago lanceolata* L. (Damarlıca) Bitki Yapraklarının *In Vitro* Antikanser Aktivitelerinin Değerlendirilmesi

Özet: Son zamanlarda bitkisel ilaçlar dışında bazı sentetik ve yarı sentetik bileşiklerin ilaç olarak kullanımları üzerine çalışmalar yapılsa da, bitki ekstraktlarından üretilen ilaçlar tüm dünyada yaygın olarak kullanılmaktadır. Bunlara ilaveten doğrudan bitkilerin çeşitli kısımlarının, basit işlemlerden geçirilerek etnofarmakolojik olarak halk arasında tedavi amacıyla yaygın olarak kullanıldıkları da bilinmektedir. Dünyadaki mevcut bitkisel çeşitlilik düşünülürse, bitkilerden elde edilen ekstrelerin çoğunun biyolojik etkileri ve etki mekanizmaları hakkındaki bilimsel veriler hala yetersiz olmakla birlikte, bu konuya olan ilgi her geçen gün artmaktadır. Plantago (sinirotu) cinsi bitkilerin çeşitli terapotik etkileri bilinmekte olup, bitki yapraklarından elde edilen ham extraktların bazı tümörler üzerinde etkili olduğu ortaya konulmuştur. Bu çalışmada ülkemizde yayılış gösteren ve halk arasında tıbbi amaçlı olarak çeşitli hastalıkların tedavisinde kullanıldığı bilinen *Plantago major* (Sinirotu) ve *Plantago lanceolata* (Damarlıca) bitkilerinin toprak üstü kısımlarından elde edilen özütlerin sitotoksik etkileri, meme kanseri hücre hattı (MCF-7) kullanılarak araştırılmıştır.
Anahtar kelimeler: Antikanser aktivite, Bitki ekstraktı, Sinirotu, Plantago.

INTRODUCTION

Cancer is the leading cause of death in the world. There is a increasing trend in the prefer of medicinal plant because of their medical effectiveness, low toxicity and the many natural anticancer agents derived from these plants (16). *Plantago* plants are old medicinal plant that has been known for centuries (2, 13, 20). They were described by the Greek physician Dioscorides in 'De materia med ica' in the first century. *P. major* was also described in the 12–13th century by the Islamic author Ibn El Beithar having adopted the knowledge from Greek medicine (4). *Plantago major* and *Plantago lanceolata* are perennial plant that belongs to the Plantaginaceae family. They can be about 15 cm high, but the size changes depending on the growth habitats. More recent ethnopharmacological studies show that *Plantago* plants are used in many parts of the world and in the treatment of a number of diseases by using as an anesthetic, antiviral, anti-inflammatory, astringent, anti-helmintic, analgesic, analeptic, antihistaminic, anti rheumatic, antitumor, anti-ulcer, diuretic, expectorant and hypotensive in traditional medicine (5, 9, 12). Also, some compounds isolated from *Plantago* spp. have been explained to excite an immunostimulating activity on human lymphocyte proliferation (3) and induce inhibitive effect on tumor and prevented tumor extension (16, 17). So, researchers have tested them for different types of biological activities. Most tests have been performed on crude extracts without examining the nature of the active compounds. In a screening of anticancer activity of stems and seeds of *P. major* had no activity in vivo against lymphocytic leukaemia in mice (1, 21). In another study, an aqueous extract was shown to have a prophylactic effect on mammary cancer in mice (11). But the detailed biological activity studies containing *Plantago* plants are not described. Also *P. major* is well known plant but *P. lanceolata* is not so popular even though they have the same morphological and structural similarity betwen two plants. Therefore, in this study antitumor effect of leaf extract of *P. major* and *P. lanceolata* collected from Sivas, on human breast adenocarcinoma carcinoma cell (MCF-7) was investigated and compared together.

MATERIAL AND METHODS

Plant materials were collected from Sivas-Turkey from following localities. 1. *Plantago lanceolata*: Cumhuriyet University Campus, 14.04.2014, *M. Tekin 1527*. 2. *Plantago major*: Eğribucak (Gerne) village vicinity, 14.04.2014, *M. Tekin 1528*. Identification of the plants were performed by Dr. Mehmet Tekin from Sivas Cumhuriyet University, Faculty of Pharmacy, Department of Pharmaceutical Botany. Voucher specimens were kept in the herbarium of Cumhuriyet University, Faculty of Science (CUFH).

Preparation of plant extracts

Plant materials were air dried at room temperature without sun light effect and ground in a mortar. Water were used as extraction solvents. A portion (100g) of dried each plant sample were extracted with deionied water (yield, 5.72 %w/w) in a soxhlet apparatus during 10 hours. The extracts were then filtered, aqueous extract was lyophilized. The freeze-dried extract was collected and stored at 4 °C until use. For bioassays, each residue was dissolved in sterile distilled water in order to obtain a final concentration of 1 mg/ml. Further dilutions were made in DMEM as 50, 100, 250, 500, 1000 µg/ml.

Cell culture and cell growing assays

Human breast adenocarcinoma (MCF-7) and normal human umbilical vein endothelial cells (HUVECs) cell lines were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). Dulbecco's modified Eagle's medium (DMEM), Fetal bovine serum and trypsin–EDTA were supplied from Gibco (Invitrogen). L-glutamine– penicillin–streptomycin solution was from Sigma–Aldrich. XTT (2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-*2H*-tetrazolium- 5-carboxanilide) cell proliferation kit was purchased from Biotium.

MCF-7 and HUVEC cells were cultured in a humidified atmosphere at 37 °C and 5% CO_2 in 25 cm^2 flasks using medium containing DMEM (High Glucose, 2mM L-glutamine and sodium pyruvate) with 10% fetal bovine serum and 100U/mL Penicillin- 100 μg/mL Streptomycin. Sterile phosphate buffer saline (PBS) solution and 0.25% trypsin-EDTA solution were employed to wash and remove from flask surface respectively. Thoma chamber was used to count the number of cells. After trypsinization with 1xtrypsin EDTA,

cells were seeded in 96 well plates (5x10³cells in 100 µl/well). After 24 h incubation of cells 100 µl aliquots of the plant extracts were added into each well.

Cell Proliferation Assay

Cytotoxic activities of the plant extracts were measured by XTT cell proliferation kit (Biotium) on MCF-7 and HUVEC cell lines. Cells were seeded in a medium with 50-1000 µg/ml concentration of the plant extracts. After 24 and 48 hours incubation, treated cells were washed with sterile PBS. XTT reagent incubated with the cells for 4 hours, the color change was measured by a microplate reader at 450- 500 nm, and the cell viability was defined.

Statistical Analysis

SPSS ver. 22.0; IBM Corparation, Armonk, New York, United States) and PAST3 (Paleontological statistics) programmes were used for evaluating the data. Univariate Variance Analyses (Anova; Robust Test: Brown-Forsythe), Pearson Correlation, Kolmogorov-Smirnov, Lilliefors regulation, Shapiro-Wilk and Levene testswere employed to evaluate differences in the mean values of measured activities. Probability values of $P<0.05$ were considered to be significant. Quantitative data were given as average ± std.(standart deviation) values in tables.

RESULTS

In this study, extracts from two *Plantago* species used in traditional medicine among them *P. major*, and *P. lanceolata* were evaluated for cytotoxic activity against the human breast adenocarcinoma (MCF-7) cell lines *in vitro*. *P. major* and *P. lanceolata* leaf extracts decreased MCF-7 cell proliferation but at the same time had some effect on normal HUVECs. We also compared the sensitivity of MCF-7 and HUVEC cells to plant extract (50, 100, 250, 500 and 1000 µg/mL) at 24 and 48 h. Both plant extract significantly inhibited MCF-7 and a little inhibition was on HUVEC cells proliferation in some dose- and time-dependent manner. Notably, the inhibitory effect of *P. major* extracts on MCF-7 cells was higher than on HUVEC cells (∗$P<$ 0.05,∗∗$P<$ 0.01; Table 1-5, Fig.1). We also investigated that the *P. lanceolata* extracts as effective as *P. major* on cytotoxic activity against cell line (Fig.1)

Figure. 1. The cell proliferation assay graphics of plant extract on MCF-7 and Huvec cell line

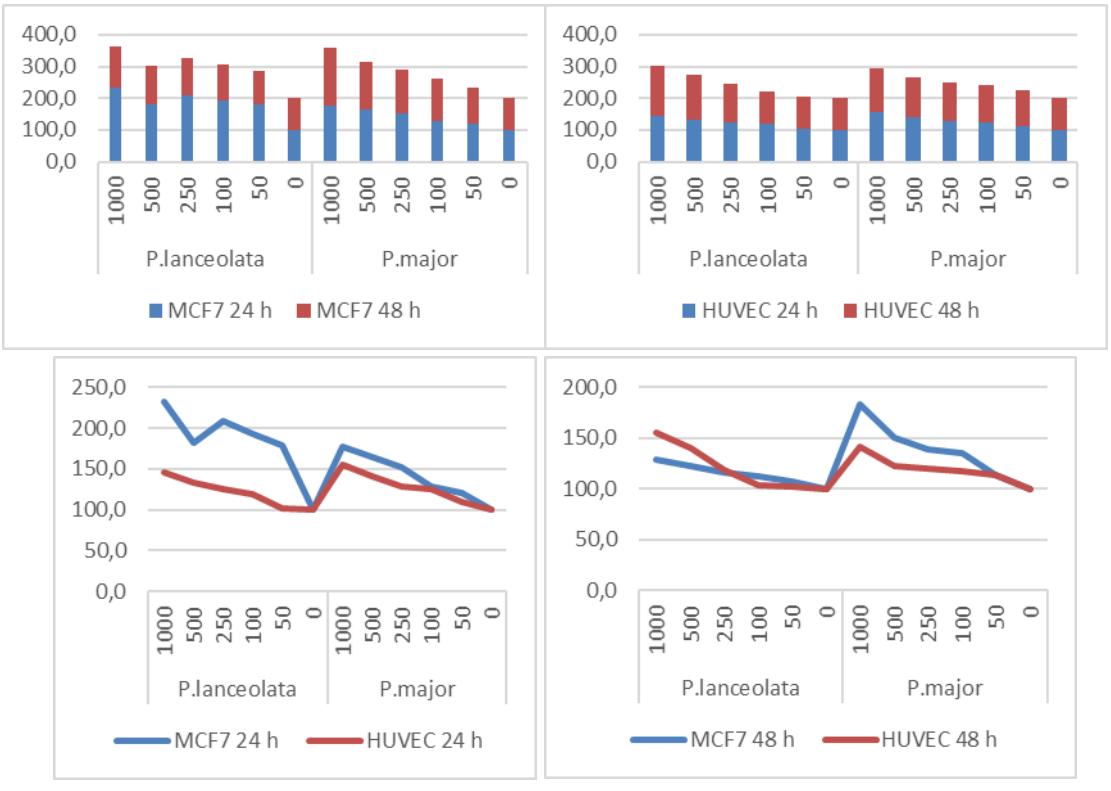


Table 1. The cell proliferation assay of plant extract on MCF-7 and Huvec cell line.

		MCF7		HUVEC		P Values			
Extract	**Doze (µg/ml)**	**24 h**	**48 h**	**24 h**	**48 h**	**MCF7 (24-48)**	**Huvec (24-48)**	**24 s (Huvec-MCF7)**	**48 s (Huvec-MCF7)**
P.lan-ceolata	1000	232,0±0,78	129,5±6,17	146,0±3,35	155,1±3,63	0,001	0,117	0,000	0,003
	500	182,4±55,08	122,0±10,64	132,9±10,23	140,8±2,29	0,254	0,233	0,258	0,040
	250	209,2±0,29	116,3±6,73	125,5±5,41	118,5±2,44	0,001	0,233	0,001	0,618
	100	192,5±5,50	112,1±8,04	118,4±1,30	103,5±1,43	0,001	0,001	0,001	0,141
	50	179,3±0,57	107,7±3,68	101,8±0,60	102,0±2,82	0,001	0,759	<0,001	0,100
	0	100,0±1,44	100,0±0,98	100,0±1,39	100,0±2,92	0,742	0,759	1	1
P.ma-jor	1000	177,0±0,56	183,5±12,85	155,1±13,37	141,0±1,35	0,39	0,267	0,047	0,005
	500	165,2±17,33	150,4±13,04	141,3±14,10	122,7±8,38	0,118	0,001	0,138	0,037
	250	152,0±2,48	139,0±1,22	129,3±9,75	120,3±0,42	0,001	0,276	0,017	<0,001
	100	129,0±2,99	134,6±2,72	124,6±9,05	117,6±0,94	0,255	0,275	0,475	0,001
	50	121,3±0,28	113,9±2,88	110,2±2,36	113,2±1,59	0,001	0,276	0,001	0,710
	0	100,0±1,08	100,0±3,56	100,0±3,97	100,0±3,80	0,749	0,798	1	1

Independent T test (Bootstrap) - Paired T Test (Bootstrap) All data were shown mean±standard deviation

Table 2. The cell proliferation results with dose response of plant extract on MCF-7 cell line in 24 **h.**

	MCF7 - 24 Hours	
Doze (mgr/ml)	*P.lanceolata*	*P.major*
1000 =VI	232,0±0,78	177,0±0,56
500 =V	182,4±55,08	165,2±17,33
250 =IV	209,2±0,29	152,0±2,48
100 =III	192,5±5,50	129,0±2,99
50 =II	179,3±0,57	121,3±0,28
0 =I	100,0±1,44	100,0±1,08
P Value	**<0,001**	**0,015**
I	0,000	0,000
I-V	0,381	0,078
I-IV	0,000	0,001
I-III	0,002	0,006
I-II	0,000	0,002

OneWay ANOVA (Brown-Forsythe) - (Method:Bootsrap) Post Hoc Test: Dunnett - Games Howell All data were shown mean±standard deviation

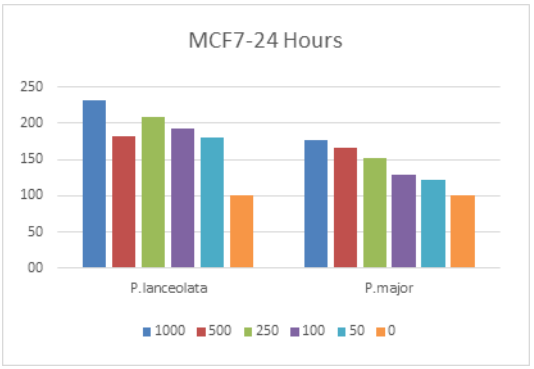


Table 3. The cell proliferation results with dose response of plant extract on MCF-7 cell line in 48 h.

	MCF7 - 48 Hours	
	P.lanceolata	*P.major*
Doze (mgr/ml)		
1000 =VI	129,5±6,17	183,5±12,85
500 =V	122,0±10,64	150,4±13,04
250 =IV	116,3±6,73	139,0±1,22
100 =III	112,1±8,04	134,6±2,72
50 =II	107,7±3,68	113,9±2,88
0 =I	100,0±0,98	100,0±3,56
P Value	**0,010**	**0,001**
I-VI	0,001	0,020
I-V	0,008	0,062
I-IV	0,047	0,005
I-III	0,166	0,002
I-II	0,520	0,038

OneWay ANOVA (Brown-Forsythe) - (Method:Bootsrap) Post Hoc Test: Dunnett - Games Howell All data were shown mean±standard deviation

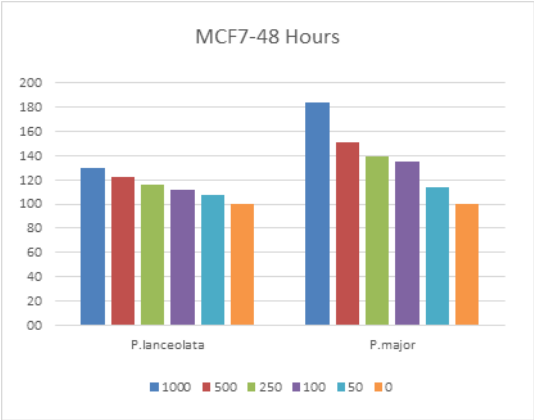


Table 4. The cell proliferation results with dose response of plant extract on HUVEC cell line in 24 h.

	HUVEC - 24 Hours	
Doze (mgr/ml)	*P.lanceolata*	*P.major*
1000 =VI	146,0±3,35	155,1±13,37
500 =V	132,9±10,23	141,3±14,10
250 =IV	125,5±5,41	129,3±9,75
100 =III	**118,4±1,30**	124,6±9,05
50 =II	101,8±0,60	110,2±2,36
0 =I	100,0±1,39	100,0±3,97
P Value	**0,002**	**0,002**
I-VI	0,002	0,000
I-V	0,102	0,001
I-IV	0,042	0,013
I-III	0,000	0,037
I-II	0,490	0,595

OneWay ANOVA (Brown-Forsythe) - (Method:Bootsrap) Post Hoc Test: Dunnett - Games Howell All data were shown mean±standard deviation

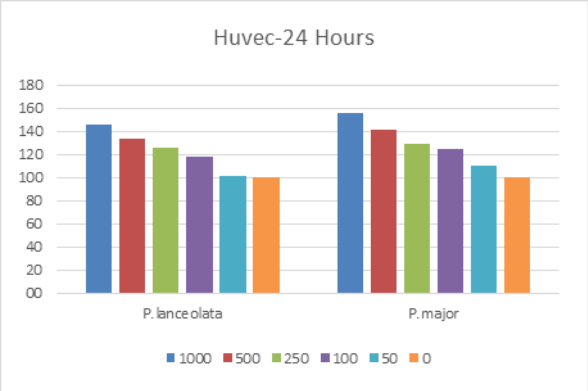
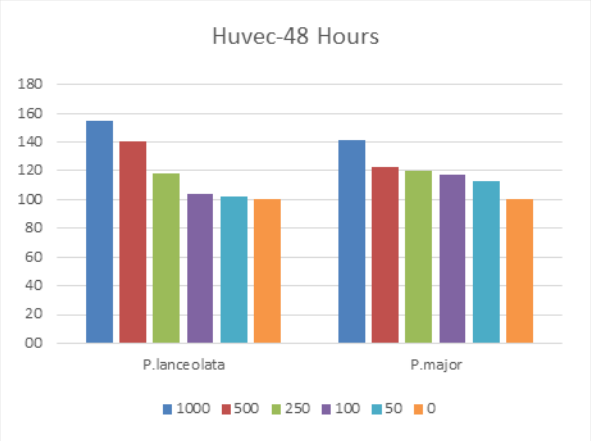


Table 5. The cell proliferation results with dose response of plant extract on HUVEC cell line in 48 h.

	HUVEC - 48 Hours	
Doze (mgr/ml)	Sıvı I	Sıvı II
1000 =VI	155,1±3,63	141,0±1,35
500 =V	140,8±2,29	122,7±8,38
250 =IV	118,5±2,44	120,3±0,42
100 =III	103,5±1,43	117,6±0,94
50 =II	102,0±2,82	113,2±1,59
0 =I	100,0±2,92	100,0±3,80
P Value	**<0,001**	**0,006**
I-VI	0,000	0,005
I-V	0,000	0,115
I-IV	0,000	0,039
I-III	0,400	0,044
I-II	0,815	0,066

OneWay ANOVA (Brown-Forsythe) - (Method:Bootsrap) Post Hoc Test: Dunnett - Games Howell All data were shown mean±standard deviation



DISCUSSION

This investigation shows that the *P. major* and *P. lanceolata* contained important biologically active compounds and different concentration of extracts showed antiproliferative activity. But the highest anticancer activity was found in concentrated extract of *P. major* leaves. The results of the cytotoxic effect from *P. major* and *P. lanceolata* leaves on MCF-7 and Huvec cells are summarized in Table 1 and Fig. 1. The differences of dose-dependent inhibition and time dependent inhibition of cell proliferation was observed for both plants in this study (Table 2-5). Finally it is clear that water extract of *P. major* leaves and *P. lanceolata* leaves had the effect on tumor cell growth. The differences between 24 h and 48 h incubation time of samples on MCF-7 cells were important statistically in both plants ($P < 0.05$; Table 1). But there was no important differences on Huvec cells between 24 and 48 h.

In past studies, it was observed that *P. major* leaf extracts activate nitric oxide and TNF-α production of macrophages-mediated lymphocyte proliferation (7). Conversely it was demonstrated that *Plantago* spp. extracts have shown growth inhibitory and cytotoxic effects on melanoma cell lines and breast adenocarcinoma (6, 10, 18, 19). Also it was shown that hot water extracts of *P. major* and *Plantago asiatica* possessed effects of immunomodulatory activity on human mononuclear cells proliferation (3). In one study, methanolic extracts from seven *Plantago* species used in traditional medicine among them *P. major*, were evaluated for cytotoxic activity against the human renaladenocarcinoma, the human breast adenocarcinoma and the human melanoma *in vitro* and *P. major* and the other six *Plantago* species showed cytotoxic activity on the breast adenocarcinoma and melanoma tumoral cell lines in a concentration-dependent manner at the recommended NCI (USA) doses. Another *in vitro* study was carried out on *Plantago* ethanolic, hot and cold water extracts of leaves and seeds separately. A dose dependent inhibition was observed for all tested extracts. The ethanolic extract of *P. major* leaves had the greatest effect on tumor cell growth follow by its hot water extract of the leaves (14, 15). Also methanolic extract of *P. major* had 80-100% cytotoxic effect (8). In our study, results support the studies above emphasizing the cytotoxic activity of Plantago plants on to MCF-7 cells. Also our results consistent with the previous studies according to the time dependent effects. In the other hand our results does not support the dose dependent inhibition, because there is important inhibition of cell proliferation in almost all concentration groups. Moreover there are many research containing *P. major* but there is not enough research about *P. lanceolata*, so our study is important for comparing the antiproliferative effect of these two plant species both. This study shows also the recent pharmacological studies based on *P. major* and *P. lanceolata* that support its traditional uses. The leaf extract is reliably nontoxic with strong hepato-protective and wound healing activities according to the literature, however data about the responsible constituents is little and further research is required and needs to be further investigated.

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