Caryologia
International Journal of Cytology, Cytosystematics and Cytogenetics

Firenze University Press
www.fupress.com/caryologia

Citation: A. Ždralović, A. Mesic, I. Eminović, A. Parić (2019) Cytotoxic and genotoxic activity of *Plantago major* L. extracts. *Caryologia* 72(3): 35-40. doi: 10.13128/caryologia-759

Published: December 13, 2019

Data Availability Statement: All relevant data are within the paper and its Supporting Information files.

Competing Interests: The Author(s) declare(s) no conflict of interest.

Cytotoxic and genotoxic activity of *Plantago major* L. extracts

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Abstract. *Plantago major* L. is a perennial, wild plant that belongs to the Plantaginaceae family, and is used as a good indicator in the assessment of destructive anthropogenic impact on the environment. The aim of the present study was to evaluate cyto/genotoxic effects of methanol extracts of *Plantago major*, collected from two locations (Tetovo and Smetovi), using *Allium cepa* test. We demonstrated that the highest concentration of *P. major* extracts from both locations reduced the mitotic index, while the lowest increased mitotic index value comparing to the positive control. As for genotoxic effects of extract from Tetovo, all concentrations increased the frequency of sticky chromosomes and chromosome missegregations in comparison with both controls, and frequency of multipolar anaphases when compared to the negative control. Higher number of cells with vagrants in comparison with positive control was detected after the treatment with 0.005 and 0.02 mg/ml concentrations. *P. major* extract from Smetovi (0.005 and 0.01 mg/ml) induced an increase in the number of vagrants as compared to the positive control, and frequency of sticky chromosomes when compared to both controls (0.01 mg/ml). Exposure to extract (0.005 and 0.02 mg/ml) caused increased number of multipolar anaphases in comparison with negative control. Apoptosis were not detected for *P. major* extract from Smetovi, while its highest concentration (0.02 mg/ml) induced increase in the frequency of necrosis as compared to the positive control. Our results demonstrated that methanol extracts of *P. major*, collected from Tetovo and Smetovi, showed cyto/genotoxic effects on *A. cepa* meristem cells.

Keywords. *Plantago major*, Cyto/genotoxicity, *Allium cepa* test, Heavy metals

INTRODUCTION

Plantago major L. is herbaceous, perennial wild plant from the family Plantaginaceae that is distributed throughout the world (Samuelsen 2000; Thome et al. 2012). Low growth and growth in the form of rosettes make this type well-adjustable on trampling, grazing and mowing (Thomet 1978). *P. major* contains biologically active compounds such as polysaccharides, lipids, phenols, flavonoids, iridoid glycosides, terpenoids (Samuelsen 2000; Chiang et al. 2003), benzoic compounds (Chiang et al. 2003), tannins, saponins and sterols (Jurišić Grubešić et al. 2005). Recent research shows that plants used in traditional medicine consumption exhibit mutagenic, geno-

toxic and cytotoxic effects *in vitro* and *in vivo* (Higashimoto et al. 1993; Schimmer et al. 1994; Kassie et al. 1996; Aşkin Çelik and Aslantürk 2007). Many chemical plant constituents have the ability to react with the DNA molecule, and may cause damage in DNA structure and/or disruption of biochemical reactions (Sofradžija et al. 1989). In order to reduce the risk of application of natural agents, plants and their parts, as well as plant extracts, it is necessary to assess their ability to induce cytotoxic, genotoxic and mutagenic effects (Askin Celik 2012). An indispensable aspect that must be noted in the modern use of plants for medicinal purposes is the increasing pollution of the environment by human activity. Plants actively participate in the circulation of nutrients and gases such as carbon dioxide, oxygen and also provide a big surface area for absorption and accumulation of air pollutants reducing the level of pollutants in the environment (Escobedo et al. 2008). Numerous studies have shown that *P. major* L. is good indicator of the degree of destructive anthropogenic impact on the environment (Montacchini and Siniscalco 1979). Furthermore, it has been demonstrated that methanol extracts of *P. major* demonstrated cytotoxic activity in different cancer cell lines (Kartini et al. 2017). *Allium cepa* assay has been described as an efficient test used for genotoxicity assessment of potential genotoxic agents in the samples taken from the environment, due to its sensitivity and good correlation with mammalian test systems *in vitro* (Firbas and Amon 2014; Prajitha and Thoppil 2016). Therefore, the aim of this research was to examine the cyto/genotoxic effects of methanol extracts of *P. major* sampled from Tetovo (polluted location) and Smetovi (control location) using *Allium cepa* test.

MATERIALS AND METHODS

Plant material

Plantago major was collected on two locations: Tetovo, which is near ironworks ArcelorMittal (exposed to daily air pollution, dust, sulfur dioxide and other pollutants) and Smetovi, located at 1.025 meters above sea level and is a popular resort and hiking destination. Plant samples were collected in October 2015. For testing were used leaves of the plant, which were carried out on the same day at both sites in PVC packaging bags and within 3 hours of collection delivered in the Laboratory of Plant Physiology, Department of Biology on Faculty of Science, University of Sarajevo. Plant material was dried at room temperature, away from direct light and stored at + 4°C until analysis. Voucher specimens (No. LRPER 383-384) were deposited in the Laboratory for research and protection of the endemic gene pool.

Extraction procedure

To prepare the plant extracts we used dry plant material and 80% methanol as solvent. 1 g of plant material was chopped and mixed with 40 ml of methanol. Incubation period was 24 h at 4°C. After filtration, supernatant was evaporated to a dry residue which was re-dissolved in 80% methanol in three concentrations: 0.02; 0.01; 0.005 mg/ml. Along three concentrations of extracts (from each location), we tested two controls: positive (80% methanol) and negative (distilled H_2O).

Allium test

For detection of cyto/genotoxic effects of *P. major* L. extracts, *Allium cepa* bulbs were used. Onion bulbs were grown in the glass vessels filled with tap water and left for germination for 48 h at room temperature with water injection as needed. We selected four bulbs for each treatment and measured their root length as previously described by Fiskesjo (1993). In this sense, the length of the root bundles from each onion bulbs was measured. The measure is taken from the point where the roots sprout, down to where the most root tips end their growth. Afterward, the bulbs were treated with *P. major* L. extracts for 24h at room temperature. At the end of the exposure period the root lengths of the bulbs were measured. For each treatment the bulb root is removed and placed into the appropriate labeled tubes containing ethanol/glacial acetic acid (3:1, v/v) fixative and kept for 24h at 4°C.

Cytogenetic analysis

A. cepa roots were hydrolyzed in 1M HCl solution for 15 minutes at room temperature. After that, the roots were transferred to distilled water. The apical 2 mm of the root were cut and placed in one drop of 2% acetorcein and squashed. Microscope slides were analyzed under the light microscope with a magnification of 400x. All photographs were made by use of Sony Cyber shoot ISO 3200 camera. To calculate the mitotic index values, 1000 cells per slide was analyzed. Chromosomal aberrations were analyzed on 100 cells in division per treatment. Counting 1000 interphase cells for each concentration, the frequency of micronuclei was determined. Apoptosis and necrosis were analyzed at 1000 interphase cells per slide.

Table 1 Roots length of *Allium cepa* (mean ± SD) before and after the treatment with different concentrations of the tested *P. major* extracts.

Concentration (mg/ml)	Tetovo		Smetovi		Methanol		H_2O	
	Roots length[a]	Roots length[b]	Roots length[a]	Roots length[b]	Roots length[c]	Roots length[d]	Roots length[c]	Roots length[d]
0,005	1.90±0.52	1.87±0.47	1.52±0.15	1.62±0.12*				
0,01	2,02±0.17	2,02±0.17	1.87±0.68	1.92±0.65	1,56±0,56	1,56±0,56	1.80±0.29	2,50±0.33
0,02	1.72±0.25	1.67±0.18*	1.70±0.73	1.70±0.74				

*Statistically significant difference compared to the negative control ($P < 0.01$).
Values are expressed in centimeters.
SD: Standard deviation.
[a] Root length in the first 48 h before treatment with different concentrations of the extracts.
[b] Root length in the next 24 h after treatment with different concentrations of the extracts.
[c] Root length of the control group in the first 48 h.
[d] Root length of the control group in the following 24 h.

Statistical analysis

To evaluate differences between tested concentrations and controls, for all analyzed parameters, Student's t-test was used. All statistical analyses were conducted by use of Microsoft Excel 2007 (Microsoft Corporation) and SPSS 20.0 software (SPSS, Chicago, IL). P values less than 0.05 were considered statistically significant.

RESULTS

The most important macroscopic parameter in *Allium* test is a root length (Fiskesjö 1985). Different concentrations of *P. major* extracts had different effects on root growth (Table 1). The extracts of *P. major* from polluted location inhibited root growth. Statistically significant effect had the highest concentration (0.02 mg/ml) of extract from mentioned location when compared to the control plants. Concentration of the extract of *P. major* (0.005 mg/ml) significantly stimulated root growth as compared to the negative control.

Table 2 The mitotic index of *Allium cepa* meristematic cells (mean ± SD) exposed to various concentrations of the samples

Concentration (mg/ml)	Tetovo	Smetovi	Methanol	H_2O
0,005	2,10±0.69	4.57±0.49**		
0,01	1.27±0.63	2.92±0.94	2.05±0.51	2.65±2.14
0,02	1.65±1.02	0.95±0.65*		

**Statistically significant difference compared to the positive control (methanol) ($P < 0.001$).
Values are expressed as a percentage.
SD: Standard deviation. MI: Mitotic index.
*Statistically significant difference compared to the positive control (methanol) ($P < 0.05$).

In order to evaluate the effect of *P. major* extracts, mitotic activity of *A. cepa* meristem cells was expressed as a percentage of cells in division in relation to the total number of analyzed cells. Statistically significant difference compared to control, was observed in the treatment with *P. major* extract from Smetovi (0.02 mg/ml, and 0,005 mg/ml) (Table 2). The highest concentrations of *P. major* extracts (0.02; 0.01 mg/ml) from Tetovo lead to the reduction in mitotic activity compared to the control, but the difference was not statistically significant.

The results of the genotoxic and cytotoxic effects of different concentrations of the *Plantago major* extract from two locations are presented in Table 3. Several genotoxic effects were observed, such as micronuclei, sticky chromosomes, anaphase bridges, vagrant chromosomes, chromosome missegregation, multipolar anaphases, as well as apoptotic and necrotic cells as cytotoxicity endpoints.

P. major extract from Tetovo (0.005 mg/ml) caused the highest number of chromosome aberrations. Similar effects were observed for extract from same location in concentration of 0.02 mg/ml. In this sense, micronuclei (Fig. 1a), multipolar anaphase (Fig. 1b), sticky chromosomes (Fig. 1c), anaphase bridges (Fig. 1d), vagrant chromosomes (Fig. 1e), chromosome missegregation, were observed. The highest number of apoptotic and necrotic cells (Fig. 1f) is observed after the treatment with the highest concentration (0.02 mg/ml) from this location, while apoptotic cells were not detected at the lowest concentration.

Unlike Tetovo, the frequency of chromosomal aberrations on meristem cells that were treated with extract of *P. major* from Smetovi (non-polluted place), was much lower. Chromosome aberrations that have reached statistical significance are: sticky chromosomes, vagrant chromosomes and multipolar anaphases. On

Table 3 The results of the genotoxicity and cytotoxicity in *Allium cepa* (mean ± SD) exposed to various concentrations of the *P. major* extract.

Tested extracts (mg/ml)	Micronuclei	Sticky chromosomes	Abnormal ana/telophases				Apoptotic cells	Necrotic cells
			Bridges	Vagrant	Chromosome missegregation	Multipolarity		
Tetovo								
0,005	0.75±0.95	5±3.16 ^{**2}	2±1.41^{*1}	2.5±1.29**	4±1.41^{**2}	9.25±2.87^{3}	n.o.	2±1.82
0,01	n.o.	4.50±3^{*1}	0.25±0.50	2.75±2.98	3±1.15^{**2}	7.75±3.50^{2}	0.50±1	1.75±1.25
0,02	0.50±0.57	2±1.15^{**2}	1.25±1.25	1.50±1.29*	2.50±1^{**2}	7.50±2.51^{3}	0.50±0.57	7±2.16
Smetovi								
0,005	n.o.	0.50±0.57	0.75±0.95	2.50±1.29*	0.25±0.50	5.25±1.50^{3}	n.o.	1.75±2.36
0,01	n.o.	2.75±2.21^{*1}	1±1.41	2.50±1.91*	1.50±0.57	5.50±0.57	n.o	1±2
0,02	0.25±0.50	0.75±0.95	0.50±0.57	3±3.82	1±0.57	7.75±6.02^{1}	n.o.	0.25±0.50*
Methanol	n.o.	n.o.	n.o.	n.o.	0.25±0.5	4.25±3.30	n.o.	4.50±3.10
H_2O	n.o.	n.o.	n.o.	1±0.81	0.25±0.5	n.o.	n.o.	5.25±4.57

Statistically significant difference compared to the negative control (H_20): ^{1} P < 0.05, ^{2} P < 0.01; ^{3} P < 0.001.
SD: Standard deviation.
Statistically significant difference compared to the positive control (methanol) after the T-test: * P < 0.05; ** P < 0.01.

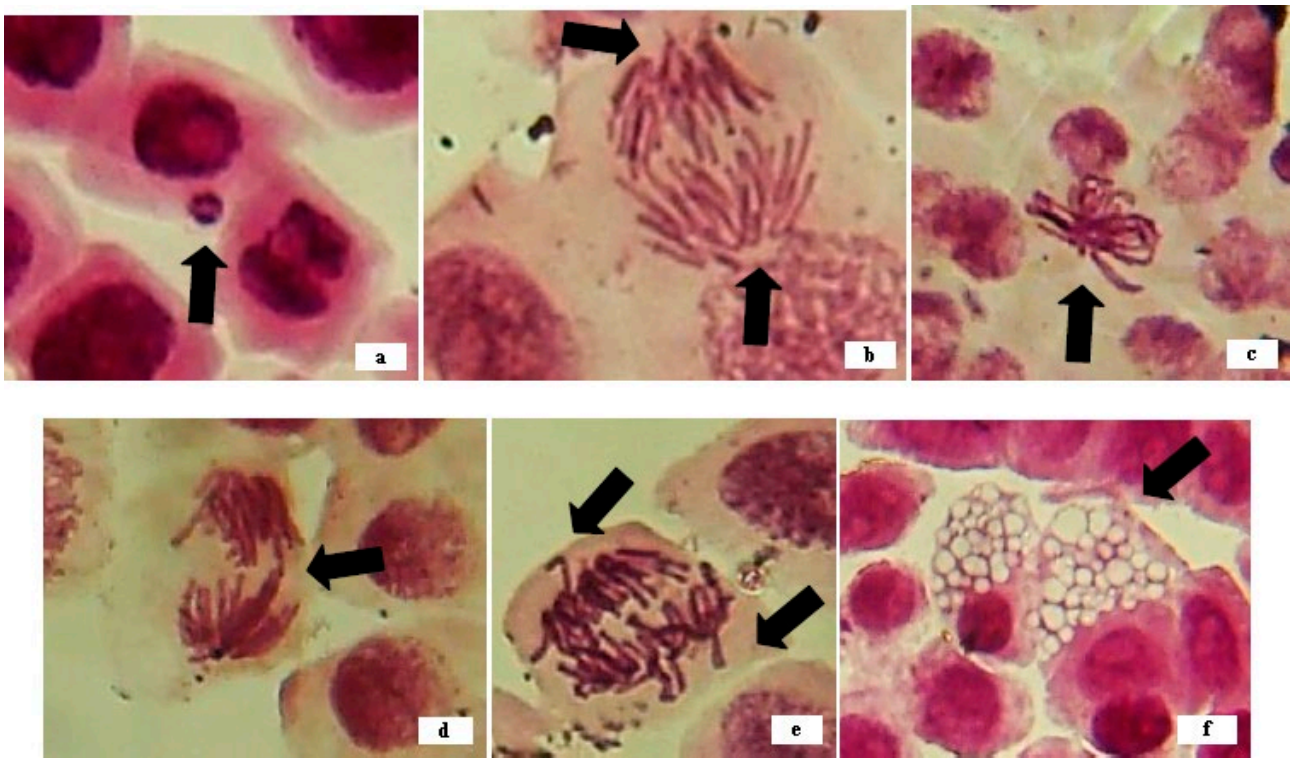

Fig. 1 Genotoxic effects of *Allium cepa* meristem cells treated with *P. major* extracts from Tetovo: a- micronucleus, b- multipolar anaphase, c- sticky chromosomes, d- anaphase bridge, e- vagrant chromosomes, f- necrotic cells.

the meristem cells treated with *P. major* extract from this location, apoptotic changes were not observed, while necrotic cells were few, but the numbers were statistically significant only for 0,02 mg/ml concentration (because of low number of necrotic cells compared to the positive control).

DISCUSSION

Plant species are an excellent source of biologically active substances, whose effects on genetic material are largely unknown (Barnes 2003). Toxicity is easy to see in inhibition of root growth, while mutagenicity correlates with chromosomal aberrations (Fiskesjö 1985). Inhibition of root growth is always parallel with the decline in cell division (Fiskesjö 1997), and can be caused by heavy metals in the plant extract. It has been found that the toxicity of extracts from plants which contain heavy metals, such as manganese, cadmium and lead is often associated with these pollutants (Boroffice 1990; Fiskesjö 1997). Recent study from locations Tetovo and Smetovi demonstrated that *P. major* is exposed to the negative impact of heavy metals, particularly in the area of Tetovo (Muratovic 2016). Heavy metals imply inhibition of *A. cepa* root growth. Early researches of plant tolerance to the heavy metals have shown that root growth is particularly sensitive to the presence of metal toxins. As a result of root growth cytokinesis, cell differentiation and extensions, metal induced inhibition of root growth is a result of toxic influences, acting on any of the three processes (Baker and Walker 1989). Rajeshwari et al. (2015) proved that aluminum nanoparticles increased the number of chromosomal aberrations in the *A. cepa* root tip cells and similar results were observed for other heavy metals such as Cu, Pb, Fe, Cd, Ni, Zn etc. (Olorunfemia et al. 2015). Therefore, our results suggest that inhibited growth of roots that were treated with *P. major* extract from polluted area of Tetovo could be due to presence of the heavy metals in plants.

The cytotoxicity of some chemical component, or plant extracts can be determined based on the increase or decrease in the mitotic index (Smaka-Kincl et al. 1996). Reduction of mitotic activity can arise as a result of inhibiting the synthesis of DNA molecules in cells or by stopping the G2 phase of the cell cycle through the action of various toxic substances present in plant extracts (Sudhakar et al. 2001). In this regard, it is important to accentuate that reduction in mitotic activity is parallel with the root growth inhibition of *A. cepa* meristem cells, which were observed after exposure to *P. major* extracts (0.02; 0.01 mg/ml) from Tetovo.

Similar results were observed by Askin Çelik & Aslantürk (2006) with reduction of mitotic index induced by *Plantago lanceolata* L. extracts, which indicates that the substance in the aqueous extract can have a cytotoxic effect. It is proven that extract of *P. major* reduce cell proliferation *in vitro* (Samuelsson 2004). Extracts of *Plantago* species have a cytotoxic effect on different tumor cell lines (Richardson 2001) due to the presence of luteolin 7-O-β-glucoside, as the main flavonoid present in most *Plantago* species (Galvez et al. 2003).

Comparing the genotoxic effects of these two locations, we can see that the total number of chromosomal aberrations was higher in cells treated with extract of *P. major* from Tetovo, which was expected because of air pollutants source. Smetovi is well known as an excursion site, and perceived aberrations (vagrant and sticky chromosomes) on this location could be explained by gasses from motor vehicles and the presence of waste material.

The toxicity of metals in the plant can be manifested with few biological markers that can be detected and analyzed at different levels of the organization, morphology of the plant as well as at the biochemical and molecular level. Therefore, they are very useful for plant monitoring and assessment of the environmental pollution (Olorunfemia et al. 2015). Among various biological effects which could be consequence of environmental pollution, genotoxicology is one aspect that is related to DNA damage and genome. According to this, our results are of great value in terms of use of *Plantago major* as an indicator of environmental pollution with heavy metals and other toxic substances.

In conclusion, the results of the present study revealed that *P. major* extracts from polluted location Tetovo reduced root growth and mitotic activity of *A. cepa* meristem cells, and that possess significant cyto/genotoxic potential. Observed chromosomal aberrations indicate that *P. major* extracts exhibit clastogenic properties with ability for mitotic spindle disruption, implying that *P. major* could be very useful in monitoring of the environmental pollution. Further toxicological studies on animal models are needed to strengthen these findings.

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