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Anti-mitotic and anti-genotoxic effects of *Plantago lanceolata* aqueous extract on *Allium cepa* root tip meristem cells

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Abstract: *Plantago* is the most important genus of Plantaginaceae family and is used in traditional medicine around the world for different purposes. *Plantago coronopus* L., *Plantago major* L., *Plantago media* L. and *Plantago lanceolata* L. are most commonly used species of *Plantago* in traditional medicine in Turkey. The main goal of this study was to investigate the eventual anti-mitotic and anti-genotoxic effects of *P. lanceolata* L. leaf aqueous extracts (15 g/L and 30 g/L) on *Allium cepa* L. root tip meristem cells which were treated with 0.7% hydrogen peroxide. For this purpose, two different experiments were performed under the same conditions. In the first experiment, *Allium cepa* onion bulbs were treated with 0.7% H_2O_2 for 1 h. After the H_2O_2 treatment, the onion bulbs were treated with two different concentrations (15 g/L and 30 g/L) of *P. lanceolata* extracts for 24 h. In the second experiment, *A. cepa* onion bulbs were treated with two different extract concentrations (15 g/L and 30 g/L) for 24 h and then with 0.7% H_2O_2 for 1 h. The test concentrations were determined according to doses which are recommended in alternative medicinal usage by people. As positive and negative control 0.7% H_2O_2 and tap water was used, respectively. As a result, it was determined that aqueous extracts reduced mitotic index and chromosome aberrations in treatment groups in comparison with controls. These results showed that *P. lanceolata* aqueous extracts have anti-mitotic and anti-genotoxic effects.

Key words: *Plantago lanceolata*; mitotic index; chromosome aberration; hydrogen peroxide; *Allium cepa*.

Introduction

Plants have been utilized as medicines for thousands of years (Ruffa et al., 2002). More recently a WHO study has shown that about 80% of the world's population still relies on traditional medicine (Cordell, 1995). These medicines take the form of crude drugs, such as tinctures, teas, poultices, powders and other herbal formulations (Balick & Cox, 1997; Ruffa et al.,2002).

A growing number of scientific concerns are focusing on the significance of natural compounds that can act as protectors against diseases (Aydin et al., 2004). Several therapeutic properties of medicinal plants are known in obstetrics and gynecology (Abo et al., 2000), respiratory disorders (Neto et al., 2002), skin disorders (Graf, 2000), cardiac diseases (Ankli et al., 2002), and mental health (Ahmad et al., 1998). In addition, several medicinal plants can be used as anti-tumor agents (Estevez et al., 1976; Mukherjee et al., 2001).

Plantago is the most important genus of Plantaginaceae family and is used in traditional medicine around the world for different purposes. *Plantago coronopus* L., *Plantago major* L., *Plantago media* L. and *Plantago lanceolata* L. are most commonly used species of *Plantago* in traditional medicine in Turkey (Baytop, 1999).

Plantago lanceolata is used internally to suppress coughs associated bronchitis and upper respiratory inflammation, to reduce skin inflammation, treatment of wounds and as a laxative (Baytop, 1999).

A recent review of plants used ethnomedically against cancer has presented the results of a query of the NAPRALET database and has reported the use of *Plantago asiatica* on Easter Island for internal cancers, *Plantago hirtella* in Mexico against cancerous diseases and *Plantago paralias* in Argentina as an anti-tumor agent (Graham et al., 2000). Furthermore, a review of traditional uses of *P. major* shows that it is used in Canary Islands, Chile, Venezuela and Panama in the treatment of tumors (Samuelsen, 2000).

Flavonoids constitute one of the most characteristic classes of compounds in *Plantago*. Kawashty et al. (1994) studied the flavonoid profile of 18 species of *Plantago* and they indicated that luteolin-7-7-*O*-β-glucoside was the major component in most *Plantago* species.

In this study, we investigated anti-mitotic and anti-

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Table 1. Average root numbers and root lengths in controls and treatment concentrations after 72 h.[a]

Concentrations	Average root number ± SD	Average root lengths (cm) ± SD
C(−)	33.8 ± 5.9	3.55 ± 0.87
C(+)	27.3 ± 9.9	4.39 ± 3.41
ME_1	28.9 ± 7.2	1.99 ± 0.37
ME_2	30.3 ± 10.4	1.75 ± 0.48*
E_1M	27.4 ± 8.2	1.09 ± 0.38*
E_2M	26.6 ± 8.4	1.07 ± 0.20*

[a]C(−): negative control; C(+): positive control (0.7% H_2O_2); M: 0.7% H_2O_2; E_1: *Plantago lanceolata* extract (15 g/L); E_2: *Plantago lanceolata* extract (30 g/L); * $p < 0.05$ in Two Way ANOVA.

genotoxic effects of *P. lanceolata* L. leaf aqueous extract on *Allium cepa* L. root tip meristem cells which were treated with hydrogen peroxide. Hydrogen peroxide is itself an oxidizing agent that induces oxidative stress (Torbergsen & Collins, 2000) and DNA breaks (Woods et al., 1999). Since the results of studies using *A. cepa* fit in well in a test battery composed of prokaryotes and/or other eukaryotes (Fiskesjö, 1993) and it is very cheap and easy to apply, we used *A. cepa* as a test material.

Material and methods

Plant collection and extraction
Plant samples were collected from vicinities of Uluköy-Afyon (Turkey) in July-August 2005. Dried and powdered leaves of *Plantago lanceolata* L. (15 g and 30 g) were extracted with 1 L boiling water for 10 min. Extracts were filtered using filter paper and freshly prepared extracts were applied daily.

Allium test
To evaluate the anti-mitotic and anti-genotoxic effects of *P. lanceolata* aqueous extract, we performed two different experiments under the same conditions.

In the first experiment, twelve commercial equal-sized *A. cepa* onion bulbs of 3–4 g per concentration were used. They were carefully unscaled, placed on top of test tubes filled with tap water and allowed to germinate in the dark at 22 °C. After 48 h two unhealthy onions with the most poorly growing roots were removed and the other healthy onion bulbs in water were treated with 0.7% H_2O_2 for 1 h. After the H_2O_2 treatment, roots were washed for 1 h and onion bulbs were treated with two different extract concentrations (15 g/L and 30 g/L) for 24 h. Ten onion bulbs were used in each treatment.

To perform the second experiment, twelve commercial equal-sized *A. cepa* onion bulbs of 3–4 g per concentration were used. They were carefully unscaled, placed on top of test tubes, including tap water and allowed to germinate in the dark at 22 °C for 24 h. After then onion bulbs were transferred into the test tubes which included two different concentrations (15 g/L and 30 g/L) of *P. lanceolata* extracts and allowed to germinate in the dark at 22 °C for 24 h. After 24 h, two unhealthy onions with the poorly growing roots were removed and the other healthy onion bulbs in extracts were treated with 0.7% H_2O_2 for 1 h. After the treatment, roots were washed for 1 h and onion bulbs were placed on top of test tubes filled with water for 24 h. Onions were germinated in dark, because plant extracts are photosensitive.

Hydrogen peroxide in the concentration 0.7% was used as positive control for 1 h and tap water was used as negative control. After the completion of treatment (72 h), the roots were counted and their lengths were measured for each onion. After then roots were fixed in 3:1 (ethanol:acetic acid). After the fixation, the roots were hydrolyzed in 1N HCl for 2 min and stained with 2% orcein stain. After removing the root caps from well-stained root tips, 1 mm of the mitotic zones were immersed in a drop of 45% acetic acid on a clean slide and squashed under a cover glass and examined microscopically. Mitotic index was expressed in terms of divided cells/total cells (Ozmen & Sumer, 2004). Chromosomal aberrations were determined by scoring cells with bridges, fragments, sticky chromosomes and polar deviation in randomly picked 3 zones and micronucleus formation in 1,000 cells per slide. Five slides were examined per onion in each group which included ten onions.

Statistical analysis of data
The mean values were calculated for each group of concentrations and controls (negative and positive control). For the determination of the significance among the means, Two Way ANOVA was applied ($p < 0.05$).

Results

The average root lengths and numbers for control and treatment groups are given in Table 1. The effect of *P. lanceolata* aqueous extracts which were applied before and after H_2O_2 treatment on root number is not significant when compared with negative and positive controls, but the extracts reduced the average root lengths significantly. The measures of average root lengths in negative and positive controls are 3.55 cm and 4.39 cm, respectively. However, the average root lengths in treatment groups (ME_1, ME_2 – extract treatments after the H_2O_2 treatment; EM_1 and EM_2 – extract treatments before the H_2O_2 treatment) are significantly lower in comparison with the negative and positive controls (Table 1).

The mitotic index, chromosomal aberrations and ‰ micronuclei for each group are given in Tables 2 and 3. The data showed that the *P. lanceolata* aqueous extracts in 15 g/L and 30 g/L concentrations reduced the mitotic index significantly. Especially, mitotic index values of aqueous extracts-treated groups after H_2O_2 treatment are significantly lower than those of other

Table 2. Mitotic values in control and in treatment concentrations.[a]

Concentrations	Total cells	Dividing cells	Mitotic index (MI) ± SD
C(−)	16226	1519	9.23 ± 0.022
C(+)	18517	1393	7.32 ± 0.029
ME_1	17952	312	1.74 ± 0.015*
ME_2	18162	80	0.04 ± 0.005*
E_1M	18157	1039	5.62 ± 0.034*
E_2M	21672	1323	6.21 ± 0.027*

[a]C(−): negative control; C(+): positive control (0.7% H_2O_2); M: 0.7% H_2O_2; E_1: *Plantago lanceolata* extract (15 g/L); E_2: *Plantago lanceolata* extract (30 g/L); * $p < 0.05$ in Two Way ANOVA.

Table 3. Percentage of chromosomal aberrations and ‰ micronuclei values in control and in treatment concentrations.[a]

Concentrations	Dividing cells	Breaks (%)	Bridges (%)	Stickiness (%)	Polar deviations (%)	Aberrant cells (%)	‰ Micronuclei
C(−)	1519	0 ± 0	0 ± 0	0.41 ± 0.66	2.41 ± 1.07	2.82 ± 1.21	1.2 ± 0.14
C(+)	1393	0.17 ± 0.28	0.05 ± 0.16	6.50 ± 3.95*	7.91 ± 3.27*	15.29 ± 6.45*	9.6 ± 0.63*
ME_1	312	0 ± 0	0 ± 0	1.80 ± 2.84	2.95 ± 3.49	4.74 ± 3.07	3.8 ± 0.18
ME_2	80	0 ± 0	0 ± 0	1.54 ± 3.42	0.27 ± 0.85	1.81 ± 3.84	1.0 ± 0.17
E_1M	1039	0.21 ± 0.66	0.11 ± 0.35	2.01 ± 1.27	3.40 ± 2.95	5.73 ± 3.41	2.4 ± 0.18
E_2M	1323	0.20 ± 0.34	0 ± 0	1.90 ± 1.49	2.90 ± 1.92	5.01 ± 2.42	1.0 ± 0.14

[a]C(−): negative control; C(+): positive control (0.7% H_2O_2); M: 0.7% H_2O_2; E_1: *Plantago lanceolata* extract (15 g/L); E_2: *Plantago lanceolata* extract (30 g/L); * $p < 0.05$ in Two Way ANOVA. The values ± SD are shown.

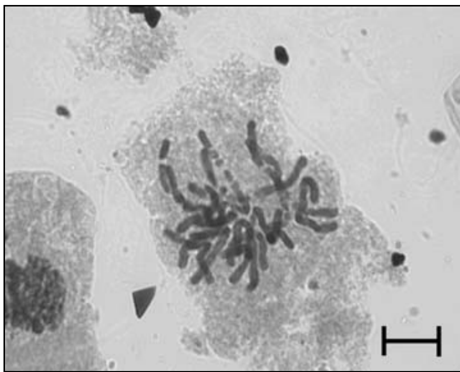

Fig. 1. Fragments; bar = 8 mm.

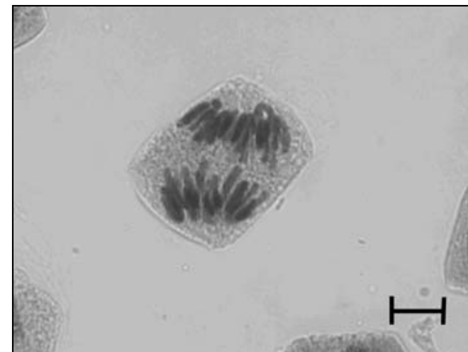

Fig. 2. Polar deviation; bar = 8 mm.

groups. The reduction in mitotic index (number of dividing cells) showed that substances in aqueous extracts may have cytotoxic effects.

The mitotic and chromosomal aberrations in terms of breaks, bridges, stickiness, polar deviations and ‰ micronuclei for each group are counted by microscopic observations for each slide (Figs 1–5). Total chromosomal aberrations showed decrease in *P. lanceolata* aqueous extracts-treated groups before and after H_2O_2 treatment, while they showed significantly increase in positive control group. Especially, aberrations in group ME_2 are significantly lower in comparison with those in other treatment groups ($p < 0.05$). Also, cells with apoptotic characters were detected in group treated with H_2O_2 (Fig. 5).

Micronucleus formation in 1,000 cells per slide (‰ micronuclei) value was also increased in positive control compared with negative control and this increase was statistically significant ($p < 0.05$). Both extract treatment before and after H_2O_2 treatment decreased

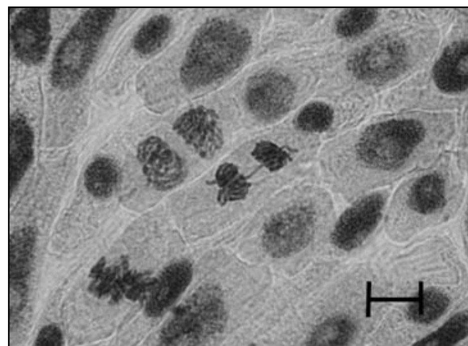

Fig. 3. Bridge; bar = 8 mm.

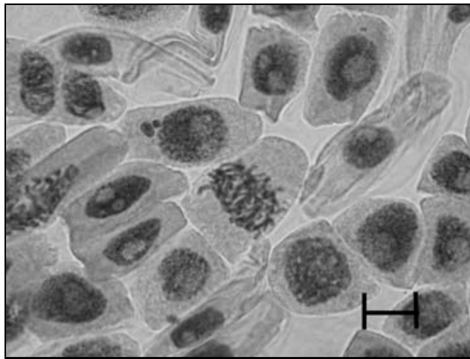

Fig. 4. Stickiness and micronucleus; bar = 8 mm.

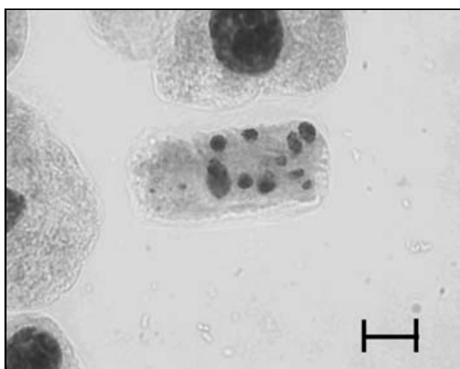

Fig. 5. Apoptotic cell; bar = 8 mm.

the micronucleus formation (Table 2). The reduction in percentage of aberrations and ‰ MN micronuclei in extract-treated groups before and after H_2O_2 treatment showed that substances in *P. lanceolata* aqueous extracts may have anti-genotoxic effect.

Discussion

Results obtained from this study showed that *P. lanceolata* aqueous extracts in 15 g/L and 30 g/L concentrations reduced the mitotic index significantly. Especially, mitotic index values of extract-treated groups after H_2O_2 treatment are significantly lower than those of other groups. The reduction in mitotic index (number of dividing cells) showed that substances in aqueous extracts may have cytotoxic effects. However, mitotic index values in the second experiment are much higher than those obtained in the first experiment (Table 2). Mitotic index values in the second experiment showed that the *P. lanceolata* aqueous extracts may have protective effect against the oxidative damage induced by H_2O_2. This protective effect may be due to antioxidant properties of flavonoids.

These results are in accordance with the literature data. *Plantago major* extracts, for example, may reduce the cellular proliferation *in vitro* (SAMUELSSON, 2004). Furthermore *Plantago* spp. extracts have cytotoxic effect on various cancer cell lines (RICHARDSON, 2001) due to luteolin-7-*O*-β-glucoside as a major flavonoid present in most of the *Plantago* species (GÁLVEZ et al., 2003). Flavonoids of *Plantago* exerting their cytotoxic potential may be thus responsible for the abovementioned effects.

Also the cells with apoptotic characters were detected in the group treated with H_2O_2 (Fig. 5). It may be possible that cells undergo a state of oxidative stress due to the H_2O_2 effect. Active oxygen forms emerging under the oxidative stress are known to affect the cytoskeleton structure (EGOROVA et al., 2001). In their turn, disorders in the cytoskeleton result in a disorganization of intracellular transport and oxygen consumption resulting in the intracellular hyperoxia and the higher active oxygen forms (LYU, 2001). Disorders in the stability of cytoskeleton proteins under oxidative stress cause the inhibition of mitotic events and result in cell death (KOZHURO et al., 2005).

It is very clear that mutations play an important role during the carcinogenesis and tumor formation. Both *in vivo* and *in vitro* experimental data showed that*Plantago* species exhibit the anti-carcinogenic and anti-tumor activity (RICHARDSON, 2001; GÁLVEZ et al., 2003; SAMUELSSON, 2004). A leaf extract prepared with 95% ethanol of *P. major* showed the anti-tumor activity when administered intra-peritoneally (0.20 mg/kg) to mice with Sarcoma type tumors during 5 days (ESTEVEZ et al., 1976).

The results of the present study are therefore important since they suggested the anti-genotoxic effect of *P. lanceolata* leaf extract. In order to reach to certain conclusions about this subject, however, further researches should be performed with different test systems.

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