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Original article

Determination of mutagenic and cytotoxic effects of *Limonium globuliferum* aqueous extracts by *Allium*, Ames, and MTT tests

Yasin Eren[a,*], *Ahmet Özata*[b]

[a]*Department of Science Education, Faculty of Education, Süleyman Demirel University, Isparta, Turkey*
[b]*Department of Biology, Science Faculty, Anadolu University, Eskişehir, Turkey*

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A B S T R A C T

Mutagenic and cytotoxic effects of roots, stems and leaves of *Limonium globuliferum* Kuntze, Plumbaginaceae, aqueous extracts were studied by *Allium*, Ames, and MTT tests. These are plant, bacterial and mammalian cell assays, respectively. The *Allium* test analyses showed that aqueous extracts of this species have dose-dependent toxicity and induce chromosomal anomalies based on defects in the spindle fibers. EC_{50} values of root stem and leaf aqueous extracts were 32.5, 50, and 50 g/l, respectively. It was observed that there was an inverse correlation between root growth and extract concentration. The lowest mitotic index value (22.72 %) was found in *L. globuliferum* root extract. As a result of the chromosome aberrations test, sticky chromosomes, anaphase bridges, laggard chromosomes, and anaphase-telophase disorders were highly detected especially in high concentration of the extract. In the Ames test, mutagenic effects were determined at all concentrations of stem and leaf aqueous extracts and only two concentrations of root extracts of *L. globuliferum*. Most of the extracts induced cytotoxic effects by the MTT test based on mitochondrial activity. Nevertheless, some of the extracts induced t cell proliferation.

Introduction

While newest physical and chemical agents facilitate human life, these agents may also cause some health problems due to their mutagenic and cytotoxic effects on living organisms. Plants have been considered as raw materials for alternative medicine and have antimutagenic effects against chemicals and enviromental factors. On the other hand, these extracts may also have mutagenic and cytotoxic effects on different organisms.

The use of plants as complementary and alternative medicine is increasing day by daydaily. The World Health Organization reported that traditional mediciness are used by some almost 60% of the world's population, and in some countries are incorporated extensively in the public health system (WHO, 2013).

Despite preliminary findings about therapeutic advantages of medicinal plants, some of their constituents may be

* *Corresponding author*.
E-mail: yasineren@sdu.edu.tr (Y. Eren).

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potentially toxic, mutagenic, carcinogenic, or teratogenic (Gadano et al., 2006). Therefore, therapeutical plants must be tested with regard to quality, safety and efficiency, like conventional drugs (Simaan, 2009).

Limonium Mill. genus is a member of the Plumbaginaceae family represented by six genera and 68 species in Turkey, and 24 genera and 800 species worldwide (Davis et al., 1982). *Limonium* species are halophyte plants which are resistant to drought and salinity (Zia and Khan, 2004). *Limonium* is called "kunduz otu", "eşekkulağı", or "deve kulağı" in Turkey.

According to records, *Limonium* chemical composition is very complex, containing amino acids, inorganic elements, vitamins, flavonoids, tannins, polysaccharides, alkaloids, organic acids and other constituents (Zhen-fa and Liang 1991; Lin and Chou 2000). This genus has been used for hematologic regulation, of bleeding, as anti-bacterial and anti-inflammatory, for bulk deposition, regulation of menstruation, for the stomach and other effects, and is currently used in clinical treatment of cervical cancer and dysfunctional uterine bleeding disorder (Bingwen et al., 1994). The latest studies suggest that *Limonium* plant also has a hepatic protection, anti-cancer, anti-viral and other pharmacologically activities (Lin and Kuo, 2000; Kuo et al., 2002).

It was determined by FTIR analysis that *Limonium globuliferum* has epigallocatechin, flavanol, gallocatechin, menthol, thymol, carvacrol and caffeic acid in methanol and aqueous root extracts; while rutin, rutinoside, myricetin, citric acid, ellagic acid, quercetin, flavanol, caffeic acid, tannins and coumarins in leaf methanol extracts. On the other hand, in *Limonium effusum* leaf methanol extracts myrcetin, menthol, thymol, carvacrol and catechol were found; while in root methanol extracts rutin, syringic acid, ellagic acid, myricetin, quercetin and flavanol were found by FTIR analysis (Avaz, 2010).

The chemical composition of *Limonium* is very rich. For example, the protein content of *Limonium sinense* is up to 14.81 g/100 g, total sugar 15.4 g/100 g, ash 13.01 g/100 g, while fat content is very low. It also contains a certain amount of vitamins, tannins, alkaloids, organic acids, flavanoids, and other substances (Zhen-fa and Liang, 1991).

According to inorganic element analysis with atomic absorption spectrophotometry, inorganic elements are highly present in *Limonium* species, and are especially rich in a variety of trace elements. It was found that constant elements (K, Na, Ca, Fe and Mg) were highly expressed in *Limonium*. Also, trace elements (Ni, Zn, Cr, Co) were largely found in *Limonium* plants. It was determined that *Limonium* genus is rich in vitamin C, B1, B2, B12, and carotenoids. In addition, *L. sinense* also contains a certain amount of vitamin D and E (Zhen-fa and Liang, 1991).

One of the previous studies about *Limonium* exhibited that *Limonium nashii* tannin extracts induced tumor formation (William et al., 1978). The aim of the present study was to determine the possible cytotoxic and mutagenic effects of *L. globuliferum* Kuntze (Plumbaginaceae). Mutagenic and cytotoxic effects of *L. globuliferum* aqueous extracts were investigated *in vitro* using the Ames, *Allium*, and MTT tests.

Materials and methods

Plant material and extraction

Limonium globuliferum Kuntze, Plumbaginaceae, samples were collected from Heybeli Thermal Spring (Afyon-Turkey) step areas and authenticated by Dr. Mustafa Kargıoğlu in Afyon Kocatepe University, Biology Department. Roots, stems, and leaves of *L. globuliferum* were dried at room temperature (25°C), and then powdered. Distilled water was used as solvent. Extraction was made according to Sofowora (1999). Plant powder (25 g) was added to 250 ml distilled water and the mixture was placed into the water bath at 80°C for 30 min. The obtained extract was filtered and stored at -20°C. Extracts were used directly and freshly for test systems.

Allium *test*

Onions (*Allium cepa*, 2 n = 16) were used in the *Allium* test system. The *Allium* test was performed according to Fiskesjö (1985). Concentrations of the plant's root extracts (6.25, 12.5, 25, 30, 32.5, 35, 37.5 and 50 g/l), and plant's stem and leaf extracts (6.25, 12.5, 25, 37.5 and 50 g/l) were used for the root growth inhibition test. $EC_{50}/2$, EC_{50} and $EC_{50}\times2$ concentrations of the extracts were used for mitotic index (MI) and aberrations studies.

Root Growth Inhibition Test (EC_{50} determination)

The onions were grown in freshly made distilled water for 24 h and then exposed for four days to the control group and other concentrations of extracts. In order to determine efficient concentration (EC_{50}) values, ten roots from each onion were cut off at the end of the treatment period, and the root's length was measured. The concentration that decreased root growth about 50% when compared to the negative control group (distilled water), was accepted as EC_{50} value. To determine the possible toxic effects on roots, $EC_{50}/2$, EC_{50} and $EC_{50}\times2$ concentrations of root, stem and leaf extracts were used in *Allium* mitotic index test.

Mitotic index (MI) determination

Onions (*Allium cepa*, 2 n = 16) were used in the *Allium* test system. Five onion bulbs were treated with distilled water and different concentrations of extracts (Table 1) for 72 h. At the end of 24, 48 and 72 h, root tips were cut and fixed in ethanol:glacial acetic acid (3:1), afterwards they were hydrolyzed in 1N HCl at 60°C for 7 min. Root tips from each concentration treatment were stained with Feulgen dye for 1 h. Five slides were prepared for each concentration and 1000 cells/per slide were counted. A total of 5000 cells were evaluated for each concentration. In the mitotic index (MI) study, about 5000 cells were counted, and MI% was determined with the following formulation.

$$MI\% = \text{divided cell number}/\text{total cell number} \times 100$$

Other aberrations were determined together with the MI study. About 500 cells were counted in the anaphase-telophase aberration study, and laggard chromosomes,

Table 1
Results of *Allium* cepa root growth inhibition test.

Limonium globuliferum root MI and aberrations results (%)							
Concentration (g/l)	MI%	Anafaz bridges	Laggard chromosome	Disordered anaphase-telophase	Sticky chromosome	Total aberrations (% ± SD)	Other aberrations % ± SD
Control - 24 h	31.98 ± 1.27a	2.6	3.4	9.2	1.40	16.6 ± 1.16a	0.23 ± 0.18a
16.25	23.71 ± 1.13b	7.59	17.14	16.16	9.54	50.43 ± 2.22b	0.26 ± 0.04a
32.5	23.11 ± 1.44b	12.42	19.30	13.83	6.9	52.46 ± 2.99b	0.16 ± 0.03a
65	23.20 ± 1.23b	11.3	14.15	11.48	16.45	53.39 ± 2.14b	0.15 ± 0.6a
Control - 48 h	31.61 ± 1.01ab	3	3.2	8.4	1.4	16 ± 1.22a	0.04 ± 0.03a
16.25	30.77 ± 1.99b	7.02	17.9	6.97	15.35	47.24 ± 3.49b	0.54 ± 0.05b
32.5	25.42 ± 2.83ac	5.24	13.96	15.68	19.25	54.12 ± 4.01bc	0.2 ± 0.09a
65	23.97 ± 2.6c	10.86	15.3	15.3	13.83	58.99 ± 3.25c	0.35 ± 0.14b
Control - 72 h	30.84 ± 1.98a	8.4	5.8	7.2	12.4	33.8 ± 1.11a	0.23 ± 0.11a
16.25	24.28 ± 1.34b	7.77	18.75	16.05	11.98	54.55 ± 3.1b	0.14 ± 0.02a
32.5	23.49 ± 0.56b	10.3	18.46	18.51	19.7	66.97 ± 8.79c	0.38 ± 0.04b
65	22.72 ± 0.38b	9.82	15.78	22.52	20.71	68.83 ± 1.46c	0.45 ± 0.08b
Control - 24 h	32.13 ± 1.14a	2.6	3.4	9.2	1.4	16.6 ± 1.6ab	0.23 ± 0.18a
25	43.28 ± 1.3b	1.2	2.52	6.57	2.32	12.61 ± 1.66a	0.16 ± 0.04a
50	53.36 ± 1.32c	-	6.91	3.03	11.4	21.34 ± 1.72b	0.2 ± 0.04a
100	36.23 ± 2.05d	2.34	0.74	7.94	5.86	16.88 ± 2.44ab	0.43 ± 0.22b
Control - 48 h	31.61 ± 1.01ab	3	3.2	8.4	1.4	16 ± 1.22a	0.04 ± 0.03a
25	32.92 ± 0.38b	2	2.6	6.2	5.1	15.9 ± 2.65a	0.06 ± 0.04a
50	31.64 ± 0.16a	6.02	5.42	6.63	3.01	21.07 ± 1.09a	0.1 ± 0.04a
100	33.43 ± 0.45b	6.8	5.2	8.8	10.53	31.33 ± 3.59b	0.16 ± 0.05a
Control - 72 h	30.84 ± 1.98a	8.4	5.8	7.2	12.4	33.8 ± 1.11a	0.23 ± 0.11a
25	27.69 ± 0.37ab	3.6	6.4	7	16	33 ± 2.3a	0.19 ± 0.06a
50	30.08 ± 0.76a	1.2	5.4	5	7	18.6 ± 2.15b	0.44 ± 0.14b
100	26.34 ± 0.63b	29	12.22	17.51	29.84	65.37 ± 2.02c	0.73 ± 0.4b
Control - 24 h	32.13 ± 1.14a	2.60	3.4	9.2	1.4	16.6 ± 1.6a	0.23 ± 0.18a
25	29.76 ± 2.38ab	4.79	15.88	15.95	20.71	57.33 ± 1.29b	0.78 ± 0.11b
50	26.68 ± 1.78ab	3.92	3.65	18.62	23.65	49.84 ± 4.63b	0.23 ± 0.04a
100	25.39 ± 1.85b	4.47	5.56	18.33	25.01	53.37 ± 1.87b	0.26 ± 0.03a
Control - 48 h	31.61 ± 1.01a	3	3.2	8.4	1.4	16 ± 1.22a	0.04 ± 0.03a
25	29.87 ± 0.55a	7.33	14.48	18.42	10.22	50.43 ± 2.17b	0.49 ± 0.17b
50	26.72 ± 1.03b	9	18.17	14.90	13.27	55.36 ± 2.6b	0.54 ± 0.09b
100	25.87 ± 0.67b	10.48	21.4	15.74	13.63	61.25 ± 1.41c	0.62 ± 0.06b
Control - 72 h	30.84 ± 1.98a	8.4	5.8	7.2	12.4	33.8 ± 1.11a	0.23 ± 0.11a
25	38.55 ± 1.53b	4	10.2	14.6	14.4	43.2 ± 1.65b	0.23 ± 0.05a
50	45.93 ± 1.02c	3.6	12.4	15.2	19.2	50.4 ± 1.98c	0.43 ± 0.19b
100	48.70 ± 0.8c	4.2	13.2	16.2	20.2	53.8 ± 1.59c	0.38 ± 0.11a

SD, Standartd deviation.
Small letters indicated stati stically significant groups according to Duncan's multiple comparison test ($p < 0.05$).

anaphase bridges, chromosome stickiness and disordered anaphase-telophase aberrations were observed. The data obtained were evaluated by One-Way ANOVA, and a Duncan multiple comparison test at $p < 0.05$ level.

Ames mutagenicity test

Salmonella typhimurium ***test strains and chemicals***

Salmonella typhimurium test strains TA98 and TA100 were obtained from Hacettepe University, Turkey. While TA98 was used for the determination of frame shifts, TA100 was used for the determination of base pair exchanges.

The S9 fraction from rat liver (Sprague-Dawley), Bacto-agar, nutrient broth n° 2 (Oxoid), 2-aminoanthracene (2AA), β-nicotinamide-adenine dinucleotide phosphate (β-NADP), glucose-6-phosphate (G6P), mitomycin-C (MMC). ampicillin, histidine and basic fuchsin were obtained from Sigma-Aldrich. Sodium azide (SA), citric acid monohydrate, NaOH, KCl, and NaCl were purchased from Riedel. 4-Nitro-*o*-phenylenediamine (NPD), 2-aminoanthracene (2AA) and 2-aminofluorene (2AF) were purchased from Fluka.

Ames test

Cytotoxic doses of the plant extracts (10 000, 1000, 100, 10, 1 and 0.1 µg/plate) were determined using the method described by Dean et al. (1985). The Ames test was performed as a standard plate incorporation assay with *S. typhimurium* strains TA98 and TA100 with or without metabolic activation (Maron and Ames, 1983). Strain selection was based on the testing and selection strategies of Mortelmans and Zeiger (2000). The strains were tested on the basis of associated genetic markers. For each test strain, a specific positive control was always used to test the experimental flaws, if any. While 4-nitro-*o*-phenylenediamine (NPD) was used for TA98, and sodium azide (SA) was used for TA100 as positive controls without metabolic activation. On the other hand, 2-aminofluorene (2AF) and 2-aminoanthracene (2AA) were used as positive controls with metabolic activation for TA98 and TA100 strains, respectively.

MTT assay

This test was performed with MDBK cells (Madin-Darby Bovine Kidney) (Sigma) according to Mosmann (1983). *L. globuliferum* root, stem and leaf aqueous extracts at different concentrations (50, 25, 12.5, 6.25, and 3.125 µg/ml) were used and distilled water was used for negative control group. Cells were incubated at different extract concentrations. The test extracts were removed at the end of the incubation period. Cells were incubated with 5 mg/ml MTT solution (Sigma) for 2 h in a CO_2 incubator to allow the transformation of MTT dye to formazan salt (not dissolved in water). Then MTT dyes were removed and 100 µl DMSO were added to the wells in order to dissolve the formazan salts formed only by living cells. Plates were analysed by ELISA at 540 nm wavelength. MTT test was repeated three times in 96 well plates for 24, 48, 72, and 96 h. Cell proliferation of the control group was accepted as "0" (Mosmann, 1983). Percentage of proliferation (Proliferation %) was calculated by the following formulation:

Proliferation % = (B-A)*100/A (Seo,2005)

Where A is the absorbance value of the control group and B is the absorbance value of plant extracts.

Results

Allium ***test results***

Allium ***root growth inhibition test*** **(EC_{50})**

This study determined the cytotoxic effects of *Limonium globuliferum* Kuntze, Plumbaginaceae, extracts on root growth. Root growth inhibition test detected the concentrations used for MI determination and chromosome aberration tests. EC_{50} was defined as the value that reduces the control group root length by half. Three experiments were designed for EC_{50} determination. EC_{50} values of root stem and leaf extracts that obtained from *L. globuliferum* were 32.5, 50, and 50 g/l, respectively. The control group change rate (%) was accepted 100%, and the other concentrations were compared to this group. Maximum inhibition (24.68%) on root growth was found at 50 g/l concentration of *L. globuliferum* root extract. On the other hand, maximum increase (108.57%) on root growth was determined in 6.25 g/l concentration of *L. globuliferum* root extract. So, these results showed that low concentrations of *L. globuliferum* have positive effects on root growth. The obtained data are shown in Table 2, and statistically significant groups were noted with small letters.

Determination of mitotic index and mitotic phases

A mitotic index study was performed in order to observe the cytotoxicity of plant extracts. In the present study, 5000 cells were counted for each concentration, and all extracts were compared to control group.

All concentrations of *L. globuliferum* root extracts decreased the root growth when compared to the control group in all treatment periods. The highest decline was found after 72 h treatment with 65 g/l concentration. *L. globuliferum* stem aqueous extracts incerased the growth at all treatment duration, except at 72 h. Aqueous extracts of *L. globuliferum* leaves induced root growth inhibition at 24 and 48 h treatment. On the other hand the 72 h treatment increased the growth clearly. These results showed that *L. globuliferum* stem and leaf extracts have adverse effects on root growth. All mitotic index data are given in Table 1.

Chromosome aberration determination

The determination of chromosome aberrations study was performed in two stages. First, 500 anaphase-telophase cells were observed and aberrations (sticky chromosomes, anaphase bridges, laggard chromosomes, and disordered anaphase-telophase) were determined. Secondly, 5000 cells were observed for each concentration, C-mitosis and polyploidy were detected. Some of the aberrations are given in Fig. 1. At the end of the study, percentages of data obtained from different extracts were compared with the control group. It was observed that there was a proportional increase in anaphase-telophase aberrations, except *L. globuliferum* stem aqueous extract. Sticky chromosomes and laggard chromosomes were seen mostly in anaphase-telophase

Table 2
Results of *Allium cepa* root growth inhibition test.

L. globuliferum root extracts			*L. globuliferum* stem extracts			*L. globuliferum* leaf extracts		
Doses (g/l)	Mean Root Length ± SD	% Change	Doses (g/l)	Mean Root Length ± SD	% Change	Doses (g/l)	Mean Root Length ± SD	% Change
Control	3.85 ± 0.42a	100.00	Control	3.85 ± 0.42a	100.00	Control	3.85 ± 0.42a	100.00
6.25	4.18 ± 0.95b	108.57	6.25	3.98 ± 0.29a	103.38	6.25	4.07 ± 0.56b	105.71
12.50	3.07 ± 0.77c	79.74	12.50	3.84 ± 0.22a	99.74	12.50	3.95 ± 0.81ab	102.60
25.00	2.32 ± 0.54d	60.26	25.00	3.04 ± 0.50b	78.96	25.00	3.24 ± 0.50c	84.16
30.00	2.16 ± 0.29e	56.10	37.50	2.28 ± 0.51c	59.22	37.50	2.46 ± 0.52d	63.90
32.50	1.97 ± 0.15ef	51.17	50.00	1.97 ± 0.31d	51.17	50.00	1.99 ± 0.33e	51.69
35.00	1.75 ± 0.16f	45.45						
37.50	1.50 ± 0.22g	38.96						
50.00	0.95 ± 0.26h	24.68						

SD, Standartd deviation.
Small letters indicate statistically significant groups according to Duncan's multiple comparison test ($p < 0.05$).

aberration test. It was not found any proportional or significant increase or decrease in the detemination of other aberrations. Aberration data and statistical analyses are given in Table 2.

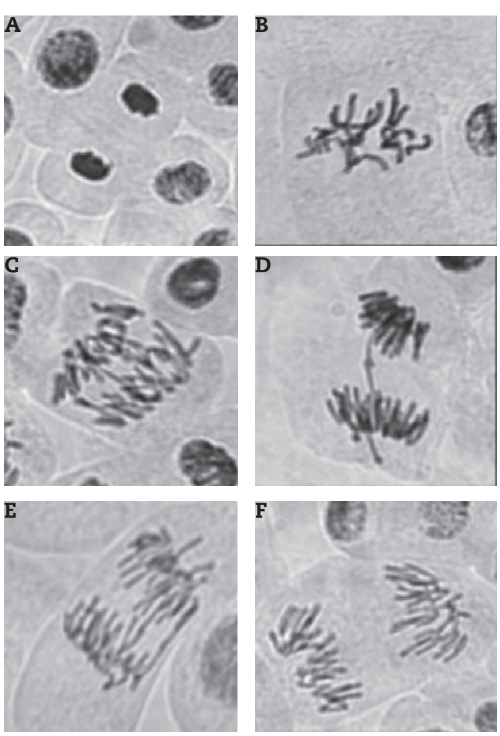

Figure 1 - Some chromosome aberrations of *Allium cepa* treated with root, stem and leaf extracts of *L. globuliferum*. A, Sticky chromosomes; B, C-mitosis; C, Three bridges; D, Anaphase bridge; E, Two bridges; F, Laggard chromosomes.

Ames test results

The Ames test was carried out to determine the mutagenicity of *L. globuliferum* extracts. Histidine mutant strains of *Salmonella typhymurium* (TA98 and TA100) were used and control group colony numbers were compared with colony numbers of plant extracts. Concentrations that generated a two-fold increase in colony numbers of negative control group were accepted as mutagenic. It was accepted that there was a weak mutagenic effect in the case of a dose-dependent increase of colony numbers (Mortelmans and Zeiger, 2000).

Determination of cytotoxic concentrations

As a result of cytotoxicity tests, it was found that only *L. globuliferum* aqueous extract of root at 10000 µg/plate was cytotoxic against *Salmonella typhymurium* strains among six tested concentrations (10000, 1000, 100, 10, 1 and 0.1 µg/plate). So, this toxic concentration was not used in the Ames test.

Ames test

4-Nitro-o-phenylendiamine (NPD), 2-aminofluorene (2AF), sodium azide (SA) and 2-aminoantracene (2AA) were used as positive controls for TA98 S9(-), TA98 S9(+), TA100 S9(-), and TA100 S9(+), respectively, while distilled water was used as negative control group. The Ames test was repeated three times with and without S9 metabolic activation. It was found that *L. globuliferum* root, stem and leaf distilled water extracts had no mutagenic activity in TA98 strain with and without metabolic activation. On the other hand, these extracts have statistically significant mutagenic effects in TA100 strain with and without metabolic activation. Moreover, all concentrations of stem and leaf and 0.1, 1 µg/plate doses of root extracts were accepted as mutagenic according to Maron and Ames (1983). These concentrations increased colony number by two compared with the control groups. Obtained data is given in Table 3.

Table 3
Results of *Allium cepa* root growth inhibition test.

Test materials	Concentration (µg/plate)	TA98 S9(-)	TA98 S9(+)	TA100 S9(-)	TA100 S9(+)
		Mean colony numbers ± SD			
(-) Control	-	28.00 ± 3.08	33.00 ± 3.61	115.60 ± 0.89	123.20 ± 1.64
SA	10			1622.80 ± 11.63[a]	
2AA	5				2647.00 ± 4.69[a]
2AF	200		2752.00 ± 43.24[a]		
NPD	200	2514.0 ± 39.75[a]			
L. globuliferum root	1000	23.60 ± 1.67	29.60 ± 2.70	135.40 ± 2.61[a]	122.20 ± 2.77
	100	22.40 ± 1.82	24.40 ± 3.36	116.00 ± 1.00	125.80 ± 1.48
	10	21.60 ± 0.55	25.40 ± 2.30	109.60 ± 1.82[a]	118.20 ± 1.10[a]
	1	22.60 ± 3.13	28.20 ± 3.11	543.40 ± 1.52[a]m	120.00 ± 1.22
	0.1	30.00 ± 2.24	24.60 ± 1.82	622.20 ± 2.39[a]m	123.20 ± 1.79
L. globuliferum stem	10000	24.00 ± 2.24	25.20 ± 3.19	479.00 ± 1.00[a]m	167.60 ± 2.51[a]
	1000	29.20 ± 4.21	30.60 ± 4.34	578.60 ± 1.14[a]m	117.20 ± 2.59[a]
	100	26.60 ± 1.52	20.00 ± 3.08	534.00 ± 2.24[a]m	133.20 ± 1.30[a]
	10	24.40 ± 4.98	25.00 ± 2.35	583.20 ± 2.95[a]m	132.00 ± 1.22[a]
	1	27.40 ± 3.05	25.80 ± 1.92	539.20 ± 4.15[a]m	139.40 ± 1.67[a]
	0.1	26.00 ± 4.74	28.60 ± 3.97	596.80 ± 2.05[a]m	125.40 ± 1.82
L. globuliferum leaf	10000	25.40 ± 2.97	25.20 ± 2.59	513.60 ± 2.97[a]m	170.80 ± 0.84[a]
	1000	25.60 ± 0.89	26.80 ± 3.03	679.40 ± 1.52[a]m	115.00 ± 0.71[a]
	100	29.80 ± 5.89	27.80 ± 1.92	695.20 ± 4.60[a]m	106.00 ± 1.41[a]
	10	30.80 ± 3.96	22.60 ± 2.30	626.80 ± 4.60[a]m	109.80 ± 0.84[a]
	1	21.80 ± 3.42	25.80 ± 3.27	526.20 ± 2.17[a]m	106.20 ± 2.17[a]
	0.1	28.80 ± 3.35	24.80 ± 1.48	587.60 ± 5.03[a]m	110.60 ± 3.29[a]

Positive controls; SA, Sodium azide; 2AA, 2-aminoantracene; 2AF, 2-aminofluorene; NPD, 4-nitro-o-phenylendiamine; m, mutagen; SD, Standartd deviation.
[a]Indicates statistically significant values according to Dunnett-t test ($p < 0.05$).

MTT test results

Root extracts of this plant had a high cytotoxic effect on MDBK cell line, except at 3.125 µg/ml concentration for the 72 h treatment. On the other hand, stem and leaf extracts showed a proliferative effect on cells, especially at low concentrations and up to the 48 h treatment period. The 50 µg/ml concentration of *L. globuliferum* stem extract decreased the cell viability more than the other extracts. *L. globuliferum* leaf extract concentrations of 6.25 and 3.125 µg/ml increased cell viability for three days. Generally a positive correlation was seen between the cell viabilities and extract concentrations or periods. The results determined the positive effects of stem and leaf extracts on cell viability up to the 48 h treatment but also identified the negative effects of root extracts on MDBK cell line. The MTT test results are presented in Figs. 2-4.

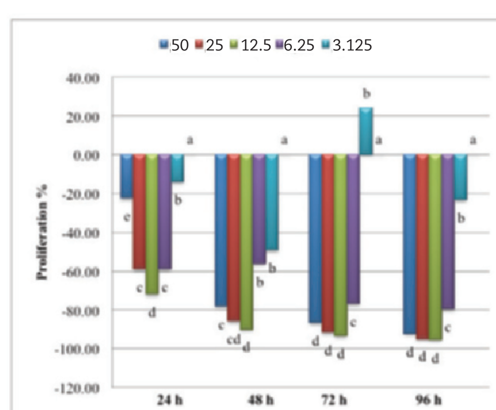

Figure 2 - Proliferation of *Limonium globuliferum* root aqueous extract.

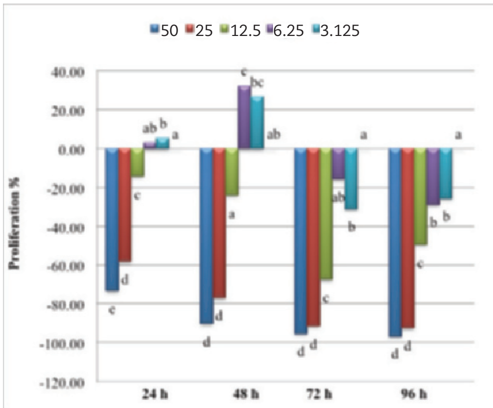

Figure 3 - Proliferations of *Limonium globuliferum* stem aqueous extract.

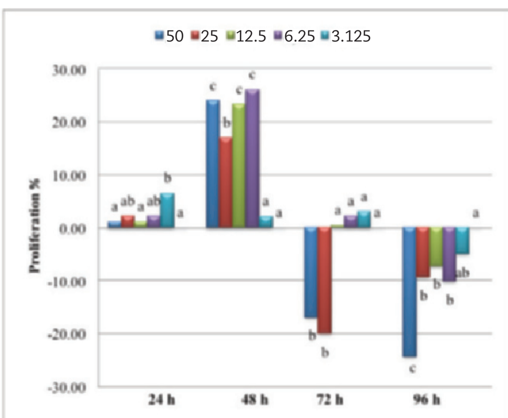

Figure 4 - Proliferations of *Limonium globuliferum* leaf aqueous extract.

Discussion

Medicinal plants have been accepted as a part of human culture. On the other hand, previous reports showed the mutagenicity and carcinogenicity of some medicinal plants (Alade et al., 2009). Many researchers have taken an interest on natural constituents and recently it has been increasing daily (Taylor et al., 1996). It was proved that some of these products have mutagenic effects on in vitro systems. (Schimmer et al., 1988; Higashimoto et al., 1993; Kassie et al., 1996)

In this study, mutagenic and cytotoxic effects of *L. globuliferum* were investigated. The study was performed using three test systems; bacterial test (Ames test), plant cell test (*Allium* test), and mammalian cell test (MTT test). These tests were previously used for mutagenicity and cytotoxicity determination (Fiskesjö, 1997; Bakare and Wale-Adeyemo, 2004; Babatunde and Bakare, 2006; Bayor et al., 2007; Alade et al, 2009; Mahavorasirikul et al., 2010).

The *Allium* test was used to determine toxicity and mutagenicity. Toxicity was easily observed by root growth inhibition while mutagenicity correlated with chromosome breaks (Fiskesjö, 1985). In this test, a concentration was accepted cytotoxic limit value and this concentration reduced the mitotic index of control group by half (Sharma, 1983). EC_{50} values of root stem and leaf aqueous extracts found were 32.5, 50, and 50 g/l, respectively. It was observed that there was an inverse correlation between root growth and concentration. The highest root growth inhibition (24.68% change) was determined at 50 g/l concentration of *L. globuliferum* root extract.

Root growth inhibiton always parallel to the decrease in dividing cells (Fiskesjö, 1997; Bakare and Wale-Adeyemo, 2004; Babatunde and Bakare, 2006), and may have occured due to the heavy metals of the extracts. It was determined that some plants contain metals like manganese (Mn), cadmium (Cd) and lead (Pb) (Al-Moaruf et al., 2004; Haider et al., 2004) and these metals cause root growth inhibition (Boroffice, 1990; Lerda, 1992; Fiskesjö, 1997). It was determined that *Limonium* species contain copper (Cu), zinc (Zn), manganese (Mn), chromium (Cr), and iron (Fe) (Xiuyun and Xian, 1991). So, their toxic effects may be related to these elements.

According to the mitotic index study in the *Allium* test, dose-dependent increase or decrease were not found opposite to the root growth inhibition test. The lowest mitotic index value (22.72%) was found in *L. globuliferum* root extract. As a result of the chromosome aberrations test, sticky chromosomes, anaphase bridges, laggard chromosomes, and anaphase-telophase disorders were highly detected especially at high concentrations of the extract. Other anomallies were rarely found.

It has been reported by different studies that naphthoquinons, specific to the Plumbaginaceae family, are very important constituents. The main cytotoxic effects of naphthoquinons are based on reactive oxygen species (ROS) formation, mitochondrial disorders and inhibition of thymine binding to DNA (Aithal et al., 2009; Babula et al., 2009).

Spindle fiber disruptions occurr because of plant alkaloids (Fasola and Egunyomi, 2005), and *Limonium* genus contains alkaloids, too. (Zhen-fa and Liang, 1991) Also, naphthoquinons (plumbagin), the specific alkaloid of the Plumbaginaceae family, highly expressed in *Limonium* species and it has cytotoxic effects. Plumbagin acts as a mitotic inhibitor on onion roots. These inhibition effects were expressed along with mitotic anomalies, like polyploidy, micronucleus, anaphase bridges, chromosome stickiness and laggard chromosomes (Krishnaswamy and Purushothaman, 1980). The data obtained from this study indicated that aberrations may be due to this component.

The Ames test is commonly used with plant extracts for possible gene mutation determination. Positive results are sufficient to classify a substance as a mutagen in any bacterial strains with and without metabolic activation (Zeiger, 2001).

The Ames test was carried out using aqueous extracts of root, stem and leaves, and mutagenicity was rarely found. In the Ames test with TA100 S9 (-), *L. globuliferum* root 0.1 and 1 µg/plate concentrations and all concentrations of stem and leaf showed mutagenic effect. It was noticed that Ames tests with plumbagin (naphthoquinon) have not shown any mutagenicity to TA98 and TA100 strains without metabolic activation. On the other hand, the tests with metabolic activation have shown conflicting results (Matsushima et al., 1986; Durga et al., 1992;

Hakura et al., 1994; Edenharder and Tang, 1997). Mutagenicity tests with *Escherichia coli* WP2/pKM101 and WP2uvrA/pKM101 strains did not show any mutagenic activity, but tests of AQ634 strain with S9 enzyme system showed mutagenic effects (Farr et al., 1985; Watanabe et al., 1998). Hydroxyl derivates of naphthoquinons show mutagenic activity in *S. typhimurium* TA2637 and TA98 strains, but it was found not to be mutagenic for *S. typhimurium* TA100 strain (Matsushima et al., 1986).

The modified Ames test was performed to some species belonging to the Plumbagineceae family, and root extracts did not show any mutagenic effects. But, in the *Allium* test with the same extract, it was determined that the extracts inhibited the root growth and decreased the mitotic index (Alade et al., 2009). Similar to this study, Fiskesjö (1985) reported that there was no correlation between the *Allium* root growth inhibition test and the Ames test.

The third test system for determination of cytotoxicity of *L.globuliferum* aqueous extracts was the MTT test with MDBK cell line. It was noticed that MTT (tetrazolium blue) colorimetric analysis may have been used with plant extracts for determination of reductions in the cell culture viability studies (Betancur-Galvis et al., 1999). Some researchers evaluated that cell proliferation could be determined by MTT (Mosmann, 1983).

Ali et al. (2007) reported that *Limonium sokotranum* leaf aqueous extract at 615.1 µg/ml concentration was moderately toxic by a cytotoxicity test with human amniotic epithelial cell line (FL-cells). They also emphasized that the cytotoxic effects of the *Limonium* genus could be a reason for traditional use as antifungal. In this study, a maximum concentration of 50 µg/ml of *L. globuliferum* extracts was used and generally most of the concentrations of this plant extracts created toxic effects at all treatment periods.

Santhakumari et al. (1980) investigated the effects of plumbagin on chicken embryo fibroblast cultures. They found that the dominant effects of plumbagin were cell growth, cell proliferation, and mitotic index reduction. They also reported that plumbagin acted as a poison of spindle fibers in low concentrations; however, at high concentrations it had nucleotoxic or cytotoxic effects. In addition to that, plumbagins can induce ROS, apoptosis and inhibition of cell cycle. In the present study, many of the extracts were found cytotoxic for all treatment periods, especially for 72 and 96 h treatments. Findings about nucleotoxic and cytotoxic effects of plumbagin were compatible with our *Allium* test chromosome aberration and mitotic index results.

Mutagenicity and cytotoxicity of *Limonium globuliferum* root stem and leaf aqueous extracts were investigated by the Ames, *Allium*, and MTT tests. In the *Allium* root growth inhibition test, there was a correlation between concentrations and cytotoxicity. EC_{50} concentrations of root stem and leaf extracts of *L. globuliferum* were found to be 32.5, 50, and 50 g/l, respectively. It was observed that many of the extracts and concentrations reduced the mitotic index in different treatment periods. Extracts mostly caused sticky chromosomes, polar disorders, laggard chromosomes and anaphase bridges that were based on spindle fiber disruptions in the *Allium* test. Similar to the mitotic index data, the MTT test indicated that many of the extracts were cytotoxic to MDBK cell line. According to the *L. globuliferum* Ames test, 1 and 0.1 µg/plate concentrations of root extract, and all concentrations of stem and leaf extracts were mutagenic. These findings may create a database about *L. globuliferum* and these results must be supported by in vivo studies.

Consequently, this is the first study on cytotoxic and mutagenic effects of *L. globuliferum* and shows that low concentrations of *L. globuliferum* extracts have proliferative effects on cells but high concentrations generally have toxic effects on cells as well as they could induce mitotic inhibition effect like plumbagin.

Authors' contributions

YE contributed in collecting plant samples, experimental design, application of the tests, and evaluation and statistical analysis of the results. AÖ designed the study, supervised the laboratory work, evaluated the results and contributed to critical reading of the manuscript. All the authors have read the final manuscript and approved the submission.

Conflicts of interest

The authors declare no conflicts interest.

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