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In vitro Toxicologic Effects of *Limonium globuliferum Acetone Extracts*

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Highlights:

- Cytotoxicity of *Limonium globuliferum*
- Mutagenicity of *Limonium globuliferum*
- acetone extract of *Limonium globuliferum*

Keywords:

- *Limonium globuliferum*
- AMES test
- MTT assay
- Cytotoxicity
- Genotoxicity
- Acetone extract

ABSTRACT:

Researching the effectiveness of plants is important for evaluating the reliability of their use in traditional medicine. Even today, various species of the Limonium plant are still used as an alternative tool in the treatment of different diseases. The aim of this study is to investigate the cytotoxic and mutagenic effects of acetone extracts of *Limonium globuliferum* to prevent the incorrect use of acetone extract of this species in alternative medicine. In this study, the mutagenicity and cytotoxicity of *L. globuliferum* acetone extracts were determined using in vitro test systems, including the bacterial AMES test and the MTT test performed with mammalian cells. According to the MTT test, the acetone extracts of the root have cytotoxic effects in all concentrations and exposure periods, stem extracts of *L. globuliferum* have a negative impact on cell proliferation except 72 h 3.125 µg/ml. Leaf extract showed positive effects on cells in all concentrations at 48 h applications. The AMES test results also indicated that the acetone extracts of the root and leaf have weak mutagenic effects as a result of the dose-dependent toxicity. On the other hand some of the concentrations has toxic effects on Salmonella strains and only 0,1 g/L leaf extract accepted as mutagenic.

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INTRODUCTION

Phytotherapy, the method of treating with plants, is used worldwide, and in countries where this treatment method is applied in a more systematic and complex manner, its usage is rapidly increasing with modernization in developed countries (Saad et al., 2006). Although some medicinal plants have significant therapeutic advantages, some components of medicinal plants can potentially be toxic, mutagenic, carcinogenic, and teratogenic (Gadano et al., 2006). The WHO encourages both developed and developing countries to include traditional plant preparations, which have been proven to be non-toxic, in their health programs (WHO, 1985).

The *Limonium* plant, which belongs to the Plumbaginaceae family, is a halophyte and has a wide distribution worldwide (Zia and Khan, 2004). The species of *Limonium* are commonly known as "beaver herb," "donkey ear," or "camel ear." The fresh leaves of these species are consumed by animals.

The chemical composition of *Limonium* is quite rich. It contains vitamins C, B1, B2, B12, D, and E, carotenoids, tannins, alkaloids, organic acids, flavonoids, and several other compounds. The *Limonium* genus is rich in essential amino acids such as valine, threonine, and phenylalanine, as well as non-essential amino acids like proline and glutamic acid. The fixed elements in *Limonium* include potassium (K), sodium (Na), calcium (Ca), and magnesium (Mg), with K and Na being the most abundant. Iron (Fe) content is also high. Trace elements such as nickel (Ni), zinc (Zn), chromium (Cr), and cobalt (Co) are also abundant (Zhen-fa and Liang, 1991). An analysis of *Limonium bicolor* showed high levels of Cu, Zn, Mn, and Fe (Xiuyun and Xian, 1991).

Acetone has moderate polarity, enabling it to dissolve both polar and nonpolar compounds. This allows for the simultaneous extraction of various chemical groups within plants (phenolics, flavonoids, terpenoids) (Tiwari et al., 2011). Especially when compared to methanol or ethanol, it demonstrates higher efficacy in separating lipophilic (fat-soluble) components from the cell wall of plant material (Eloff, 1998). In studies examining the antibacterial effects of plant extracts, acetone is the most recommended solvent. The reason for this is that acetone exhibits very low “intrinsic” toxicity toward the tested microorganisms; thus, the results accurately reflect the potency of the plant extract (Eloff, 1998). Additionally, acetone isolates compounds that inhibit microbial growth (alkaloids, anthraquinones) more efficiently than water (Cowan, 1999). Polyphenol oxidase enzymes released when plant tissue is disrupted can rapidly oxidize and discolor the plant’s secondary metabolites. Acetone rapidly denatures (inactivates) these enzymes, thereby preserving phenolic compounds in their original form (Boeing et al., 2014). Compared to other organic solvents such as chloroform, benzene, or methanol, acetone is a less toxic option for laboratory workers, and its environmental impact is more manageable (Tiwari et al., 2011). These properties of acetone have made it effective for use as a solvent in this study.

Researching the efficacy of plants is important for evaluating the reliability of their uses in the community. This study aims to investigate the cytotoxic and mutagenic effects of acetone extracts of *Limonium globuliferum*, a non-endemic species for our country. In this study, the in vitro tests used to determine the mutagenicity and cytotoxicity of *Limonium globuliferum* acetone extracts were the bacterial AMES test and the MTT test with mammalian cells.

MATERIALS AND METHODS

Plant Extraxtion Method

The *Limonium globuliferum* plant was collected from the Heybeli Hot Springs (Afyonkarahisar) region and was taxonomically classified at the Herbarium of the Biology Department at Afyon

Kocatepe University. The dried plant samples were separated into roots, stems, and leaves, and each part was powdered separately. Acetone was used as the solvent in this study. Acetone extracts were obtained by modifying the extraction protocols of Sirohi et al. (2009), where 25 grams of each sample were placed in 250 ml of solvent and left to stand at room temperature for 24 hours. After adding 250 mL of acetone to 25 gram plant samples, the powdered plants were kept in a water bath at 80°C for 30 minutes, and the extract were then centrifuged at 3000 rpm for 10 minutes. The resulting supernatant was collected and filtered through filter paper. The solvent from the final extracts were evaporated under vacuum in a rotary evaporator at 45°C. Extracts from which part of the solvent has been evaporated were used from 100 g/L stock solution.

AMES Test

Cytotoxicity test

The cytotoxicity assay was performed according to Dean et al. (1985). To determine the lethal dose of the test compounds for the test bacteria, 0.1 mL of bacterial culture and 0.1 mL of extracts at various concentrations were added to 2 mL of top agar. First, extract concentrations of 0.1 µg/plate, 1 µg/plate, 10 µg/plate, 100 µg/plate, 1000 µg/plate, and 10,000 µg/plate were added to the test tube. The mixture in the tube was poured onto 3 separate Nutrient agar plates, which were then incubated at 37°C for 24 hours. After incubation, the average number of colonies on the plates was determined, and toxic and non-toxic doses were identified by comparing them with control plates.

Procedure for the Ames Mutagenicity Test

The purpose of the experiment is based on the observation that oxotrophic strains, which previously required the amino acid histidine for growth, become capable of synthesizing histidine again when exposed to the test compounds used. The Ames test was performed according to the method described by Maron and Ames (1983). The number of colonies formed by bacteria capable of producing histidine was compared to the colony count of the negative control group (solvents of plant extracts). Accordingly, the concentration value that doubles the colony count of the negative control group is considered the mutagenic concentration. If there is a dose-dependent increase in colony count, the substance is considered to have a weak mutagenic effect (Mortelmans and Zeiger, 2000).

S9 negative (-) experiment

For this purpose, test tubes containing 2.5 mL of top agar, to which 0.25 mL of histidine-biotin solution had been added, were heated in a 45°C water bath, and 0.1 mL of the test substance and 0.1 mL of a 5-hour-old fresh bacterial culture were added to them. The tubes were shaken and poured onto MGA plates preheated to 37°C; the agar was spread evenly over the plates by quickly making an “8” shape with the pipette. After allowing the agar to set for 15 minutes, the plates were inverted and incubated at 37°C for 48–72 hours. At the end of incubation, the colonies on the plates were counted. The experiment was set up with three separate plates for each dose, and two independent experiments were conducted; to evaluate the results, spontaneous controls, solvent controls, and positive controls were used in parallel with the experiments.

S9 positive (+) experiment

In the plate incorporation assay, the test compound, bacterial test strain, and S9 mixture were mixed into top agar and poured onto minimal glucose agar plates. The plates were incubated at 37°C for 48–72 hours. At the end of this period, the histidine-positive revertant bacterial colonies on the plates were counted.

In this method, 0.25 mL of histidine-biotin was added to 2.5 mL , 0.1 mL of the test strain culture, 0.1 mL of the chemical to be tested, and 0.5 mL of the S9 mixture were added, vortexed at low speed for 3 seconds, and spread onto minimal glucose agar plates at room temperature. To ensure the top agar spread over the entire surface of the plate before solidifying, the entire process of mixing, pouring, and spreading was completed in less than 20 seconds. In each experiment, positive mutagenic effect controls were performed using a positive mutagen to verify the recovery rates of each strain and the effect of the S9 mixture. Negative control plates containing the bacteria, the S9 mixture, and the solvent used but not the test chemical were used to determine the number of spontaneously reverting bacteria for each strain. The experiment was performed using 3 separate plates for each dose, and two independent experiments were conducted.

MTT Test

Preparation of cells for testing

The MDBK (Madin-Darby Bovine Kidney) cells (Sigma) used in this study were cultured in Earle's BSS (Balanced Salt Solution), 2 mM L-glutamine, 0.1 mM non-essential amino acids, and 1.5 g/L sodium bicarbonate, and 10% fetal bovine serum.

Cells were allowed to proliferate in an incubator at 37°C and 5% CO_2; when they completely or 90% covered the flask surface, they were treated with Trypsin-EDTA (Sigma) and detached from the flask bottom. The cells, stained with Trypan blue (Sigma), were counted three times using a Thoma slide and suspended in medium to ensure an adequate number of cells in each well of the 96-well cell culture plates for the MTT assay; 100 µl of the cell suspension was then transferred to the 96-well cell culture plates. The plates were incubated at 37°C for 24 hours to allow the cells to adhere to the plate surfaces. At the end of the 24-hour incubation period, the medium was removed from the cells. To determine the cytotoxic effects of the test compounds, fresh medium containing the desired concentrations of the test compounds was added to the cells, and the plates were incubated at 37°C in a CO_2 incubator for 24, 48, 72, and 96 hours. Acetone was used as the negative control group. Acetone was added to the medium at concentrations not exceeding 0.1%, and the extract concentrations were set at 50, 25, 12.5, 6.25, and 3.125 µg/ml (Mosmann, 1983).

The statistical analysis of the data was performed using SPSS 22.0, employing a one-way ANOVA with a Dunnet's t-test.

MTT Assay

At the end of the incubation period, the culture media were removed from the cells treated with the test compounds for 24, 48, 72, and 96 hours. The cells were incubated in a 5 mg/ml MTT solution (Sigma) for 2 hours in a CO_2 incubator to allow the MTT dye to be converted into the water-insoluble formazan salt as a result of the metabolic activity of live cells. At the end of this period, the MTT dye was removed from the cells. To dissolve the formazan salts produced by the live cells, 100 µl of DMSO was added to each well. The optical densities of the cells in the plates were measured at a wavelength of 540 nm using an ELISA reader. The averages of the measured absorbance values were calculated, and from these averages, the percentage proliferation values representing the ratio of the number of live cells to the control were determined. The percentage proliferation rate was calculated using the following formula (Seo, 2005):

Percentage proliferation rate = (B – A) × 100 / A, Here, A represents the absorbance value of the control group, and B represents the absorbance value of the groups to which the sample was applied.

The proliferation rate of the experimental cells was expressed as a percentage, with the proliferation of control cells (untreated with the test substance) set to zero. This test was repeated three times (Mosmann, 1983).

Statistical analysis of the obtained proliferation data was performed using one-way ANOVA with the Duncan multiple range test in SPSS 22.0 at a significance level of $p < 0.05$.

RESULTS AND DISCUSSION

In recent studies, it was indicated that some of the Limonium species have positive effects on cancer and antivirus treatments. In a study conducted with *Limonium bicolor*, the role of its inorganic elements in the hemostatic effect showed a positive correlation (Xiuyun and Xian, 1991). The ethanol extracts and decoctions of *Limonium bicolor* have been found to shorten bleeding time in rats (Bingwen et al., 1994). The Limonium genus has antibacterial, anti-inflammatory, anti-swelling, and menstruation and digestion regulating effects. It is also used in clinical applications for uterine cancer and uterine bleeding disorders (Chen Xinmin, 1991; Bingwen et al., 1994). Recent studies have shown that Limonium plants have hepatoprotective effects and exhibit anticancer and antiviral properties (Kuo et al., 2002; Chaung et al., 2003). In rats with induced liver damage, water extracts from the roots and alcohol-chloroform extracts from the leaves of Limonium were found to play a role in protecting the liver (Chaung et al., 2003). The LD50 value in mice for acute toxicity tests was determined to be 777.6 mg/kg, indicating that the plant has low toxicity. Cell studies with water-soluble polysaccharides from *Limonium bicolor* showed that these polysaccharides significantly inhibited the proliferation of HeLa cells (Ka-Lin and Li, 2004). Another study found that Limonium plants are rich in flavonoids, with compounds like quercetin showing antioxidant and anticancer effects (Jinming, 2003; Zhenheng and Hafei, 2005). Ethanol extracts of *Limonium sinense* were found to inhibit Herpes simplex virus type I, with Samarangenin B, a tannin compound, significantly inhibiting Herpes simplex I virus (Kuo et al., 2002).

In addition to studies on the anticancer and antiviral activity of Limonium species, certain plants within the Plumbaginaceae family have cytotoxic and mutagenic effects. Limonium species contain high levels of naphthoquinones (plumbagin) and their derivatives—one of the primary alkaloids characteristic of the Plumbaginaceae family and that these compounds exhibit cytotoxic effects (Krishnaswamy and Purushothaman, 1980). In our study a dose-dependent increase in colony count was observed with root and leaf extracts. Therefore, it can be said that root and leaf acetone extracts have weak mutagenic effects.

On the other hand, Limonium or some species in Plumbaginaceae family have no cytotoxic and mutagenic effects. In a modified AMES test study, *Plumbago zeylanica* root extracts at concentrations of 10, 5, 1, and 0.1 mg/mL did not induce any changes in biochemical markers indicative of mutagenicity (Alade, 2009). The mutagenicity of methanol extracts from the roots of *Plumbago zeylanica* (Plumbaginaceae) was investigated using the AMES test preincubation method at concentrations of 25, 50, and 100 µg/plate with strains TA97, TA100, TA102, and TA104, and no toxic effects were observed (Aqil et al., 2008). According to the results of the AMES test conducted on dichloromethane and methanol extracts of *Plumbago auriculata* Lam. (Plumbaginaceae), no mutagenic effect was observed in strain TA98 (Elgorashi E.E., 2003). According to results of this study, any concentration that doubled the colony number was not detected in the presence or absence of the S9 enzyme fraction. Stem 0.1 µg/plate dose inTA98 strain without S9 was determined the highest revertant colony number according to negative control group.

In conclusion *Limonium globuliferum* acetone extracts have no mutagenic activity. These results also showed that except for the 10,000 µg/plate concentration of *Limonium globuliferum* root extracts, no cytotoxic effect was observed on Salmonella strains. Because of this result, experiments were conducted using five concentrations, excluding 10,000 µg/plate, for root extracts. Other extracts were tested with all six concentrations. Statistically, highest doses of root, stem and leaf extracts were found important in TA100 with and without S9 treatments by Dunnett's test. The results of the Ames test are presented in Table 1.

According to the MTT test, some extracts and exposure time showed proliferative effects to the cells, but it was mostly observed that acetone extracts had cytotoxic effects. Statistically, most of the root extracts and 3.125 µg/ml concentration of stem extracts were found important by Duncan test. The results of the MTT test are shown in Figures 1, 2, and 3.

In stem acetone extracts, 50 µg/ml for 24 hours and 3.125 µg/ml for 72 h and in leaf acetone extracts, all concentrations increased cell viability after 48 h treatment increased cell proliferation. Similar to these results, effect of the total ethanol extract of the *Limonium tetragonum* (Thunb.) plant on murine RAW 264.7 macrophage cells was investigated, and cell viability increased by 114.92% compared to the control group (Yang et al., 2009). The proliferative effects of 200 µg/ml concentrations of *Limonium tetragonum* on mouse spleen and thymus cells were examined; the methanol extract was found to have a proliferative effect of 32.46% on spleen cells, while the dichloromethane extract had an effect of 235.09%. In thymus cells, the methanol extract was shown to affect cell proliferation at a level of 17.34%, and the dichloromethane extract at 174.62% (Seo, 2005).

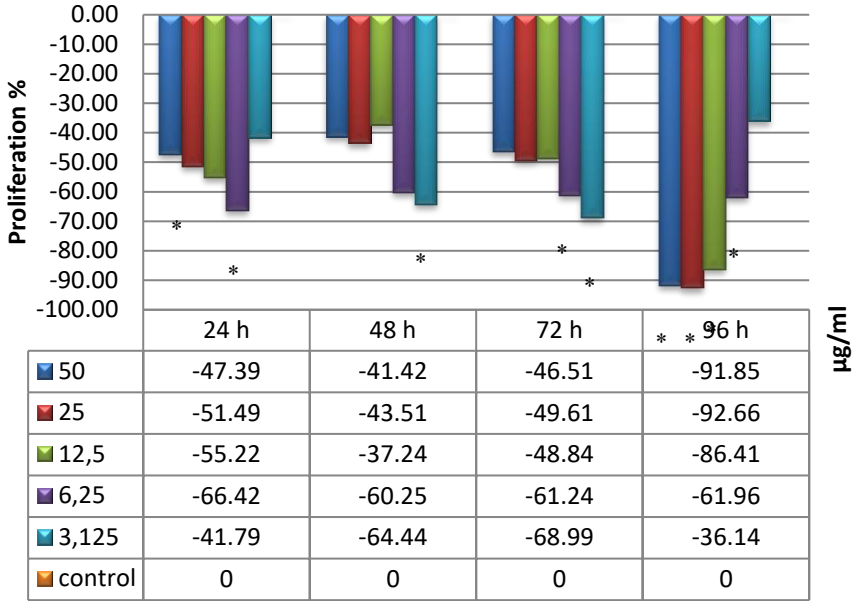
Except the proliferative doses as indicated before all treatment doses had toxic activity on MDBK cells. The highest toxic effect was observed at 25 µg/ml after 96h in root extracts. In all time and dose applications of *Limonium globuliferum* root acetone extracts showed cytotoxic effects. In a similar manner to this study Ali et al. (2007), investigated the effects of distilled water, methanol, and chloroform extracts of *Limonium sokotranum* leaves on the FL cell line, a human amniotic epithelial cell line, and reported that concentrations of 615.1 µg/ml in distilled water and 522.1 µg/ml in methanol produced moderate toxic effects and Santhakumari et al. (1980) investigated the effects of plumbagin (naphthoquinone) on chicken embryo fibroblast cultures. They noted that the most prominent effect was the inhibition of cell growth and proliferation, as well as the accumulation of cells in metaphase and a reduction in the mitotic index. These researchers reported that at low concentrations, plumbagin behaves like a spindle-polygonum toxin and at high concentrations, it exhibits nucleotoxic and cytotoxic effects.

Table 1. Ames test results *: p< 0.05 (Dunnet's t test)

Material		Concentration (µg/plate)	TA98 -S9 Avg. number of colonies ± Std. Deviation	TA98 +S9	TA100 -S9	TA100 +S9
(-) Control Acetone			25.6±2.30	31±3.77	138±6.4	141.2±5.62
(+) Positive controls	SA	10			2922.4±8.51*	
	2AA	5				3011.12±27.4*
	2AF	200		2931.42±55.11*		
	NPD	200	2614±29.24*			
L. globuliferum root		1000	29.22±5.27	33.18±2.82	150.61±8.3	199.3±19.88*
		100	29.15±5.34	32.3±2.16	145.14±6.45	175±20.14*
		10	28.2±5.2	30.7±4.25	143.4±10.18	160.22±14.32
L. globuliferum root		1	26.71±3.42	28.26±312	140.1±7.22	152.42±16.23
		0.1	25.44±6.12	28.2±2.88	138.9±6.73	146.4±15.26

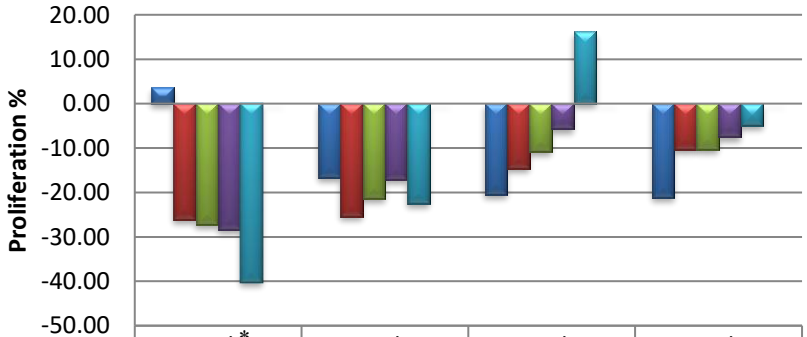
Table 1. Ames test results *: p< 0.05 (Dunnet's t test) (Continued)

L. globuliferum stem		10000	25.84±2.68	25.1±2.54	115.6±9.33*	150.2±22.32
		1000	27.5±3.52	26.1±2.88	119.3±8.64*	155.4±18.12
		100	28.3±3.46	28.23±3.38	129.1±8.52	158.6±11.99
		10	30.5±3.27	29.8±3.72	133.3±11.23	163.1±17.35
		1	32.21±5.33	31.8±5.14	135.7±11.12	166.2±19.58
		0.1	43.4±6.72*	33.42±6.16	139.1±8.24	169±7.30*
L. globuliferum leaf	10000	33.4±5.17	28.64±3.15	173±9.12*	177±12.54*	
	1000	32±4.34	27±2,14	135.6±10.14	155.3±14.45	
	100	30.5±5.23	26.2±1.48	127.9±7.8	152±11.12	
	10	30.6±3.51	26.16±3.1	125.14±6.74	150.4±10.24	
	1	29±2.72	25.32±2.42	122.6±4.71	148±9.89	
	0.1	28±5.16	24.42±3.12	120.3±3.07	147.1±10.40	



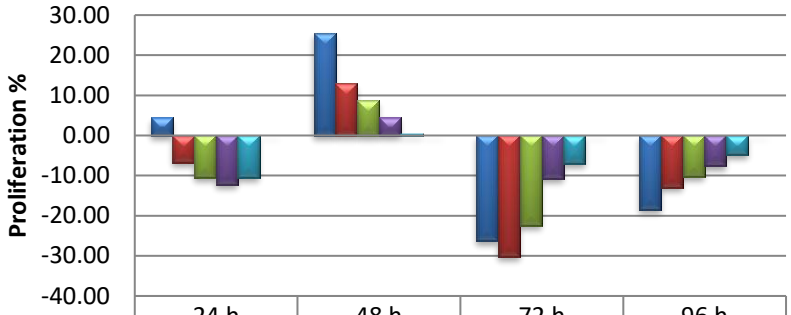
	24 h	48 h	72 h	96 h
50	-47.39	-41.42	-46.51	-91.85
25	-51.49	-43.51	-49.61	-92.66
12,5	-55.22	-37.24	-48.84	-86.41
6,25	-66.42	-60.25	-61.24	-61.96
3,125	-41.79	-64.44	-68.99	-36.14
control	0	0	0	0

Figure 1. Proliferation % of *Limonium globuliferum* root acetone extracts * : p< 0.05 (Duncan test)



	24 h*	48 h	72 h	96 h
50	3.73	-16.74	-20.54	-21.20
25	-26.12	-25.52	-14.73	-10.33
12,5	-27.24	-21.34	-10.85	-10.33
6,25	-28.36	-17.15	-5.81	-7.61
3,125	-40.30	-22.59	16.28	-4.89
control	0	0	0	0

Figure 2. Proliferation % of *Limonium globuliferum* stem acetone extracts *: p< 0.05 (Duncan test)



	24 h	48 h	72 h	96 h
50	4.48	25.52	-26.36	-18.48
25	-6.72	12.97	-30.23	-13.04
12,5	-10.45	8.79	-22.48	-10.33
6,25	-12.31	4.60	-10.85	-7.61
3,125	-10.45	0.42	-6.98	-4.89
control	0	0	0	0

Figure 3. Proliferation % of *Limonium globuliferum* leaf acetone extracts *: p< 0.05 (Duncan test)

CONCLUSION

In conclusion, the MTT test showed that the acetone extracts of *Limonium globuliferum* roots and stems had a negative effect on cell proliferation, while the leaf extracts had positive effects in some applications. The results of the AMES test also determined that root and leaf acetone extracts showed weak mutagenic effects. To draw conclusions regarding the mutagenicity and cytotoxicity of the acetone extract, this study should be supported by other in vitro test systems and in vivo studies.

Conflict of Interest

The authors have no relevant financial or non-financial interests to disclose. The corresponding author states that there is no conflict of interest.

Author's Contributions

Dr. Yasin EREN conceived and designed the analysis, collected the data, contributed data or analysis tools, performed the analysisand wrote the paper.

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