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ACCUMULATION OF CR^{6+}, PB^{2+} AND CD^{2+} AND ULTRAVIOLET RADIATION ALTER METHYLATION AND GENOMIC DNA STATUS IN *RAMALINA FARINACEAE*

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ABSTRACT. In this study was aimed to determine the genotoxic effect of *Ramalina farinacea* lichen species against stress sources at the molecular level. After applying three different heavy metals (Pb^{2+}, Cd^{2+}, and Cr^{6+}) to the *R. farinacea*, the extent to which the lichen sample absorbed these metals was determined by Flame Atomic Absorption Spectroscopy. RAPD and MSAP-AFLP assays were also used to determine the status of DNA damage. The heavy metal analysis showed that *R. farinacea* had high levels of Pb^{2+}, Cd^{2+}, and Cr^{6+} content. According to the results obtained from molecular analyses, band changes were observed against seven primers heavy metal stresses and three primers against UV stress. An increase in Genomic Template Stability (GTS) was determined during the time in *R. farinacea* treated with all heavy metal concentrations. The effect of UV radiations in *R. farinacea* revealed the highest polymorphism and the lowest GTS rate depending on the dose. Among all methylation combinations, Type II was found to show altered in *R. farinacea* in response to Pb^{2+}, Cd^{2+}, and Cr^{6+} contents and UV radiations. *R. farinacea* can be used at the molecular level as a biomarker of suitable genotoxic effect. This is the first study to reveal DNA damage against stress sources using a sample of *R. farinacea* lichen species.

Keyword and phrases. Ramalina farinacea, heavy metals, ultraviolet radiation, genotoxicity

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1. INTRODUCTION

The developments in the field of industry led to a serious increase in environmental pollution by releasing most of the heavy metals and various harmful pollutants [1,2]. The increase in the amount of heavy metals in the environment together with many other contaminants effects the living cells by disrupting the balance between reactive oxygen species (ROS) & antioxidant systems, and in this case, it causes diseases that negatively effects life [2]. In particular, soil, water and air analyzes, which are the physical elements of the natural environment, cannot provide sufficient data to reveal heavy metal genotoxicity. Lichens are important biological organisms that do not have organs such as root, stem, leaves and cuticles. Thanks to these properties, lichens have the ability to absorb elements such as heavy metals emitted from pollutant sources [3]. In recent studies, lichen species have been identified as suitable organisms that can be used to reflect atmospheric rates by measuring pollutants such as metals, elements and radionuclides accumulated in their thalli [4,5,6]. Particularly, epiphytic lichens are considered to be one of the important bioindicators of air quality because they easily obtain water and essential nutrients from the air [7-10].

It is known that lichens show different reactions to the accumulation of heavy metals. Some types of lichen can even tolerate high doses of some heavy metals such as Cu [11], Fe [12], Pb [12], Cd [13] and Mn [13]. With the accumulation of metals in lichen samples at a level that cannot be tolerated, problems such as decreased photosynthesis rate, decreased nitrogen fixation, thylakoid and plasma damage, chlorophyll deterioration can be observed. Environmental pollution of heavy metals has increasingly become of serious concern around the world [14-16]. Among all heavy metals, cadmium (Cd^{2+}), chromium (Cr^{3+} and Cr^{6+}) and lead (Pb^{2+}), in particular, continue to be a world concern [16]. The increased concentrations of Cr^{6+}, Cd^{2+}, and Pb^{2+} in polluted areas may pose a variety of problems and hazards [17]. In the study conducted by Liu et al. [18], *Orzya sativa* was treated with Cd and DNA damage was evaluated. According to the Random Amplified Polymorphic DNA (RAPD) analysis results, they stated that cadmium pollution has serious effects on DNA level. In the study, the researchers found that some bands disappeared and changes occurred in some bands compared with DNA of the control sample (unpolluted sample).

Ultraviolet (UV) radiation has vital importance as it has negative effects on humans, animals and plants. Plants have responded to the stress by repairing and enhancing the affected efficient systems and by incorporating the UV protective substances that prevent damage. While the substances used by different groups of

organisms are chemically various, the screening of UV radiation is used universally [19,20]. Numerous colourless lichen compounds absorb UV-B strongly, and some, such as parietin also assimilate photosynthetic active radiation (PAR) [21]. Studies have shown that lichens may generally vary in their responses to UV exposure by increasing the production and accumulation of secondary metabolites that block UV penetration [20,22]. Hall et al. [23] have found that secondary metabolites such as phenolic compounds accumulate at a high level in the outer layers of the medulla as a result of the short and long-term effects of UV-B radiation in some lichen species, and this accumulation reduced the transmission of UV radiation to thalli, and aromatic components in lichens played a protective role against UV radiation.

Rapid advances in molecular biology in recent years have provided new methods to detect DNA damage [23-25]. With the help of these sensitive molecular markers used, any genotoxic damage occurring in the biological organism can be easily detected [24-26]. The contamination of soil with heavy metals has a genotoxic effect on plants that have to grow in these areas, causing changes in the DNA profile, such as mutation [27]. Recently, RAPD and Amplified Fragment Length Polymorphism (AFLP) techniques have been successfully utilized to identify temporary DNA changes caused by heavy metal stress [6,16,28-32]. Current molecular genetics and genomic studies have provided insight into the importance of cytosine methylation, which plays a significant role in gene regulation [33]. Methylation creates differences in plants by causing DNA polymorphisms or epigenetic variations. The plant genome can respond to environmental and genetic stresses that result dynamically in both genetic and epigenetic methylation polymorphisms. Stress-triggered genotypes can contribute significantly to phenotypic innovation and the development of biological organisms [33].

Another method that makes it possible to indicate the effect of environmental pollutants on DNA size is comet assay. Single-cell gel electrophoresis (Comet) assay is a sensitive and simple tool capable of specifying DNA damage in the cells of biological organisms [34-36]. Moreover, RNA sequence and quantitative Real-Time-PCR techniques have been also used to determine the genetic damage that occurred in the samples collected from some polluted areas. In recent years, the genotoxic effect of pollutants in biological organisms other than lichen species has been identified with these two techniques [35]. However, the fact that lichens are a biological organism consisting of algae and fungi makes it difficult to apply these techniques based on RNA in the lichens. In this situation, the RAPD technique is still the best option for determining the molecular size genotoxic effect of pollutants using lichens, which is the best bio-indicator organism. However, in

order to make our results more detailed and reliable, a study was also carried out with the MSAP-AFLP technique [34-36].

The main objective of the present work was to elucidate whether the genotoxic effect of lichens, one of the best biomonitor organisms against pollutants, by using two different molecular techniques. *R. farinaceae* lichen species was exposed for heavy metals (Pb^{2+}, Cd^{2+}, and Cr^{6+}) and UV radiations (UVA, UVB, UVC, daylight, UVA+UVB, UVA+day light) for evaluating the impact of pollution. In this study, RAPD and MSAP-AFLP assays were used in *R. farinacea* for possible changes in DNA status after the exposure to different stress factors. Thus, this study will provide a more comprehensive understanding of the molecular mechanisms of cellular protection against different stress factors on *R. farinacea* lichen specimen. According to our knowledge, this study could be the first to evaluate the genotoxic effects on DNA of *R. farinacea*.

2. MATERIALS AND METHODS

2.1. Lichen samples and stress treatment

Ramalina farinacea was collected from Yenice Forest in Karabük, Turkey (41°10'N, 32°23'E). Three heavy metals and different UV radiation stress were applied to lichen samples at different time intervals in the study.

Thallus sample was placed in a petri dish in laboratory condition. It was exposed to different doses of UV radiation using a dose-meter (352 nm, 50Hz, 0.60 Amps) at 25 °C. The different levels of UV radiations were obtained with the UV irradiation chamber BS-03 (Dr. Gröbel UV-Electronic GmbH) and a dosimeter to determine the exposure of different UV radiation rates.

2.2. Determination on heavy metal content

Lichen sample exposed to Cd^{2+}, Cr^{6+} and Pb^{2+} heavy metals were analyzed by Flame Atomic Absorption Spectroscopy (FAAS; Instrument PM Avarta, GBC Scientific Equipment, Australia). The heavy metal content was determined according to Hamutoğlu et al. [16] studies.

2.3. Genomic DNA extraction and RAPD assay

DNA extraction was carried out under the protocol developed by Aras and Cansaran [37]. DNA purity was measured using nanodrop (NanoDrop ND-1000

Spectrophotometer, Thermo Scientific, Wilmington, USA). 10 primers were used in RAPD analysis and all primers showed amplified clear & reproducible bands, seven with metal and UV stress, respectively. PCR components were determined according to the protocol of Hamutoğlu et al. [16]. PCR products were loaded and visualized on agarose gel stained with ethidium bromide. The samples unexposed stress sources were used as control samples Negative control was also used to verify that the presence of any contaminating nucleic acid has been introduced into the master mix during the process.

2.4. MSAP-AFLP analysis

The genomic DNA (200 ng) of samples exposed to the heavy metal and UV stress was cut separately with *Eco*R I/*Msp* I and *Eco*R I/*Hpa* II restriction enzymes at 37 °C for 2 h (Table 1). MSAP-AFLP analysis was performed according to the protocol of Hamutoğlu et al. [16]. AFLP-PCR experiments were repeated at least twice for each primer and faint bands were not recorded in this study.

TABLE 1. Types of methylation produced by the cleavage of HPAII and MspI restriction enzymes.

Type	Methylation pattern	HpaII	MspI
Type I	CCGG C<u>C</u>GG GGCC GGCC	Active	Active
Type II	<u>C</u>CGG GGCC	Active	Inactive
Type III	C<u>C</u>GG GGCC	Inactive	Active
Type IV	<u>C</u>CGG GGCC	Inactive	Inactive

2.5. Statistical analysis

The results of data analysis were done with the multifactor analysis of variance (univariate ANOVA). The experiments were independently repeated three times. (n=3).

2.5.1. Estimation of profiling scoring and data analyses for RAPD assay

The rate of polymorphism was calculated by the disappearance or appearance of bands formed when the sample was exposed to stress [16,29,38].

2.5.2. Estimation of profiling scoring and data analyses for methylation analyses

All the amplified bands that are identified with the MSAP-AFLP analysis were classified into four categories based on the presence or absence of each amplicon as indicated by Li et al. [39].

3. RESULTS

3.1. The determination of heavy metal contents

The highest absorption ratio was determined as about 92.2 %, 95.1 % and 95.5 % using 30, 60 and 120 mg/L Cr^{6+} for 18 h, respectively. According to the results of 30, 60 and 120 mg/L Pb^{2+} application in lichen specimen, the absorption capacity percentage decreased from 43.4 % to 34.5 % as *R. farinacea* lichen specimen was applied to 30 mg/L Pb^{2+} for 24 h. 60 mg/L Pb^{2+} heavy metal absorption decreased from 58.1 % to 35.1 % for 24h in *R. farinacea*. At 120 mg/L Pb^{2+}, the absorption efficiency was found to be lower than 30 and 60 mg/L Pb^{2+} in *R. farinacea* (82.5 %) for 24 h ($p<0.05$) (Table 2). The highest removal efficiency (21 % for 2 h, 73.3 % for 24 h and 83.3 % for 24 h) was determined 30, 60 and 120 mg / L Cd^{2+} in *R. farinacea* lichen species ($p<0.05$).

TABLE 2. The measurements of heavy metal contents with AAS after exposure of the *R. farinacea* lichen sample to 120 mg / L Pb^{2+} (ANOVA analysis was performed and the same letters in a column indicate no significant differences with ANOVA test at $p < 0.05$).

Samples	***N***	***Mean***	***Std. Deviation***	***Std. Error***	***95% Confidence Interval for Mean***		***Min***	***Max***
					Lower Bound	***Upper Bound***		
Control	3	119.9(a)	.20	.11	119.4	120.3	119.7	120.1
30 min	3	96.7(b)	.20	.11	96.2	97.1	96.5	96.9
1 h	3	88.2(c)	.20	.11	87.7	88.6	88.0	88.4
2 h	3	79.6(d)	.10	.05	79.3	79.8	79.5	79.7
6 h	3	72.2(e)	.10	.05	71.9	72.4	72.1	72.3
18 h	3	71.8(f)	.40	.23	70.8	72.7	71.4	72.2
24 h	3	70.9(g)	.10	.05	70.6	71.1	70.8	71.0
48 h	3	70.9(h)	.20	.11	70.4	71.3	70.7	71.1
72 h	3	70.9(i)	.10	.05	70.6	71.1	70.8	71.0
Total	27	82.3	16.16	3.11	75.9	88.7	70.7	120.1
	Sum of Squares	**df**	**Mean Square**	**F**	**Sig.**			
Between Groups	6796.0	8	849.5	21237.5	.000			
Within Groups	.70	18	.04					
Total	6796.7	26						

3.2. Determination of heavy metals and UV radiations on RAPD profiles in *R. farinacea*

R. farinacea control sample obtained a total of 42 and 15 bands in heavy metal and

UV stress respectively by using RAPD analyses (Table 3). Total number of bands were more in *R. farinacea* variety treated with Cr^{6+} (42 in control, 30 mg/L Cr^{6+}: 132; 60 mg/L Cr^{6+}: 139; 120 mg/L Cr^{6+}: 192 band) when compared to *R. farinacea* treated with Pb^{2+} (28 in control, 30 mg/L Pb^{2+}: 85; 60 mg/L Pb^{2+}: 69; 120 mg/L Pb^{2+}: 83 band) and Cd^{2+} (42 in control, 30 mg/L Cd^{2+}: 122; 60 mg/L Cd^{2+}: 113; 120 mg/L Cd^{2+}: 91 band) (Table 3-5). After 120 mg/L Cr^{6+} treatment, 11 extra bands appeared and 20 extra bands disappeared at 30 min in *R. farinacea* variety, and this species showed the highest levels of change in bands treated with Cr^{6+} (Figure 1) (Table 3). The lowest number of band changes (7 bands) was detected at 60 mg/L Pb^{2+} after 1 h treatment in *R. farinacea* lichen specimen (Figure 2) (Table 4).

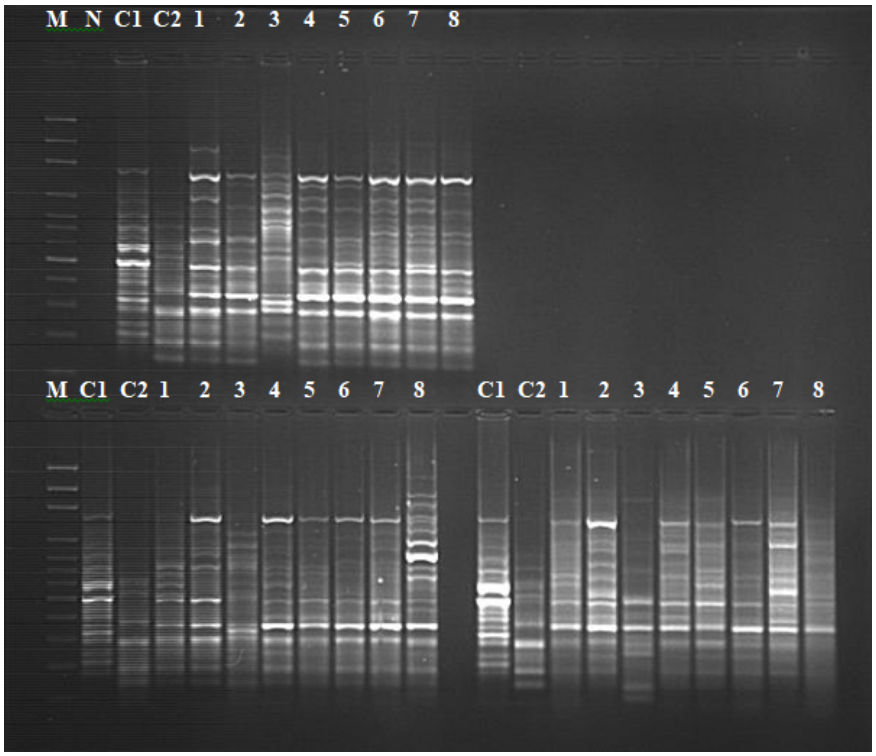

FIGURE 1. DNA band profile of 30 mg/L (top-left), 60 mg/L (lower-left) and 120 mg/L (lower-right) concentration of Cr^{6+} in *Ramalina farinacea* using RAPD with primer OPC7 primers. (M: Marker, C1-C2: Control, N: Negative control, 1: 30 min, 2: 1 h, 3: 2 h, 4: 6 h, 5: 18 h, 6: 24 h, 7: 48 h, 8: 72 h).

TABLE 3. Varying band number using OPC 02, OPC 04 and OPC 07 primers as a result of treating 30, 60 and 120 mg/L Cr^{6+} heavy metal stress in *Ramalina farinacea* lichen specimen.

30 mg/L Cr^{6+}																	
		30 min		**1 h**		**2 h**		**6 h**		**18 h**		**24h**		**48 h**		**72 h**	
Primer	**C**	**a**	**b**	**a**	**b**	**a**	**b**	**a**	**b**	**a**	**b**	**a**	**b**	**a**	**b**	**a**	**b**
OPC 02	**14**	3	4	1	7	4	5	2	5	3	3	3	4	3	5	1	3
OPC 04	**14**	2	4	2	2	1	2	2	2	1	0	0	2	1	3	1	4
OPC 07	**14**	2	6	3	5	1	6	3	3	2	6	3	2	1	1	2	2
	42	7	14	6	14	6	13	7	10	6	9	6	8	5	9	4	9
	a+b	**21**		**20**		**18**		**17**		**15**		**14**		**14**		**13**	
60 mg/L Cr^{6+}																	
		30 min		**1 h**		**2 h**		**6 h**		**18 h**		**24h**		**48 h**		**72 h**	
Primer	**C**	**a**	**b**	**a**	**b**	**a**	**b**	**a**	**b**	**a**	**b**	**a**	**b**	**a**	**b**	**a**	**b**
OPC 02	**14**	2	3	1	3	3	4	2	3	1	4	1	0	2	7	2	5
OPC 04	**14**	1	4	2	1	3	2	2	5	2	5	1	4	3	2	1	1
OPC 07	**14**	4	6	6	4	2	4	3	1	1	4	4	3	0	5	4	6
	42	7	13	9	8	8	10	7	9	4	13	6	7	5	14	7	12
	a+b	**20**		**17**		**18**		**16**		**17**		**13**		**19**		**19**	
120 mg/L Cr^{6+}																	
		30 min		**1 h**		**2 h**		**6 h**		**18 h**		**24h**		**48 h**		**72 h**	
Primer	**C**	**a**	**b**	**a**	**b**	**a**	**b**	**a**	**b**	**a**	**b**	**a**	**b**	**a**	**b**	**a**	**b**
OPC 02	**14**	5	6	4	7	4	6	5	3	4	4	2	4	2	3	5	0
OPC 04	**14**	4	6	5	1	3	2	2	6	3	3	1	1	2	5	1	6
OPC 07	**14**	2	8	4	8	5	8	4	3	2	6	5	7	2	5	1	7
	42	11	20	13	16	12	16	11	12	9	13	8	12	6	13	7	13
	a+b	**31**		**29**		**28**		**23**		**22**		**20**		**19**		**20**	

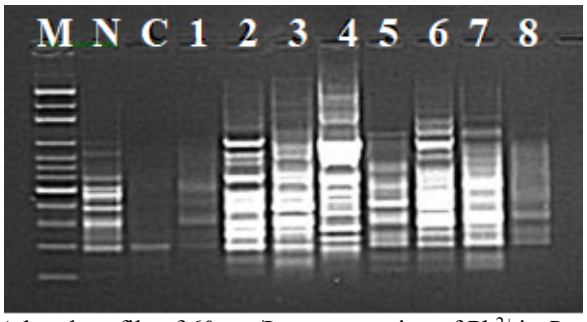

FIGURE 2. DNA band profile of 60 mg/L concentration of Pb^{2+} in *Ramalina farinacea* using RAPD with primer OPC10 primers. (M: Marker, C: Control, N: Negative control, 1: 30 min, 2: 1 h, 3: 2 h, 4: 6 h, 5: 18 h, 6: 24 h, 7: 48 h, 8: 72 h).

TABLE 4. Varying band number using OPC 04 and OPC 10 primers as a result of treating 30, 60 and 120 mg/L Pb^{2+} heavy metal stress in *Ramalina farinacea* lichen specimen.

30 mg/L Pb^{2+}																	
		30 min		**1 h**		**2 h**		**6 h**		**18 h**		**24h**		**48 h**		**72 h**	
Primer	**C**	**a**	**b**	**a**	**b**	**a**	**b**	**a**	**b**	**a**	**b**	**a**	**b**	**a**	**b**	**a**	**b**
OPC 04	**14**	2	5	2	4	1	2	1	4	3	2	2	5	2	2	1	4
OPC 10	**14**	3	2	2	3	2	5	3	2	2	1	1	0	0	3	1	3
	28	5	7	4	7	3	7	4	6	5	3	3	5	2	5	2	7
	a+b	**12**		**11**		**10**		**10**		**8**		**8**		**7**		**9**	
60 mg/L Pb^{2+}																	
		30 min		**1 h**		**2 h**		**6 h**		**18 h**		**24h**		**48 h**		**72 h**	
Primer	**C**	**a**	**b**	**a**	**b**	**a**	**b**	**a**	**b**	**a**	**b**	**a**	**b**	**a**	**b**	**a**	**b**
OPC 04	**14**	3	5	1	6	5	3	1	4	4	2	2	4	4	1	3	2
OPC 10	**14**	1	1	2	2	2	0	2	2	0	1	0	2	1	1	0	2
	28	4	6	3	8	7	3	3	6	4	3	3	2	6	5	3	4
	a+b	**10**		**11**		**10**		**9**		**7**		**8**		**7**		**7**	
120 mg/L Pb^{2+}																	
		30 min		**1 h**		**2 h**		**6 h**		**18 h**		**24h**		**48 h**		**72 h**	
Primer	**C**	**a**	**b**	**a**	**b**	**a**	**b**	**a**	**b**	**a**	**b**	**a**	**b**	**a**	**b**	**a**	**b**
OPC 04	**14**	4	5	3	2	2	5	3	1	2	3	0	4	2	3	1	1
OPC 10	**14**	2	4	4	3	4	1	2	5	0	4	3	1	1	2	2	4
	28	6	9	7	5	6	6	5	6	2	7	3	5	3	5	3	5
	a+b	**15**		**12**		**12**		**11**		**9**		**8**		**8**		**8**	

In terms of Cd^{2+} stress in *R. farinacea*, the highest GTS value was observed expose to 30 mg/L Cd^{+2} heavy metal stress at 48 h and 72 h (69.0 %), 60 mg/L Cd^{2+} at 48 h (73.8 %) and 120 mg/L Cd^{2+} at 48 h (83.3 %) treatments. The lowest GTS values (54.7, 59.5 and 59.5 %) were obtained at 30 min treatments in all Cd^{2+} heavy metal stress concentrations, respectively (Table 6). As regards Cr^{6+} stress in *R. farinacea*, the highest GTS value was seen expose to 30 mg/L Cr^{6+} heavy metal stress at 72 h (69.0 %), 60 mg/L Cr^{6+} stress at 18, 48 and 72 h (54.7 %) and 120 mg/L Cr^{+6} stress at 48 h (54.7 %). The lowest GTS value (50.0, 38.0 and 26.1 %) was obtained at 30 min treatments in all Cr^{6+} concentrations (Table 6).

TABLE 5. Varying band number using OPC 01, OPC 04 and OPC 08 primers as a result of treating 30, 60 and 120 mg/L Cd^{2+} heavy metal stress in *Ramalina farinacea* lichen specimen.

30 mg/L Cd^{2+}																	
		30 min		**1 h**		**2 h**		**6 h**		**18 h**		**24h**		**48 h**		**72 h**	
Primer	**C**	**a**	**b**	**a**	**b**	**a**	**b**	**a**	**b**	**a**	**b**	**a**	**b**	**a**	**b**	**a**	**b**
OPC 01	**14**	1	6	1	5	3	2	5	3	0	4	2	4	3	3	3	2
OPC 04	**14**	2	3	1	3	0	5	1	1	2	1	2	0	2	1	0	2
OPC 08	**14**	2	5	4	3	3	2	1	5	4	4	3	3	1	3	2	4
	42	5	14	6	11	6	9	7	9	6	9	7	7	6	7	5	8
	a+b	**19**		**17**		**15**		**16**		**15**		**14**		**13**		**13**	
60 mg/L Cd^{2+}																	
		30 min		**1 h**		**2 h**		**6 h**		**18 h**		**24h**		**48 h**		**72 h**	
Primer	**C**	**a**	**b**	**a**	**b**	**a**	**b**	**a**	**b**	**a**	**b**	**a**	**b**	**a**	**b**	**a**	**b**
OPC 01	**14**	3	5	2	4	3	2	1	5	2	5	1	3	1	4	0	3
OPC 04	**14**	2	3	1	2	2	3	1	1	0	3	0	1	2	1	0	1
OPC 08	**14**	2	2	4	3	6	1	1	5	0	4	2	5	1	2	3	5
	42	7	10	7	9	11	6	3	11	2	12	3	9	4	7	3	9
	a+b	**17**		**16**		**17**		**14**		**14**		**12**		**11**		**12**	
120 mg/L Cd^{2+}																	
		30 min		**1 h**		**2 h**		**6 h**		**18 h**		**24h**		**48 h**		**72 h**	
Primer	**C**	**a**	**b**	**a**	**b**	**a**	**b**	**a**	**b**	**a**	**b**	**a**	**b**	**a**	**b**	**a**	**b**
OPC 01	**14**	4	2	3	5	2	2	0	5	3	2	1	2	1	1	2	2
OPC 04	**14**	3	1	2	2	3	1	2	2	0	1	0	0	1	1	0	0
OPC 08	**14**	2	5	1	2	0	7	2	1	0	3	1	4	1	2	0	4
	42	9	8	6	9	5	10	4	8	3	6	2	6	3	4	2	6
	a+b	**17**		**15**		**15**		**12**		**9**		**8**		**7**		**8**	

In terms of Pb^{2+} stress in *R. farinacea*, the highest GTS values were seen expose to 30 mg/L Pb^{2+} heavy metal stress at 48 h (75.0 %), 60 mg/L Cr^{6+} stress at 18, 48 and 72 h (75.0 %) and 120 mg/L Cr^{6+} stress at 24, 48 and 72 h (71.4 %). The lowest GTS values were obtained at 30 min treatments in 30 and 120 mg/L Pb^{2+} and 1 h treatments in 60 mg/L Pb^{2+} heavy metal stress (Table 6).

Varying band-number using primers as a result of UVA, UVB and UVC radiation samples in *R. farinacea* was also shown in Table 7. As regards UVA stress in *R. farinacea* lichen species, the highest GTS value (96.6 %) was observed exposed to 4 j/cm^2 UVA radiation (Table 8). UVC treatments in *R. farinacea*, the highest GTS value (88.6 %) was determined at 4 j/cm^2 UVC radiation. UVB treatments in *R. farinacea*, the highest GTS value (66.6 %) was revealed at 8 j/cm^2 UVB radiation. The lowest GTS value (43.3 %) was observed at 40 j/cm^2 UVB radiation (Figure 3) (Table 8).

TABLE 6. The rates of GTS values using Cd^{2+}, Cr^{6+}, and Pb^{2+} heavy metal stress in *Ramalina farinacea* lichen specimen.

Samples		Rates of GTS (%)	Samples		Rates of GTS (%)	Samples		Rates of GTS (%)
30 ppm Cd^{2+}	30 min	54.76	60 ppm Cd^{2+}	30 min	59.52	120 ppm Cd^{2+}	30 min	59.52
	1 h	59.52		1 h	61.90		1 h	64.28
	2 h	64.28		2 h	61.90		2 h	64.28
	6 h	61.9		6 h	66.66		6 h	71.42
	18 h	64.28		18 h	66.66		18 h	78.57
	24 h	66.66		24 h	71.42		24 h	80.95
	48 h	69.04		48 h	73.80		48 h	83.33
	72 h	69.04		72 h	71.42		72 h	80.95
Samples		**Rates of GTS (%)**	**Samples**		**Rates of GTS (%)**	**Samples**		**Rates of GTS (%)**
30 ppm Cr^{6+}	30 min	50.0	60 ppm Cr^{6+}	30 min	38.09	120 ppm Cr^{6+}	30 min	26.19
	1 h	52.38		1 h	42.85		1 h	30.95
	2 h	57.14		2 h	45.23		2 h	33.33
	6 h	59.52		6 h	50.0		6 h	45.23
	18 h	64.28		18 h	54.76		18 h	47.61
	24 h	66.66		24 h	50.0		24 h	52.38
	48 h	66.66		48 h	54.76		48 h	54.76
	72 h	69.04		72 h	54.76		72 h	52.38
Samples		**Rates of GTS (%)**	**Samples**		**Rates of GTS (%)**	**Samples**		**Rates of GTS (%)**
30 ppm Pb^{2+}	30 min	57.14	60 ppm Pb^{2+}	30 min	64.28	120 ppm Pb^{2+}	30 min	46.42
	1 h	60.71		1 h	60.71		1 h	57.14
	2 h	64.28		2 h	64.28		2 h	57.14
	6 h	64.28		6 h	67.85		6 h	60.71
	18 h	71.42		18 h	75.0		18 h	67.85
	24 h	71.42		24 h	71.42		24 h	71.42
	48 h	75.0		48 h	75.0		48 h	71.42
	72 h	67.85		72 h	75.0		72 h	71.42

3.3. Methylation DNA and polymorphism in examined lichen species to different levels of stress condition

Band changes after exposure to heavy metals and UV radiation were compared with untreated control samples. 413 to 691 bands were obtained in the untreated sample and a total of 217 bands were determined with an average of 11 per primer in the MSAP-AFLP analysis. The total number of band changes was 117 bands for the heavy metal and lichen species exposed to UVB stress, respectively. The highest methylation was observed for the heavy metal stressed *R. farinacea* at 6 h (91.2 %) and for the UV radiations stressed *R. farinacea* at 30 min and 1 h (89.7 %). The lowest levels of methylation polymorphism were detected at 1 h in the heavy metal stressed *R. farinacea* (30 %) and at 12 and 24h in the UV radiations

TABLE 7. Determination of the genotoxic effect after UVA, UVB and UVC radiation treatment in *Ramalina farinacea* using OPC01, OPC02, OPC04, OPC07, OPC10, OPA16 and TubeA05 primers.

UVA											
		4 j		**8 j**		**12j**		**20 j**		**40 j**	
Primer	**C**	**a**	**b**	**a**	**b**	**a**	**b**	**a**	**b**	**a**	**b**
OPC01	**15**	1	2	2	4	1	3	2	3	2	1
OPC02	**15**	1	1	1	1	2	1	1	0	0	3
OPC04	**15**	0	0	2	0	1	1	0	1	0	2
OPC07	**15**	1	1	3	3	1	5	2	4	3	7
OPC10	**15**	1	1	1	1	2	3	0	2	2	2
TubeA05	**15**	1	3	1	0	0	4	3	6	4	5
	90	5	8	10	9	7	17	8	16	11	20
	a+b	**13**		**19**		**24**		**24**		**31**	
UVB											
		4 j		**8 j**		**12 j**		**20 j**		**40 j**	
Primer	**C**	**a**	**b**	**a**	**b**	**a**	**b**	**a**	**b**	**a**	**b**
OPC 01	**15**	2	3	1	3	2	3	3	6	1	4
OPC 02	**15**	1	4	3	5	3	2	3	5	5	6
OPC 04	**15**	2	3	1	3	1	5	7	2	3	7
OPC 07	**15**	1	4	2	3	2	4	4	5	7	4
OPC10	**15**	2	5	3	2	1	2	1	5	2	8
Tube A05	**15**	1	3	0	4	2	3	2	2	2	5
	90	9	22	10	20	11	19	20	28	17	34
	a+b	**31**		**30**		**39**		**48**		**51**	
UVC											
		4 j		**8 j**		**12 j**		**20 j**		**40 j**	
Primer	**C**	**a**	**b**	**a**	**b**	**a**	**b**	**a**	**b**	**a**	**b**
OPC01	**15**	1	2	2	1	1	5	3	5	4	4
OPC02	**15**	1	3	1	3	1	2	1	4	4	3
OPC04	**15**	1	0	1	1	1	2	2	4	2	3
OPA16	**15**	1	1	2	3	1	3	3	1	4	2
TubeA05	**15**	0	0	1	2	2	4	5	6	3	4
	90	4	6	7	10	6	16	14	20	17	16
	a+b	**10**		**17**		**22**		**34**		**33**	

stressed *R. farinacea* (40 %). MSAP - AFLP analysis results were evaluated according to methylation types in samples exposed to heavy metal stress. In this context, the maximum methylation level (33.3%) was observed in Type II and the lowest methylation level (63.3%) was revealed in Type III status. After the first 6 h of heavy metal exposure, there was no change in the type II methylation level, but then there was a change in methylation rate of 34.3% in 12 and 24 h. The highest methylation rate (79.6%) was determined in *R. farinacea* lichen species exposed to 12 j/cm^2 UVB stress. The lowest methylation rate (39.6%) was observed after UV exposure at 12 j/cm^2. As shown in Figure 4, the methylation rate decreased at 12 j/cm^2 and started to increase again in other UV radiations (20 and 40 j/cm^2) applied after this joule value. The differences were observed in methylation status in lichen samples not exposed to UV stress and *R. farinacea* exposed to 12 j/cm^2 UV stress.

The methylation status of 34.3% was determined in *R. farinacea* exposed to 4 j/cm^2. According to the MSAP-AFLP analysis data obtained from UVB stress, Type II methylation level reached the maximum level (71.2%) at the 6th h. While the minimum rate of Type IV methylation (33.6%) was observed in 24 h, the maximum level of Type III methylation (77.2%) was also detected.

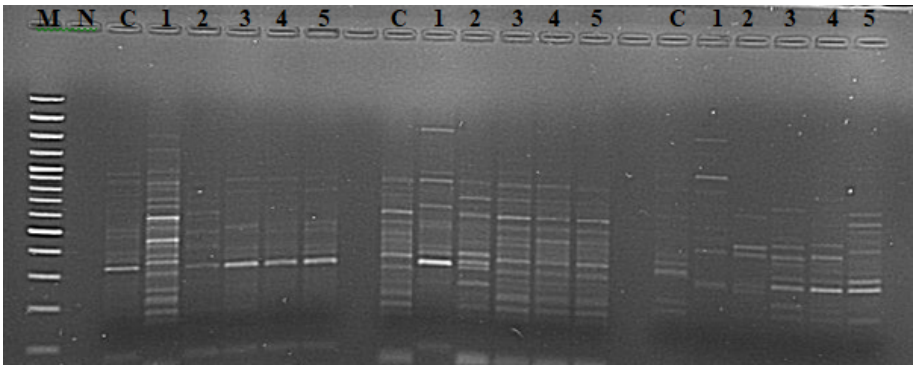

FIGURE 3. The results of RAPD-PCR treating at UVB radiation in *Ramalina farinacea* lichen specimen (left OPC01, center OPC02; right OPC04 primers). (M: Marker, N: Negative control; C: Control, 1: 4 j/cm^2, 2: 8 j/cm^2, 3: 12 j/cm^2, 4: 20 j/cm^2, 5: 40 j/cm^2).

TABLE 8. The rates of GTS values using UV radiations in *R. farinacea* lichen specimen.

	Samples	Rates of GTS (%)		Samples	Rates of GTS (%)		Samples	Rates of GTS (%)
UVA	4 j/cm^2	96.66	UVB	4 j/cm^2	65.55	UVC	4 j/cm^2	88.69
	8 j/cm^2	80.0		8 j/cm^2	66.66		8 j/cm^2	80.86
	12 j/cm^2	75.75		12 j/cm^2	56.66		12 j/cm^2	77.39
	20 j/cm^2	73.33		20 j/cm^2	46.66		20 j/cm^2	67.82
	40 j/cm^2	62.42		40 j/cm^2	43.33		40 j/cm^2	62.60
	Samples	**Rates of GTS (%)**		**Samples**	**Rates of GTS (%)**		**Samples**	**Rates of GTS (%)**
UVD	4 j/cm^2	81.33	UVA+UVB	4 j/cm^2	66.66	UVA+UVD	4 j/cm^2	84.76
	8 j/cm^2	80.0		8 j/cm^2	53.33		8 j/cm^2	79.04
	12 j/cm^2	70.66		12 j/cm^2	53.33		12 j/cm^2	72.38
	20 j/cm^2	60.0		20 j/cm^2	33.33		20 j/cm^2	68.57
	40 j/cm^2	54.66		40 j/cm^2	26.66		40 j/cm^2	65.71

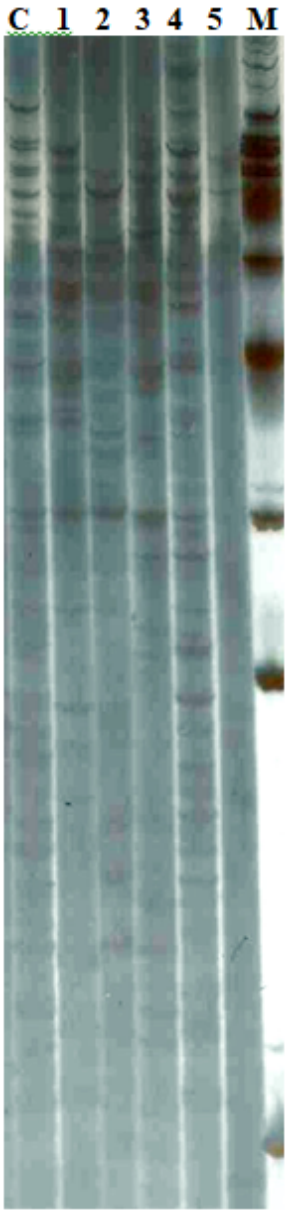

FIGURE 4. AFLP profiles resulting from Type II methylation in *Ramalina farinacea* lichen species exposed to UVB radiation. (M: Marker, C: Control, 1: 4 j/cm^2, 2: 8 j/cm^2, 3: 12 j/cm^2, 4: 20 j/cm^2, 5: 40 j/cm^2).

4. DISCUSSION

Environmental stressors such as heavy metal pollution can induce mutations and toxic effects, leading to the disruption of DNA integrity [40]. Therefore, the determination of DNA damage in terrestrial organisms living in polluted areas is a considerable exponent in ecotoxicology studies [6,41]. As a result of the comparing of band differences of RAPD and Coupled Restriction Enzyme Digestion-Random Amplification (CRED-RA) analysis, it was determined that the rate of change in the methylation model of the samples exposed to heavy metal stress varies more than the samples exposed to UV radiation. It was observed in the present study that the samples with heavy metal stress had a high GTS rate in the RAPD-PCR results, but the GTS rate decreased due to increasing radiation doses in the samples with UV radiation. Accordingly, the effect of UV stress on lichen DNA stability is genetically thought to be higher than heavy metals. On the other hand, heavy metal samples are epigenetically considered to have less stability of the methylation pattern.

Several studies have been published to determine the genotoxicity with DNA molecular markers of heavy metals exposure in lichens. Hamutoğlu et al. [6] observed that there were 19, 45 and 51 bands in *P. furfuracea* (control band number 83), respectively, after the 1st, 2nd and 3rd regions, located 50, 100 and 200m away from the cement factory that was exposed to contaminants. Sorrentino et al. [42] investigated with ISSR molecular markers in moss *Sphagnum palustre* for showing both Cd and Pb salts a genotoxic effect in a dose-dependent manner. They observed a total of 169 reproducible bands using 12 primers, ten of which yielded polymorphisms, pointing out a clear genotoxic effect caused by the metals. Batir et al. [43] determined the effect of different concentrations of copper (Cu) solutions on maize (*Zea mays* L.) seedlings by using physiological parameters and RAPD analysis. In this study revealed band increase and or loss in the RAPD profiles of the samples. They found that the RAPD band profiles of the samples and the GTS ratios were compatible with each other. Our study significantly determined the maximum change of band intensities. The highest number of the band that appeared and disappeared was observed in the *R. farinacea* for 120 mg/L. We determined a total of 42 bands using three primers to evaluate Cr^{6+} heavy metal samples in *R. farinacea*. It could be easily said that these species could be used as bioindicator organisms for genotoxicity. As a result of the present study, it could be used as a novel organism for remediation of polluted sites.

Bajpai et al. [44] revealed that the exposure of Cr^{6+} (0, 10, 25, 50, 75, and 100 μM) for several days under controlled conditions caused a major reduction in physiological parameters with increasing metal stress in *Pxine coces* lichen

specimen. They pointed out that genetic changes could be used as a tool to investigate ecological stress and polymorphisms because of genotoxicity [44]. When this lichen specimen was compared with *R. farinacea* according to their genotoxic capacity and band differences, *R. farinacea* was a higher accumulator of Cr^{+6} than *P. coces*. In the RAPD band profile created after exposure to Cr^{+6} heavy metal in *R.farinacea* lichen specimen, differences in the number of band were detected compared to the control.

As observed in the present study that *R. farinacea* has a considerably higher accumulation compare to the other organisms studied on heavy metal accumulation and this result is compatible with many remarkable studies in the literature [10,45,46,47]. In our previous study, *Hypogymina physodes* lichen specimen was evaluated with the genotoxic effect of pollutants. It was concluded that changes in RAPD assay and DNA methylation analysis observed that homologous nucleotide sequences in the genome from untreated and treated species with pollutants showed different band and methylation patterns [16]. It revealed that thallus heavy metal accumulation in the contaminated areas was particularly higher for Cr^{6+}, Cd^{2+} and Pb^{2+}, and UV radiations compared to the control [16]. However, it was determined that *R. farinacea* had more heavy metal accumulation capacity than *H. physodes*. Chetia et al. [48] also reported that Pb, Cd, Zn, Cu, Co, Ni and Cr concentrations were found to be higher in the lichen species collected from the polluted areas compared to the control. This may be due to the wider surface of the lichen tallus.

Zulaini et al. [5] evaluated two lichen species as a bioindicator for the accumulation of heavy elements in Malaysia. Their results imply that *P. tinctorum* was found suitable bioindicator of air pollution due to the higher capability to accumulate heavy metals compared to *U. diffracta*. Sujetoviene et al. [10] also investigated the physiological response of lichens *Evernia prunastri* and *R. farinacea*, which were transported near a landfill in the center of Lithuania, and evaluated airborne contamination of heavy metals using these lichens. They found that the concentrations of heavy metals (Cd, Fe, Cr, Mn) varied between the study sites. The study showed an increased accumulation of some heavy metals in lichens transplanted to the sites downwind from the factory. The measured values in the samples collected in the study sites showed moderate air pollution. In the current study, we showed that we collected lichen samples from the clean area and treated with different heavy metal. In particular, Cr^{6+}, Cd^{2+}, and Pb^{2+} examined at the highest concentrations caused a GTS reduction of about 25-40 %. This result determined us the level of genotoxic effect depending on GTS level.

It is cellular DNA that is most affected by UV radiation [49] and UV radiation mainly causes mutations to occur in DNA [49, 50]. The RAPD-PCR assay is applied to analyze the genetic damage caused by UV and X-rays in plants and macroalgae species [51]. The results obtained show that determining the genotoxic effect with the RAPD and MSAP-AFLP techniques in *R. farinacea* has enabled us to get detailed information on the level of pollution.

Garty et al. [52] evaluated the effects of UV-B radiation combined with $NaHSO_3$ solution on stress ethylene production of two lichen species, including the same genus, under laboratory conditions. *Ramalina lacera* was found to be more sensitive to the effect of UV-B radiation combined with $NaHSO_3$ compared to *R. maciformis*, an epiphytic Mediterranean lichen. The adaptation of *R. maciformis* to UV-B radiation appears to be related to the photoreactive abilities of lichen components. The findings show that *R. lacera* is in greater danger due to the lack of photoreactive components involved in UV-B radiation compared to *R. maciformis*. *R. lacera* has been found to be less at risk from intense air pollution and severe UV-B radiation increase than *R. maciformis*. With the data obtained as a result of Garty's study [52] and our study, it was confirmed that both epiphytic lichen species that belong to *Ramalina* genus are a good bioindicator organism in determining the genotoxic effect.

5. CONCLUSION

This study determined that the *R. farinacea* lichen sample can be predicted as a key organism to monitor the genotoxic effect at the molecular level against different stress sources. It is the first study to determine the genotoxic effect by using *R. farinaceae*, which is a cheap, more easily obtainable, useful biological organism to determine the negative effects of environmental pollutants. However, the results of the study need to be investigated in detail with the use of advanced molecular biological methods.

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Declaration of Competing Interests The authors declare no conflict of interest.

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HIGHLIGHTING THE MELISSOPALYNOLOGICAL AND PHYSICOCHEMICAL CHARACTERISTICS OF AYDER-RİZE (TURKEY)

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ABSTRACT. The aim of this research is characterizing the honey produced in Ayder/Rize-Turkey. In this context 20 honey samples were collected from Ayder in 2018. The melissopalynological and physicochemical analysis of the honey samples were done by this research. According to the melissopalynological analysis 40 taxa belong to the 22 plant families were identified as botanical sources of the investigated honey samples. Nine of the investigated samples (sample no 1-9) were evaluated as monofloral and the others as multifloral honeys. As a result of melissopalynological analysis, while pollen belong to the C*astanea sativa* taxon were found in dominant ratios in some of the investigated samples, the pollen belongs to the *Castanea sativa, Trifolium repens, Lotus corniculatus, Coronilla orientalis* taxa were found as secondary in some other honey samples. Total pollen number in 10 gram honey (TPN 10) values of the samples were found between 7 732 and 167 147 by melissopalynological analysis. The first step of the physicochemical analsyis was the moisture analysis and the values found between 15,8% and 18,8% (mean value:7,01±0,98 %). The fructose/glucose analysis were done by High Performance Liquid Chromatography (HPLC) and the values for fructose found as: 26,43-35,57g/100g (mean: 31,39±2,41 g/100g), glucose values as; 20,11-30,58 g/100g (mean: 26,83±2,16 g/100g). Also fructose/glucose ratio was found as 1,03-1,34 (mean: 1,17±0,10). Hydroxymethylfurfural (HMF) and total phenolic acids analysis were done also by HPLC. The results for HMF analysis is between 0,7- 11,31 ppm (mean: 3,8±2,6 ppm), for total phenolic acids the value are found between 88,92±0,04 mgGAE/kg - 196,17±0,10 mgGAE/kg (mean: 121,98±0,1 mgGAE/kg). As a step of chemical analysis; the volatile compounds of the honey samples were determined by Gas Chromatography and Mass

Keyword and phrases. Honey, melissopalynology, TPN10, HMF, GC-MS, total phenolic

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Spectrometry (GC-MS). The compounds belong to the aldehydes, aliphatic acids and esters, alcohols, hydrocarbons, carboxylic acids and their esters, ketones, terpenes, fatty acids and their esters groups were found as a result of GC-MS analysis. The compounds belong to the carboxylic acids and their esters, fatty acids and their esters identified in higher ratios compare to the other compounds. Since, there is little detailed published information about the quality and properties of Ayder-Rize honey, the current study aims to characterize the honey belong to the this region.

1. INTRODUCTION

Honey is a natural product that is produced by honey bees, especially by the species of *Apis mellifera.* Two types of honey is present: one kind as blossom honey comes from nectars of flowers and the second kind as honeydew honey (forest honey) is a type of honey made from honeydew secreted by plant-sucking insects such as aphids [1].

The chemical composition of honey is variable, owing to the numerous parameters. Its constituents are carbohydrates, water, organic acids, enzymes, amino acids, pigments, pollen and wax; some are added by the bees and some of them are sourced from the plants [2].

Sugars are the major components of honey. It consists mostly glucose and fructose [2]. Honeydew honeys have lower contents of glucose and fructose while have higher levels of oligosaccharides [3].

In many countries, honey is considered more as a medicine or special tonic, rather than as a food. Honey has medicinal properties that are acknowledged increasingly by modern medicine. Besides, honey is used as a source of sugars for making honey wines and beers, and in the manufacture of many secondary products: breakfast cereals, bakery goods, and a multitude of other value-added products. It is also, applied to wounds, burns, ulcers and promotes faster healing [4].

As is seen from the literature; there are so many published scientific researches about Turkish honey. The researches mainly based on characterization of honey on the basis of region or qualified the honey types according to their melissopalynological and physicochemical features. The studies in Turkey that contains melissopalynological analysis of honey samples began with Quistani [5-11].

Nowadays, the researches are mostly comprised the physicochemical analysis of honey samples collected from different regions of Turkey. Can et al. (2015)

investigated 62 Turkish honey samples (11 unifloral honeys; chestnut, heather, chaste tree, *Rhododendron*, common eryngo, lavender, Jerusalem tea, *Astragalus*, clover and *Acacia*, two different honeydew honeys; lime and oak and seven different multifloral honeys) on the basis of physico-chemical and biochemical characteristics. They mentioned that physico-chemical and biological characteristics of honeys are closely related to their floral sources [12].

Kıvrak et al. (2017) investigated the 54 honey samples from eighteen different locations of Turkey (cedar from Konya, eucalyptus from Muğla, multifloral from Hakkari, *Rhododendro*n from Kastamonu, *Vitex* from Aydın, carob from Muğla, clover from Diyarbakır, pine and heather from Muğla, sunflower from Konya, citrus from Antalya, *Sideritis* and thyme from Muğla, chestnut from Düzce, *Acacia* from Burdur, lavender from Isparta, cotton from Adana, linden from Artvin) [13].

Derebaşı et al. (2014) searched characteristics of honey samples collected from different cities of Black Sea Region of Turkey. Owing to the physicochemical results, they mentioned that Black Sea Region honeys indicate a good quality level, adequate processing, good maturity and freshness [14].

Malkoç et al. (2019) evaluated the honey samples collected from Anzer-Rize according to the melissopalynological analysis, total phenolic contents (TPC), total flavonoid contents (TFC), and total antioxidant activities [15].

Kanbur et al. (2021) searched the physicochemical parameter changes, aroma, melissopalynological properties, and heavy metal content of honey produced from different types of flora (chestnut and highland) in the Senoz Valley from Rize [17].

Despite so many scientific researches are existing about Turkish honey and especially produced in Anzer plateu - Black Sea Region of Turkey, there are little scientific publications about honey produced in Ayder plateu of Rize-Turkey [10,14,15,16,17].

The aim of this study was characterizing the honey samples collected from Ayder plateu of Rize-Turkey according to their botanical sources and physochemical characteristics. Ayder plateu has an important role for Turkish beekeeping owing to its virgin nature, floral circumstances and climatical conditions. Since there is little detailed data is available about the honey of Ayder plateu, the results will be a data source for the region.

2. MATERIALS AND METHODS

2.1. Honey samples

Honey samples were collected from Ayder-Rize that is located in the East of Black Sea Region. Twenty honey samples were collected between August- September of 2018 from different localities (Köy location-sample no 1-5, Kedmeç location-sample no 6-10, Galer location - sample no 11-15, Yayla location- sample no 16-20) of Ayder plateu/ Rize (Black Sea Region of Turkey) (Figure 1).

FIGURE 1. The map of Ayder plateu (http://gezilecekyerler.com/camlihemsin/)

2.2. Melissopalynological analysis

Preparation of honey samples for qualitative and quantitative mellisopalynological analysis was performed by Louveaux et al. (1978) [18]. The total pollen number (TPN) of all samples was calculated according to the method described by Moar (1985) [19]. The honey samples (10g) were classified according to total pollen number (TPN) as Group I: TPN<20000; Group II: 20000<TPN<100000; Group III: 100000<TPN<500000; Group IV: 500000<TPN<1000000 and Group V: TPN>1000000 [19]. The terms used for the evolution of the frequency classes in honeys were: dominant pollen (>45%), secondary pollen (16-45%), minor pollen (3-15%), and trace pollen (<3%) [20].

2.3. Physicochemical characterization of honey

Moisture Measurement

Moisture analyses were done by a portable refractometer and determined as % (w/v) ratio.

GC-MS Analysis

A GC 6890 N instrument from Agilent (Palo Alto, CA, USA) coupled with a mass detector (MS5973;Agilent) was used for the analysis of honey samples. Organic compounds in honey samples were identified in Wiley's NIST Mass Spectral Library, if they obtained comparison scores were higher than 95%.

Sugar Analysis

Sugar (Fructose/glucose) content was determined according to the harmonised methods of international honey commission's suggestions (2009). The samples were analysed by HPLC (Agilent Technologies, USA) with RID detector (Agilent Technologies, USA) and Zorbax (4.6x250mm, 5-Micron) carbohydrate column (Agilent Technologies, USA) [21].

HMF Analysis

HMF content were analysed by HPLC. It was determined according to the harmonised methods of international honey commission's suggestions (2009). The samples analysed by HPLC (Agilent Technologies, USA) with UV detector Agilent Technologies, USA) and C18-reversed phase column (Agilent Technologies, USA) [21].

Total Phenolic Compound Estimation

Total phenolic compound of honey extracts estimated according to the Folin-Ciocalteu method described by Slinkard and Singleton (1977). The absorbances of samples were measured at 760 nm with UV/VIS spectrophotometer [22].

3. RESULTS AND DISCUSSION

3.1 Melissopalynological analysis results

By palynological analysis of the investigated honey samples the pollen of taxa belong to the Apiaceae, Asteraceae, Berberidaceae, Betulaceae, Boraginaceae, Brassicaceae, Campanulaceae, Caryophyllaceae, Cistaceae, Cyperaceae, Ericaceae, Fabaceae, Fagaceae, Lamiaceae, Plantaginaceae, Poaceae, Polygonaceae, Ranunculaceae, Rosaceae, Rubiaceae and Salicaceae families were identified (Table 1).

Nine of the 20 samples were found as chesnut honey and the other 11 samples were evaluated as multifloral honey. With regard to the results, all investigated five samples of Köy location were detected as monofloral–chesnut honey. Five samples were collected from Kedmeç location and four of the samples qualified as monofloral-chesnut honey and the last one as multifloral. Five samples were collected from each Galer and Yayla locations and the samples were defined as multifloral.

Total pollen number in 10 gram honey (TPN 10) values of the samples were found between 7 732 and 167 147 by melissopalynological analysis (Table 1).

3.2 Physicochemical analysis results

Moisture analysis

The moisture values of the investigated samples were found between 15.8-18.8% (mean:17.01%) (Table 2). These results are suited to Codex Alimentarius (2001) and Turkish Food Codex, Honey Directive (2020) [23,24].

Sugar content analysis

According to the sugar analysis, the values for fructose found as: 26,43-35,57g/100g (mean: 31,39±2,41 g/100g), glucose values as; 20,11-30,58 g/100g (mean: 26,83±2,16 g/100g). Also the fructose/glucose ratios were found between 1.03-1.34 (mean:1.17) (Table 2). These results fit with the sugar values that is mentioned by Codex Alimentarius (2001) and Turkish Food Codex, Honey Directive (2020) [23,24].

Total phenolic contents

The total phenolic content of the samples are measured as minimum 88,92±0,04 mgGAE/kg and maximum 196,17±0,10 mgGAE/kg (mean: 121,98±0,1 mgGAE/kg) (Table 2).

HMF analysis

The HMF values are determined as minimum 0,7 ppm, maximum 11,31 ppm (mean: 3,8±2,6 ppm) (Table 2). Since, Codex Alimentarius (2001) and Turkish Food Codex, Honey Directive (2020) allow until 40 ppm, our findings are suitable in terms of HMF values.

GC-MS analysis

The compounds belong to the aldehydes, aliphatic acids and esters, alcohols, hydrocarbons, carboxylic acids and their esters, ketones, terpenes, fatty acids and their ester groups were found as a result of GC-MS analysis. The compound belong to the carboxylic acids and their esters, fatty acids and their esters identified in higher ratios compare to the other compounds (Table 3).

Owing to the palynological analysis, taxa belong to the Apiaceae, Asteraceae, Berberidaceae, Betulaceae, Boraginaceae, Brassicaceae, Campanulaceae, Caryophyllaceae, Cyperaceae, Ericaceae, Fabaceae, Fagaceae, Lamiaceae, Plantaginaceae, Poaceae, Polygonaceae, Ranunculaceae, Rosaceae, Rubiaceae and Salicaceae families were found as botanical sources of honey samples. These findings overlap with the flora of the study area. To confirm the palynological observations, plant samples were collected from the environments of the apiaries that samples collected during the field study. Our results are compatible with this plant list too.

Demir (2013) investigated 41 honey samples from Ayder-Rize and 21 samples from Kedmeç location. By this research, the pollen of taxa belong to the Apiaceae, Asteraceae, Boraginaceae, Brassicaceae, Caryophyllaceae, Chenopodiaceae, Ericaceae, Fabaceae, Fagaceae, Gentianaceae, Geraniaceae, Lamiaceae, Lauraceae, Malvaceae, Polygonaceae, Primulaceae, Ranunculaceae, Rosaceae, Salicaceae, Scrophulariaceae families were found in honey samples of Kedmeç location. These palynological findings are similiar with our results [25].

Also, Demir (2013) mentioned that all the samples are chesnut honey owing to dominant *Castanea sativa* pollen contents in honey samples. Similarly, we evaluated, four of five Kedmeç samples as chesnut honey [25].

Malkoç (2019) mentioned that honey samples from Anzer-Rize contains pollens from different family types at levels less than 45%, including; Lamiaceae, Fabaceae, Apiaceae, Rocaceae, Asteracea, Ericaceaa, and Liliaceae. Also mostly detected pollen defined as *Thymus, Rumex, Onobrychis, Cistus, Plantago, Ranunculus, Rhododendron, Myosotis* and *Geranium* [15]. In our research, the families contain determined pollens are very similiar with the results of Malkoç (2019) owing to the research area.

As a part of melissopalynological analysis, we calculated the "total pollen number in 10 gr honey (TPN 10) values are between 7732 and 167147 by melissopalynological analysis. Demir (2013) observed these values between 3 438-85 285 for honey samples collected from Kedmeç location. Our TPN 10 values for Kedmeç honeys are found between 11 046 - 36 247. Also Demir (2013) mentioned the identified taxa number vary from 4 to 14 for Kedmeç samples. We found this rate between 5-8 [25].

Moisture rate of the honey gives an idea about the harvesting of the honey. With regard to our results, the moisture values are between 15.8-18.8% (mean:17.01%). These results are suited to Codex Alimentarius (2001) and Turkish Food Codex, Honey Directive (2020). By previous researches; moisture of chesnut honey found as 19.70±1.33%, 16.21% [12, 13], and also Kedmeç-Ayder honey (evaluated as chesnut honey) as 16.6-20.6% [25]. Derebaşı et al. (2014) found the mean moisture value as 16.6±0.12% from different honey samples of Black Sea Region-Turkey and 18.57±0.72% for honey samples collected from Rize [14].

Hydroxymethylfurfural (HMF) content is a marker for freshness and overheating of honey (Book of honey 2009). According to Codex Alimentarius (2001) and Turkish Food Codex, Honey Directive (2020), permitted value for HMF is maximum 40 ppm. Our HMF results are in this limitation. By previous researches HMF values for chesnut honey were found as 9.28±7.13 ppm, 1.66ppm [12,13] and of honey samples collected from Rize as 9.19±1.12ppm, as 8.86±0.38 ppm from different honey samples of Black Sea Region-Turkey [14].

Some previous researches about total phenolic contents of chesnut honey reflect the values as; 98.26±1.77 mgGAE/100g, 97,66 mgGAE/100g, 430±68 mgGAE/100g [12,13,17], also honey samples from Anzer as 240±52 mg GAE/100g [17]. Malkoç

et al. (2019) found the total mean phenolic content of Anzer honey as 26.92 mg GAE/100 g [15]. Hepsağ (2019) found the total phenolic content of the honey samples from Anzer plateau in Rize between 802.6- 1352.6 μg GAE / g honey [26]. The total phenolic content of the samples are measured in our research as minimum 88,92±0,04 mgGAE/kg and maximum 196,17±0,10 mgGAE/kg (mean: 121,98±0,1 mgGAE/kg). It is clear that from the results our findings lower than the values from the previous researches.

The investigation about the invert sugar of honey samples collected from Black Sea Region of Turkey found for; Rize honey as 72.874±2.12%, fructose of chesnut honey as: 38.44±2.72% , glucose of chesnut honey as: 19.35±3 % fructose of Kedmeç-Ayder honey as 16.31-65.28 %, glucose of Kedmeç-Ayder honey as 17.23-64.47 % [12,14,25].

The biological effects of the Black Sea Region honey also searched and Çakır et al. (2020) found that Anzer – Rize honey effected on *Staphylococcus aureus*, *Saccharomyces cerevisiae* and *Escherichia coli* [27].

4. CONCLUSION

As understood from the literature there are so many researches about characterization of honey collected from Black Sea Region of Turkey. While there are so many investigation about Anzer honey (Rize-Black Sea Region of Turkey), there are limited researches about honey collected from Ayder (Rize- Black Sea Region of Turkey). So our findings will be helpful for characterizing Ayder honey and light the way for the geographical indication surveys of Ayder honey.

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Author Contribution Statement Ö.G.Ç., A.Ö., Ç.Ö., and N.M performed the analysis, K.S., A.Ö., Ç.Ö., G.Z., N.M. carried out the field study, Ö.G.Ç. wrote the manuscript, All authors reviewed the manuscript

Declaration of Competing Interests The authors declare no conflict of interest.

Table 1. Melissopalynological analysis results of investigated honey samples

			Honey Number																		
			Köy location					Kedmeç location					Galer location					Yayla location				
Taxa no	Plant fanily	Plant taxon	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
1	Apiaceae											M										
2	Asteraceae	*Anthemis* spp.										T		M	M	M	T					
3		*Centaurea spp.*																		T		
4		*Petasites albus*											T									
5		*Tanacetum sorbifolium*											M									
6		*Taraxacum spp.*										T					M		T			
7	Berberidaceae	*Berberis* spp.																T				
8	Betulaceae	*Betula* spp.									T						T					
9	Boraginaceae	*Cerinthe* spp.															T					
10		*Myosotis* spp.							T									M	M	T	M	M
11	Brassicaceae													T					T			
12		*Cardamine impatiens*			T													T				
13	Campanulaceae	*Campanula latifolia*																T				
14	Caryophyllaceae																		M		T	
15	Cistaceae									T								T				
16	Cyperaceae	*Carex* spp.													T							
17		*Epigaea gaultherioides*										M										
18	Ericaceae	*Rhododendron* spp.	T				T	T	M	T	T		M	M	M	M	M	M	T	M	M	M
19	Fabaceae																			M		
20		*Trifolium repens*	T		T	T	T	T	T			T	M	M	M	M	M	M	S	M	S	S
21		*Coronilla orientalis*																S				
22		*Lotus corniculatus*									M	T		T	T		M				T	S
23		*Medicago* spp.												T						T		M

Table 1. continued.

			Honey Number																		
			Köy location					Kedmeç location					Galer location					Yayla location				
Taxa no	**Plant family**	**Plant taxon**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**
24	Fagaceae	*Castanea sativa*	D	D	D	D	D	D	D	D	D	S	S	S	S	S	S	E	S	S	M	M
25	Lamiaceae	*Ajuga orientalis*																M				
26		*Nepeta* spp.									T								T	T		
27		*Teucrium spp.*																		T		
28		*Thymus praecox*																T				
29	Plantaginaceae	*Plantago lanceolata*						T												T		
30	Poaceae									T												
31	Polygonaceae	*Rumex* spp.												T	T		T	M			M	M
32	Ranunculaceae	*Ranunculus spp.*										T								T	M	
33	Rosaceae	*Fragaria vesca*																T				
34		*Prunus* spp.											E									
35		*Rubus idaeus*		T				T	T	T			M	M	M	M	M		M	T	T	M
36		*Rosa* spp.							T													
37													T					M				
38	Rubiaceae	*Galium* spp.												T						T		
39	Salicaceae	*Salix* spp.	T										M	M	T	T				T		
40	Tiliaceae	*Tilia* spp.		T	T	T	T															
	TPN10 values		53083	1E+05	89729	2E+05	46099	11 192	36247	30848	16110	11046	8 699	9 205	21089	15920	20878	18043	**7 732**	28998	19332	12888

TABLE 2. Physicochemical analysis results of honey samples

Honey number	Location	Moisture (%)	Fructose (g/100g)	Glucose (g/100g)	Fructose/ Glucose	HMF (ppm)	Total phenolic content (mgGAE/kg)
1	KÖY	16,3	35,57	26,54	1,34	6,5	178,91±0,32
2	KÖY	16,1	33,95	25,59	1,32	8,6	164,12±0,17
3	KÖY	16,5	33,61	26,42	1,27	5,3	179,63±0,20
4	KÖY	16,6	33,13	24,84	1,33	3,4	186,42±0,15
5	KÖY	16,6	31,92	26,08	1,22	3,7	196,17±0,10
6	KEDMEÇ	16,3	32,97	28,55	1,15	1,6	112,06±0,08
7	KEDMEÇ	16,2	30,00	24,97	1,20	1,5	108,04±0,08
8	KEDMEÇ	16,2	31,44	27,36	1,14	4,4	104,33±0,09
9	KEDMEÇ	15,8	28,86	27,81	1,03	1,9	99,52±0,06
10	KEDMEÇ	16,0	31,09	29,71	1,04	1,6	106,93±0,02
11	GALER	18,7	26,43	20,11	1,31	11,31	104,58±0,08
12	GALER	18,3	30,03	27,25	1,10	0,7	104,41±0,01
13	GALER	18,8	29,18	26,86	1,08	3,1	95,85±0,13
14	GALER	18,7	28,57	27,60	1,03	1,7	116,09±0,03
15	GALER	18,3	28,14	25,43	1,10	1,6	105,29±0,08
16	YAYLA	16,9	35,32	28,41	1,24	3,2	97,01±0,10
17	YAYLA	17,0	32,80	28,34	1,15	6,2	88,92±0,04
18	YAYLA	17,0	32,16	26,96	1,19	4,4	94,35±0,02
19	YAYLA	16,8	31,02	27,19	1,14	3,1	93,44±0,05
20	YAYLA	17,1	31,66	30,58	1,03	1,7	103,55±0,19
	Mean value	**17,01±0,98**	**31,39±2,41**	**26,83±2,16**	**1,17±0,10**	**3,8±2,6**	**121,98±0,1**

TABLE 3. GC-MS analysis results of investigated honey samples (% ratio)

Chemical compounds	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
Aldehydes	5.85	5.73	5.63	5.53	4.4	7.03	3.31	7.26	2.49	1.68	5.33	2.72	2.63	3.68	7.1	6.43	2.43	5.4	1.52	0.98
Aliphatic acids and esters	8.89	3.53	1.77	10.34	3.52	3.56	3.95	5.6	4.4	1.46	9.17	0.09	4.21	2.87	6.85	5.63	3.99	3.21	3.65	2.85
Alcohols	5.02	3.57	6.86	6.7	3.32	3.58	2.19	5.21	4.79	2.96	5.91	7.74	3.32	6.89	0.25	4.5	7.46	3.45	2.1	3.32
Hydrocarbons	1.47	1.2	4.51	0.17	2.27	6.28	0.26	0.12	1.15	2.09	3.43	2.05	0.19	4.74	-	0.13	0.28	2.64	0.2	0.32
Carboxylic acids and their esters	22.24	21.88	19.52	25.57	19.53	25.34	17.34	23.22	33.27	22.28	11.25	17.06	17.98	26.59	16.29	40.13	14.34	18.72	20.14	24.85
Ketones	6.9	3.25	6.22	7.21	11.95	8.18	4.02	0.91	8.73	7.09	5.51	31	4.98	8.46	8.69	6.16	9.58	6.62	29.23	10.15
Terpenes	0.72	0.44	4.36	0	0.85	1.8	2.61	1.79	2.23	1.92	3.91	0.92	2.16	0.88	2.34	6.52	2.04	1.91	2.21	-
Fatty acids	8.99	3.22	8.78	13.51	16.76	19.12	11.32	15.24	20.03	13	33.75	7.2	13.91	16.75	29.46	9.67	15.49	11.73	10.34	12.39

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IN SILICO PROOFS FOR PHLORIDZIN, NARINGENIN, AND CINNAMIC ACID AS ALPHA-AMYLASE ACTIVATORS, WHICH IS IMPORTANT IN INDUSTRIAL MICROBIOLOGY OR BIOCHEMICAL ENGINEERING

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ABSTRACT. Enzymes are commonly defined as biological catalysts, regulating particular biochemical reactions. α-Amylase (EC 3.2.1.1) is one of the industrially important enzymes, which are extensively used in starch hydrolyzing processes, such as brewing, fermentation, detergent production, food processing, etc. This enzyme breaks down α-1,4 glycosidic bonds in amylose or amylopectin. The end products from amylose are maltotriose and maltose. Maltose, glucose, and limit dextrin are formed from amylopectin. There are many studies in the literature regarding the α-amylase inhibitors, which have the potentials of being used in diabetes and obesity. However, there is a very limited number of studies in the literature about the activation of this enzyme, which could be harmful to such diseases. This study aims to support the activation activity of phloridzin, naringenin, and cinnamic acid for α-amylase, which was previously proved experimentally, with some *in silico* tests.

1. INTRODUCTION

Enzymes are commonly defined as biological catalysts, regulating particular biochemical reactions. α-Amylase (EC 3.2.1.1) is one of the industrially important enzymes, which are extensively used in starch hydrolyzing processes, such as brewing, fermentation, detergent production, food processing, etc. [1,2]. This enzyme breaks down α-1,4 glycosidic bonds in amylose or amylopectin chains [3]. α-Amylase cleaves long polysaccharide chains. The end products from amylose are maltotriose and maltose. Maltose, glucose, and limit dextrin are formed from amylopectin. [2].

Keyword and phrases. Alpha amylase, activator, phloridzin, naringenin, cinnamic acid, *in silico*

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Several industrially important crops, such as potato, maize, rice, and wheat, contain starch as a storage polysaccharide. The industries based on a large scale of starch processing increased in the last century. As a reason for this, the importance of these crops increased too.

α-Amylase has several applications in the industry. And the bread industry is one of them. In the bread industry, right after the dough was prepared, a fermentation process starts. In this process, the yeasts convert sugar to alcohol and CO_2. This process helps the dough to rise. In the process of preparing the dough, adding α-amylase will hydrolyze the starch present in the wheat flour. This hydrolyzation directly affects the fermentation process. It leads to an improvement in the taste, the volume of bread, the texture of crumbs, toasting qualities, and the crust color [4,5].

α-Amylase is widely used in the production of glucose and fructose syrups, specifically at the liquefaction step to partially hydrolyze starch into short-chain dextrins, so that the viscosity of the starch suspension is reduced [5,6].

Besides, α-amylase is also used in the paper industry. During the paper production process, starch is used to coat paper for increasing strength and smoothness, and to ease writing and erasability. However, the viscosity of the natural starch suspension is considerably high and α-amylase is used to decrease the viscosity of the starch solution to make it suitable for a continuous paper production process [6,7].

Enzymes have been commonly using in detergents for laundry and dishwashing for a long time. Because they can considerably improve the potential of the detergent to remove the stain. The aim of using α-amylase in detergents is to digest food particles containing starch to form smaller oligosaccharides soluble in water [8].

Starch obtained from potatoes or corn is frequently used in ethyl alcohol production and using α-amylase converts starch into fermentable sugars. Adding this enzyme increases the rate of conversion for microorganisms producing their starch degrading enzymes and it is necessary for microorganisms, which are not able to produce any starch decomposing enzyme by themselves, to utilize this carbon source for alcohol production [9]. Since ethyl alcohol is the most commonly used liquid biofuel, α-amylase also has great importance in producing fuel alcohol [10].

There are many studies in the literature regarding the α-amylase inhibitors because they have the potentials of being used in diabetes and obesity. However, there is a very limited number of studies in the literature about the activation of this enzyme,

which could be harmful in diseases such as diabetes and obesity [11]. One of these studies shows that phloridzin, naringenin, and cinnamic acid, which are plant secondary metabolites, are acting as α-amylase activators [12].

Although α-amylase inhibitors are proposed to have great importance in the literature, it is clear that the activation of α-amylase could potentially lead to improvements in several industrial processes.

This study aims to support the activation activity of phloridzin, naringenin, and cinnamic acid for α-amylase, which was previously proved experimentally, with some *in silico* tests.

2. MATERIALS AND METHODS

2.1 Target enzyme preparation

The X-ray crystal structure of human salivary amylase (PDB ID: 1SMD) [13] was downloaded from the Protein Data Bank (http://www.rcsb.org/structure/1SMD). In the structure of amylase, a Cl^- ion and a Ca^{2+} ion were present. The Cl^- ion was interacting with Arg195, Asn298, and Arg337. The Ca^{2+} ion was interacting with Asnl00, Arg158, Asp167, and His201. Therefore, before performing molecular docking, Cl^- ion, Ca^{2+} ion, and water molecules in the structure of the enzyme were deleted from the X-ray crystal structure of the enzyme by Discovery Studio Visualizer v.20.1.0.19295 [14]. The 3D structure of the enzyme was further modified by adding charges and H atoms by AutoDock 4.2 [15].

2.2 Compound preparation

The 3D structures of phloridzin, naringenin, and cinnamic acid were downloaded from PubChem (National Institute of Health). Their structures were protonated at pH 7.4, Gasteiger charges, and 3D coordinates were assigned by Open Babel v.3.1.1 [16,17].

2.3 Prediction and analysis of active sites

The pockets and their amino acid sequences were determined by CASTp v.3.0 [18]. The potential pockets were also visualized by UCSF Chimera v.1.14 software [19]. Potential binding pockets, which are suitable for the interaction between the enzyme and the activators, obtained from CASTp were used in molecular docking analysis.

2.4 Virtual screening by molecular docking

To obtain possible docking conformations and orientations of phloridzin, naringenin, and cinnamic acid, when binding to α-amylase, virtual screening by molecular docking was performed by AutoDock 4.2 [15]. The best binding location and position of the compounds were determined by their binding affinities.

In this process, firstly a virtual screening for compounds was performed by rigid molecular docking with a grid box covering the possible pocket. The central coordinates grid box was set to 8.349, 58.709, and 19.099 with a grid point spacing of 0.450 Å. In performing molecular docking, phloridzin, naringenin, and cinnamic acid kept flexible, while α-amylase was rigid. As a final point, the lowest binding energies of all possible conformations and orientations were chosen for further analysis.

In addition to AutoDock 4.2, the interaction scores of phloridzin, naringenin, and cinnamic acid were determined by iGEMDOCK v.2.1 (Graphical Drug Design System for Docking, Screening, and Post-analysis) [20].

2.5 Visualization

The 2D interactions of the compounds with α-amylase were analyzed by using LigPlot+ v.2.2 software [21]. This software gives better visualization to understand the nature of interactions between the compound and enzyme in the docking, indicating the hydrogen bonds, hydrophobic bonds with the length of bonds.

3. RESULTS

3.1 Target enzyme and compound preparation

Target enzyme and compounds, which were prepared according to the methods given in the previous section, are given in Figure 1.

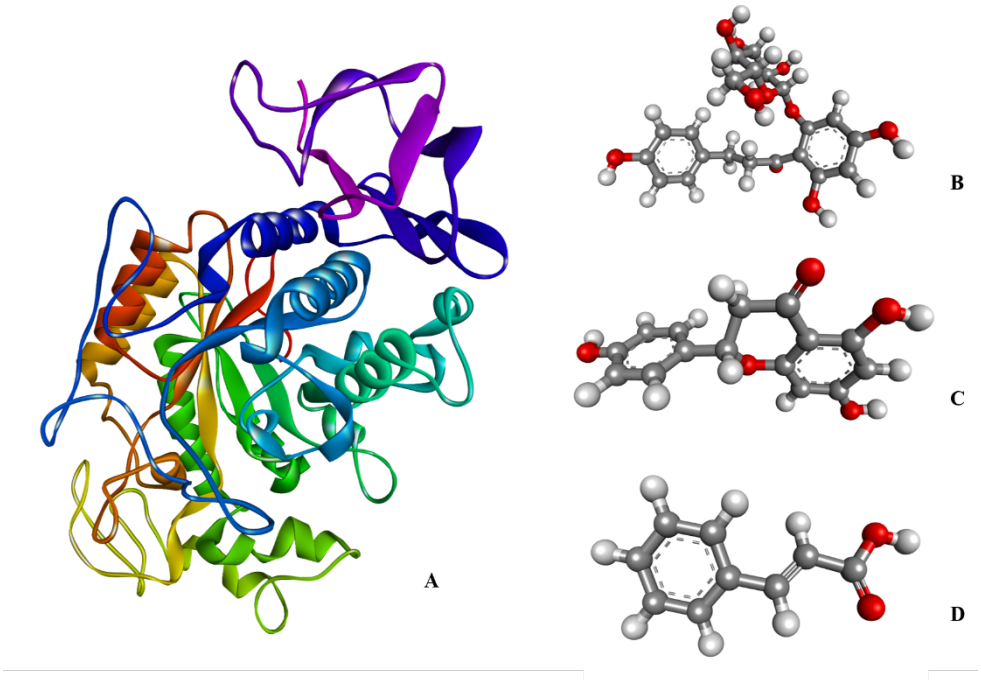

FIGURE 1. 3D Structure of a. α-amylase, b. phloridzin, c. naringenin, d. cinnamic acid.

3.2 Prediction and analysis of active sites

The pockets, where phloridzin, naringenin, and cinnamic acid can bind were predicted by CASTp v.3.0 [18]. According to the results, 86 possible pockets are present in α-amylase. In these pockets, solvent-accessible (SA) areas are observed to be ranging between 159.662 and 0.000 $Å^2$ and solvent-accessible (SA) volumes between 177.930 and 0.000 $Å^3$.

However, seven of them can be accepted as major pockets having volume (SA) higher than 10.000 $Å^3$. The locations of these seven major pockets are shown in Figure 2a and the data regarding these pockets are given in Table 1.

The pockets, where the substrate binds, were visualized by UCSF Chimera v.1.14 software [19] (Figure 2b).

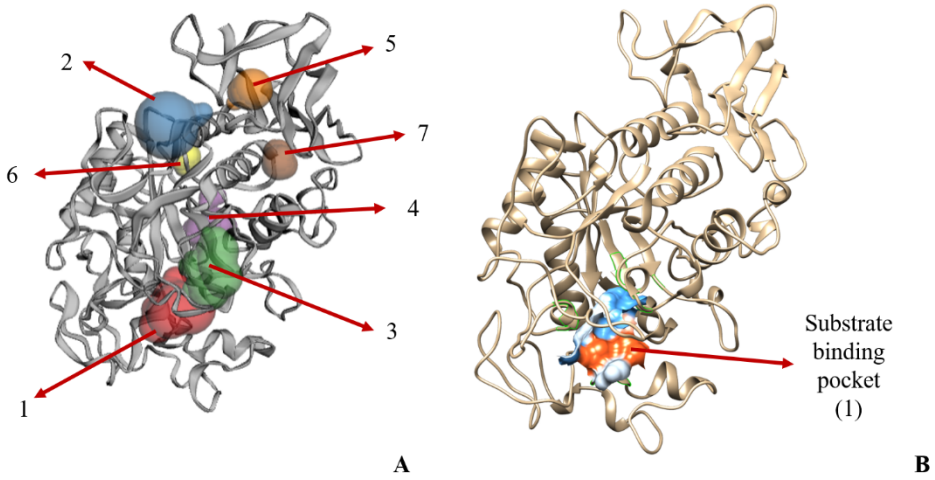

FIGURE 2. a. Major pockets of α-amylase (Numbers are showing pocket IDs), b. Substrate binding pocket.

TABLE 1. Data about major pockets.

ID	MS Volume	SA Volume	Pocket MS Area	Pocket SA Area	# openings	Mouth MS Area	Mouth SA Area
1	457.9	177.9	241.0	159.7	1	130.3	61.2
2	226.9	97.4	111.6	80.0	1	138.7	68.1
3	343.4	93.9	234.8	136.8	1	110.2	43.7
4	220.1	43.2	177.4	89.9	3	112.9	33.1
5	106.2	18.7	91.1	38.5	1	50.2	15.2
6	72.6	12.8	61.7	25.9	1	54.1	14.3
7	81.0	11.2	75.9	26.8	1	32.0	7.9

MS volume - pocket volume based on the molecular surface
SA volume - pocket volume based on the solvent-accessible surface
pocket MS area - pocket molecular surface area
pocket SA area - pocket solvent-accessible surface area
openings - number of mouths, or openings to the external molecular surface
mouth MS area - total area of mouth opening(s) based on the molecular surface
mouth SA area - total area of mouth opening(s) based on the solvent-accessible surface

3.3 Molecular docking analysis

In molecular docking analysis, phloridzin, naringenin, and cinnamic acid were docked in pockets of α-amylase to predict the best possible binding pose of these

compounds with higher binding scoring. The 3D and 2D interactions between phloridzin and α-amylase is given in Figure 3a and 3b respectively.

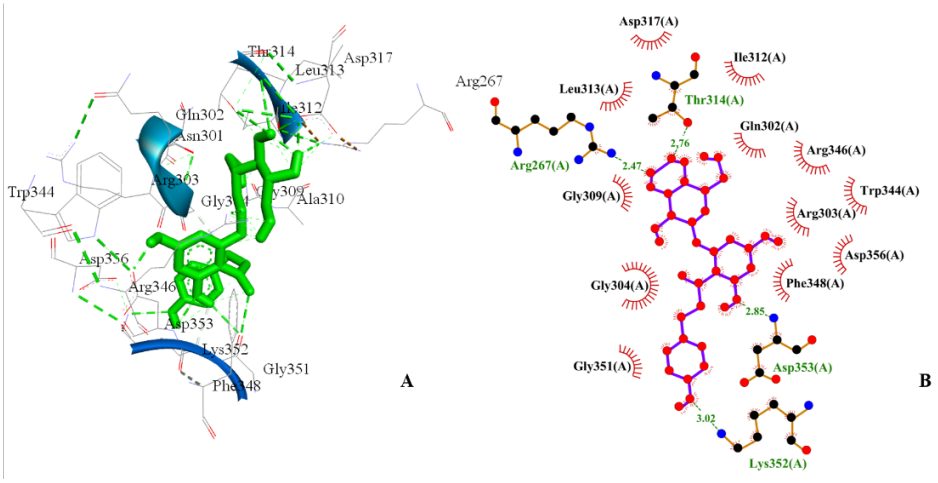

FIGURE 3. The interaction between phloridzin and α-amylase a. 3D, b. 2D.

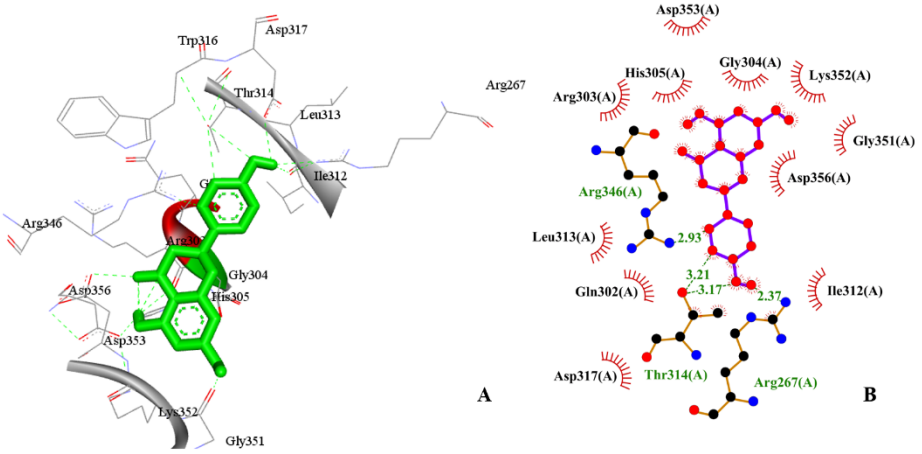

FIGURE 4. The interaction between naringenin and α-amylase a. 3D, b. 2D.

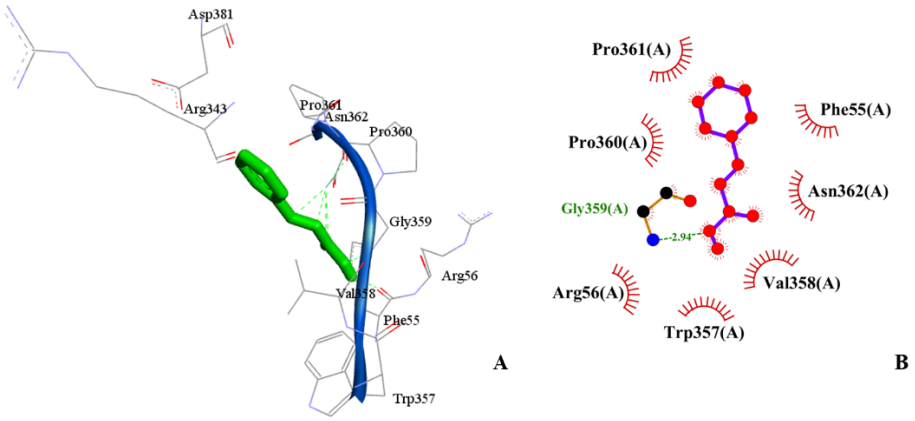

FIGURE 5. The interaction between cinnamic acid and α-amylase a. 3D, b. 2D.

The interactions between naringenin and cinnamic acid with α-amylase are given in Figure 4 and Figure 5 respectively.

The best docking possibilities binding to the major pockets are calculated and ten lowest binding energies for the compounds are given in Table 2.

The molecular docking results are mainly based on the binding energies of activators to α-amylase for all possible interactions. The lowest binding energy of phloridzin was found as -10.90 kcal/mol. This value was -9.61 kcal/mol for naringenin and -6.26 kcal/mol for cinnamic acid. Phloridzin showed slightly less binding energy, which means better binding affinity than naringenin and cinnamic acid (Table 2).

The post-analysis was performed for phloridzin, naringenin, and cinnamic acid by iGEMDOCK. Tested compounds were ranked by using both scores based on energies and pharmacological interactions. If a negative value for binding energy was observed, it means this interaction will be spontaneous. In addition, if this negative value is higher, the chance of being accepted as a drug candidate will be higher too [22]. The lowest binding energies in an enzyme-compound interaction mean the compound is fitting to the target enzyme.

TABLE 2. Ten lowest binding energies of phloridzin, naringenin, and cinnamic acid (kcal/mol) to the major pockets according to AutoDock.

	Phloridzin		Naringenin		Cinnamic Acid	
	Location	Binding Energy	Location	Binding Energy	Location	Binding Energy
1	10.832816 49.702500 34.319368	10.90	10.014391 50.022522 33.974087	9.61	-6.508833 51.789833 32.205000	6.26
2	-8.484868 49.323789 1.942500	10.78	10.109304 49.859130 34.176913	9.46	8.428250 48.213917 34.076167	6.17
3	10.948658 49.839184 35.124105	10.22	10.049913 49.873478 34.189913	9.45	8.504333 48.417750 34.221750	6.15
4	13.770711 70.880921 8.066816	9.43	10.026652 49.791565 34.320826	9.42	8.331667 48.276250 34.041750	6.12
5	12.006921 51.628395 35.093316	9.29	8.375673 58.626996 19.156482	9.41	8.542833 48.553250 34.300250	6.12
6	5.442263 44.231526 21.051895	8.78	10.034609 49.802565 34.336565	9.40	8.365744 58.647360 19.114551	6.10
7	-14.476605 51.435658 20.360789	8.72	10.019870 49.771478 34.356087	9.39	18.432917 68.508750 12.722417	5.95
8	15.198237 67.743842 8.683421	8.67	10.279739 49.822043 33.458652	9.27	18.444417 68.570167 12.626167	5.93
9	11.109500 54.599237 -2.566579	8.65	10.555043 49.672130 33.804870	9.04	3.142667 44.169167 20.271250	5.64
10	9.845132 44.627816 19.466105	8.51	3.795130 43.357913 21.198739	9.02	4.586167 45.664917 20.692333	5.52

Among the screened compounds, phloridzin (-103.02 kcal/mol) has the lowest binding energy and cinnamic acid (-58.29 kcal/mol) has the highest binding energy (Table 3).

TABLE 3. Binding energies of phloridzin, naringenin, and cinnamic acid according to AutoDock and iGEMDOCK.

		Phloridzin	Naringenin	Cinnamic Acid
AutoDock	Estimated Free Energy of Binding (kcal/mol)*	-10.90	-9.61	-6.26
	Final Intermolecular Energy (kcal/mol)	-15.07	-10.80	-7.15
	vdW + Hbond + desolv Energy (kcal/mol)	-15.07	-10.80	-7.15
	Electrostatic Energy (kcal/mol)	0.00	0.00	0.00
	Final Total Internal Energy (kcal/mol)	-4.88	-1.05	-0.25
	Torsional Free Energy (kcal/mol)	+4.18	+1.19	+0.89
	Unbound System's Energy (kcal/mol)	-4.88	-1.05	-0.25
	Estimated Inhibition Constant (nM)	10.31	90.90	25.90
iGEMDOCK	Total Energy (kcal/mol)	-103.02	-96.14	-58.29
	vdW (kcal/mol)	-78.78	-75.43	-54.79
	H-bond (kcal/mol)	-24.24	-20.71	-3.5
	Electrostatic Energy (kcal/mol)	0.00	0.00	0.00

* Estimated Free Energy of Binding = Final Intermolecular Energy + Final Total Internal Energy + Torsional Free Energy-Unbound System's Energy
- nM: nanomolar, μM: micromolar
- vdW: Van der Waals, H-bond: Hydrogen Bond

The lowest binding energy of phloridzin means phloridzin can bind easier to the enzyme than cinnamic acid. This can also be explained by comparing Figures 3 and 5. Figure 5 shows that cinnamic acid binds to the location (-6.508833, 51.789833, 32.205000) by only one hydrogen bond with **Gly 359**, but phloridzin binds to the location (10.832816, 49.702500, 34.319368) by four hydrogen bonds with **Arg 267**, **Thr314**, **Lys352** and **Asp353**. Table 3 also shows that the hydrogen bonding energy for cinnamic acid was -3.5 kcal/mol, where this value for phloridzin was -24.24 kcal/mol. In addition, the energy for Van der Waals interactions was lower in the binding of phloridzin to the enzyme. These data clearly present that phloridzin can bind to the enzyme easier than cinnamic acid.

4. DISCUSSION

In this current study the activation activities of phloridzin, naringenin, and cinnamic acid for α-amylase, which were previously proved experimentally, with some *in silico* tests.

According to the current literature, there are very few studies regarding the effect of phloridzin, naringenin, and cinnamic acid on α-amylase.

Menshaz and Altuner [12] previously proved that phloridzin, naringenin, and cinnamic acid are acting as activators for α-amylase.

In addition, Yusoff et al. [23] observed that phloridzin significantly reduced glucose absorption rate in rats.

Previous studies also proved that naringenin increases the α-amylase activity [24]. On contrary, some studies proposed naringenin as an α-amylase inhibitor [25,26]. As can be seen, sometimes consistent and sometimes inconsistent results were found in the literature. There are too many parameters in enzyme activity studies that can affect these inconsistencies. A detailed analysis should be done to understand where these inconsistencies arise.

Whether these compounds inhibit or activate α-amylase, *in silico* proofs in this recent study clearly show that they can bind to the enzyme to the locations rather than the substrate binding active site.

Figure 3 shows the interaction between phloridzin and α-amylase. The amino acid residues taking the role in this interaction are **Arg267**, Gln302, Arg303, Gly304, Gly309, Ile312, Leu313, **The314**, Leu317, Trp344, Arg346, Phe348, Gly351, **Lys352**, **Asp353**, and Asp356, where the amino acid residues written in bold are involving in active interaction with phloridzin.

Figure 4 shows the interaction between naringenin and α-amylase. The amino acid residues taking the role in this interaction are Phe55, **Arg56**, Trp357, Val358, Gly359, Pro360, Pro361, and Asn362, where the amino acid residue written in bold is involving in active interaction with naringenin.

Figure 5 shows the interaction between cinnamic acid and α-amylase. The amino acid residues taking the role in this interaction are **Arg267**, Gly302, Arg303, Gly304, His305, Ile312, Ile313, **The314**, Asp317, **Arg346**, Gly351, Lys352, Asp353, and Asp356, where the amino acid residues written in bold are involving in active interaction with naringenin.

Qian et al. [27] proposed that His101, His201, His299, and His305 are some of the important amino acid residues forming hydrogen bonds with inhibitors. The analysis showed that cinnamic acid interacts with His305 amino acid residue. This probably

causes a change in the active site of the enzyme. Thus, the substrate can bind effectively.

The results showed that phloridzin and cinnamic acid can bind to similar locations, where both of the compounds interact with **Arg267** and **The314** amino acid residues. The interaction location of naringenin is very close both to phloridzin and cinnamic acid, but not with the same amino acid residues.

As a result, it can be proposed that although all compounds activate the enzyme, the mechanisms of action of activation are different from each other.

Declaration of Competing Interests: The author declares no conflict of interest.

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A NEW RARE MACROPHYTE RECORD FROM SW ASIA AND ECOLOGICAL CONDITIONS AT ITS HABITAT

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ABSTRACT. *Aldrovanda vesiculosa* L. is a cosmopolitan but very rare carnivorous aquatic angiosperm species from the family Droseraceae. The species was widespread and more common few decades ago but unfortunately it is being extinct in so many former distribution areas due to aquatic pollution and eutrophication. During the field trip to Karakuyu Lake we recognized a remarkable free-floating plant, after detailed investigation we identified the specimens as *Aldrovanda vesiculosa* which is a new record from Turkey and also SW Asia. In this publication, we provided detailed description of the species based on Turkish specimens, its habitat description, water physiochemical attributes, co-occurring macrophyte species list and botanical illustration of the species.

1. INTRODUCTION

Aldrovanda vesiculosa L. is a rare monotypic aquatic carnivorous plant that belongs to Droseraceae family. Even though it is a cosmopolitan aquatic plant, it is rare and under risk of extinction with "EN" IUCN threat category (1). The plant known from Europe, Africa, Australia, and East and Central Asia where it is native (1). It is also introduced to America (2).

During our studies of aquatic flora of Karakuyu Lake, we have collected a remarkable free-floating plant, after identification we realized that we have been collected *A. vesiculosa*. There is no *A. vesiculosa* record from Turkey except Kamiński (3) mentioned that there was a record from northern Turkey stated as "Turkey – in the north (herbarium of Institute of Plant Biology of Wroclaw University – no further detail)." There are two herbaria in Wroclaw University, WRSL and BRSL, we contacted with the both herbaria for the specimen but it could

Keyword and phrases. Aldrovanda vesiculosa, Droseraceae, macrophytes, carnivorous plant

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not be found on both herbaria. There is no *Aldrovanda* record from SW Asia except Melamed et al. (4) recorded it in Israel from 750.000 years old sediments.

This article documents the first record of *A. vesiculosa* from Turkey and SW Asia. The habitat characteristics including water chemical and physical attributes, co-occurring macrophytes and morphological features of the species are presented. Scientific illustration based on collected specimens is also provided.

2. MATERIALS AND METHODS

Karakuyu Lake which is also known as Çapalı Lake (5) is located in Dinar district of Afyonkarahisar province at lat 38°15' N, long 39° 22' E. It is a wetland originated artificial lake that created for irrigation purposes by the State Waterworks (DSI). The lakes elevation is 1006 meters, its total area is 1277,04 ha, average depth is ca. 2 m and its deepest point is 4 m.

Karakuyu Lake was visited two times in vegetation season in 2014 and two times for physicochemical analysis in 2014. The plant specimens were collected and preserved using a plant drying press than stored in herbarium ANK. Collected plant material investigated under stereo zoom microscope and identified using identification keys and descriptions on relevant literature (6, 7). Physicochemical parameters (Table 1) were analyzed according to American Public Health Association (APHA) methods (8). CO_2 concentration in the water calculated by using the pH, alkalinity and the temperature according to Prietto and Millero (9) on hamzas reef freshwater CO_2 level calculator on web (10).

3. RESULTS

A. vesiculosa recorded first time from Turkey and SW Asia. Vegetation of Karakuyu Lake where the *A. vesiculosa* recorded is dominated by *Schonoplectus lacustris* (L.) Palla and *Phragmites australis* (Cav.) Trin. Ex Steud. communities. *Aldrovanda vesiculosa* is common in reedbed clearings with *Ceratophyllum demersum* L., *Chara vulgaris* L., *Lemna trisulca* L., *Myriophyllum spicatum* L., *Utricularia australis* R. Br. and *Utricularia minor* L.. Other aquatic plants determined from the lake are *Alisma gramineum* Lej., *Nymphaea alba* L. *Potamogeton lucens* L., *Potamogeton natans* L., *Potamogeton berchtoldii* Fieber, *Ricciocarpus natans* (L.) Corda, *Sparganium erectum* L. and *Stuckenia pecctinata* (L.) Böerner.

***Aldrovanda vesiculosa* L., Sp. Pl. 281 (1753).**
Aquatic rootless, free floating submerged plants with filiform somewhat brunching stem from 8 to 20 cm long; leaves 8-14 mm, in dense whorls of 6-8; each with a cuneate basal part, with 0.5—1 cm long petioles and terminating in 4 setaceous segments (4-6 mm long) and an orbicular lobe (5-6 mm long and 6-9 mm broad), leaf blade of 2 semicircular lobes folding up along the midrib which slightly exceeds the semicircular lobes, Turkish specimens do not have flowers.

We have not observed any flowering *A.vesiculosa* individuals in the field which is actualy not surprising because like many other aquatic plants *Aldrovanda* also relying mainly on vegetative propagation and displays reduced generative reproduction (1).

Examined specimen: TURKEY: B3 Afyon province, Dinar district, Karakuyu Lake, 1006 m. a.s.l, 09.06.2014, Yaprak & Körüklü BM169 (ANK).

According to Güner *et al.* (11) there are 9 carnivorous plant taxa in Turkey and one of them *Pinguicula habilii* Yıldırım, Şenol & Pirhan is endemic. With this new record the number of carnivorous plants of Turkey reaches 10 taxa from 4 genus and two families. The list of carnivorous plants of Turkey can be seen from below:

Droseraceae
Drosera intermedia Hayne
Drosera longifolia L.
Drosera rotundifolia subsp. *rotundifolia* L.
Aldrovanda vesiculosa L.

Lentibulariaceae
Pinguicula crystallina Sm.
Pinguicula balcanica Casper
Pinguicula balcanica subsp. *pontica* Casper
Pinguicula habilii Yıldırım, Şenol & Pirhan

TABLE 1. Results of water physicochemical parameters in the Karakuyu Lake.

Parameters	**Unit**	**June 2014**	**August 2014**
Temperature	°C	22,3	19,8
pH		7,75	7,69
Electrical Conductivity	µS/cm	34	35,6
Dissolved Oxygen	mg/L	7,34	7,65
Turbidity	NTU	1,42	3,25
Suspendend solids	mg/L	4	18
Alkalinity	mg/L	178,5	176,5
Total hardness	mg/L	174,5	174,6
Biochemical Oxygen Demand (BOD)	mg/L	5	4
Chemical Oxygen Demand (COD)	mg/L	13,4	8
Total Organic Carbon (TOC)	mg/L	3,99	1,33
Total nitrogen	mg/L	1,478	1,394
Ammonium nitrogen	mg/L	0,033	0,011
Ammonia	mg/L	0,031	0,010
Nitrites	mg/L	0	0,001
Nitrates	mg/L	1,609	< 0,085
Total Kjeldahl nitrogen	mg/L	1,114	1,387
Organic nitrogen	mg/L	1,089	1,378
Total phosphorus	mg/L	0,112	0,04
Ortho Phosphate	mg/L	< 0,2	< 0,2
CO_2	mM/L	0,28	0,34

FIGURE 1. The habitat of *Aldrovanda vesiculosa* L.

FIGURE 2. *Aldrovanda vesiculosa* L. photograph in the field.

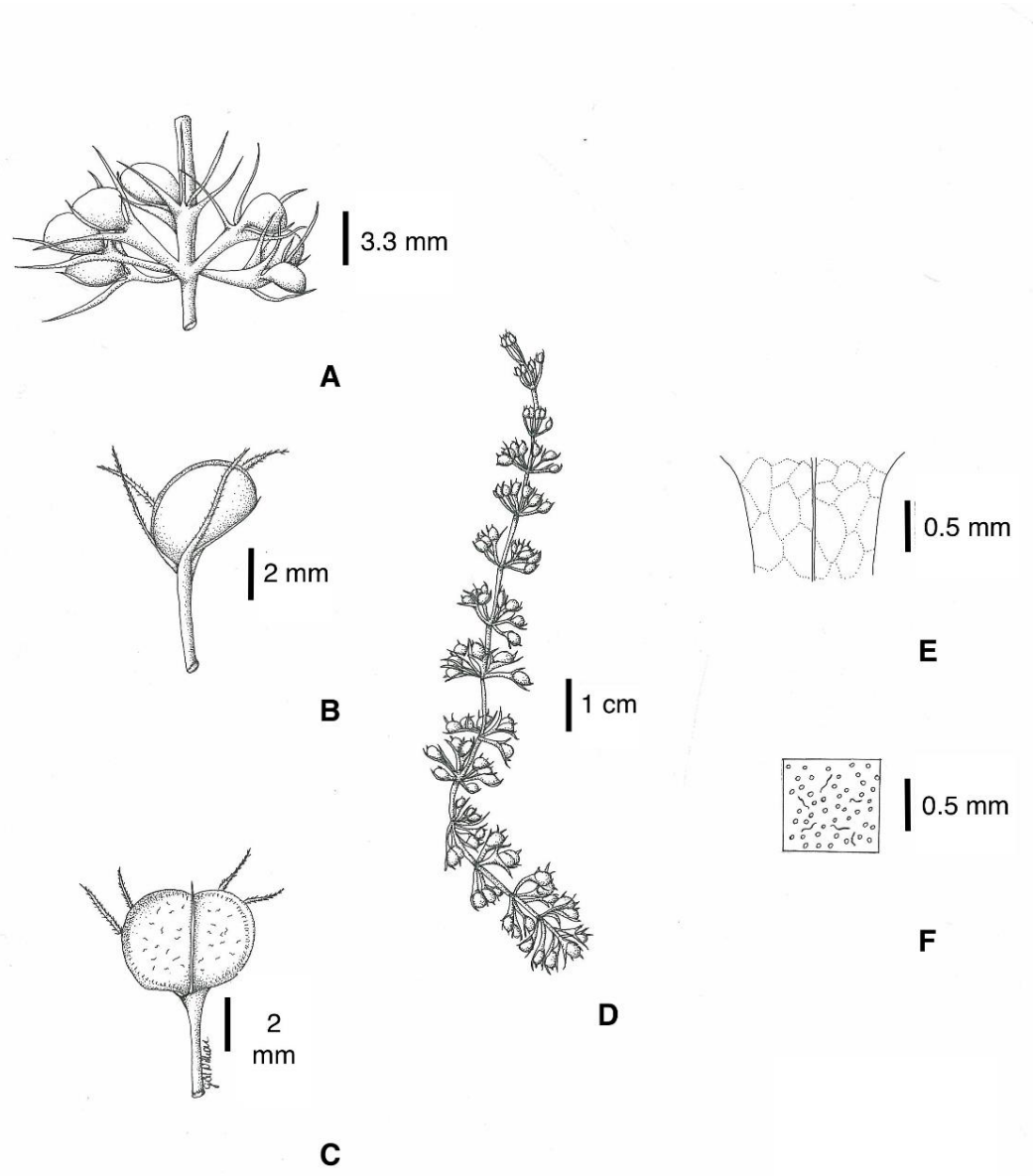

FIGURE 3. Scientific illustration of *Aldrovanda vesiculosa* L.
A: Whorl of leaves, B: A closed trap, C: A trap with open blades, D: Whole plant, E: Petiole, F: Inner surface of a trap

4. DISCUSSION

Adamec (7) reported that *A. vesiculosa* is clearly a photosynthetic strict CO_2 user and needs minimum ca. 0.1 mM and optimum 0.3 to 1 mM CO_2 levels. In the Turkish population, we have calculated dissolved CO_2 levels as 0.24 mM in June and 0.34 mM in August, the former is higher than the minimum level and the latter is in between optimum values. Even though the average deepth of the lake is 2 m, we only observed *A. vesiculosa* in shallow littoral parts (0,2 to 0,6 m deep) of the lake where the bottom covered with thick layer of dead *Schonoplectus lacustris* and *Phragmites australis* material. Water physicochemical parameters in the Karakuyu Lake generally compatible with formerly reported ecological requirements for *A. vesiculosa* populations such as low turbidity, pH range, water temperature and the nutrient concentrations except for Nitrates. We measured 1,609 mg/L Nitrate concentration in Karakuyu Lake on June which is approximately 80 times higher than the optimum concentrations reported by Adamec (7) but Kamiński (12) reported 1.63 mg/L Nitrate concentration from "Staw Nowokuźnicki" Pond which is slightly higher than our measurement.

Flora and vegetation of the lake was studied on 1982 (13) and *A. vesiculosa* was not recorded in the study. It is most likely the plant introduced to the lake after that. There are some studies supported that it may spread by migratory waterbirds to new sites (14, 15, 16). Nergiz and Tabur (17) reported 50 migratory waterbirds (7 winter migrants, 22 summer migrants, and 21 transit migrants) from the Karakuyu Lake which may be the reason of the introduction.

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Author Contribution Statement AEY-specimen collection and identification, data analysis and manuscript writing. STK-specimen collection and identification. GNT-scientific illustration and manuscript writing.

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EFFECTS OF LIGHT AND SALINITY ON THE GERMINATION OF CLOSELY RELATED THREE *SALSOLA* TAXA

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ABSTRACT. Three closely related *Salsola* taxa (*Salsola boissieri* Botsch. subsp. *serpentinicola* (Freitag & Özhatay) Freitag & Uotila, *Salsola boissieri* Botsch. subsp. *boissieri*, *Salsola turcica* Yıldırımlı (halophytic ecotype), *Salsola turcica* Yıldırımlı (gypsicole ecotype)) from different edaphic conditions were studied according to changing light and salinity conditions. Seeds of target taxa were collected in 2017 and their weights were determined. The perianth segments were removed before the experimental trials and all the trials were conducted at 9°C/22°C which is the mean night and day temperatures of germination season. For the determination of the influence of light, one set of seeds for distilled water trial were kept at complete darkness. Different NaCl concentrations (distilled water, 100, 200, 300 mM NaCl) were used to evaluate the effects of salinity on germination. Viability of the seeds were determined by Triphenyl Tetrazolium Chloride (TTC) test which was applied to the seeds that did not germinate during the trials. As a result, it was found out that light stimulates germination of the taxa and *Salsola* seeds showed better germination ratio at light. The most tolerant taxa against salinity are the halophytic and gypsicole ecotypes of *S. turcica*, and the most susceptible one is *S. boissieri* subsp. *serpentinicola*. Both of the species show reduced germination ratios with increasing salinity. Salinity tolerance of *S. boissieri* subsp. *serpentinicola* and *S. boissieri* subsp. *boissieri* are very low, according to the Decreasing Germination Percentage (DGP) values. Although they show different germination response against increasing salinity, there is not any statistically meaningful difference between these three taxa according to germination percentages at different salinities, germination rates, last germination ratios and seed viabilities (F=1.818 p>0.05) (One Way ANOVA, SPSS 25).

Keyword and phrases. Amaranthaceae/Chenopodiaceae, germination, Gypsicole, halophyte, salinity, Salsola, serpentine

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1. INTRODUCTION

Soil salinity became an important problem as a result of global warming, lack of precipitation and also wrong irrigation policies, especially in arid and semi-arid areas increase [1] so halophytes became an important plant group whose biology and germination ecology attracts attention in last few decades [2-6]. Halophytes have some special adaptation mechanisms that make them germinate, survive and complete their life cycles at high salinities where the most of the plants can not survive [5, 7]. Although they have these adaptation they show better germination abilities at non-saline or less saline conditions [8-20], and increase in salinity cause decrease in their germination rate and ratio and/or retard germination [3, 12-19, 21-23].

The genus *Salsola* L. is represented by 18 species and 23 taxa in Turkey [24]. The members of the genus are generally adapted to saline or semi-saline areas of arid regions [25, 26]. Although *Salsola turcica* Yıldırımlı, *Salsola boissieri* Botsch. subsp. *boissieri* and *Salsola boissieri* Botsch. subsp. *serpentinicola* (Freitag & Özhatay) Freitag & Uotila are phylogenetically closely related, they are adapted to different edaphic conditions and became habitat specialists which make them more vulnerable against habitat loss and degradation [27].

S. turcica is endemic to Turkey and has two ecotypes spread over gypsaceous and saline steppes between altitudes 950 m and 1000 m [28]. The subspecies of *S. boissieri* are geographically separated from each other. *S. boissieri* subsp. *boissieri* prefers rocky and stony slopes at 900-2500 m altitudes around Kahramanmaraş and Sivas provinces [25]. *S. boissieri* subsp. *serpentinicola* is also endemic to Turkey and, prefers serpentine rocks and rock cracks between 1600 and 2000 m around Muğla and Burdur provinces [29]. If populations of a species is adapted to local conditions and have genetically preserved morphological and physiological differences, they are called as ecotypes [30]. *S. turcica* has a wide distribution area and populations from both halophytic and gypsicole areas were studied and because of this differentiation they may have different physiological adaptations. The germination trials of these ecotypes can provide some information about the differentiations of them.

Most of the *Salsola* taxa are salt tolerant during the germination phase of their life cycles, as supported by several studies on different *Salsola* species like: *S. kali* L. [21], *S. baryosma* (Schult.) Dandy [31], *S. villosa* Del. ex Roem. et Schult [32], *S. iberica* Sennen & Pau [3], *S. chandharyi* L. [33], *S. imbricata* Forssk. [34, 35],

S.affinis C.A.Mey. ex Schrenk [23], *S. vermiculata* L. [26], *S. ferganica* Drobow [36], *S. grandis* Freitag, Vural & N. Adıgüzel [37].

In this study, three closely related *Salsola* taxa adapted to different edaphic conditions were studied to understand the influence of their salinity tolerance on their distribution and also the germination characteritics of them under different NaCl concentrations. These taxa are *S. boissieri* subsp. *serpentinicola*, *S. boissieri* subsp. *boissieri*, *S. turcica* (halophytic ecotype), *S. turcica* (gypsicole ecotype).

2. MATERIALS AND METHODS

2.1 Collection of fruits and seeds

During vegetation period of 2017, populations were evaluated at their natural distribution areas and, fruits and seeds were collected according to the current population sizes of the taxa and at least 10 individuals were sampled. Until the start of experiments specimens were stored at 4°C. All the fruit and seed specimens were evaluated by BAB stereo binocular microscope and BAB image processing and analysis system (Bs200Pro). Mean weight of fruits and seeds were measured. The locations and collection numbers of specimens were given in Table 1.

TABLE 1. Taxa with their subspecies and ecotype information, collection number of specimens and localities

Taxon	Collection number of specimen (IBÇınar)	Locality
***S. boissieri* subsp. *serpentinicola* (serpentinicole subspecies)**	**1139**	Burdur, Altınyayla, Dirmil pasture, serpentine soils, 1669 m
***S. boissieri* subsp. *boissieri* (glycophyte subspecies)**	**1141**	Sivas, Yıldızeli, Yusufoğlan village, 1400 m
***S. turcica* (halophytic ecotype)**	**1142**	Konya, Cihanbeyli, Bolluk Lake, saline alkaline areas, 943 m
***S. turcica* (gypsicole ecotype)**	**1143**	Ankara, Beypazarı, about 14 km west of Beypazarı, on the right side of Beypazarı-Nallıhan highway, 948 m

2.2 Germination trials

Periant segments of seeds were removed before the trials and seeds were sterilized with 0,1% sodium hypochlorite. During the trials, the mean night and day temperatures of the germination period of the distribution areas were used, 9°C / 22°C with 12 h photoperiodism (light intensity 12000 lux ± %10). For the determination of the influence of salinity on germination distilled water and 100, 200, 300 mM NaCl solutions were used. At each trial, 25 seeds with 4 replicates were monitored for 10 days and the emergence of radicula was accepted as germination. Ungerminated seeds were taken into TTC test for detection of their viability under binocular microscope [38-39]. Stained red at TTC test is the indicator of viability which detects the cellular level respiratory activity [40]. Red stained seeds were accepted as viable and the ones stained green were accepted as unviable [15].

After the trials germination percentages, germination rates (Timson index) [41] and viability percentages were calculated.

Germination rate: ΣG/t [41]
Last germination: (a / c) × 100
Seed viability: [(a+d) / c] × 100 [15]
ΣG: sum of the germination percentages of every 2 days
t: total germination period.
a: total germinated seed number at the end of the trials (number of germinated seeds after recovery + number of germinated seeds at salinity)
c: Total number of seeds
d: seeds stained red after test- viable seeds

DGP: [(Germination percentage at distilled water − Germination percentage at salinity) / Germination percentage at distilled water] × 100 [42].

2.3 Statistical Analysis

All the data were arcsin transformed and then evaluated by SPSS (IBM SPSS Statistics Version 25) with One Way ANOVA for the comparison of the influence of trials. T test was used for importance control ($p< 0.05$).

3. RESULTS AND DISCUSSION

Mean weight and sizes of all the taxa were given at Table 2 and the examples of fruits and seeds are provided in Figure 1 and 2.

TABLE 2. Taxa, collection number of specimens and localities

Taxon	Mean weight of a fruit (g)	Mean weight of a seed (g)	Mean diameter of a fruit (mm)	Mean diameter of a seed (mm)
***S. boissieri* subsp. *serpentinicola* (serpentinicole subspecies)**	0.615 ± 0.029	0.297 ± 0.008	5.53 ± 0.74	1.40 ± 0.18
***S. boissieri* subsp. *boissieri* (glycophyte subspecies)**	0.389 ± 0.019	0.225 ± 0.025	4.22 ± 0.64	1.23 ± 0.15
***S. turcica* (halophytic ecotype)**	0.862 ± 0.039	0.415 ± 0.016	4.34 ± 0.54	1.50 ± 0.15
***S. turcica* (gypsicole ecotype)**	0.583 ± 0.028	0.362 ± 0.035	4.63 ± 0.75	1.42 ± 0.17

Halophytic ecotype of *S. turcica* has the heaviest fruits and seeds according to the mean weights, and the lightest fruits and seeds belong to *S. boissieri* subsp. *boissieri*. *S. boissieri* subsp. *serpentinicola* has the largest mean fruit diameter and *S. boissieri* subsp. *boissieri* has the smallest. However, in general there is no big difference between the weights and diameters of fruits and seeds. Even though the values were close to each other; the largest seed diameter was measured at halophytic ecotype of *S. turcica* and the smallest at *S. boissieri* subsp. *boissieri.*

All the germination results, germination percentages, germination rates, last germination percentages, viability percentages after TTC test DGP ratios at changing salinities were given at Table 3 and Figure 2.

TABLE 3. Germination results of taxa

S. boissieri* subsp. *serpentinicola					
	Germination percentage	**Germination rate**	**Last germination (%)**	**Viability (%)**	**DGP**
Darkness (Distilled water)	59	-	-	-	-
Photoperiodism (Distilled water)	65	12.8	73	73	-
100 mM NaCl	46	12.7	52	52	29.2
200 mM NaCl	40	10.1	43	43	38.4
300 mM NaCl	33	12.9	33	33	49.2
S. boissieri* subsp. *boissieri					
	Germination percentage	**Germination rate**	**Last germination (%)**	**Viability (%)**	**DGP**
Darkness (Distilled water)	76	-	-	-	-
Photoperiodism (Distilled water)	100	24.9	100	100	-
100 mM NaCl	59	17.3	60	60	41.0
200 mM NaCl	57	16.2	65	65	43.0
300 mM NaCl	55	13.8	59	59	45.0
***S. turcica* (halophytic ecotype)**					
	Germination percentage	**Germination rate**	**Last germination (%)**	**Viability (%)**	**DGP**
Darkness (Distilled water)	69	-	-	-	-
Photoperiodism (Distilled water)	77	22.9	80	80	-
100 mM NaCl	74	22.5	82	82	3.8
200 mM NaCl	70	21.6	84	84	9.1
300 mM NaCl	45	12.5	55	55	41.5
***S. turcica* (gypsicole ecotype)**					
	Germination percentage	**Germination rate**	**Last germination (%)**	**Viability (%)**	**DGP**
Darkness (Distilled water)	90	-	-	-	-
Photoperiodism (Distilled water)	96	35.4	96	96	-
100 mM NaCl	85	32.4	89	89	11.4
200 mM NaCl	88	29.3	91	91	8.3
300 mM NaCl	83	25.2	86	86	13.5

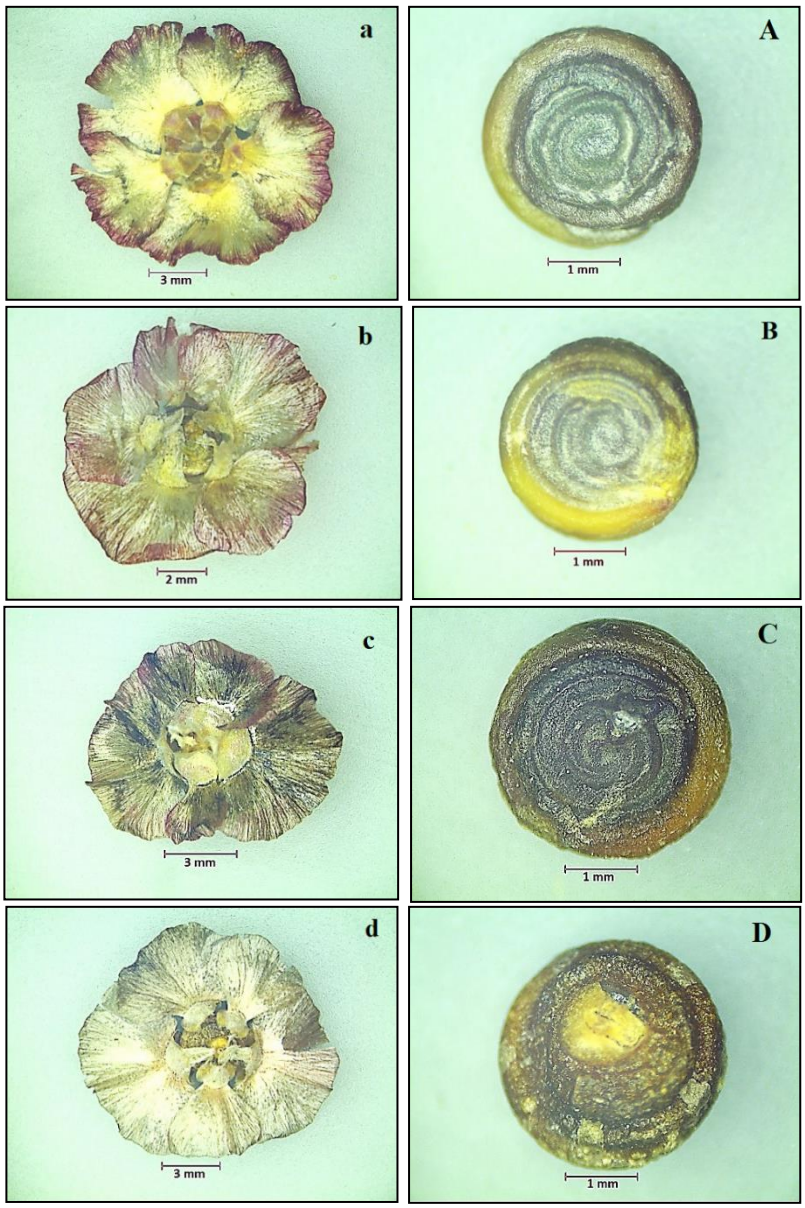

FIGURE 1. Fruits of analysed taxa (**a.** *S. boissieri* subsp. *serpentinicola*, **b.** *S. boissieri* subsp. *boissieri,* **c.** *S. turcica* (halophytic ecotype)*,* **d.** *S. turcica* (gypsicole ecotype)) and seeds of taxa (**A.** *S. boissieri* subsp. *serpentinicola*, **B.** *S. boissieri* subsp. *boissieri,* **C.** *S. turcica* (halophytic ecotype),**D.** *S. turcica* (gypsicole ecotype))

All of the previous studies about halophyte germination showed that the seeds showed better germination ratios at distilled water [8-20]. Our results are consistent with the former results and we also found better germination ratios at distilled water (Figure 2, Table 3); and increasing NaCl concentrations inhibited the seed germination [18, 26, 37, 43-44].

Also, when the results of complete darkness were evaluated, it was found that light stimulates the germination and for this reason 12/12 h photoperiodism was used during the germination trials. The comparisons of the germination percentages at light and complete darkness and also changing NaCl conditions can be seen in Figure 2.

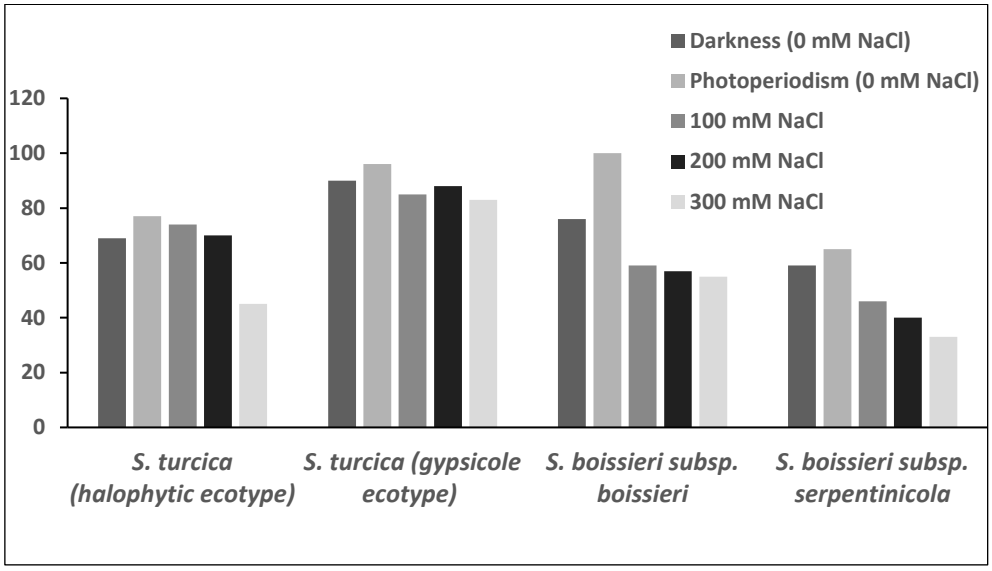

FIGURE 2. Germination percentages of each taxa at distilled water and at changing NaCl concentrations

The presence of halophyte taxa at their habitats is shaped by their salinity tolerance [45] and germination phase of their life cycles defines edaphic conditions that they are facing with throughout their life span [46] so salinity, light, photoperiodism etc. influence germination ability at time and space [6].

According to our results, not only the germination percentages and rates but also the seed viabilities of all taxa decreased with increasing salinity (Table 3). Likewise, there is a negative correlation between last germinations and increasing salinities.

Halophytes show species specific salinity tolerance at germination and seedling phases [42] but for our study this specificity is not so obvious which may be caused by very close relations of the studied taxa. Formulize DGP (decreasing germination percentage) to quantify the negative correlation between salinity tolerance and germination percentages, higher the DGP lower the salinity tolerance.

According to the statistical analysis, even though these closely related taxa prefer different edaphic conditions, there is not any statistically important difference between germination percentages at different salt concentrations, last germination percentages, germination rates and seed viabilities (F=1.818 p>0.05)(One Way ANOVA, SPSS 25).

Seeds did not germinate after salinity treatments were taken into viability test. At the end of TTC test colour change denotes viability as can be seen from Figure 3. According to the classification used by [47-51] seeds stained totally red are accepted as viable.

FIGURE 3. Un-viable (partially stained) and viable seeds (completely satined red) after TTC

According to our results, we concluded that light stimulates germination better than complete darkness at both of the taxa. The highest salinity tolerance among these three taxa was observed at both halophytic and gypsicole ecotypes of *S. turcica* and according to salinity tolerance the most vulnerable one is the *S. boissieri* subsp. *serpentinicola*. Both of the examined taxa showed better germination at distilled water which is consistent with the general knowledge about salinity tolerance mechanism of halophytes [12, 52-53]. Even though these three taxa are closely

related, their salinity tolerances are different from each other, especially according to calculated DGP values. The studied taxa are phylogenetically close to each other [25, 28-29] but because of their habitat specifications they evolved some physiological adaptations at germination phase and ecotypes of *S. turcica* are more tolerant than the subspecies of *S. boissieri* are glycophytes.

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Conflict of interest The authors declare that they have no conflict of interest.

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