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PEG-STABILIZED COLLAGEN-CHITOSAN MATRICES: PHYSICOCHEMICAL AND *IN VITRO* BIOLOGICAL ASSESSMENT OF HYDROGELS

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ABSTRACT. In this study, some of the physicochemical and *in vitro* biological properties of hydrogel matrices formed under different crosslinking conditions, consisting of the structural protein collagen and chitosan polysaccharide were investigated. The chemical compositions of the developed matrices were verified, their surface morphologies were examined by SEM and AFM, and their light transmittance status was determined. Next, the *in vitro* cytocompatibility of the hydrogels was demonstrated based on one week of interaction with mesenchymal stem cells. As a result, highly transparent and cytocompatible hydrogel matrices with mechanical stability in aqueous conditions were obtained.

1. INTRODUCTION

Hydrogels are polymeric materials that can swell and maintain their three-dimensional structure in aqueous conditions. Since they have close similarities with the physicochemical properties of various tissues of the human body, they have been used in numerous biomedical applications as biomaterials [1-3]. Among the shortcomings of hydrogels are their poor mechanical properties, which are often overcome by methods such as crosslinking copolymerization, crosslinking of reactive polymer precursors, and crosslinking via polymer-polymer reactions [1,2].

Hydrogels can be of synthetic or natural origin. The lack of bioactivity in synthetic polymers has led to the use of natural-origin polymers or their combinations in the development of hydrogel biomaterials. Proteins and polysaccharides can be considered as the major classes of natural polymers. Thus, structural proteins such as collagen, gelatin, albumin, elastin, keratin,

Keywords. Hydrogel, collagen, chitosan, PEG-stabilized matrix, biomaterials
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silk, and polysaccharides such as hyaluronic acid, chitosan, chitin, alginate are some major macromolecules of interest in the development of natural-origin hydrogels [4-7].

Studies have been carried out using collagen and collagen-chitosan to develop hydrogel matrices [6,7]. It is necessary to achieve the mechanical, biological and various other properties of the products in an application-oriented manner [7,8]. Various crosslinking methods are in use to provide the required mechanical properties. Water soluble carbodiimide, in conjunction with NHS allows, for two-step coupling of two proteins without affecting the carboxyl groups of the second protein. Homobifunctional crosslinkers, for example cross linkers with polyethylene glycol (PEG) spacers, are suitable for covalent conjugation between amine-containing molecules. This study is based upon the investigation of the physicochemical and *in vitro* biological properties of hydrogel matrices formed under different crosslinking conditions, consisting of the structural protein collagen and chitosan polysaccharide with structural similarity to glycosaminoglycans.

2. MATERIALS AND METHODS

2.1. Chemicals

Chitosan [(1-4) linked 2-amino-2-deoxy-β-D-glucopyranose] (Mr ~400,000 Da; >85% deacetylation) was supplied from Fluka (Milwaukee, WI). Collagen was isolated from rat tail tendons using the standard method. EDC [N-ethyl-N′-(3-(dimethylamino)propyl) carbodiimide], NHS (N-hydroxysuc cinimide) and all other chemicals and solvents were purchased from Sigma-Aldrich (St. Louis, MO), unless otherwise stated. NHS-PEG-NHS [polyethylene glycol bis(succinimidyl succinate)] with a molecular weight of 3400 Da was obtained from Nanocs Inc. (New York, NY). Sterile disposable tissue culture plasticware was purchased from Corning (Corning, NY). Cell culture medium, serum and supplements were obtained from Lonza (Basel, Switzerland).

2.2. Fabrication of matrices

In this study, collagen-chitosan-based hydrogel biomaterials were created using three different cross-linking methods (M1-M3). Collagen-based membranes without chitosan were fabricated as controls (C1-C3). The

compositions of the groups and the applied crosslinking methods are given in Table 1.

Collagen solution was prepared at 4°C at a concentration of 1% (w/v). Three percent (w/v) chitosan solution was prepared at 4°C with 0.2 N HCl. The collagen-chitosan-based hydrogel matrices (M1-M3) were fabricated as follows: 0.06 mL of 3% (w/v) chitosan solution and 3 mL of 1% (w/v) collagen solutions were mixed with 0.2 mL of 0.625 M 2-(N-morpholino) ethanesulfonic acid (MES) buffer. Then, 57 µL of the cross linker solution was added and mixed well. After, the mixture was poured into disc-shaped (r= 20 mm, h= 1.5 mm) molds having a volume of 500 µL each, incubated for 16 h at room temperature, and at 37°C for 5 h, both under 85% humidity conditions. The obtained hydrogel matrices were washed in 0.1 M Na_2HPO_4 and stored at 4°C in 10 mM PBS.

For cell culture experiments, the steps were carried out under aseptic conditions and the matrices were sterilized by keeping in 70% ethanol for 6 h, and then washing with sterile PBS [9]. The contents of the cross linkers used are as follows; for M1: 57 µL of EDC/NHS (25 mg EDC and 15 mg NHS dissolved in 0.125 mL of 0.625 M MES buffer), for M2: 57 µL of NHS-PEG-NHS (Figure 1) solution (42 µL NHS-PEG-NHS dissolved in 0.125 mL of PBS), and for M3: 57 µL of EDC/NHS & NHS-PEG-NHS (37.5 mg EDC, 22.5 mg NHS, and 25 mg NHS-PEG-NHS dissolved in 0.125 mL of 0.625 M MES buffer). All conditions applied in the synthesis of the collagen-based control matrices (C1-C3) were the same as those of the experimental groups (M1-M3).

TABLE 1. Compositions of the groups

	Biopolymer		**Crosslinker**		
Groups	Col	Col-Chi	EDC/NHS	NHS-PEG-NHS	EDC/NHS & NHS-PEG-NHS
C1	+		+		
C2	+			+	
C3	+				+
M1		+	+		
M2		+		+	
M3		+			+

2.3. ATR-FTIR analysis

Structural analysis of the formed matrices was performed by using attenuated total reflection Fourier transform infrared spectroscopy (ATR-FTIR Spectrum 100, Perkin Elmer, Shelton, CT). The sample compartments were thoroughly dried overnight. The samples were dried and powdered finely. Spectral measurements were performed in the range of 650–4000 cm^{-1} with an accumulation of four scans at resolutions of 4.00 wavenumbers, under identical conditions.

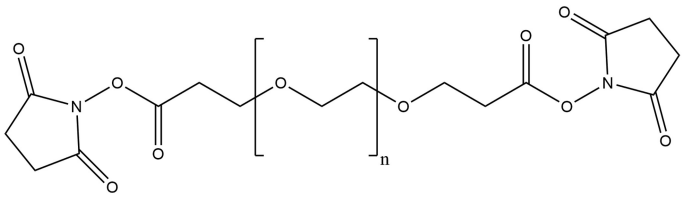

FIGURE 1. Polyethylene glycol bis(succinimidyl succinate) (NHS-PEG-NHS)

2.4. Water content analysis and stability

The difference in water contents of the matrices were determined using deionized water. The weighted equilibrium hydrated mass ($m_{hydrated}$) and dry mass (m_{dry}) values were used to calculate % water contents according to Eq.1.

$$\text{Water content (\%)} = [(m_{hydrated} - m_{dry}) / m_{hydrated}] \times 100 \quad (1)$$

To determine the mechanical stability of the matrices, samples were kept in 0.1 M phosphate buffered saline (pH 7.4) for up to 3 months and mass changes were monitored with weekly weigh-ins and Eq.2.

$$\text{Weight loss (\%)} = [(m_{initial} - m_{time\ point}) / m_{initial}] \times 100 \quad (2)$$

2.5. SEM evaluation

The surface morphology of the matrices was examined by scanning electron microscopy (SEM). Prior to examination, the samples were first lyophilized at -56ºC for 5 h, dehydrated by passing through an ethanol series (50-95%), attached to stubs and then sputter-coated with a thin layer of gold. Matrices were evaluated using the Quanta 200 FEG (Portland, OR) instrument at 10 kV.

2.6. AFM evaluation

For atomic force microscopy (AFM) analysis, biopolymer samples were formed on a mica substrate by spin coating (Primus SB15) at 3000 rpm, crosslinked by the methods described above for each matrix type, and dried in a vacuum oven at 30ºC. Analyses were carried out with a NI-AFM model (Nano Magnetics, Ankara, Turkey) device operated in dynamic mode in air using a Tap300A1 model cantilever. The surface was randomly scanned at 10μm x 10μm.

2.7. Light transmittance analysis

Optical properties of the developed matrices based on light transmission were investigated in the visible region. Matrices fabricated on glass coverslips were placed in the wells of a 12-well culture plate, and kept in PBS overnight prior to analysis [10]. The light transmittance and back-scattering measurements of the samples were carried out with a UV/visible spectrophotometer (Shimadzu, Tokyo, Japan) at room temperature. Refractive indices were measured with an Abbe refractometer (Bergman, Berlin, Germany) after the device was calibrated with alpha bromonaphthalene.

2.8. MSC culture

To determine the *in vitro* cytocompatibility of the developed matrices, human bone marrow-derived mesenchymal stem cells (MSCs; ATCC) were seeded on the matrices and cultured for up to 7 days [11]. MSCs were cultured under standard conditions; *i.e.* in α-MEM supplemented with 10% FBS, and Pen/Strep (100 U/mL penicillin, 100 μg/mL streptomycin), inside an incubator set to 37ºC, 5% CO_2, >95% humidity. Relative cell viability was assessed based on metabolic activity by the MTT (3-[4,5-dimethylthiazol-2-yl]-diphenyltetrazolium bromide) assay.

2.9. Statistical analysis

Cell viability data was presented as mean ± SD (n=3). Statistical analysis was performed via one-way Anova test followed by Tukey's post hoc test, using GraphPad Prism 7 (GraphPad, La Jolla, CA). Significance levels were set as ** $p \leq 0.01$, and ****$p < 0.0001$.

3. RESULTS AND DISCUSSION

3.1. Chemical characterization of matrices

The chemical composition of the developed matrices was evaluated by ATR-FTIR analysis, between 3800-800 cm^{-1}; findings are given in Figure 2. The formation of amide bonds between collagen and chitosan was expected as a result of different cross-linking methods applied in the study. The presence of Amide 1 and 2 bands, and also Amide 3 were monitored to confirm amide bond formations [12]. These bands show asymmetric and symmetrical stretching of NH at ~3300-3450 cm^{-1}. M3 showed a stronger peak at ~3300 cm^{-1} compared to M1 and M2. Amide 1 band stretch of C=O was observed at ~1650 cm^{-1}; H ~3050 cm^{-1} was due to the NH stretch. The band at~1530-1570 cm^{-1} was associated with Amide 2, ~1260-1300 cm^{-1} band with Amide 3, and ~2850-2960 cm^{-1} was attributed to the CH_2-CH_3 groups.

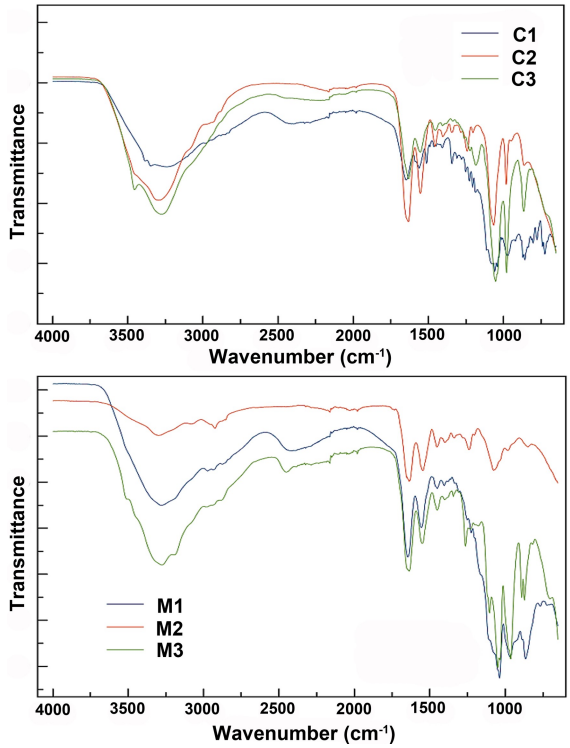

FIGURE 2. FTIR spectral analyses of (a) collagen-based control matrices (C1-C3), and (b) collagen-chitosan-based matrices (M1-M3)

3.2. Water content and stability of matrices

Water content analysis indicated that the difference between the water uptake of the matrices was insignificant (<3%). All matrices retained their structural integrity in PBS over a three-month period (data not shown). This finding indicates that collagen-chitosan matrices may be suitable for prospective long-term use in aqueous media.

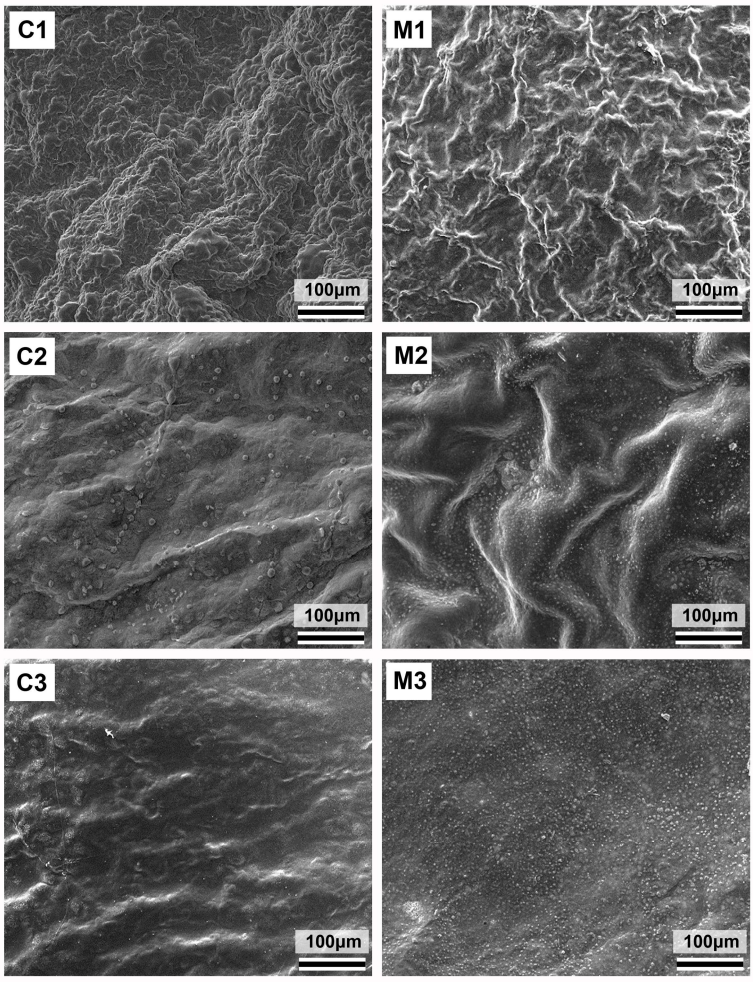

FIGURE 3. SEM evaluations of the surfaces of the matrices

3.3. Morphological characterization of matrices

Scanning electron microscopy analysis was performed to characterize the surface morphology of the developed matrices. Figure 3 contains the SEM findings of the developed matrices. Micrographs revealed that M1 and M2 type matrices (as well as C1 and C2) had rougher surface morphologies compared to M3 (also C3). M3 had the smoothest surface topography among all matrices.

Figure 4 shows high-resolution surface topographies of the matrices in the 10µm x 10µm area. The AFM images of the matrices generally revealed a homogeneous distribution of nanometer-sized (150-200 nm) clusters on the substrates.

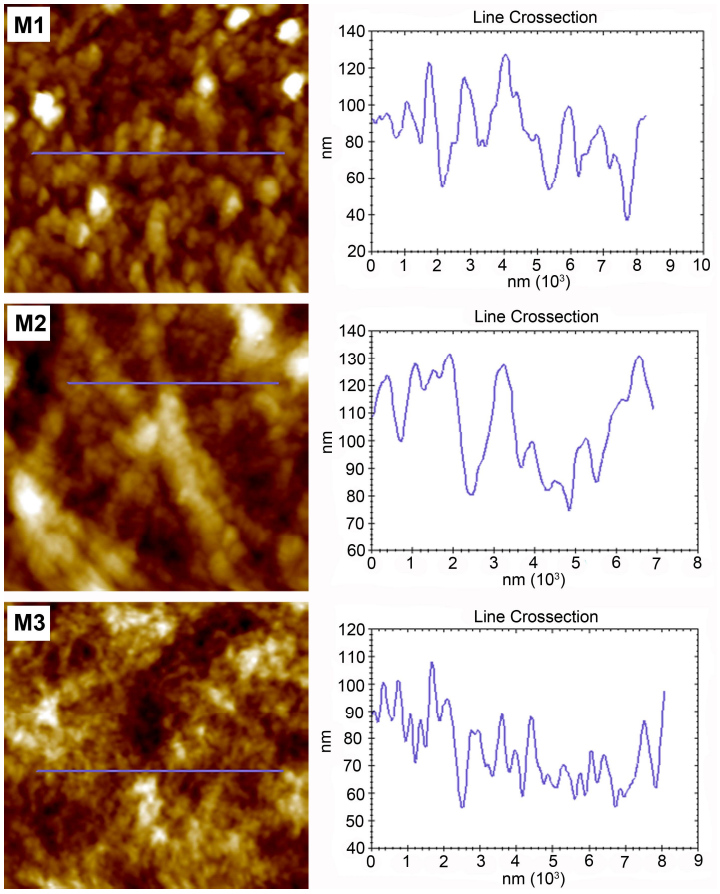

FIGURE 4. AFM cross-sectional analysis of collagen-chitosan-based surfaces

3.4. Light transmittance of the matrices

Analyses were carried out to determine the light transmittance of the developed matrices in the visible region. As can be seen in Figure 5, M2 showed the lowest light transmittance values (~50-70%) (similarly control C2, ~40-53%). Among all matrices, M3 had the highest light transmittance value of ~92-93%, compared to ~80% for M2. Transparency can be considered an important property for potential ophthalmic applications of hydrogel biomaterials, such as soft contact lenses, intraocular lenses, and ophthalmic drug delivery devices [13,14].

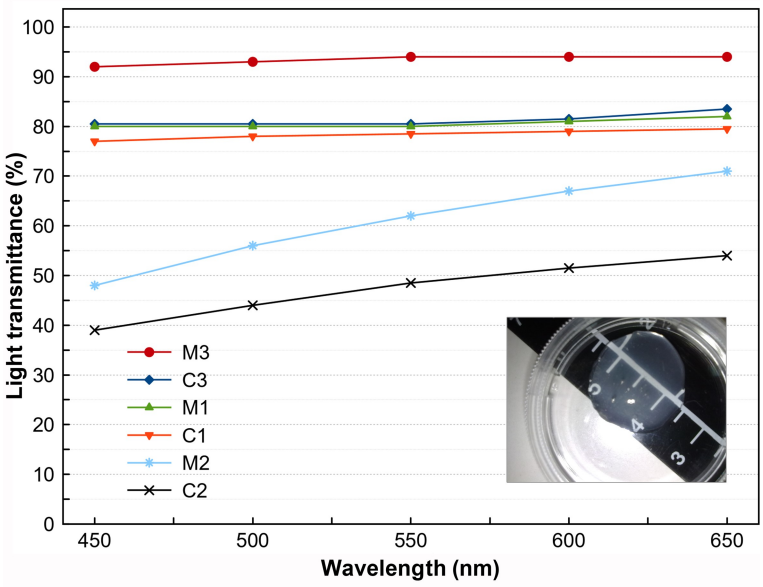

FIGURE 5. Light transmittance of the hydrogels in the visible region. Inner image: macrograph demonstrating the transparency of a collagen-chitosan hydrogel matrix

3.5. *In vitro* cytocompatibility of the matrices

To determine the *in vitro* cytocompatibility of the developed hydrogel matrices, mesenchymal stem cells were seeded on substrates and cultured for up to 7 days. The relative viability and proliferation capacity of MSCs on the matrices are presented in Figure 6. From the figure, it is clear that none of the matrices had any adverse effect on the metabolic activity of the seeded cells and the MSCs in culture proliferated over time. Relative cell viability

increased significantly between days 3 and 7 of cell culture (Figure 6). Among the groups, M3 was found to be a more suitable growth substrate for mesenchymal stem cells compared to M1 and M2.

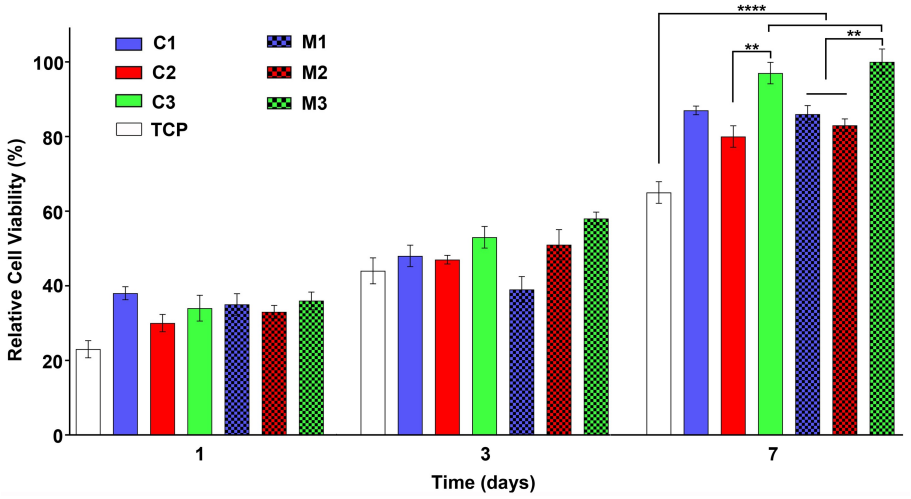

FIGURE 6. Relative viability and proliferation capacity of MSCs on the matrices assessed by MTT measurements upto 7 days in culture (**p < 0.01, and ****p < 0.0001)

4. CONCLUSIONS

In this study, collagen-chitosan-based hydrogel matrices were successfully created using three different cross-linking methods. Some physicochemical and *in vitro* biological properties of the matrices were studied in comparison with those of collagen-based hydrogels synthesized under the same conditions. All matrices retained their structural stability in a buffered aqueous environment for more than three months; this result is promising for potential future applications of matrices. Morphological analysis revealed that the formed hydrogels had relatively smooth surface features, while a smoother topography was detected for M3. *In vitro* cell culture experiments demonstrated that the substrates supported the metabolic activity of mesenchymal stem cells. The seven-day culture experiment revealed that M3 (*i.e.* PEG-stabilized collagen-chitosan) was a more suitable substrate for cells than M1 and M2. PEG-stabilized collagen-chitosan matrices also showed the highest visible light transmittance, which could be useful for

potential ophthalmic applications. However, further studies will be required to confirm this idea.

Author Contribution Statements SZ- conceptualization, investigation, data curation, formal analysis, writing. YME- supervising, resources, conceptualization, writing–review & editing. Both authors have read and approved the manuscript

Declaration of Competing Interests The authors declare no conflict of interest in relation to this particular article.

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REDISCOVERY OF AN ANCIENT HERBARIUM FOR TURKISH BOTANICAL HISTORY: BOLU GIRLS VILLAGE INSTITUTE HERBARIUM

NURSEL İKİNCİ

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ABSTRACT. Bolu Girls' Village Institute Herbarium (Bolu Kız Köy Enstitüsü Herbaryumu) was founded in 1953 by Muhittin Fehmi Özgen. It is one of the earliest herbaria of the Turkish Republic. The herbarium was part of the natural history collections of the school which also included insects, minerals, and fossils. The herbarium initially contained around 2500 specimens within 72 cardboard boxes. Additionally, specimens from this herbarium were sent to İstanbul University Department of Biology Herbarium (ISTF) and around 600 specimens to the herbarium of Royal Botanic Garden Edinburgh (E). In 2006, we received 567 plant specimens in five boxes. The plants were mainly collected from Bolu and neighboring provinces between 1953 and 1958. The herbarium collection contains one bryophyte specimen and three seedless vascular plants. The remaining taxa belong to gymnosperms and angiosperms. The herbarium labels were prepared both in Latin and Turkish. Majority of the specimens have identification up to family and genus level and a small number of them up to species level. This herbarium remained largely unknown to Turkish botanists. Therefore, we think it is valuable to provide detailed information about the history and the content of the herbarium. The information given here not only will contribute to our knowledge about the natural history collections of Turkey but also to the education history of the Turkish Republic.

1. INTRODUCTION

1.1. Historical development of herbaria during the Ottoman Empire

Herbaria are systematically arranged collections of dried plant specimens which allow scientists to study plants simultaneously regardless of their

Keywords. Botanical history, Kızılçullu, Savaştepe, Turkey, Village Institutes
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growth time and locality. Historical herbaria are especially important as they provide us information about the distribution of plants in time and space. They can be useful for tracing the origin of cultivated plants and tracking the spread of invasive species. The earliest herbaria of the world were founded in Italy during the first half of the 16th century. Botanist Luca Ghini working at the University of Pisa is known to be the inventor of the herbarium. Later, the practice of storing pressed and dried plants in herbaria spread rapidly to the rest of Europe [1]. However, the earliest herbaria in the Ottoman Empire began to be built around mid-19th century. The oldest herbaria of the Ottoman Empire were founded mainly by American and French Colleges and in few other Turkish schools mainly by foreign teachers. According to Baytop [2], the first official herbarium of the Ottoman Empire was "Mekteb-i Tıbbiye" or "Herbier de l'École Impériale de Médecine de Galata-séraï" (Imperial School of Medicine of Galata-Serai). The herbarium was founded by the German pharmacist Friedrich Wilhem Noë in 1845. It was a short-lived herbarium since all the collections, associated materials, and equipment along with other natural history collections were lost during the great fire of Beyoğlu in 1848 [3]. While in Turkey, F. W. Noë made several botanical excursions and sent specimens to European herbaria [3]. Later in 1870, Dr. Abdullah Bey whose original name was Carl Eduard Hammerschmidt (1799-1874) was assigned to restore the natural history collections of the school. In 1871, the school's herbarium collection included 2725 specimens. However, these herbarium specimens could not reach today [2]. One of the other earliest herbaria of the Ottoman Empire was established in 1869 within the Robert College as part of the Natural History Museum. The herbarium collections were mainly provided by Georges Vincent Aznavour and George E. Post. There were 13000 plant specimens in the herbarium [4]. According to Demiriz [5] thousands of specimens collected by G. V. Aznavour were transported to Geneve Herbarium by B. V. D. Post and his wife. The remaining specimens were transferred to Saint-Joseph School in İstanbul [6]. Another school with herbarium collections was Central Turkey College Aintab which was founded in 1875 and closed in 1924. It had a natural history museum named as Mary A. Dickinson Museum. In 1905, teachers and their pupils at Saint Joseph High School in İstanbul began to collect plants from İstanbul-Kadıköy and its environs until the mid-1970s. The collection which remains today includes 2253 herbarium specimens belonging to 1006 species, 436 genera and 87 families [7]. Another school with herbarium collections was Museum of Anatolia College, Merzifon (1913–1938) which contained botanical collections by Manissadjian. In 1914, the herbarium had 7000

botanical collections. In 1938, the museum collections were transferred to Tarsus American College and part of the herbarium collections were sent to herbarium of Ankara University. Later, the collections at the Tarsus American College were sent to Robert College in İstanbul [8]. Other than the above-mentioned herbaria, several other schools had some plant collections. Akyıldırım [9] found natural history collections in 47 schools of İstanbul. However, only 16 of them were well preserved. In terms of botanical collections, a few of them contain complete herbarium specimens as most of them had either only leaf or seed collections.

1.2. The First Herbaria of the Turkish Republic

After the foundation of Turkish Republic and with the higher education revolution new herbaria were founded in Ankara and İstanbul in 1933. German botanist Prof. Dr. Kurt Krause was assigned as the director of the botanical department of the Ankara Higher Agricultural Institute (Ankara Yüksek Ziraat Enstitüsü) between 1933 and 1939. This department was firstly attached to the Ministry of Agriculture. In 1933, Prof. Dr. Krause founded the herbarium of Ankara Yüksek Ziraat Enstitüsü, also called as "Herbarium Turcicum" (ANK). In addition to the foundation of the herbarium, Prof. Dr. Krause travelled around Turkey and collected plant specimens. In 1948, the institute and the herbarium were joined to the Science Faculty of Ankara University. Körüklü [10] stated that there are currently specimens in this herbarium collected by Turkish botanists Kadri Ahmet and Hikmet Birand dated from 1927. The herbarium also contains older specimens received from European herbaria as a gift and specimens from the first herbarium of the Ottoman Empire, Imperial School of Medicine of Galata-Serai which were collected by the director Dr. F.W. Noë in 1844 [10]. In the meantime, İstanbul University Science Faculty Herbarium (ISTF) was founded by Prof. Dr. Alfred Heilbronn in 1933. Later, two additional herbaria were opened under the University of İstanbul belonging to Pharmacy Faculty (ISTE) in 1945 and Forestry Faculty (ISTO) in 1950. At present, these four herbaria are still active and are among the herbaria with the largest collections in Turkey.

Later, Central Anatolia Forestry Research Institute Herbarium (ANKO) was opened in 1959. Then, Ege University herbarium (EGE) was founded in 1962, followed by Ankara University, Faculty of Pharmacy herbarium (AEF) in 1967, Hacettepe University, Faculty of Pharmacy herbarium (HUEF) in 1969 and Atatürk University, Faculty of Science herbarium

(ATA) in 1969. During the 1970s with the foundation of new universities several new herbaria were opened under biology, pharmacy, or forestry departments.

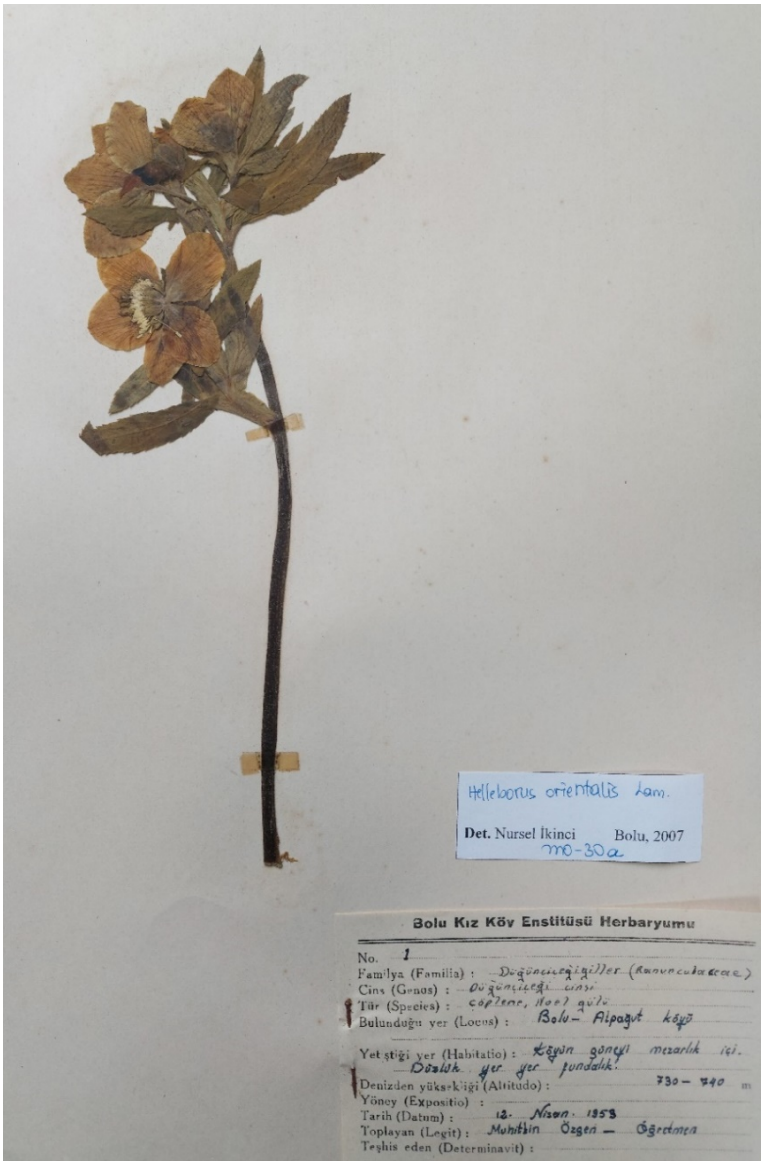

FIGURE 1. One of the specimens from Bolu Girls' Village Institute Herbarium collected in 1953.

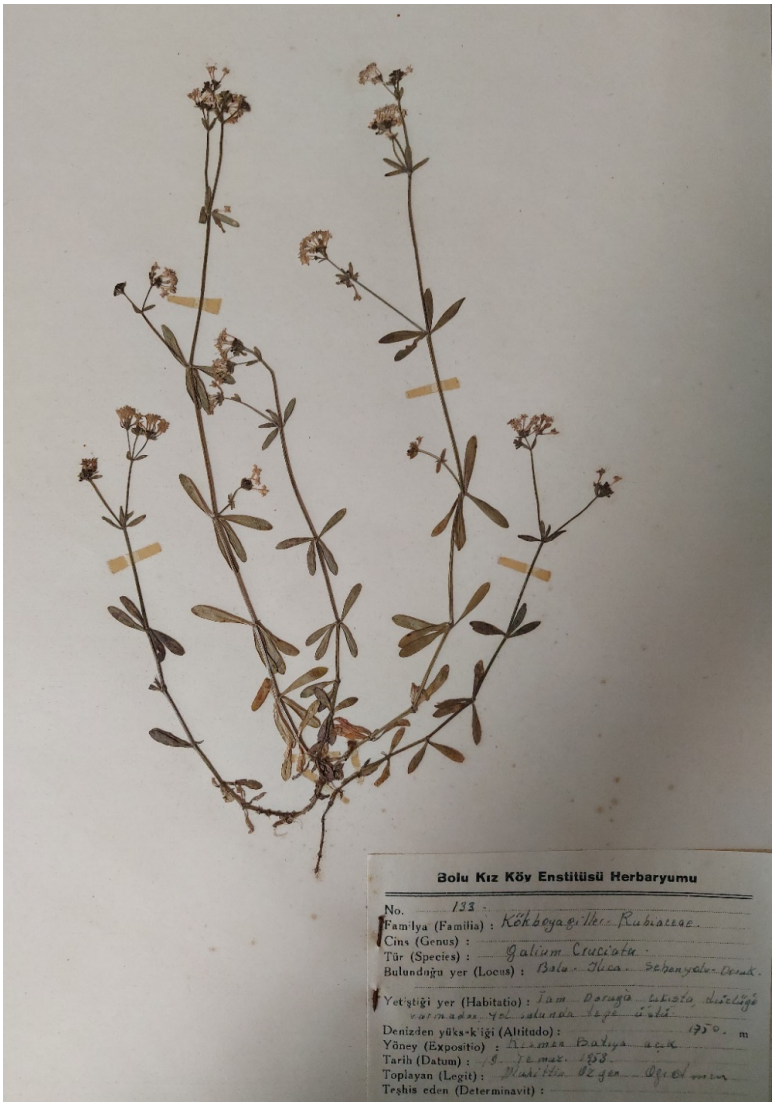

FIGURE 2. One of the specimens with identification up to species level.

1.3. Rediscovery of Bolu Girls' Village Institute Herbarium

In 2006, we received 567 herbarium specimens within five boxes (Figure 1-3). The specimens were collected between 1953 and 1958. These were carefully prepared herbarium specimens with accurate labels written in both

Latin and Turkish. Majority of the specimens were collected by Muhittin Özgen (teacher and the founder of the herbarium) but there were also many specimens collected by the pupils. Later in 2013, Mehmet Tunçkol (a retired teacher in Bolu who also brought us the five herbarium boxes) found an additional set of around 50 herbarium specimens belonging to the same herbarium collections. These later specimens were collected from Abant in 1953 [11]. According to Demiriz [5], specimens from Bolu Girls' Village Institute Herbarium were also sent to İstanbul University Science Faculty Herbarium (ISTF). Some of his specimens were cited in the Flora of Turkey [12]. Additionally, during that time, around 600 specimens were sent to the herbarium of Royal Botanic Garden Edinburgh (E).

FIGURE 3. Five boxes we received in 2006 containing the herbarium specimens.

Bolu Girls' Village Institute did not only own herbarium collections but also zoological, geological, and fossil collections (Figure 4). Together with the pupils, they also built a weather station in the schoolyard to record some of the environmental data. Part of the zoological collection also saved but not in good condition and stored at the Abant Izzet Baysal University Entomology Museum (AIBUEM) (Figure 5 and 6). Aim of this paper is to

provide details about the history of the Bolu Girls' Village Institute collections and its founder Muhittin Fehmi Özgen and to give the list the of specimens stored at AIBU herbarium with all the details attached to them including identifications performed by us.

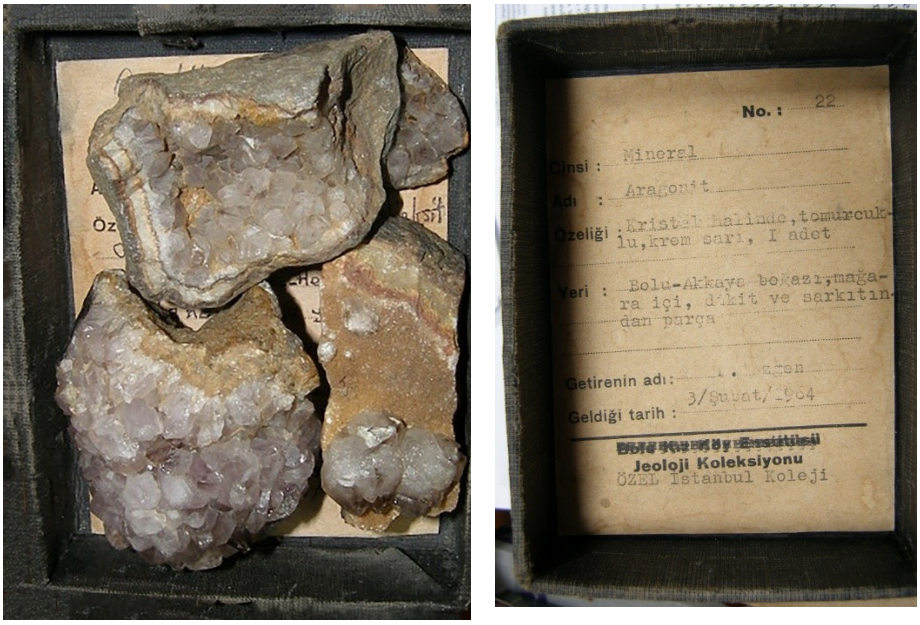

FIGURE 4. An example from the mineral collection of the school (left). Muhittin Özgen continued to use the labels prepared for Bolu Girls' Village Institute when he moved to different schools by crossing out "Bolu Kız Köy Enstitüsü" and replacing it with "Özel İstanbul Koleji" (right).

2. MATERIALS AND METHODS

We identified and prepared a list of the 567 received specimens. Identification of the herbarium specimens were done using "Flora of Turkey and the East Aegean Islands [12-13] and recent publications related with the region [14-22]. All the herbarium specimens are kept in the Herbarium of Bolu Abant İzzet Baysal University (AIBU). Taxa in the floristic list are given in alphabetical order of families (Appendix). In this list, only plant identifications were added by us. All the other data provided were obtained from the original herbarium labels. There are several duplicates for almost

every specimen. Therefore, we have given the label details of one specimen and added the total number of duplicates.

FIGURE 5. Part of the insect collection of Bolu Girls' Village Institute which are stored at the Abant Izzet Baysal University Entomology Museum (AIBUEM).

FIGURE 6. Second part of the insect collection.

3. RESULTS AND DISCUSSION

3.1. History of Bolu Girls' Village Institute Herbarium

Bolu Girls' Village Institute Herbarium was founded in 1953. The herbarium initially contained 2500 herbarium specimens in 72 cardboard boxes [5; 23]. Other than these specimens, the founder of the herbarium Muhittin Özgen and his pupils had also collected specimens which were sent to İstanbul University Department of Biology Herbarium (ISTF). An additional set of 600 specimens were sent to the herbarium of Royal Botanic Garden Edinburgh (E). In 2006, we received five cardboard boxes containing 567 herbarium specimens. This was what was left of the original 72 herbarium boxes.

Regarding the history of the school, before the Bolu Girls' Village Institute, the school served as Bolu Forestry School (Bolu Orman Mektebi) for 15 years between 1937 and 1952 [24]. The school converted to Bolu Girls' Village Institute in the fall 1952 and the forestry school was moved to Düzce

together with all the staff (teachers and civil servants) and the furniture [24]. The first pupils of the Bolu Girls' Village Institute came from İzmir Kızılçullu Village Institute. İzmir Kızılçullu Village Institute was opened in 1937 with the name of "village teachers' training school". The school used the American College buildings and its campus purchased from the Americans by the Turkish State. The institute was closed in 1952 because its buildings and the campus wanted to be used as NATO headquarters. In fall 1952, 400 pupils of the school and 25 teachers were transferred to Bolu Girls' Teacher School and the remaining 400 pupils were sent to Beşikdüzü Girls' Village Institute [25].

The institute in Bolu has continued to this day but under different names and formats. Firstly, the name of the school was changed as "Bolu Girls' Teacher College" (Bolu Kız Öğretmen Okulu) in 1954 because all the "Village Institutes" were closed throughout Turkey. Later, Bolu Girls' Teachers College was closed in 1980. Then, in 1982, when all the materials of the school were distributed to other schools in the vicinity, Mehmet Tunçkol (teacher and school principal at that time in Bolu) saved the above-mentioned herbarium specimens in 20 boxes [11]. Then, Tunçkol put all the herbarium specimens into five boxes and kept them until 2006. In this time, our university also received two boxes of insect collections (Figures 5 and 6). These specimens are stored at the Abant Izzet Baysal University Entomology Museum (AIBUEM). In 2007, during his visit to our university Muhittin Özgen brought us several geological collections but as no one is studying in this field in our faculty we did not keep them. The rest of the zoological and geological collections are probably either destroyed or thrown away. Therefore, we can conclude that the school had different type of natural history collections in addition to herbarium specimens ranging from zoological and geological collections to fossils. The pupils were involved in every step of specimen preparation. All the materials required for different type of collections were produced at the schools' ateliers and carpentry. They also built a weather station in the schoolyard to record some of the environmental data.

3.2. The founder of the herbarium Muhittin Fehmi Özgen

The founder of the Bolu Girls' Village Institute Herbarium, Muhittin Fehmi Özgen was born in Malatya, Hekimhan, in 1921 (Figure 7 and 8). He attended primary school in Hekimhan and secondary school in Elazığ. Then he entered Balıkesir Necatibey Teachers College in 1937 and graduated in

1940 as a teacher. His first teaching place was Tunceli, Hozat where he worked one and a half year. Then, he completed his compulsory military service between 1942 and 1944 in İzmir and İstanbul. In October 1944, he entered the Natural Sciences Department of Ankara Gazi Education Institute (Ankara Gazi Terbiye Enstitüsü Tabii Bilimler Şubesi). There he studied botany, zoology, and geology. He graduated from the Biology Department of this institute in 1946 and was assigned as a teacher at Balıkesir, Savaştepe Village Institute (Savaştepe Köy Enstitüsü) and worked there until the end of 1952. He already started to collect plants and make herbarium specimens during his six years of stay in Balıkesir, Savaştepe and involved students in every step of natural history collection from preparing materials to field work and specimen preparation [23]. He was especially happy when students returned from their summer holidays with geological and other collections. According to Demiriz [5], there was about 500 herbarium specimens in this collection prepared carefully comparable to university herbarium collection standards. Muhittin Özgen moved to Bolu at the beginning of 1953 and continued working as a teacher at Bolu Girl's Village Institute (Bolu Kız Köy Enstitüsü) until the end of 1963. Then, he moved to İstanbul to continue his teaching career until his retirement in 1977. He passed away in İstanbul in 2017. His wife Süeda Özgen (?-2007) was a physical education teacher. They had two children: Mine and Savaş.

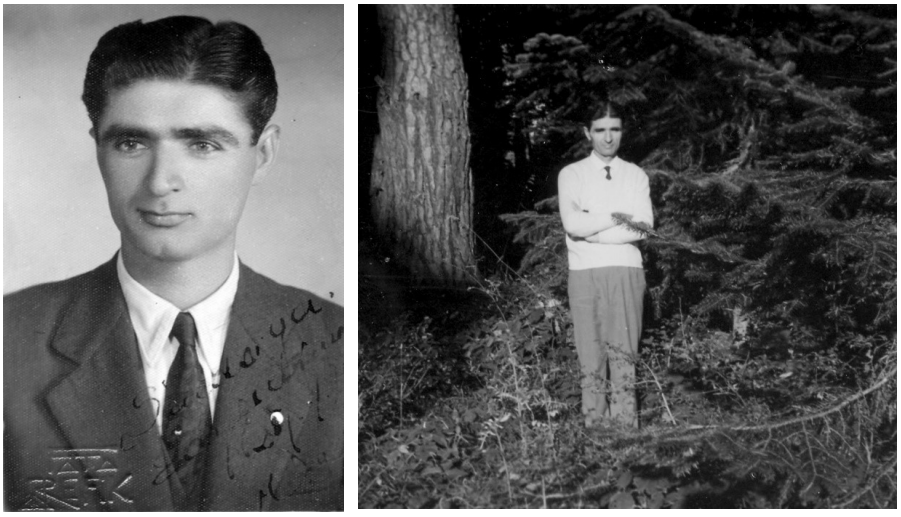

FIGURE 7. Muhittin Fehmi Özgen when he was a teacher in Balıkesir, Savaştepe Village Institute in 1947 (left) and during an excursion to Lake Abant in 1964 (right).

FIGURE 8. Muhittin Fehmi Özgen with his family in front of the lodging of Bolu Girls' Teacher College in 1961.

3.3. Content of the botanical collections

In Appendix, we provide the list of the 567 specimens (stored at AIBU herbarium) with their original label information. The original labels were written both in Turkish and Latin. In Figure 9 and 10 we provide examples of different labels. It can be seen from the labels that many students were involved in plant collection and herbarium label writing. The specimens were collected between 1953 and 1958. Nearly all the specimens have

family identifications given in Turkish and Latin (Figure 9). On the other hand, some specimens had genus names written on their labels and there are also several specimens with complete identification up to the species level. The specimens are in good condition regarding any infections. The only problem is with the poor quality of the strapping tape used which caused many loose specimens. Therefore, specimens need to be remounted.

The collection of 567 herbarium specimens represents 163 taxa as most of them have several duplicates. There is only one bryophyte specimen and three seedless vascular plants. The remaining 159 taxa belong to the flowering plants (Figures 11-17). Demiriz [5] indicated that part of the collection was identified by İstanbul University, Department of Biology staff. Muhittin Özgen informed us that he was searching for botany books in İstanbul bookstores to help him in plant identification (pers. comm.).

Considering the geographical distribution of the plants, we see that most of the specimens were collected from Bolu and neighbouring provinces. However, there are some specimens from different parts of Turkey. An example is a cotton specimen (*Gossypium herbaceum* L.) from Adana province collected by a pupil during the summer holiday.

The collection is composed of mainly wild plants but also includes ornamental plants like *Dahlia* sp., *Philadelphus coronarius* L. and several other cultivated plants, like *Prunus avium* (L.) Moench, *Pyrus elaeagnifolia* Pallas subsp. *elaeagnifolia*.

In terms of species richness of the collection, Caryophyllaceae has the highest number of species with 14 different species followed by Asteraceae (13 taxa) and Rosaceae (10 taxa). Boraginaceae (9 taxa), Fabaceae (8 taxa) and Campanulaceae (8 taxa) are the other families with high number of species. *Campanula* (6 species), *Geranium* (4 species) and *Dianthus* (4 species) are the genera with the highest number of species.

FIGURE 9. Label examples-I.

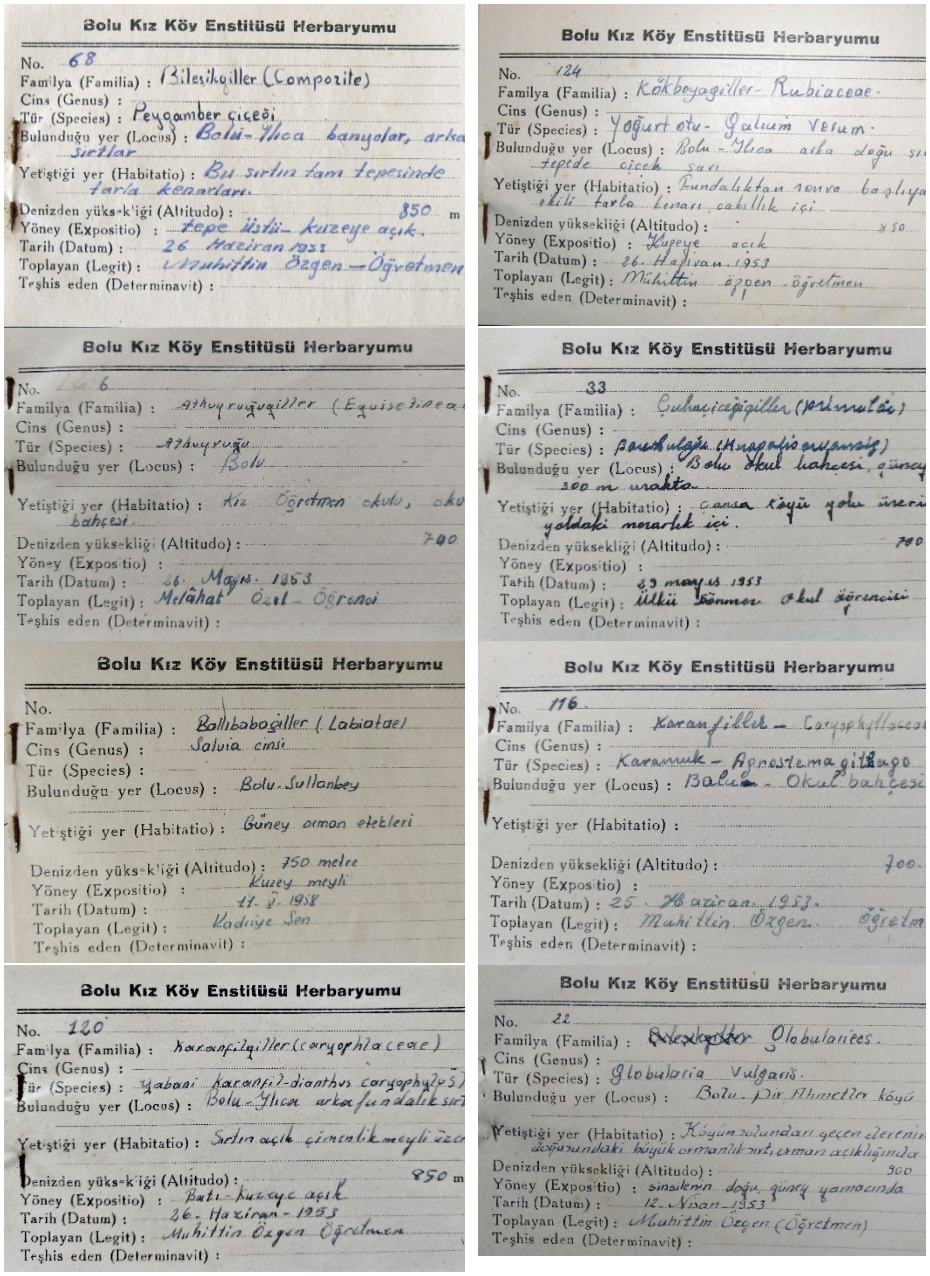

FIGURE 10. Label examples-II.

4. CONCLUSIONS

Although mentioned earlier in the publication of Demiriz [5], Bolu Girls' Village Institute Herbarium remained largely unknown to most Turkish botanists. It is important to notice that such a carefully prepared herbarium existed in a country school of Turkey much before the Flora of Turkey was started to be published [12-13]. The herbarium collections of the school also contributed to the Flora of Turkey. Therefore, it is important to document the history and the content of this herbarium which will not only improve our knowledge about natural history collections in Turkey but also the education history of the Turkish Republic.

The Village Institutes were opened in 1940 to supply the urgent teacher demand of the young Turkish Republic. The pupils to be educated in these schools were selected from villages and rural areas. After graduation they were going to be employed again in villages. There were 40 Girls' Institutes in Turkey in 1947 [26]. Pupils were accepted to Girls' Institutes after finishing five years of elementary school education and they study five years in the institutes. Secondary school graduates (finishing 5+3 years of education) were also accepted, and they could graduate afters two years of education at the institutes [26]. The Village Institutes were closed in 1954 due to ideological reasons. They were converted to teacher-training schools. In this paper, we provide solid information about the quality of education and types of methods involved in the Village Institutes.

Acknowledgement. The author thanks Mehmet Tunçkol (Bolu) who stored the herbarium specimens between 1982 and 2006 and made them available to us and Prof. Dr. Okan Külköylüoğlu who reached Muhittin Özgen and invited him to our university so that we could learn the details of the natural history collections of the school from the first hand. Finally, I would like to thank Prof. Dr. Mustafa Ünal for the information about the insect collection.

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

FIGURE 11. A specimen from Bolu Girls' Village Institute Herbarium.

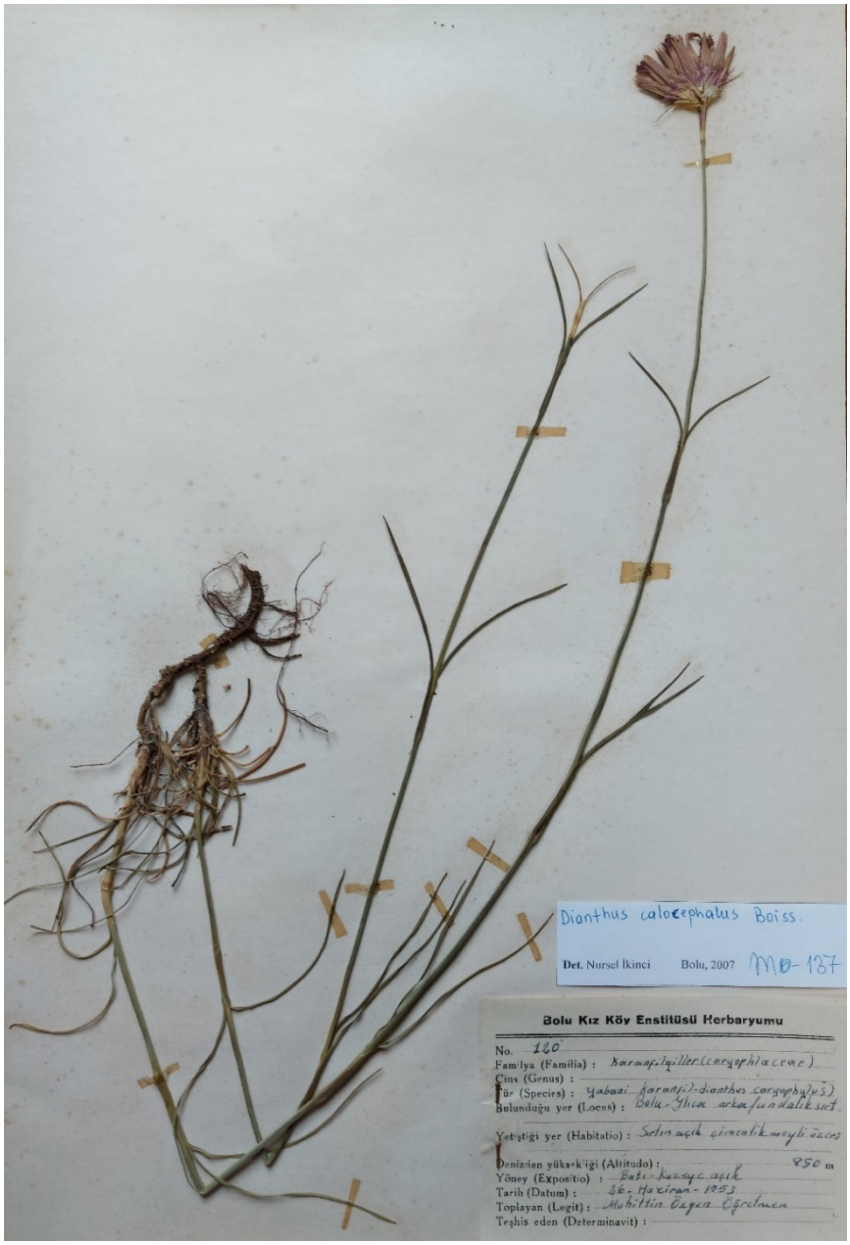

FIGURE 12. A specimen from Bolu Girls' Village Institute Herbarium.

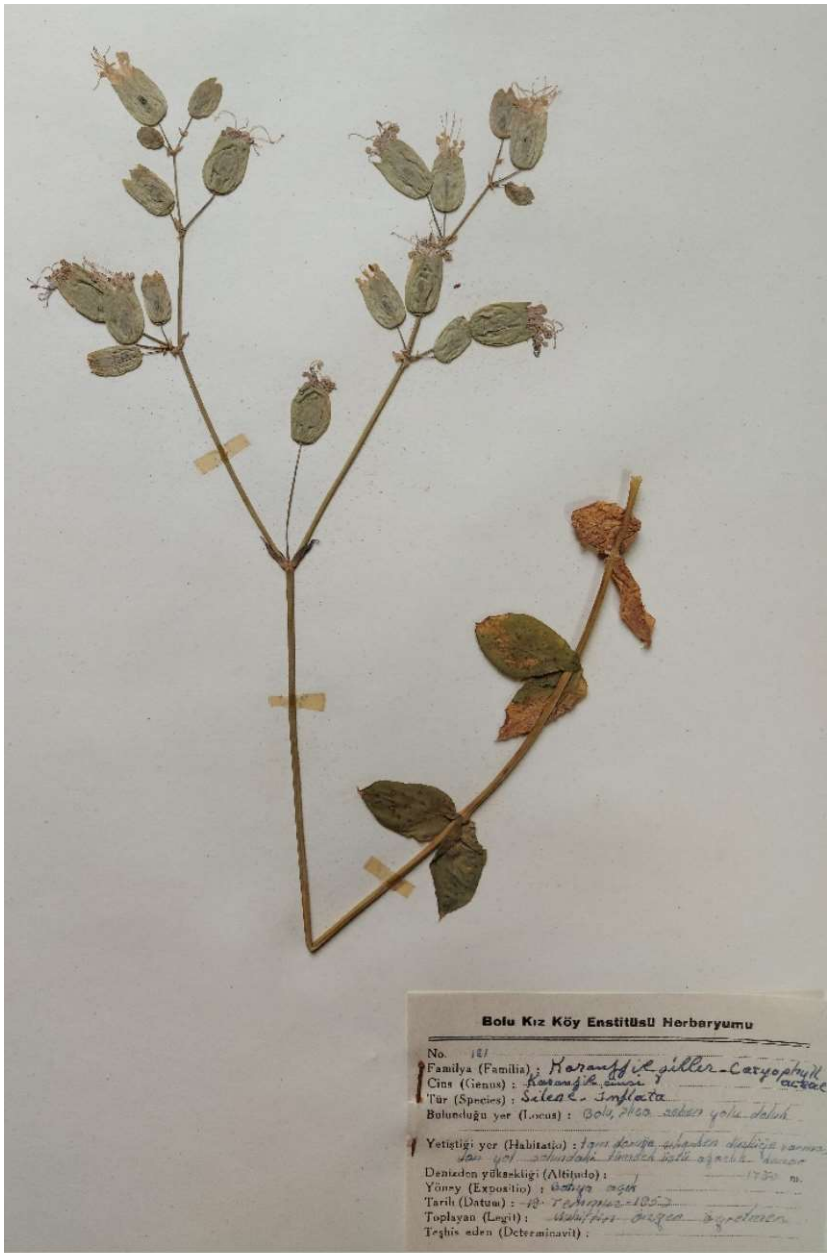

FIGURE 13. A specimen from Bolu Girls' Village Institute Herbarium.

FIGURE 14. A specimen from Bolu Girls' Village Institute Herbarium.

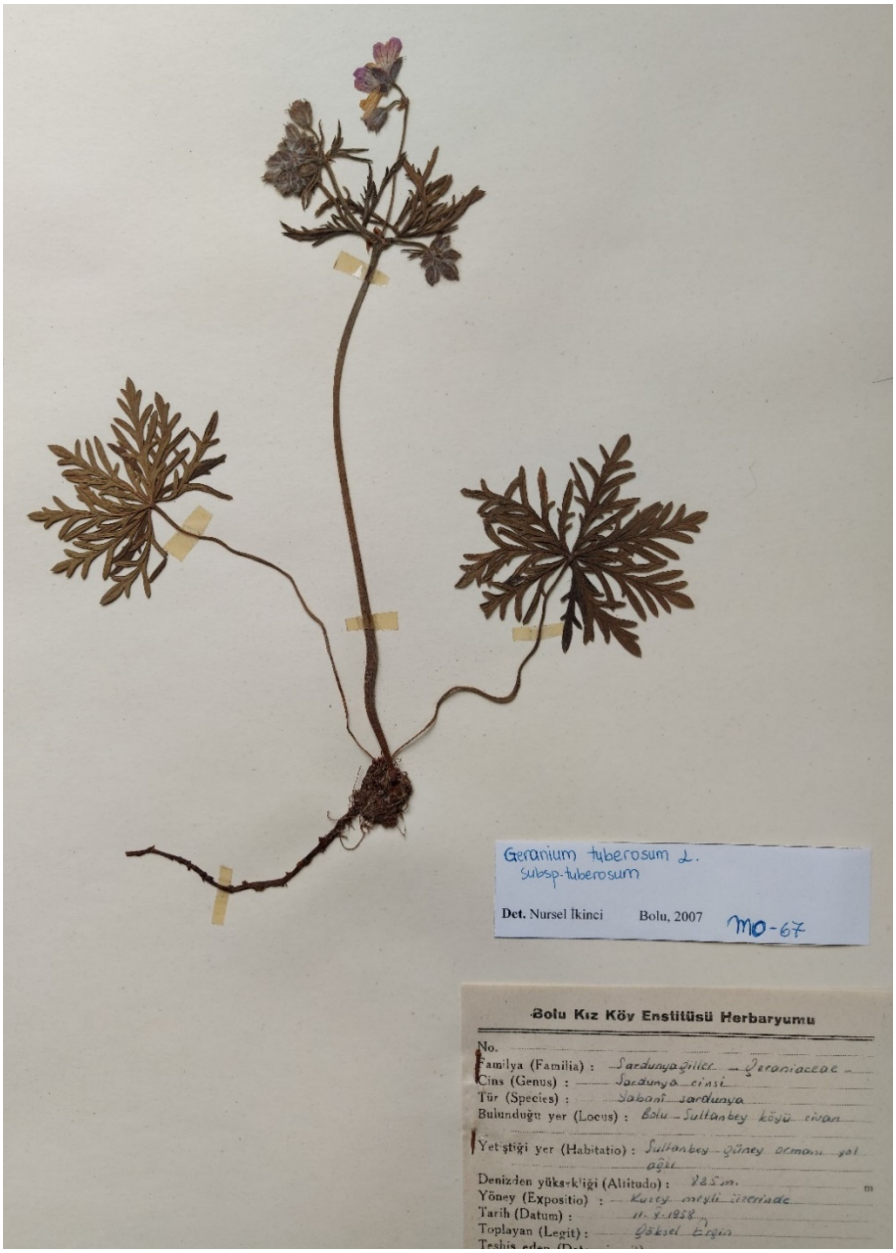

FIGURE 15. A specimen from Bolu Girls' Village Institute Herbarium.

FIGURE 16. A specimen from Bolu Girls' Village Institute Herbarium.

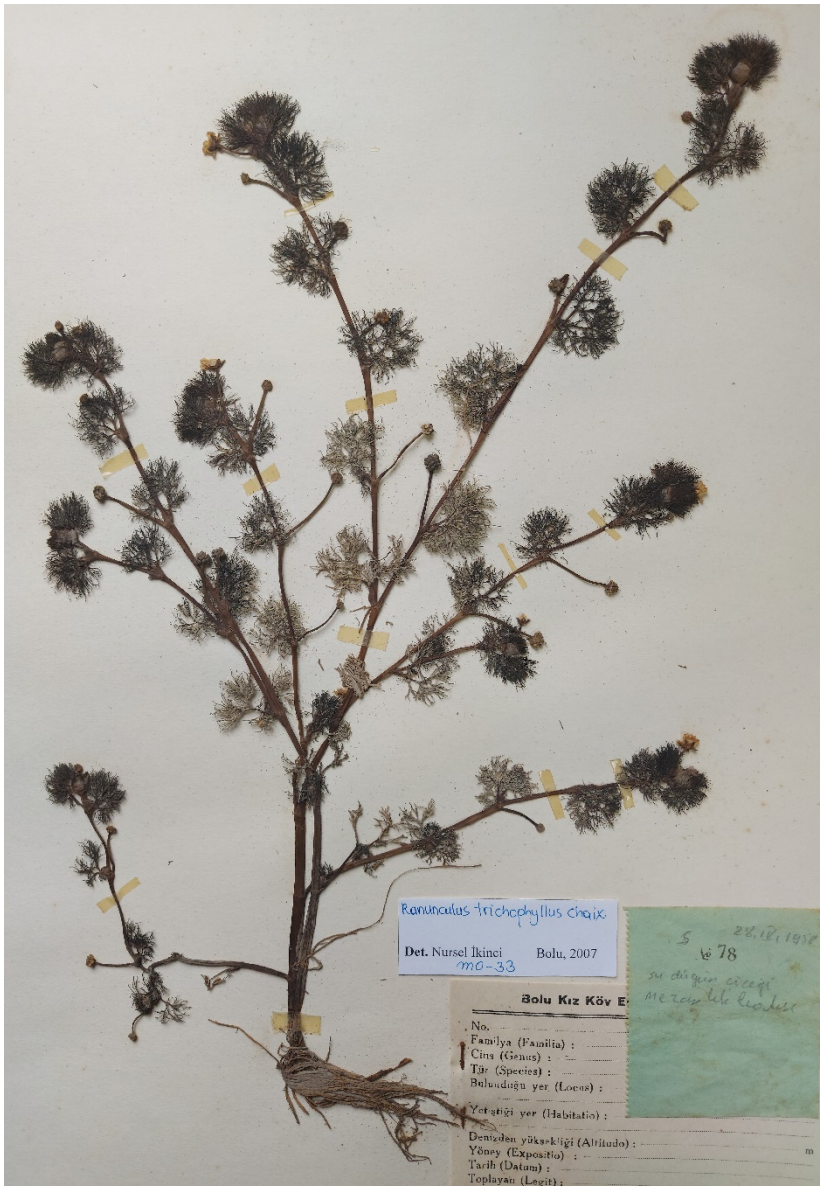

FIGURE 17. An example of an aquatic species, *Ranunculus trichophyllus* Chaix ex Vill., Turkish name written as “su düğün çiçeği”.

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APPENDIX. List of taxa belonging to Bolu Girls' Village Institute Herbarium which are stored at AIBU. First seedless plants and then seed plants are given according to alphabetical order of the families. The numbers are given by us to identity each specimen together with its duplicates (the first coloumn). The province name is only given when it is different than Bolu.

No	**Taxon**	**Locality, habitat, altitude, collector** (as on the original label)	**Date**	**Turkish name** (as on the original label)
144	**Bryophyta**	Ilıca-Seben yolu, doruktan 500 m kadar beride oluklu çeşme, yolun sol kenarında meyli üzerinde, toprak sulu, altı çamur, silsilenin kuzey meyli, 1500 m, M. Özgen	19.07.1953	Yosun
	Equisetaceae			
145	*Equisetum telmateia* Ehrh.	Kız Öğretmen Okulu, okul bahçesi, 740 m, Melahat Özal	26.05.1953	Atkuyruğugiller atkuyruğu
	Dennstaedtiaceae			
142	*Pteridium aquilinum* (L.) Kuhn	Abant, kuzey, Y. Özyavuz	14.06.1956	

No	**Taxon**	**Locality, habitat, altitude, collector** (as on the original label)	**Date**	**Turkish name** (as on the original label)
	Dryopteridaceae			
143	*Dryopteris filix-mas* (L.) Schott	Karabük		Eğrelti otu
	Aceraceae			
21	*Acer negundo* L.	Kız Öğretmen Okulu bahçesi	02.07.1955	
	Apiaceae			
151	*Smyrnium perfoliatum* L.	Okul bahçesi güneyi 50 m uzakta Çamşa yolu kenarı, bu yol kenarında-düzlük, 740 m, Nazire Ovalı	29.05.1958	
152	*Torilis leptophylla* (L.) Rchb.f.	Sultanbey Köyü civarı, kuzey meyli, güney ormanları eteği, 750 m, Gülşen Öztürk	11.05.1958	
153		Okul bahçesi, sürülmemiş tarla içi, 700 m, M. Özgen	16.06.1953	
	Aquifoliaceae			
12	*Ilex aquifolium* L.	Karabük, Büyükdüz Yaylası ormanı, orman içi (funda), 1550 m, M. Özgen	15.06.1954	Çoban püskülü
	Aristolochiaceae			
13	*Aristolochia pontica* Lam.	Kız Öğretmen Okulu bahçesi, 740 m, Binnaz Akbudak	26.05.1953	Lohusaotugiller Lohusa otu
	Asteraceae			
154	*Dahlia* sp.			
117	*Bellis perennis* L.	Sultanbey Köyü civarı, kuzey meyli üzerinde, Güney ormanları eteği, 750 m, Gül Yıldırım	11.05.1958	Bileşikgiller papatya
116	*Cichorium intybus* L.	Okul bahçesi, ön kısmı, Okul önü, solda sürülmemiş tarla içi, 700 m, M. Özgen	15.07.1953	Bileşikgiller şahin otu
124	*Carduus nutans* L. subsp. *nutans*	Çakmaklar Köyü yolunda, kuzeyde, 700 m, Ayhan Başaran	08.06.1955	
121a	*Centaurea depressa* M.Bieb.	Karacasu Ilıca sırtı güneydoğu sırtı kuzey meyli, güney, 780 m, Ayhan Başaran & Aysel Akarsu	04.06.1955	
121b	*Centaurea depressa* M.Bieb.	Ilıca, Banyolar, arka sırtlar, bu sırtın tam tepesinde, tarla kenarı, tepe üstü-kuzeye açık, 850 m, M. Özgen	26.06.1953	
121c	*Centaurea* depressa M.Bieb.	Okul bahçesi güneyi Çanşa yolu 50 m uzakta, yol ve tarla kenarı, tamamen açıklık, 700 m, Ayşe Kiraz	29.05.1953	

No	**Taxon**	**Locality, habitat, altitude, collector** (as on the original label)	**Date**	**Turkish name** (as on the original label)
123	*Lapsana communis* L. subsp. intermedia (M.Bieb.) Hayek	Ilıca Seben yolu doruk, tam doruğa çıkarken düzlüğe varmadan yol solundaki tepe, sırt üstü, batıya açık, 1750 m, M. Özgen	19.07.1953	
119	*Achillea millefolium* L. subsp. *pannonica* (Scheele) Hayek	Okul bahçesi güneyi Çanşa yolu 200 m uzakta, Tarla ve yol kenarı, tamamen düzlük, 700 m, Ayla Arat	29.05.1953	
122	*Carduus acanthoides* L. subsp. *canthoides*	Okul bahçesi aşağıda çam fidanlığı kenar, 2 m'ye kadar yükselmiş çam fidanlarının içinde, 700 m, M. Özgen	18.06.1953	Onofordom
120	*Cirsium hypoleucum* DC.	Ilıca-Seben yolu, otomobil durağı oluklu çeşme, bu çeşmeden 200 m ileride yolun sol meyli üzerinde, kuzeye dönük meyil üzerinde, 1500 m, M. Özgen	19.07.1953	
125	*Tanacetum* sp.	Karabük, Kuzey ormanı, Uzun Göveç Yaylası bölge binası civarı, Orman içi açıklığı güneye dönük meyil üzeri, 1500 m, M. Özgen	16.06.1954	
127	*Anthemis cotula* L.	Okul bahçesi, spor salonu inşaat yerinde, sık ve zengin bitki topluluğu içinde, açık düzlük, 700 m, M. Özgen	12.06.1953	
115	*Doronicum orientale* Hoffm.	Okul bahçesi, Okulun doğusu çam fidanlığı, 700 m, M. Özgen	25.05.1953	
126	*Pilosella hoppeana* (H.Schult.) C.H. & F.W.Schultz subsp. *isaurica* Hub.-Mor.	Çakmaklar Köyü arka sırtı çamlığında, kuzeyde, 800 m, Naime Vardar	08.06.1955	
	Boraginaceae			
107b	*Lithospermum purpurocaeruleum* L.	Sultanbey Köyü civarı, Sultanbey güney ormanları eteği, kuzey meyli, 750 m, Pakize Soğancı	11.05.1958	Hodangiller
108	*Myosotis alpestris* F.W.Schmidt subsp. *alpestris*	Ilıca-Seben yolu, doruk, doruğa tam çıkışta yol solundaki tepecik üstü, batıya açık, 1750 m, M. Özgen	19.07.1953	
109	*Myosotis lithospermifolia* (Willd.) Hornem.			
111	*Cynoglossum creticum* Miller	Okul bahçesi, binanın batısında çit kenarı, Düzlük, 700 m, M. Özgen	16.05.1953	
110	*Cynoglossum*	Kuzey, Muazzez Uluöz		

No	**Taxon**	**Locality, habitat, altitude, collector** (as on the original label)	**Date**	**Turkish name** (as on the original label)
	officinale L.			
113	*Onosma mutabile* Boiss.	Yıldız Özyavuz		
114	*Trachystemon orientalis* (L.) G.Don			
112	Boraginaceae	Çanşa Köyü, Tarla kenarı, sulak yerde, düzlük, 700 m, Saniye Korkusuz	28.04.1958	
107a	*Lithospermum purpurocaeruleum* L.	Sultanbey, güney etek ormanları	02.07.1955	
	Brassicaceae			
155	*Cardamine impatiens* L. var. *impatiens*	Karabük		
156	*Nasurtium officinale* R.Br.	Gönül Mumcu		
	Campanulaceae			
55	*Campanula persicifolia* L.	Ilıca-Seben yolu, Gölcük oluklu çeşme arası, bu iki nokta arasında devam eden şosenin doğu sırtları-orman etekleri, kuzeye kısmen açık, 1500 m, M. Özgen	19.07.1953	Çançiçeğigiller çan çiçeği
56	*Campanula olympica* Boiss.	Ilıca Banyolar seben yolu doruk, tam doruk düzlüğünde çok bodur funda içinde, 1850 m		
59	*Campanula rapunculus* L. var. *rapunculus*	Ilıca-Seben yolu, Gölcük oluklu çesme arkası, bu iki nokta arasında devam eden şosenin doğu sırtları-orman etekleri, kuzeye kısmen açık, 1500 m, M. Özgen	19.07.1953	Çançiçeğigiller
54	*Campanula latifolia* L.	Ilıca-Seben yolu, Gölcük üstü oluklu çeşme kenarı funda içi, otomobil durağının dahada yukarısındaki oluklu çeşmenin hemen yanında, batı kuzeye açık, 1500 m, M. Özgen	19.07.1953	Çançiçeğigiller
57	*Campanula glomerata* L. subsp. *hispida* (Witasek) Hayek	Ilıca Banyolar arka fundalık sırt daha doğuda. Yarenler düzlüğü, düzlük, sulak, 900 m, M. Özgen	26.06.1953	
58	*Campanula pterocaula* Hausskn.	Karabük, Kuzey ormanına giden yol, değirmen üstü şase kenarı, fundalık, 400 m, M. Özgen	18.06.1954	
52	*Legousia falcata* (Ten.) Fritsch	Okul bahçesi güneyi, Çanşa yolu 50 m ilerde, yol kenarı, 700 m, Mürüvvet Özer	29.05.1953	

No	**Taxon**	**Locality, habitat, altitude, collector** (as on the original label)	**Date**	**Turkish name** (as on the original label)
53	*Legousia pentagonia* (L.) Thell.	Okul bahçesi, düzlük, 700 m, M. Özgen	11.06.1953	
	Caprifoliaceae			
9	*Sambucus nigra* L.	Seben yolu, Gölcük üstü, oluklu çeşmeden biraz beride, yol kenarı, kuzey batıya açık, 1450 m,	19.07.1953	Mürüver
10	*Sambucus ebulus* L.	Ilıca-Seben yolu, Gölcük daha yukarıda oluklu çeşme altı, çeşme akıntısının sağ açık ve kıraç sırtları üzerinde fundamsı, batıya tamamen açık, 1500 m, M. Özgen	19.07.1953	Mürüver
76	*Viburnum lantana* L.	Sultanköy civarı, Güney orman etekleri, kuzey meyli, 725 m, Sevim Savaş	11.05.1958	Gülgiller
	Caryophyllaceae			
132	*Agrostemma githago* L.	Okul bahçesi, 700 m, M. Özgen	25.06.1953	Karanfilgiller karamuk
134	*Cerastium tomentosum* L.	Kız Öğretmen Okulu bahçesi, 740 m, Nalezar Tofur	26.05.1953	
158	*Stellaria media* (L.) Vill. subsp. *postii* Holmboe	Okul bahçesi güneyi, Çanşa yolu 300 m mesafede mezarlık içi, düzlük, 740 m, Ülkü Sönmez	29.05.1953	
158b	*Stellaria media* (L.) Vill. subsp. *postii* Holmboe			
161	*Minuartia anatolica* (Boiss.) Woron. var. *anatolica*	Okul bahçesi 50 m güney Çanşa Köyü yolu, yol ve tarla kenarı ağaçsız açık, 700 m, Nurten Temel	27.05.1953	
160b	*Stellaria media* (L.) Vill. subsp. *media*	Şehir içi, Mudurnu Caddesi Tekel Binaları önü, 700 m, M. Özgen	25.05.1953	
160	*Stellaria media* (L.) Vill. subsp. *media*	Okul bahçesi 50 m güney Çanşa Köyü yolu, yol ve tarla kenarı, 700 m, Ayşe Kiraz & Mürvet Özer	29.05.1953	
160c	*Stellaria media* (L.) Vill. subsp. *media*	Okul bahçesi güneyinden geçen Çanşa yolu 100 m uzakta, tarla ve yol kenarı düzlük, 700 m, Ayla Arat	29.04.1953	
159	*Moenchia mantica* (L.) Bartl. Subsp. *mantica*	Ilıca-Seben yolu, doruk, güney sırtlar, doruktan 2 km güneyde orman açıklığı çimenlik bir meyili yaylalık, doğu-güneye açık, 1650 m, M. Özgen	26.07.1953	

No	**Taxon**	**Locality, habitat, altitude, collector** (as on the original label)	**Date**	**Turkish name** (as on the original label)
157a	*Minuartia hirsuta* (M.Bieb.) Hand.-Mazz. subsp. *falcata* (Gris.) Mattf.	Abant, gölün hemen kuzey doğu sırtın gerisi, sırtın kuzey meğilinde ağaçsız hafif çimenlikler arası, 1350 m, M. Özgen	07.06.1953	Karanfilgiller
157b	*Minuartia hirsuta* (M.Bieb.) Hand.-Mazz. subsp. *falcata* (Gris.) Mattf.	Bolu-Seben yolu, yolun tam tepe noktasına vardığı yer, doruk, doruğa çıkışta karşılaşılan ilk çimenlik alan üzerinde, açık düzlük, etraf muhafazalı, 1850 m, M. Özgen	19.07.1953	Karanfilgiller
136	*Dianthus barbatus* L.	Okul bahçesi, Dikiş atölyesi bahçesi, 700 m, M. Özgen	16.06.1953	
137	*Dianthus calocephalus* Boiss.	Ilıca arka fundalık sırt, sırtın açık çimenlik meyli üzeri, Batı-kuzey açık, 850 m, M. Özgen	26.06.1953	Karanfilgiller yabani karanfil
137b	*Dianthus calocephalus* Boiss.	Karacasu, Ilıca sırtı, Güneydoğu sırtı, kuzey meyli, güney, 800 m, Aysel Akarsu	04.06.1955	
138	*Dianthus ancyrensis* Hausskn. & Bornm.	Kızık yaylası, Aladağ orman işletmesi civarı, orman açıklığı, yayla, yaylalık, 1350 m, Emine Coşkun	23.06.1955	
139	*Dianthus corymbosus* Sibth. & Sm.	Karabük, Büyükdüz Ormanı, Yol kenarı, 1600 m, M. Özgen	16.06.1954	
133	*Vaccaria pyramidata* Medik.	Ilıca banyolar, arka sırt, tepe üstü, ekilmiş tarla içi, kuzeye açık, 850 m, M. Özgen	26.05.1953	
140	*Silene compacta* Fischer	Ilıca-Seben yolu otomobil ihmal yeri, Oluklu çeşme ile Gölcük arasında yol kenarı orman eteği, kuzeye açık (kısmen), 1500 m, M. Özgen	19.07.1953	
141a	*Silene vulgaris* Huds. va *vulgaris*	Ilıca-Seben yolu, doruk, tam doruğa çıkarken düzlüğe varmadan yol solundaki tümsek üstü ağaçlık kenarı, batıya açık, 1750 m, M. Özgen	19.07.1953	Nâhul Çiçeği
141b	*Silene vulgaris* Huds. var. *vulgaris*	Okul bahçesi, Okul altı çam fidanlığı kenarında, 700 m, M. Özgen	18.06.1953	
	Chenopodiaceae			
4	*Chenopodium foliosum* (Moench) Aschers.	Kız Öğretmen Okulu bahçesi		
	Cistaceae			
6	*Helianthemum nummularium* (L.) Mill. subsp. *nummularium*	Karacasu Ilıca sırtı güneydoğu sırtı kuzey meyli, güney, 780 m, Mükerrem Atay	04.06.1955	

No	Taxon	Locality, habitat, altitude, collector (as on the original label)	Date	Turkish name (as on the original label)
7	*Cistus laurifolius* L.	Karabük Kuzey ormanına giden yol değirmen üstü şose kenarı, kuzey doğuya dönük tarafta, 400 m, M. Özgen		
	Convolvulaceae			
24	*Calystegia silvatica* (Kit.) Griseb.			
	Corylaceae			
51	*Corylus avellana* L. var. *avellana*	Akçakoca, Akçakoca deniz sahili, 25 m, M. Özgen	12.07.1954	
	Cupressaceae			
16	*Juniperus oxycedrus* L. subsp. *oxycedrus*	Ilıca-Seben yolu, doruk 1000 m uzaktaki arka sırt, bu kısımda bulunan orman arasındaki açık kısım, yayla, doğuya açık, 1500 m, M. Özgen	26.07.1953	Selvigiller bodur ardıç
	Dipsacaceae			
19	*Dipsacus laciniatus* L.	Ilıca-Seben yolu, otomobil ikmal yeri daha yukarı oluklu çeşme, bu çeşmenin üstü yol kenarında yolun doğu meyili üzerinde açık alan, Kuzey batıya açık, 1500 m, M. Özgen	19.07.1953	
149	*Scabiosa columbaria* L. subsp. *columbaria* var. *columbaria*	Ilıca arka sırtlar, Banyoların hemen arkasındaki fundalık kısmın üst kısmı kaplayan tarla kenarlıkları, 800 m, M. Özgen	26.06.1953	Tarakotugiller uyuzotu
	Ericaceae			
60	*Rhododendron ponticum* L. subsp. *ponticum*			
61	*Arbutus unedo* L.			
	Euphorbiaceae			
28	*Euphorbia amygdaloides* L. var. *amygdaloides*	Karabük, Uzun Göveç Yaylası, Yanıklık orman deneme sahası, kuzeye dönük meyil üzerinde, kuzeye dönük meyil, 1500 m	13.06.1954	
27	*Euphorbia helioscopia* L.	Kız Öğretmen Okulu bahçesi güneyi, 300 m ileride soldaki mezarlık içi, sık çimenli sulakca açık bir düzlük, 700 m, Ruşen özer	29.05.1953	Sütliyengiller sütliyen
	Fabaceae			
81	*Dorycnium graecum* (L.) Ser.	Ilıca seben yolu doruktan 200 m geride orman kenarı, yolun kenarında bitki ile funda arasındaki koyda, batı kuzeye açık, 1700 m, M. Özgen	19.07.1953	

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80	*Chamaecytisus supinus* (L.) Link	Ilıca seben yolu doruktan 200 m geride orman kenarı, tarif edilen mahalde yolun sol kanarında çok kısa funda, batı kuzeye açık, 1750 m, M. Özgen	19.07.1953	
85	*Genista lydia* Boiss. var. *lydia*	Kızık Yaylası-Aladağ Orman İşletmesi civarı, Orman içi yayla, yaylalık, 1350 m, Emine Coşkun	23.06.1955	
85b	*Genista lydia* Boiss. var. *lydia*	Sultanbey köy civarı, Güney orman etekleri, kuzey meyli üzeri, 825 m, Gülhan Efe	11.05.1958	
82	*Lathyrus nissolia* L.			
83	*Lathyrus laxiflorus* (Desf.) O.Kuntze subsp. *laxiflorus*			
84a	*Lathyrus digitatus* (M.Bieb.)	Sultanbey Köyü civarı, Sultanbey güney orman eteği, kuzey meyli, 750 m, Benar Uçkun ve Perihan Ersoy	11.05.1958	Baklagiller
84b	*Lathyrus digitatus* (M.Bieb.)	Yüksel Canan		
87	*Trifolium campestre* Schreb.	Çayırköy civarı, tarla kenarı, dere kenarı, düzlük, 700 m, Pakize Soğancı	28.04.1958	
88	*Medicago sativa L. subsp. sativa*	Okul bahçesi, açık düzlük, 700 m, M. Özgen	16.06.1953	
	Fagaceae			
17	*Fagus orientalis* Lipsky	Karabük, Büyükdüz ormanı, Orman, 1600 m, M. Özgen	09.06.1954	Kayıngiller kayın
18	*Castanea sativa* Miller			
	Geraniaceae			
68	*Geranium robertianum* L.	Ilıca Seben yolu dorutan yol boyunca 2 km kadar ilerde, güneyde yolun solundaki çimenlik açık alanda orman açıklığında, 1650 m, M. Özgen	26.07.1953	
67	*Geranium tuberosum* L. subsp. *tuberosum*	Sultanbey köy civarı, güney orman etekleri, kuzey meyli, 750 m, Ayşe Özkaya	11.05.1958	Sardunyagiller yabani sardunya
66	*Erodium cicutarium* (L.) L'Hérit. subsp. *cicutarium*	Ilıca Seben yolu doruk 3 km güney yol solu, orman içerisi çimenlik açık düzlük yayla, güneye açık, 1650 m, M. Özgen	26.07.1953	Sardunyagiller
69	*Geranium sylvaticum* L.	Ilıca Seben yolu, doruk, tam doruğa çıkıldığında karşılaşılan düzlükte funda içi, kısmen doğuya açık, 1750 m, M. Özgen	19.07.1953	Sardunyagiller

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65b	*Geranium pyrenaicum* Burm.	Okul bahçesi, okulun doğusunda duvar dibi, 700 m, M. Özgen	18.06.1953	Sardunyagiller
65a	*Geranium pyrenaicum* Burm.	Kız Öğretmen Okulu bahçesi, 740 m, Nazende Kaçmaz	26.05.1953	Sardunyagiller turnagagası
	Globulariaceae			
26a	*Globularia trichosantha* Fisch. & Mey.	Pirahmetler Köyü, köyün solunda geçen derenin doğusundaki büyük ormanlık sırt, orman açıklığında, silsilenin doğu, güney yamacında, 900 m, M. Özgen	12.04.1953	
26b	*Globularia trichosantha* Fisch. & Mey.			
	Iridaceae			
41	*Gladiolus italicus* Miller	Karacasu-Ilıca güneydoğu sırtı tepesinde, güney, 800 m, Meliha Baskın	04.06.1955	
39	*Iris sintenisii* Janka	Karacasu-Ilıca güneydoğu sırtı kuzeyi meyili, güney, 750 m, Ayhan başaran	04.06.1955	
	Juncaceae			
47	*Luzula campestris* (L.) DC.	Karabük, Büyükdüz, orman içi, 1600 m, M. Özgen	17.06.1954	
49	*Luzula forsteri* (Sm.) DC.	Karabük, Kuzey ormanı Büyükdüz mevkii, Bölge Binası, Orman içi açıklığı, kuzey-doğu meyili, 1500 m, M. Özgen	15.06.1954	
48b	*Luzula sylvatica* (Hudson) Gaudin			
48	*Luzula sylvatica* (Hudson) Gaudin	Abant, Gölün akıntı köprüsü karşı çıplak sırtlar (hemen geride orman başlıyor), bu tepenin doğu meyili üzerinde, 1350 m, M. Özgen	31.05.1953	
	Lamiaceae			
93b	*Ajuga reptans* L.	Sultanbey Köyü civarı, Güney orman etekleri, kuzey meyli üzerinde, 825 m, Yüksel Duman	11.05.1958	
93a	*Ajuga reptans* L.	Kızık Yaylası-Aladağ Orman İşletmesi civarı, Orman kenarı-yayla, yaylalık, 1350 m, Adile Özbay	23.06.1955	
92	*Ajuga orientalis* L.			
95	*Lamium purpureum* L. var. *purpureum*			
94	*Salvia tomentosa* Mill.	Okul bahçesi, Okul önü, sol kısım düzlük, 700 m, M. Özgen	18.06.1953	

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90	*Sideritis montana* L. subsp. *montana*	Ilıca, banyolar arka sırtlar, bu sırtın üst kısmında fundalığın arkasındaki çimenlik meğil üzerinde, 850 m, M. Özgen	26.06.1953	
89c	*Thymus longicaulis* C. Presl subsp. *longicaulis* var. *subisophyllus* (Borbás) Jalas	Çakmaklar Köyü çamlığı doğu sırtı, kuzeyde, 800 m, Naime Vardar	08.06.1955	
89	*Thymus longicaulis* C. Presl subsp. *longicaulis* var. *subisophyllus* (Borbás) Jalas	Okul bahçesi güneyi, Çansa yolu kenarı, okuldan 200 m uzakta, yol ve tarla kenarı düzlük, 740 m, Nazire Ovalı	29.05.1953	
89b	*Thymus longicaulis* C. Presl subsp. *longicaulis* var. *subisophyllus* (Borbás) Jalas	Ilıca-Seben yolu doruk., tam doruğa çıkıldığında başlıyan çimenlik-düzlük içinde, yolun sağı, 1750 m, M. Özgen	19.07.1953	
91	*Calamintha grandiflora* (L.) Moench	Ilıca-Seben yolu doruktan 300 m beride, ormanlık içi Seben yolu kenarı, 1700 m, M. Özgen	19.07.1953	
	Lauraceae			
96	*Laurus nobilis* L.			
	Hyacinthaceae			
44a	*Muscari armeniacum* Leichtlin	Karabük düz ormanı, orman içi, 1600 m, M. Özgen	13.06.1954	
44b	*Muscari armeniacum* Leichtlin	Sultanbey Köyü civarı, Kireç ocağı kuzeyi, çeşme kenarı, kuzey meyli, 725 m, Gülşen Öztürk	11.05.1958	Zambakgiller zambak, sümbül
44c	*Muscari armeniacum* Leichtlin			
	Liliaceae			
40	*Fritillaria pontica* Wahlenb.	Sultanbey Köyü civarı, Güney orman etekleri, kuzey meyil üzerinde, 825 m, Saadet Ocabaş	11.05.1958	Zambakgiller
	Amaryllidaceae			
43	*Allium paniculatum* L. subsp. *paniculatum*	Ilıca Banyolar, arka doğu fundalık sırtı, sırt üstü tarla kenarı, kuzeye açık, 850 m, M. Özgen	26.06.1953	
	Convallariaceae			
42	*Polygonatum orientale* Desf.	Karabük kuzey ormanı, Büyükdüz yanıklık orman sahası, vadi tabanı ormanlık içi sulak, vadi güneye mail, 1500	17.06.1954	Mühürü süleyman

No	Taxon	Locality, habitat, altitude, collector (as on the original label)	Date	Turkish name (as on the original label)
		m, M. Özgen		
	Linaceae			
148b	*Linum hirsutum* L. subsp. *anatolicum* (Boiss.) Hayek var. *anatolicum*	Karacasu-Ilıca güneydoğu sırtı, güney, 780 m, Naime Vardar	04.06.1955	
148c	*Linum hirsutum* L. subsp. *anatolicum* (Boiss.) Hayek var. *anatolicum*	Çakmaklar Köyü çamlığı arka sırtı, kuzey, 800 m, Meliha Baskın ve Naime Vardar	08.06.1955	
148a	*Linum hirsutum* L. subsp. *anatolicum* (Boiss.) Hayek var. *anatolicum*			
146	*Linum tenuifolium* L.	Çakmaklar Köyü arka sırtı, kuzey, 800 m, Ayhan Başaran	08.06.1955	
147	*Linum flavum* L. subsp. *scabrinerve* (P.H.Davis) P.H.Davis	Çakmaklar Köyü arka sırtı çamlığında, kuzeyde, 800 m, Meliha Baskın	08.06.1955	
147b	*Linum flavum* L. subsp. *scabrinerve* (P.H.Davis) P.H.Davis	Kızık Yaylası-Aladağ Orman İşletmesi civarı, Orman açıklığı-yayla, yaylalık, 1350 m, Adile Özbay	23.06.1955	
	Lythraceae			
25	*Lythrum salicaria* L.			
	Malvaceae			
1	*Gossypium herbaceum* L.	Adana, Çukurova, Yurdanur Özkan	25.08.1957	Ebegümecigiller pamuk
63	*Althaea hirsuta* L.	Okul bahçesi, 700 m, M. Özgen	16.06.1953	Çançiçeğigiller
64	*Lavatera punctata* All.			
62	*Alcea pallida* Waldst. & Kit	Kız Öğretmen Okulu bahçesi	02.07.1955	
	Orchidaceae			
46	*Orchis* sp.	S. Gökmen		
45	*Anacamptis pyramidalis* (L.) L.C.M. Richard	Ilıca, banyolar arka sırtları, fundalık sırtın tam tepesindeki tarla kenarı, çakıl içerisi, Kuzeye meyilli, 850 m, M. Özgen	26.06.1953	
	Papavenaceae			
37	*Fumaria officinalis* L.	Okul bahçesi, açık düzlükçe, 700 m, M. Özgen	16.06.1953	
38c	*Papaver rhoeas* L.	Ilıca, binaların arkası, Buğday ekili tarla içinde fundalık sırtı eteğinde, kuzeye açık, 700 m,	26.06.1953	Gelincikgiller

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		M. Özgen		
38b	*Papaver rhoeas* L.	Sultanbey köy civarı, Güney orman etekleri, kuzey meyli üzeri, 725 m, Necla Sönmez	11.05.1958	Gelincikgiller
38	*Papaver rhoeas* L.	Okul bahçesi, Tarla kenarı ağaçlar arası, 700 m, M. Özgen	18.06.1953	Gelincikgiller
	Poaceae			
50	*Poa bulbosa* L.	Karabük kuzey ormanı, Büyükdüz mevkii bölge binası kuzeydoğu meyili orman içi, orman içi açıklığı, 1500 m, M. Özgen	15.06.1954	
	Polygalaceae			
	Polygala anatolica Boiss. & Heldr.			
	Polygonaceae			
5	*Rumex acetosella* L.	Karabük, Kuzey ormanı, Büyükdüz Orman bakım evi ön meyili üzerinde, orman açıklığı meyil üzeri, 1500 m, M. Özgen	15.06.1954	Kuzu kulağı
	Primulaceae			
130	*Anagallis foemina* Miller	Kız Öğretmen Okulu, okulun ön kısmındaki açıklıkta, 700 m, M. Özgen	16.06.1953	Çuhaçiçeğigiller fare kulağı
131	*Anagallis arvensis* L. var. *arvensis*	Okul bahçesi güneyi 300 m mezarlık içi, Çanşa Köyü yolu üzerinde soldaki mezarlık içi, 700 m, Fatma Özkan	29.05.1953	Çuhaçiçeğigiller fare kulağı
128	*Cyclamen coum* Mill. subsp. *coum*	Abant, otel arkası 50 m geride, orman içi, ormanlık, ağaçlar arası, 1350 m, Türkan Gültekin	31.05.1953	Çuhaçiçeğigiller Siklemen, Buhurumeryem
135	*Androsace maxima* L.	Okul bahçesi güneyi, Çanşa yolu, 200 m uzakta yol kenarı, bu yol kenarında düzlük, 740 m, Suna Sertçetin	29.05.1953	
14	*Lysimachia verticillaris* Sprengel			
M0-22	*Lysimachia atropurpurea* L.	Karacasu Ilıca sırtı, Kuzey meyli güneydoğu sırtı, güneyde, 800 m, Meliha Baskın	04.06.1955	
129	*Primula vulgaris* Huds. subsp. *vulgaris*			
	Ranunculaceae			
34	*Adonis aestivalis* L. subsp. *aestivalis*	Okul bahçesi güneyi, Çanşa yolu, 200 m ilerde, yol ve tarla kenarı, Açık düzlük, 700 m,	29.05.1953	Düğünçiçeğigiller Avcuotu

No	Taxon	Locality, habitat, altitude, collector (as on the original label)	Date	Turkish name (as on the original label)
		Yüksel Kaçar		
30b	*Helleborus orientalis* Lam.			
30a	*Helleborus orientalis* Lam.	Alpağut Köyü, Köyün güneyi mezarlık içi, düzlük, yer yer fundalık, 730-750 m, M. Özgen	12.04.1953	Düğünçiçeğigiller Çöpleme, Noel gülü
33	*Ranunculus trichophyllus* Chaix	Mezarlık	28.04.1958	Su düğün çiçeği
35	*Ranunculus brutius* Ten.			
36	*Ranunculus oxyspermus* Willd.			
31	*Consolida regalis* S.F.Gray subsp. *paniculata* (Host) Soó var. *paniculata*	Karacasu Ilıca sırtı kuzey meyli güneydoğu sırtı, güney, 800 m, Naime Vardar	04.06.1955	
32a	*Consolida orientalis* (Gay) Schröd.	Çakmaklar Köyü yolunda, kuzeyde, 700 m, Nezahat Kaymak	08.06.1955	
32b	*Consolida orientalis* (Gay) Schröd.	Okul bahçesi, düzlük, 700 m, M. Özgen	25.06.1953	Düğünçiçeği Şakayık
	Resedaceae			
23	*Reseda lutea* L. var. *lutea*	Okul bahçesi, İşlenmiş toprakta, düzlük, 700 m, M. Özgen	25.06.1953	
	Rosaceae			
72	*Filipendula vulgaris* Moench	Karabük kuzey ormanı, Baklabostanı bölge binası ön alanı, Ormanlık su yolu kenarı-sulak, doğu kuzeye meyil, 850 m, M. Özgen	18.06.1954	
75	*Potentilla crantzii* (Crantz) G.Beck var. *crantzii*	Karacasu Ilıca sırtı güney doğu sırtı kuzey meyli, güney, 800 m, Meliha Baskın	04.06.1955	
70	*Fragaria vesca* L.	Ilıca seben yolu oto duragı ile doruk arasındaki oluklu çeşmeden 300 m ileride şasenin kenarı, orman içi (funda), kuzeye dönük, 1500 m, M. Özgen	19.17.1953	Yabani çilek
71a	*Potentilla micrantha* Ramond	Pirahmetler Köyü, Köyün doğu kuzeyinde derin vadi., Bu derenin doğu kuzeyi meyili üzerinde, taşlık, fundalık, sarp bir meyil, dereye yakın., derin bir vadi içi, 850 m, M. Özgen	12.04.1953	Gülgiller yabani çilek

No	**Taxon**	**Locality, habitat, altitude, collector** (as on the original label)	**Date**	**Turkish name** (as on the original label)
71b	*Potentilla micrantha* Ramond	Kızık Yaylası-Aladağ Orman İşletmesi civarı, Orman içi-yayla, yaylalık, 1350 m, M. Özgen	23.06.1955	
73	*Potentilla reptans* L.	Okul bahçesi, Cümle kapısı önü, çimenlik, 700 m, M. Özgen	11.06.1953	Düğünçiçeğigiller Beş parmakotu
74	*Potentilla umbrosa* Stev.	Ilıca, banyolar arka sırt, doğu sırtlar, bu fundalık sırtın üstündeki tarlaların kenarı, kuzeye dönük, 850 m, M. Özgen	26.06.1953	Düğünçiçeğigiller Beş parmakotu
78	*Prunus avium* (L.) Moench	Çayırköy civarı, köy içi, düzlük, 700 m, Pakize Soğancı	11.05.1958	Gülgiller
79	*Prunus mahaleb* (L.) Miller var. *mahaleb*	Çayırköy civarı, köy içi, düzlük, 700 m, Pakize Soğancı	11.05.1958	
77	*Pyrus elaeagnifolia* Pallas subsp. *elaeagnifolia*	Çanşa Köyü, köy tarla kenarı, düzlük, 700 m, Saniye Korkusuz	28.04.1958	Gülgiller
162	*Spiraea crenata* L.	Okul bahçesi, binanın arkasındaki düzlük. Ağaççık halinde, 700 m, M. Özgen	25.06.1953	
	Rubiaceae			
104	*Asperula involucrata* Wahlenb.	Karabük, Büyükdüz ormanı, orman açıklığı, 1600 m, M. Özgen	15.06.1954	Kökboyagiller yoğurtotu
106	*Asperula taurina* L. subsp. *taurina*	Ilıca-Seben yolu, doruk, tam doruğa çıkışta, düzlüğe varmadan yol solunda tepe üstü, kısmen batıya açık, 1750 m, M. Özgen	19.07.1953	Kökboyagiller
105	*Galium verum* L. subsp. *verum*	Ilıca arka doğu sırtlar tepede, fundalıktan sonra başlayan ekili tarla alanları çakıllık içi, kuzeye açık, 850 m, M. Özgen	26.06.1953	Kökboyagiller yoğurtotu
103	*Galium odoratum* (L.) Scop.	Karabük, Kuzey ormanı, yanıklık ve düz orman sahası, sık orman içi, 1500 m, M. Özgen	17.06.1954	Kökboyagiller belumotu
	Hydrangeaceae			
86	*Philadelphus coronarius* L.	Okul bahçesi batı kısmında, 15 m^2'lik bir alanda 1,5-2 m boyunda ağaççık halinde, 700 m, M. Özgen	25.06.1953	
	Scrophulariaceae			
99	*Veronica jacquinii* Baumg.	Karacasu, Ilıca sırtı, Güneydoğu sırtı, kuzey meyli, güney, 750 m, Meliha Baskın	04.05.1955	
101	*Veronica chamaedrys* L.			

No	**Taxon**	**Locality, habitat, altitude, collector** (as on the original label)	**Date**	**Turkish name** (as on the original label)
102	*Veronica persica* Poiret	Okul bahçesi, Ağaçlık önü, 740 m, M. Özgen	26.05.1953	Fare kulağı
100	*Veronica anagallis-aquatica* L.	Okul bahçesi müsamere salonu arkası, açıklık ve düzlük, 700 m, M. Özgen	25.06.1953	
97	*Verbascum mucronatum* Lam.			
98	*Melampyrum arvense* L. var. *arvense*	Ilıca-Banyolar, arka sırt, bu sırtın tam tepesinde bulunan tarlaların üstü, yol ve arık kenarı, kuzeye açık, 800 m, M. Özgen	26.06.1953	
	Tamaricaceae			
11	*Tamarix parviflora* DC	Kız Öğretmen Okulu bahçesi, 740 m, Güner Akçaylı	26.05.1953	Ilgıngiller ılgın
	Taxaceae			
15	*Taxus baccata* L.	Karabük, Yanıklık Ormanı, orman, 1650 m, M. Özgen	09.06.1954	Porsukgiller porsuk
	Thymelaeaceae			
20	*Daphne pontica* L.			
	Tiliaceae			
8	*Tilia argentea* Desf.	Okul bahçesi, 700 m, M. Özgen	17.07.1953	Ihlamurgiller ıhlamur
	Valerianaceae			
150	*Valerianella coronata* (L.) DC.	Okul bahçesi güneyi Çanşa yolu 200 m uzakta, bu yol kenarı üzerinde, 700 m, Ayşe Kiraz	29.05.1953	
118	*Valeriana alliariifolia* Adams	Ilıca-Seben yolu, tam doruğa çıkışta, düzlüğe varmadan yol solu tümsek üzeri, 1750 m, M. Özgen	19.07.1953	Çuhaçiçeğigiller
	Violaceae			
2	*Viola alba* Besser	Abant, G. Mumcu	14.06.1956	
3	*Viola tricolor* L.	Karabük, Kuzey Ormanı, Uzun Göveç Yaylası, Bölge binası civarı, orman içi açıklık, güneye dönük meyil üzeri, 1500-1800 m, M. Özgen	16.06.1954	

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HISTOCHEMICAL EFFECTS OF BRODIFACOUM ON RAT SPLEEN

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ABSTRACT. In this study, the histochemical effects of Brodifacoum, an anticoagulant used against rodents, on the spleen are examined under a light microscope using CD4 and CD8 histochemical staining methods. A single dose of 0.2 mg Brodifacoum was dissolved in Dimethyl Sulfoxide (DMSO) and was given orally to mature male rats. Spleen samples were collected under ether anesthesia after 24 h, 72 h, 14 days, and 30 days from the rats in the experimental groups and after 14 days from the rats in the control group. In this light microscope study, it was observed that the capsule, white pulp, and red pulp zones in the rat spleen were constructed normally and as their natural structures primary and secondary follicles (germinal center) they were few, and CD4 and CD8 lymphocytes were spherically structured. In the 24 h spleens of the rats, the diameters of germinal centers were expanded and deterioration of the structure of CD4 and CD8 cells was observed. Related to the increase in time (72 h and 14 days) it was determined that primary follicles increased in number and the diameters of germinal centers expanded. In addition to this, after30 days, the rate of CD4:CD8 of the brodifacoum applied rat spleens were approximately the rate of the control group, and the improvement of the structures of the cells was reported as an effect of regeneration. As a result of this study, it was found that Brodifacoum caused immunohistochemical abnormalities in the rat spleen, affected the morphological structure of CD4 and CD8 T lymphocytes and created an immune response in rats. It is thought that the obtained results will be a source for the studies on Brodifacoum.

1. INTRODUCTION

Colloquially known as rat poison, Rodenticides are a chemicals used for pest and rodent population control [1]. There are two groups of rodenticides

Keywords. Brodifacoum, Rattus norvegicus, spleen, histochemistry, anticoagulant, CD4 T lymphocyte, CD8 T lymphocyte, light microscope

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commercially available. These are acute and anticoagulant rodenticides. Anticoagulant rodenticides are among the most important pesticides used for the control of harmful rodent populations [2]. The hydroxycoumarine derivatives Difenacoum, Brodifacoum, and Bromadiolone belong to the group of second-generation anticoagulant rodenticides. Second-generation anticoagulants (4-anticoagulants) inhibit one or more enzymes related to blood IX, II, VII, and K synthesis. The lipophilic structure of long-acting anticoagulant rodenticides facilitates the tight binding of the enzymes to the cell membranes they target [3]. Anticoagulant rodenticides are stored in the liver until the microsomal binding sites are saturated [4]. It has been reported many times that rodenticides have toxic effects on non-target organisms. Therefore, it is imperative to investigate the possible effects of these dangerous chemicals on organisms. Second-generation anticoagulant rodenticides have been reported multiple times to contaminate non-target wildlife, particularly poisoning primary predator birds and mammals, and their routes of exposure are not yet understood [5]. However, it has been reported that the poison accumulates in the body because of its consumption [6]. In a study with rabbits, one of the non-target animals, the clearance times of Warfarin, Brodifacoum and Difenacoum from plasma were compared and it was shown that Brodifacoum was cleared more slowly than warfarin, but Difenacoum spread to larger volumes [7]. Predators are at a risk of secondary poisoning due to the persistent accumulation of anticoagulants in the liver [8]. In mammalian predator population studies, for example, anticoagulant rodenticide residues were found in 84% of the red fox (*Vulpes vulpes*) population [9]. The presence of anticoagulant rodenticides was investigated in liver samples taken from red-tailed hawk (*Buteo jamaicensis*) and great-horned owls (*Bubo virginianus*), and they were found in 81% of red-tailed hawks and 82% of great-horned owls [10].

The spleen plays an important role in host defense. It is the organ where antigens from the blood are cleared. The spleen is an essential organ for blood homeostasis [11]. These functions are performed by two structurally and cellularly different components of the spleen, white pulp, and red pulp. The spleen is a vascular organ as it acts as a blood filter. The arterial branches were surrounded by lymphoid tissues. The main arteries feed the red pulp, and the smaller arteries feed the white pulp [12]. Lymphocytes involved in the immune system consist of two main groups, B and T. T lymphocytes get their name from the Thymus from which they are formed about 80% of all lymphocytes in the peripheral blood. T lymphocytes have several subgroups. One of these groups, helper T lymphocytes, is recognized by the CD4 marker protein on their surface, while another group of cytotoxic

T lymphocytes is recognized by the CD8 marker protein. These cells play important tasks in the immunity of the organism. Helper T lymphocytes secrete the cytokine hormone, which initiates immune system reactions. Cytotoxic T lymphocytes, on the other hand, neutralize pathogenic organisms or molecules with some enzymes they secrete [13]. The development of the primary immune response involving T lymphocytes may take up to 14 days, depending on the antigen and the region where it enters the organism [14]. There are 30%-40% T lymphocytes in the spleen. According to Langeveld et al.'s (2006) study, it has been suggested that CD8 T lymphocytes are more abundant than CD4 T lymphocytes in the human spleen. It has been determined that active cells are in most populations of these cells and CD8 T cells are mostly in the cytotoxic CD27(-) CD45RA(+) memory cell phenotype [15]. It has been shown that the fact that the lymphocyte subgroups in the spleen differ according to the lymphocyte subgroups in the peripheral blood means that CD4 and CD8 T lymphocyte activation in the spleen has an important and distinct place [15]. It has been reported that CD4+ and CD8+ T cells play an important role in the cytokine response to virus infection, and the release of antiviral and regulatory cytokines is shared between these two-T lymphocyte subgroups [16]. In previous studies, the effects of Brodifacoum on the histological structure of the spleen have been observed. In this study, both structural and immunological changes in the membranes of rodent spleen cells, CD4 helper T lymphocytes, and CD8 cytotoxic T lymphocytes and how Brodifacoum affected the immunity of rats were determined. In this study, it was observed that Brodifacoum affected rat spleen structures and an immune response occurred in rats on the 7th and 14th days. It was thought that the results of the study would be a source and support for studies on the resistance of rats to rodenticides.

2. MATERIALS AND METHODS

2.1. Experimental animals

After the approval of the Ankara University Ethics Committee (Decision number: 2014-18-133), 30 male albino rats (*Rattus norvegicus*, 7–9 weeks old, 200–250 g) were used in the experiments, 24 of which were in the experimental group and 6 of them are in the control group (Table 1). Before starting the experiments, the experimental animals were quarantined for seven days. Each animal was kept in separate cages under appropriate laboratory conditions (22±2℃; 12h:12h photoperiod, 60% relative

humidity). Animals were fed ad libitum with solid food throughout the experiment.

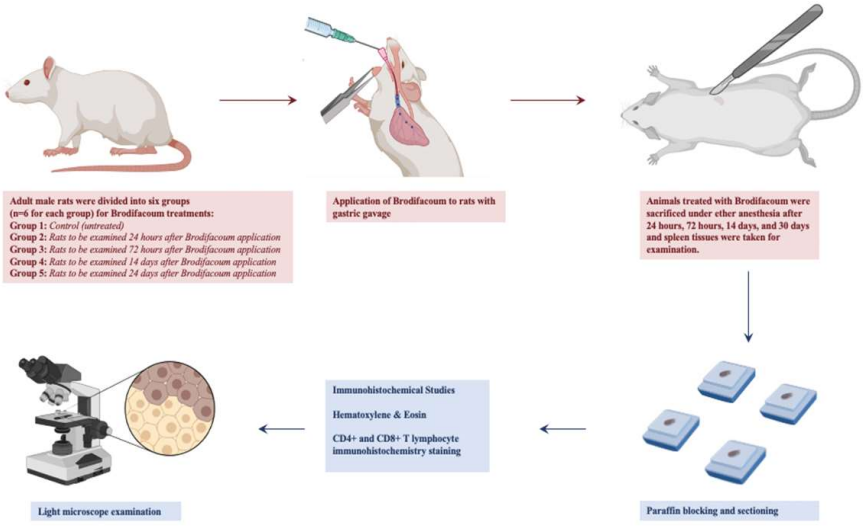

FIGURE 1. Schematic representation of the implementation stages of the experiment (Created with BioRender.com)

2.2. Brodifacoum Application

Experimental animals were divided into five groups (Table 1). Once groups of animals were determined, optimum drug doses (below LD50 dose to determine chronic cytotoxic effects) were calculated by measuring the weight of each rat.

TABLE 1. Experiment groups

Group 1	*Control group rats*	n: 6
Group 2	*Rats to be examined 24 h after Brodifacoum application*	n: 6
Group 3	*Rats to be examined 72 h after Brodifacoum application*	n: 6
Group 4	*Rats to be examined 14 days after Brodifacoum application*	n: 6
Group 5	*Rats to be examined 30 days after Brodifacoum application*	n: 6

A dose of 0.2 mg/kg BW (Body Weight) Brodifacoum prepared with DMSO (Dimethyl sulfoxide)/polyethyleneglycol-400/water (0.02ml/1ml/1ml)

solution was given orally to rats by gastric gavage method [4]. 1ml/kg BW DMSO (Dimethyl sulfoxide)/polyethyleneglycol-400/water (0.02ml/1ml/1ml) solution was administered to the control group animals by gastric gavage. Spleen samples were taken from the rat groups under ether anesthesia 24 h, 72 h, 14 days, and 30 days after Brodifacoum was applied, while spleen samples were taken 14 days later from the control group [17].

2.3. Light Microscope Preparation

Spleen samples taken from rats in the control group (after 14 days) and Brodifacoum (0.5 mg/kg) administered (24 h, 72 h, 14 days, and 30 days) under ether anesthesia were prepared for examination under a light microscope. The removed spleen samples were weighed on a precision balance, the samples were washed with distilled water, and the samples were taken in 2.5% Formaldehyde (prepared in 0.1 M Sodium phosphate buffer) and their first determinations were made (2 h, +4 °C). After fixation, the samples were washed with the same buffer (washed with 3 solution changes in 1 h) and then incubated for 5 min in alcohol series (80, 90, 96, and 100%) to dehydrate the samples. The samples were embedded in paraffin blocks by passing through xylol and 5μ thick sections were taken using a microtome [18].

2.4. Immunohistochemical Analysis

Hematoxylin-eosin staining was performed to examine the general structure of the spleen. The sections taken were treated with hematoxylin and then washed under running water. After being treated with 95% ethyl alcohol, it was dyed with Eosin and the excess paint was removed by passing it through ethyl alcohol again. Special antibody staining kits (Santa Cruz Biotech, USA) were used to examine CD4 and CD8 T lymphocytes. The instruction manual in the kit was followed for CD4 and CD8 T lymphocytes immunohistochemical staining. The stained sections were examined under a light microscope (Olympus BX43, Tokyo, Japan).

3. RESULTS

This study investigated how Brodifacoum affected the spleen weights and histological structure of the spleen in rats. According to the spleen weight measurements (Figure 2.a.), although the spleen weight of the rats treated with Brodifacoum for 24 h, 72 h, and 14 days compared with the control

group decreased, a weight close to the control group was detected during the 30-day application period. The effect of Brodifacoum on CD8 and CD4 lymphocyte counts is given in Figure 2. b and 2. c. It was determined that CD4 and CD8 levels increased at the same rate in terms of the effect of brodifacoum on T lymphocyte types.

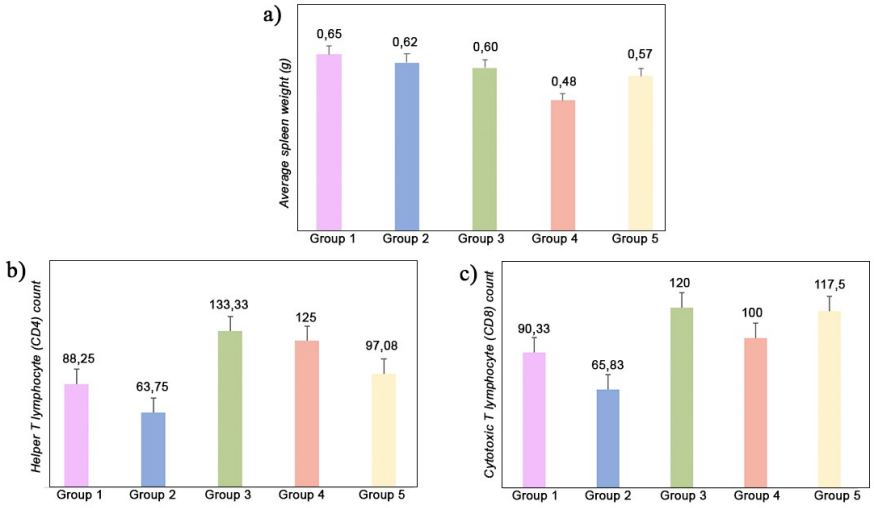

FIGURE 2. a) Spleen weights of rats treated with Brodifacoum b) Helper T lymphocyte (CD4) count of Brodifacoum-treated rat groups c) Cytotoxic T lymphocyte (CD8) count of rat groups treated with Brodifacoum

In this study, the effect of Brodifacoum on CD4 (helper T) lymphocytes, which stimulate other cells by secreting cytokines in the presence of immune antigen in the white pulp region of the spleen of rats, and on CD8 (cytotoxic T) lymphocytes, which eliminate foreign organisms and toxic substances in immunity with their enzymes, were examined by the histochemical method. When the control group was examined histologically, it was found that the capsule, white pulp, and red pulp regions in the spleen were in the normal structure and that the primary and secondary follicles (germinal center) were few in number as in their natural structures (Figure 3). A large number of normal erythrocytes, lymphocytes, and macrophage cells were found in the red pulp area. In terms of helper T lymphocytes, it was observed that the spleen of CD 4 Control group rats had a normal structure and white pulp, red pulp, and primary follicles were properly located. Additionally, it was noted

that there were a few primary follicles in the spleen samples taken from the control group of rats. In the spleen of the rats in the control group, CD4 T lymphocytes were found to have a spherical shape in the white pulp region where thymus-derived T lymphocytes were located. It was determined that CD4s were more numerous in this region compared to other cells. In the CD8 control group, white pulp and red pulp regions were prominent in the spleen structure, and there were hardly any primary and secondary follicles. It was observed that CD8 T lymphocytes in the control group were round or nearly angular and found in the same proportion as other cells.

In the spleen samples taken from rats treated with Brodifacoum for 24 h in the study, it was observed under the light microscope that large-scale germinal centers were formed and erythrocytes proliferated (Figure 4). In the spleen sample taken at the end of 24 h, it was determined that the lymphocyte count increased, and the erythrocytes were smaller than normal compared to the control group. As a sign of immunity, many newly developing primary follicles and a few secondary follicles (germinal centers) were found, in which primary follicles and germinal centers became prominent. It was observed that in the white pulp region, CD4 T lymphocyte cells also deteriorated and decreased in number compared to the control group, while in the red pulp region in the spleen of the CD8 group, the morphology of CD8 cytotoxic T lymphocytes changed from a round shape to a triangular or pentagonal shape compared to the control group and decreased compared with other cells.

The presence of primary and secondary follicles and the width of their diameters were noted in the spleen sample taken from the rat 72 h after the application of Brodifacoum, it was observed that erythrocytes were more numerous than lymphocytes and that they were smaller than their normal structure (Figure 5). While small-diameter primary follicles were seen in the areas close to the capsule, secondary follicles were not found and germinal center diameters were observed to be large. It was noted that the diameters of CD4s were smaller and in different shapes compared to the control group, and simultaneously, the number of cells other than CD4 was less. It was observed that CD8s, in which other cells were reduced, changed shape, were large and small, and macrophages were more numerous.

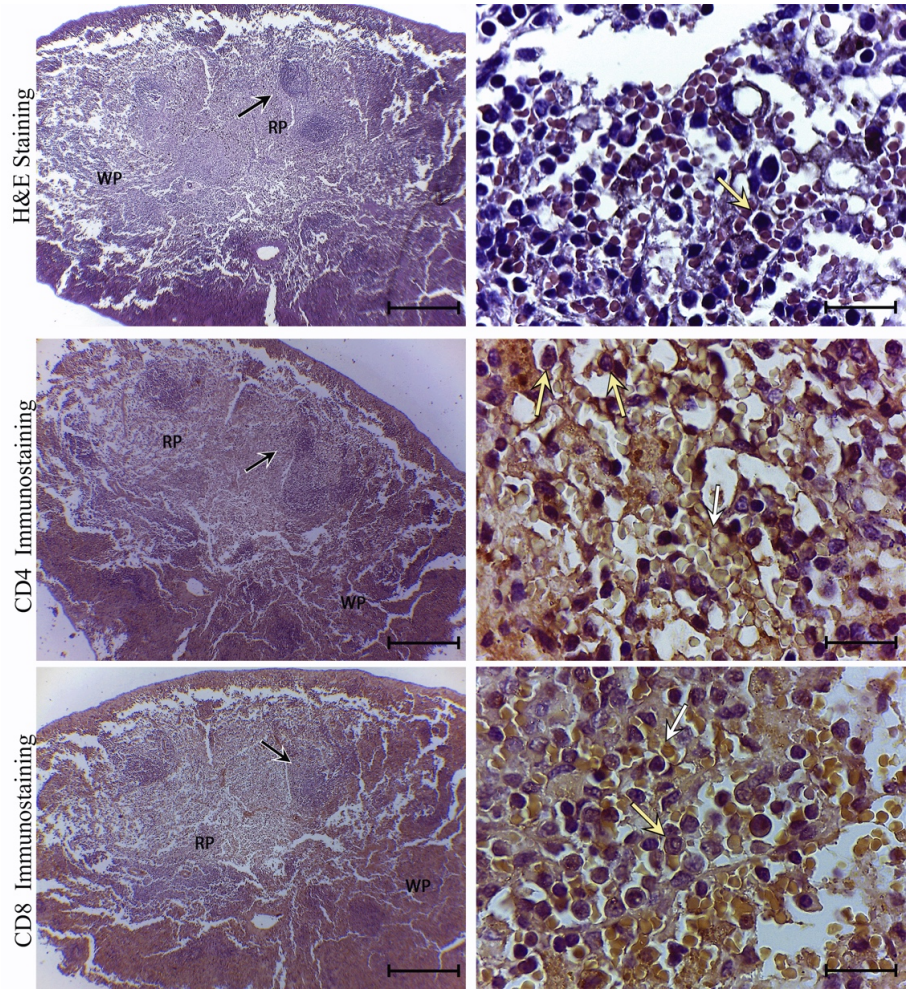

FIGURE 3. The general view of the control group rat spleen. White pulp region (WP), Red pulp region (RP), Primary follicle (black arrow), macrophage (yellow arrow), T lymphocytes (white arrow) (Scale bar left: 200 μm, right: 50 μm)

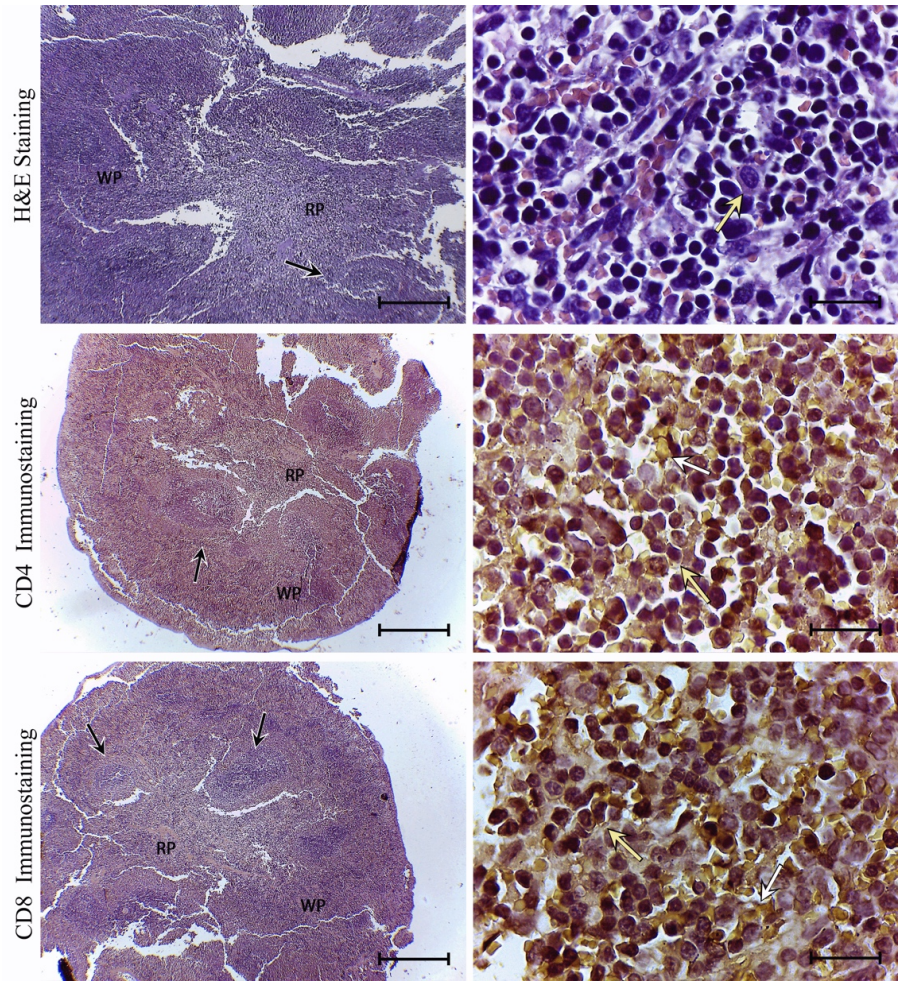

FIGURE 4. The general view of rat spleen taken 24 h after Brodifacoum application. White pulp region (WP), Red pulp region (RP), Primary follicle (black arrow), macrophage (yellow arrow), and T lymphocytes (white arrow) (Scale bar left: 200 μm, right: 50 μm)

It was observed that the primary and secondary follicles in the spleen covered the entire organ on the day when the immunity was most intense 14 days after the application of Brodifacoum, which was the fourth experimental group (Figure 6). We observed that the erythrocytes, which should be of the same size as the lymphocytes, were gradually getting smaller compared to the control group. In some places, lysis and cell groups

were observed in the tissue, it was observed that CD4s changed into triangular or rectangular shapes in the white pulp region compared to the control group, and they fused in some regions. It was noted that CD8s were few, shrunk by changing their shape and formed groups, whereas cells other than CD8s were more numerous.

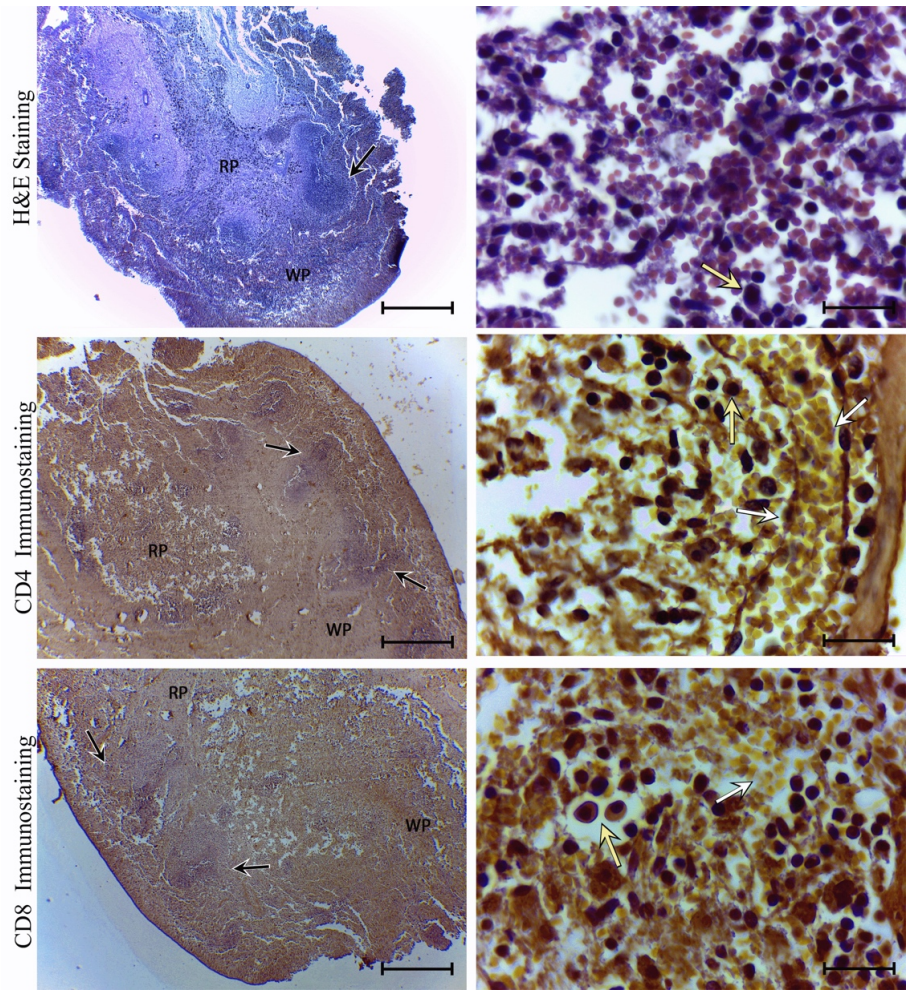

FIGURE 5. The general view of rat spleen taken 72 h after Brodifacoum application. White pulp region (WP), Red pulp region (RP), Primary follicle (black arrow), macrophage (yellow arrow), and T lymphocytes (white arrow) (Scale bar left: 200 µm, right: 50 µm)

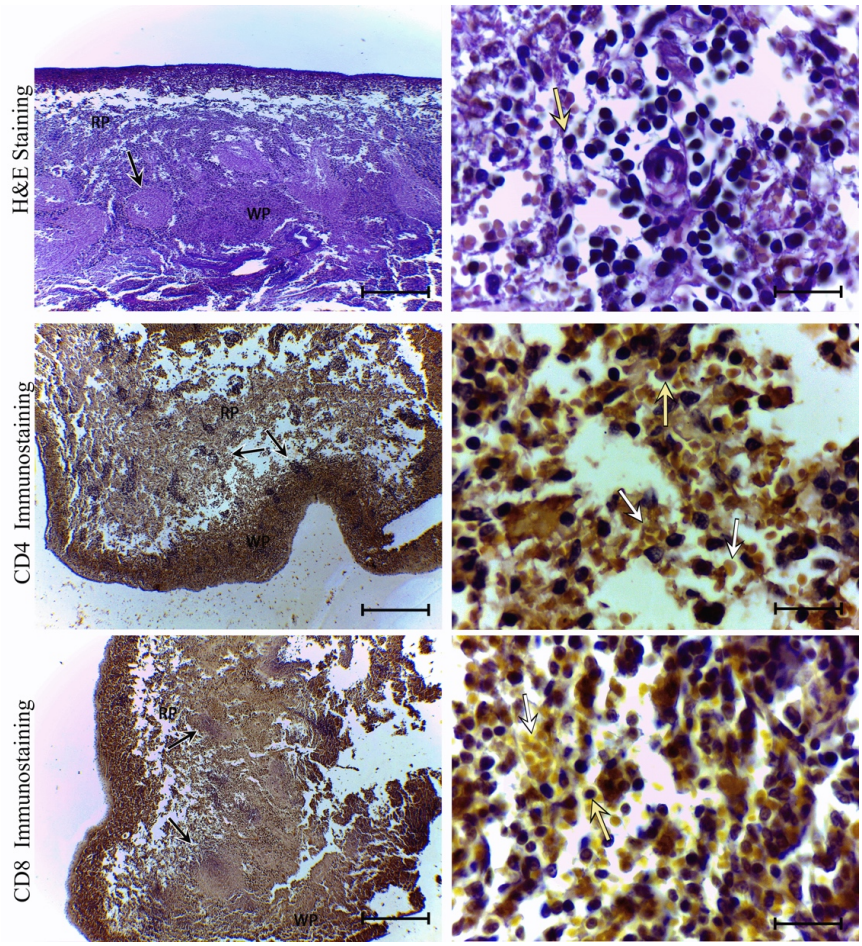

FIGURE 6. The general view of rat spleen taken 14 days after Brodifacoum application. White pulp region (WP), Red pulp region (RP), Primary follicle (black arrow), macrophage (yellow arrow), and T lymphocytes (white arrow) (Scale bar left: 200 μm, right: 50 μm)

In the spleen samples taken 30 days after the application of Brodifacoum, which is the fifth and last of the experimental groups, it was observed that there were few primary and secondary follicles in the spleen like in the control group (Figure 7). A large number of lymphocytes and a smaller number of irregularly adhered erythrocytes compared with lymphocytes were observed. In the general structure of the spleen, the germinal centers

were almost non-existent, whereas the large and small diameter primary follicles were numerous, which was accepted as a sign of recovery. As in the control group, CD4 T lymphocytes were observed to be round in shape and all of approximately the same size. This situation was thought to be a sign of retrograde recovery, and few follicles were observed as another sign of recovery. It was observed that CD8 T lymphocytes were close in size to the control group and were in the same shape as the cells in the control group.

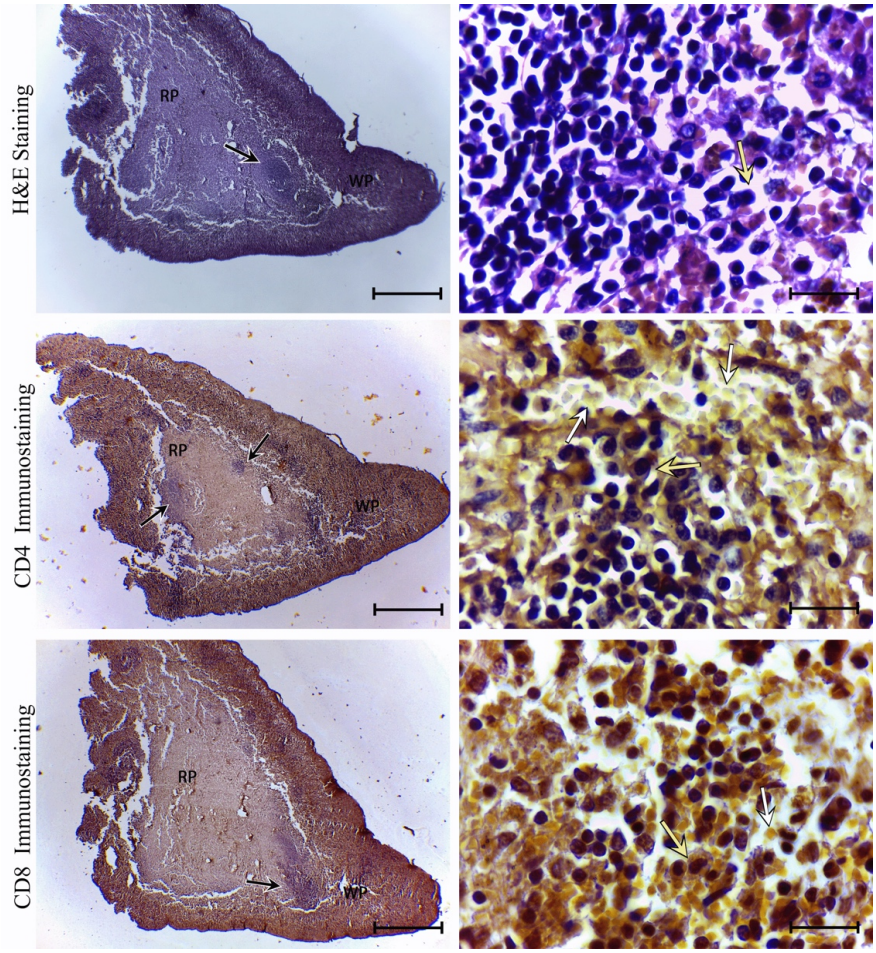

FIGURE 7. The general view of rat spleen taken 30 days after Brodifacoum application. White pulp region (WP), Red pulp region (RP), Primary follicle (black arrow), macrophage (yellow arrow), and T lymphocytes (white arrow) (Scale bar left: 200 μm, right: 50 μm)

4. DISCUSSION

T cells are divided into 6 groups: helper T cells (CD4+ T cells), cytotoxic T cells (CD8+ T cells), effector T cells, suppressor T cells, memory T cells, and delayed-type hypersensitive (DTH) T cells. CD4+ T cells and CD8+ T cells are T cells involved in the immune system. CD4+ T cells assist cellular immune function, while CD8+ T cells can destroy target cells labeled with specific antigens [19]. Therefore, CD4 and CD8 T-cell counts are an important indicator of cellular immunity [20]. In a study on the effect of boron on spleen T lymphocytes in rats, the results obtained from immunohistochemical staining showed that the cytoplasm of CD4+ and CD8+ cells was yellowish brown, and the cells were scattered in and around the splenic periarteriolar lymphoid sheaths [21]. It is possible to interpret the immunohistochemical staining results of our study similarly. Likewise, immunohistochemical staining showed that the number of CD4 cells increased as the boron concentration increased [21]. Our study observed that Brodifacoum increased the number of CD4 cells on the 14th day, which is the most intense day of immunity. Because of immunohistochemical staining, deterioration in lymphocyte shapes was observed on the 72nd and 14th days. In the study examining the effect of Cyclosporine A on rats, structural deterioration was observed in lymphocytes in the spleen tissues, similar to our study [22]. The effect of ethanol on the immune system was investigated in a study on mice [23]. In this study, the ETOH diet was applied to mice and CD4 and CD8 cells in the thymus and spleen were examined by immunohistochemical staining at certain time points. According to the findings of the study, T cell loss was detected in mice on the ETOH diet. In our study, when the first 14 days are examined, the decrease in the numbers of CD4 and CD8 shows compatibility with this study. When lymphocytes in mouse spleens treated with ETOH were examined by immunohistochemical staining, it was revealed that T lymphocytes were structurally smaller, which is similar to our study.

Humoral and cellular immunity was investigated by administering Propoxur (PPX) insecticide to C57-bl/6 mice. Because of this study, it was reported that PPX at a dose of 10 mg/kg suppressed the delayed-type hypersensitivity response and increased the percentage of CD4(-)/CD8(+) T cells [24]. In another study, it was reported that cocaine increased cytokine-secreting CD4(+) T lymphocytes in the spleen in vitro [25]. In our study, it was observed that the CD4/CD8 ratio increased on the 14th day, which is the immune response was most intense.

It was administered to rats and the changes were observed to examine the changes in the spleen as a result of the toxicity of chlorophem. The spleen weights of the experimental rats increased significantly compared to the control group of rats, and fibrosis in the capsule and lesions in the parenchyma was observed [26]. In our study, it was observed that the weight decreased in groups 2, 3, and 4 in which Brodifacoum was applied and increased in group 5. It has been interpreted as a part of the healing process.

By the current research, Brodifacoum causes various clinical symptoms in rabbits and rats, including drowsiness, fatigue, anorexia, decreased movement, and rapid and easy exhaustion after administration [27]. Because of our experiments, male rats treated with Brodifacoum experienced fatigue, loss of appetite, etc., similar to previous studies. Weight loss was observed in Brodifacoum-administered rats because of anorexia. Likewise, studies have reported that Brodifacoum causes weight loss. Behavioral disorders and mouth and nose bleeding were observed in rats after Brodifacoum administration [28].

5. CONCLUSION

In this study, the damaging effect of Brodifacoum on the spleen of rats in the mammalian group, including humans, was demonstrated histochemically. It has been understood that Brodifacoum even disrupts the structure of CD4 and CD8, which shows an immune reaction, and therefore is effective against rats. It is thought that the results obtained from this study will contribute and be a source for chemical control studies against rodents. In addition, this study reveals that the spleen structure will histologically deteriorate in humans and non-target organisms that accidentally come into contact with Brodifacoum.

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Author Contribution Statements BBÖ - Conceptualization, Investigation, Data curation, Formal analysis, Writing. NG – supervising, resources, conceptualization, writing–review & editing. Both authors have read and approved the manuscript.

Declaration of Competing Interests The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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THE EFFECT OF MELATONIN ON RAT SOLEUS MUSCLE TREATED WITH CARBON TETRACHLORIDE

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ABSTRACT. Antioxidants are known to restrains various tissue damage caused by the administration of carbon tetrachloride (CCl_4). This study examined whether melatonin (MEL), a molecule known to have antioxidant properties, has a protective effect on the rat soleus muscle, where toxic damage is caused by the application of CCl_4. In the study, eighteen albino-type male Wistar rats were used and divided into three groups as Control Group (group 1), CCl_4 group (group 2) and CCl_4 + MEL group (group 3). End of the 12 weeks, blood samples were taken as intracardiac from the rats under ketamine/rompun anesthesia, and the soleus muscles of the rats were removed. Tissue samples were subjected to routine preparation procedures for light microscopy. Sections 5 µm thick taken and stained with hematoxylin-eosin (HE) for histopathological examinations and Masson's Trichrome stain for fibrosis formations. In conclusion, the CCl_4 group displayed muscular hypertrophy, fiber orientation dysfunction and atrophy in some areas. In addition, fibrosis was spotted around the venous and nerve plexuses. In contrast to the CCl_4 group, the melatonin group displayed no fibrosis and maintained tissue integrity. Therefore, when comparing CCl_4+MEL and CCl_4 groups, it was observed that melatonin had a stabilizing or even curative effect on the injuries.

Keywords. Skeletal muscle, soleus muscle, melatonin, CCl_4
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1. INTRODUCTION

Carbon tetrachloride (CCl_4) is a volatile, non-flammable and colorless liquid chemical that has been known to cause a variety of tissue damage via its free radical, trichloromethyl, which is produced in the liver [1]. It is a well-known model and causes toxicity in many tissues such as liver, kidney, heart, lung, testes, brain, and blood [3,4] as a result of the production of CCl_4 free radicals, which are also produced chemically [2]. In humans, about 50-80% of CCl_4 is absorbed by the lungs. Approximately 4% of the metabolized CCl_4 is directly converted to CO_2 and exhaled from the lungs [5]. The toxic effect of CCl_4 occurs after its conversion to its free radical trichloromethyl by P-450, which is where the lipid peroxidation process begins [10-12].

Melatonin is a neurohormone derives from N-acetylated serotonin [13] and synthesized by bone marrow cells, intestine, lens, pineal gland, retina, Harderian glands, ovary, testicle, and skin [1,9]. Since melatonin is both fat and water soluble, it easily enters every cell in the body, including cytosol and intracellular structures, therefore it is much more effective than vitamin and mineral antioxidants. [14]. Thus, the nuclear DNA, cytosolic proteins and membrane lipids are protected by melatonin against diseases that cause degenerative and proliferative changes [14,15]. This pleiotropic hormone synthesized mainly in the pineal gland, showed positive affects in organism as if regulation of cell apoptosis, inflammation, circadian rhythms, oxidative stress and metabolic disorders [16-18]. In order to diverse uses of melatonin, it has gained all interest as a functional and strategic therapeutic biochemical in an effort to prevent and treat some illness related to muscle [19-21]. Melatonin is a hormone that belongs to the indolamine family and has significant antioxidant properties as a result of its electron donor ability [22-24].

Skeletal muscle plays an important role in energy metabolism, insulin resistance and movement in adults, and accounts for almost 40% of all body mass [25]. The most remarkable feature of the skeletal muscle is the ability to regenerate quickly after injury, made possible by the resident population of stem cells (also called satellite cells (SC)) located between the sarcolemma and the basal lamina of myofibers [26-28].

The soleus muscle is located in the posterior compartment of the lower leg and above the Achilles tendon and has slow-contracting muscle fibers [29]. Soleus is a powerful muscle group that functions while walking and running and it prevents the body from falling forward while standing [30]. Muscle tissue is susceptible to different types of injuries, and the process of

regeneration often leads to the formation of scar tissue. By applying exogenous substances, such as antioxidants, the regeneration processes can be improved, preventing muscle strength loss and scarring [1,31]. Numerous studies have shown that antioxidants prevent different tissue damage caused by CCl_4 administration [32,33]. In this study, melatonin, which is known to have strong antioxidant properties, was investigated for the curative effects on the soleus muscle damaged by carbon tetrachloride (CCl_4).

2. MATERIALS AND METHODS

Eighteen Wistar albino-type male rats were housed in rooms with a 12:12 hour light-dark cycle and a constant temperature for the study. Three groups are formed as control group (group 1), CCl_4 group (group 2), and CCl_4+MEL group (group 3). In group 1 (Control group), corn oil, which dissolves CCl_4, was injected subcutaneously in a volume of 1.5 ml/kg twice a week for 12 weeks. In order to cause muscle injury, the second group was injected subcutaneously in a volume of 1.5 ml/kg with sterile CCl_4 dissolved in corn oil at a ratio of 1:1, 2 times a week for 12 weeks. Group 3 received the same CCl_4 treatment as Group 2, but in addition, 10 mg/kg per day of melatonin was given subcutaneously to the Group 3 starting after the CCl_4 treatment. At the end of the 12^{th} week, intracardiac blood samples and soleus muscle tissues were taken from the rats under ketamine-rompus anesthesia. Standard light microscopy preparation techniques were taken on the muscle tissue specimens. Cross sections 5 μm thick were taken and stained with hematoxylin-eosin and Masson's Trichrome staining technique.

3. RESULTS

It was observed that the morphological structure of the soleus muscle cells in the control group stained with hematoxylin-eosin was smooth and the muscle integrity was normal (Figure 1). Additionally, collagen fibers were found to be at normal levels in Masson's Trichrome stained muscle fibers (Figure 2). Hematoxylin-eosin-stained soleus muscle fibers of Group 2 (CCl_4) displayed protein loss, fibrosis around the blood vessels, atrophy, and hypertrophy in some places (Figure 3 and 4). Also in this group, Masson's Trichrome staining revealed increased connective tissue, atrophy in muscle fibers, and increased collagen fibers around the blood vessels (Figure 5 and 6). Examining the hematoxylin-eosin-stained soleus muscle specimens of the CCl_4 groups treated with melatonin revealed that, in comparison to the CCl_4 group, the tissue integrity was partially preserved, and the morphological

structure of the cells was close to normal (Figure 7). Again, in the same group, Masson's Trichrome stained samples showed that there was a decrease in collagen fibers and a decrease in fibrosis, especially around the blood vessels (Figure 8).

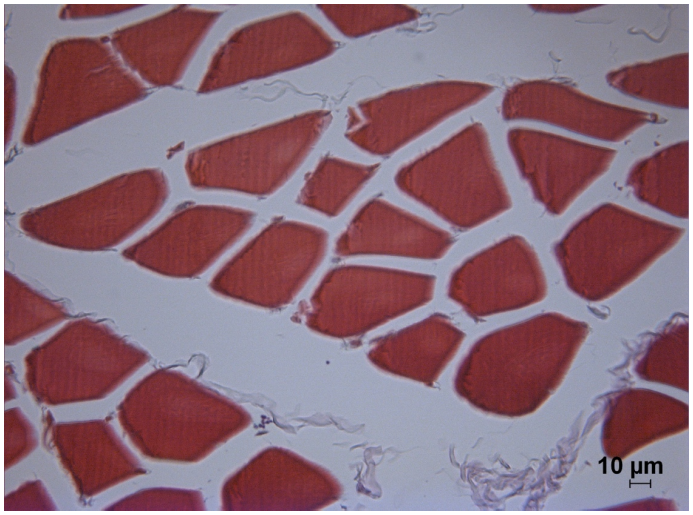

FIGURE 1. View of the soleus muscle tissue of the control group rats, 100µm.

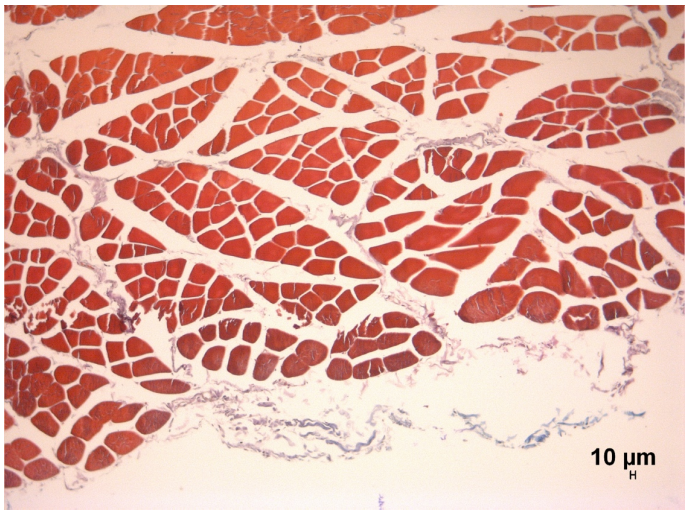

FIGURE 2. Normal levels of collagen fibers in Masson's Trichrome-stained muscle fibers of the control group rats.

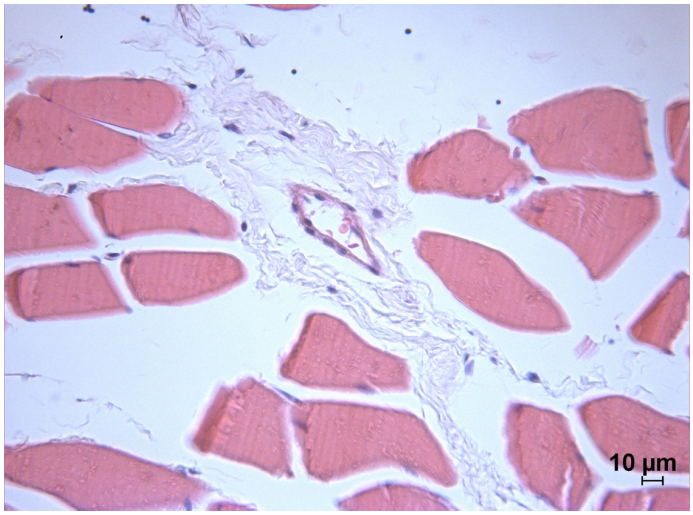

FIGURE 3. Loss of protein, atrophy in some places and fibrosis around the vessels of the CCl_4 group soleus muscle stained with hematoxylin-eosin.

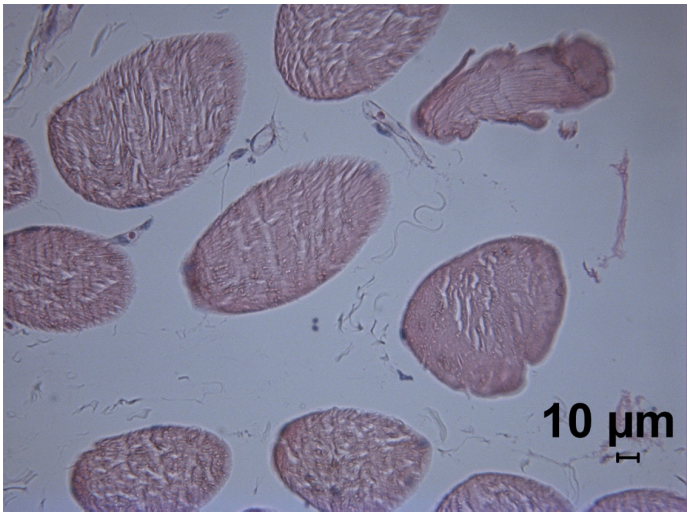

FIGURE 4. Hypertrophy of the muscle fibers in the CCl_4 group.

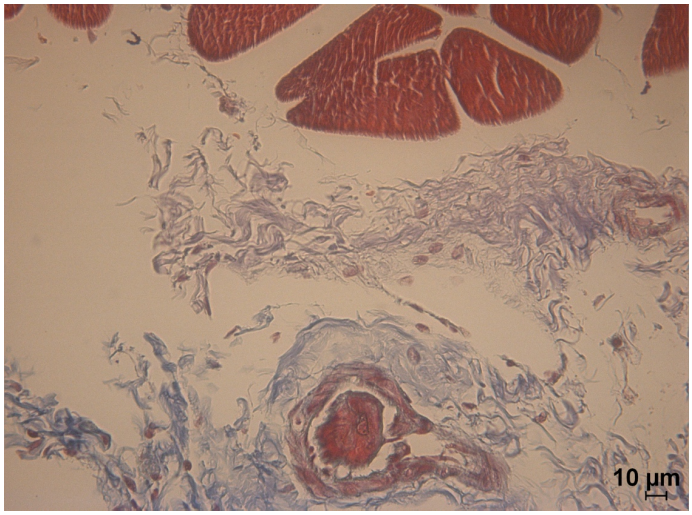

FIGURE 5. Increased collagen fibers around the blood vessel in the CCl_4 group (stained with Masson's Trichrome).

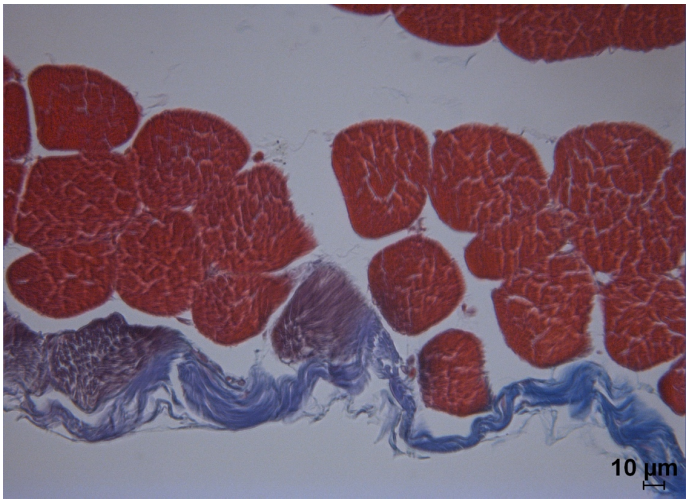

FIGURE 6. Atrophy of muscle fibers and increase in the connective tissue in the CCl_4 group.

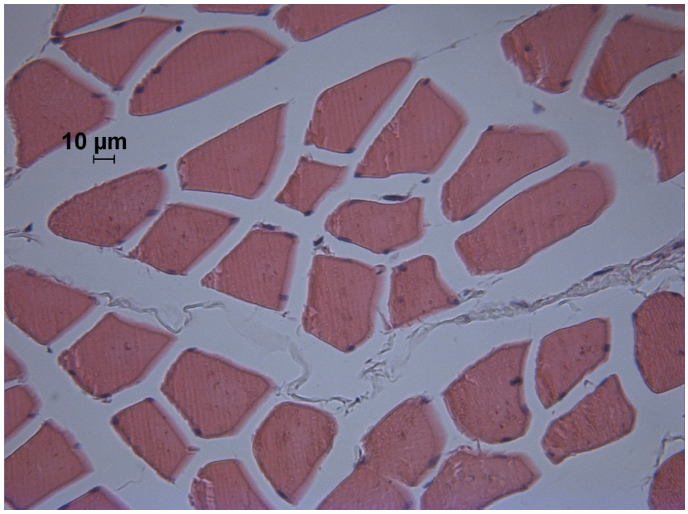

FIGURE 7. Hematoxylin-eosin staining of the CCl_4+MEL group.

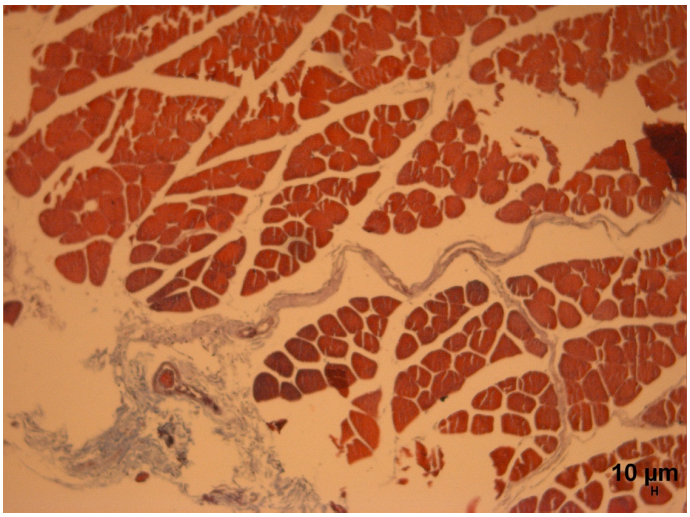

FIGURE 8. Masson's Trichrome staining of the CCl_4+MEL group.

4. DISCUSSION

Carbon tetrachloride (CCl_4) is a molecule produced synthetically in an effort to use in industrial agents such as fire extinguishers, solvents and paints [5].

It has a quite large half-life between 30 to 100 years. It can enter the body through digestion, respiration and dermal absorption and then distributed to tissues such as the brain, kidney, muscle, lung and testes, especially liver. This chlorine-containing substance has been used in animal experiments to cause oxidative damage to several tissues, including the liver, kidneys, brain, muscles, lungs and testicles [33].

Research by Pope and Rall [5] stated that, the general population may be exposed to CCl_4 through ambient air because CCl_4 can evaporate easily and the emissions of CCl_4 as chemical waste into the air, water and soil are not controlled properly. Numerous studies have shown that melatonin has a protective effect against diseases that cause proliferative and degenerative changes to nuclear DNA, membrane lipids and cytosolic proteins. Kuş et al. [36] reported that melatonin reduced kidney and liver damage after CCl_4 intoxication. In another study by Erdem et al. [37] emphasized that melatonin has an important role on striated muscle tissue as a protectant from ischemia-reperfusion injury.

Sokolovic et al. [38] investigated the potency of melatonin to prevent biological and clinical changes in the rat biceps muscle following acute exposure to CCl_4. Microscopic analysis of the biceps muscle from animals exposed to this synthetic chemical come up significant muscle fiber irregularity and intense inflammatory cell infiltration, while significant improvement was observed in the group receiving melatonin. They also investigated serum and tissue biochemistry and revealed that melatonin has a significant effect on preventing CCl_4-induced skeletal muscle damage.

In the study where Chen et al [25] summarized the latest research about the role of melatonin in regulating muscle growth and regeneration, they reported that due to its wide biological functions, application of melatonin is important in regulating muscle and fat metabolism to treat muscle diseases or to improve health in order to its broad-spectrum antioxidant, anti-apoptotic, and anti-tumor properties.

The results of this study were found to be consistent with the afore-mentioned studies. In the CCl_4 group, fibrosis, atrophy in some areas, increase in connective tissue around the nerve plexus, disorientation and hypertrophy in muscle fibers were observed, while in the CCl_4+MEL group, a near-normal appearance was observed with a decrease in fibrosis. In conclusion, when the group given CCl_4+MEL is compared with the group given CCl_4, it is possible to say that melatonin has a stabilizing or even healing effect on the CCl_4-induced muscle injuries.

Author Contribution Statement DFV and DO-experimental design and performance, DFV and HME-manuscript writing. SC-manuscript editing. All authors have read and approved the manuscript.

Declaration of Competing Interests The authors declare no conflict of interest. This study was presented as an oral presentation at the "1st National Zoology Congress", 28-31 August 2013, NEVŞEHİR, TURKEY

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COI GENE ANALYSES OF THE DAGHESTAN PINE VOLE (*Microtus daghestanicus* Shidlovsky, 1919) POPULATION FROM NORTHEASTERN TURKEY

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ABSTRACT. Daghestan pine vole (*Microtus daghestanicus* Shidlovsky, 1919) is spread in Caucasia, Turkey and Northwestern Iran and distribution of this species is limited to Northeastern Anatolia in Turkey. Few molecular studies on *M. daghestanicus* have been performed so far, and it was analysed in this study with the mitochondrial cytochrome oxidase subunit I (*COI*) region and compared with other *Terricola* species (*Microtus subterraneus* and *Microtus majori*) and other *Microtus* species found in its distribution area (*Microtus arvalis* and *Microtus mystacinus*). For this purpose, mean genetic distance values and fixation index values were calculated. Also, Bayesian Inference tree and Median-joining network were constructed. The acquired results showed that *M. daghestanicus* was clearly separated in the Pleistocene Period and was closer to *M subterraneus* than *M. majori* in the subgenus *Terricola*.

1. INTRODUCTION

Microtus Schrank, 1798 is one of the largest rodent genus and due to the unsolved taxonomic problems, high number of studies have been performed on this genus [1,2,3,4,5,6,7]. *Microtus daghestanicus* Shidlovsky, 1919 whose type location is Daghestan is a species of pine vole (subgenus *Terricola*) recorded from Southwestern parts of European Russia, Georgia, Armenia, Northeastern Turkey, Azerbaijan and Northwestern Iran [8,9]. Although *M. daghestanicus* and *M. majori* Thomas, 1906 were considered as subspecies of *M. subterraneus* de Selys Longchams, 1836 before [10,11], differences of these species were determined by karyological studies and they were accepted as valid species [12]. Conducted molecular studies showed that *M. daghestanicus* split from *M. subterraneus* and *M. majori* as

Keywords. *Microtus daghestanicus*, Daghestan pine vole, *Microtus subterraneus*, *Microtus majori*, cytochrome oxidase subunit I, Anatolia

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closer to *M. subterraneus* in mitochondrial cytochrome-*b* (*CYTB*) and nuclear *IRBP, BRCA1* and *XIST* analyses [3,13,14].

Mitochondrial cytochrome oxidase subunit I (*COI*) is a barcod gene frequently used in eukaryotes. Since *COI* gene has a fast rate of evolution [15], few insertions and deletions, provides enough variation between species [16], this gene has been often preferred for species determination [17] and phylogeny construction of recently diverged species [18], including studies on rodents [6,7,17,19,20,21]. It was also suggested that species identification of rodents belong to the Murinae and Arvicolinae subfamilies is difficult due to rapid radiation, high morphological similarity, and high intraspecies and interspecies diversity [22]. Therefore, effective and reliable methods are required for the identification of these species, and the *COI* gene region could be remarkably effective to solve this problem.

In this paper, *M. daghestanicus* specimens obtained from Northeastern Turkey (Rize Province) were compared with other *Terricola* species (*M. subterraneus* and *M. majori*) as well as *Microtus arvalis* Pallas, 1778 and *Microtus mystacinus* de Filippi, 1865 species found in Daghestan pine vole's distribution area using *COI* marker. It was attempted to obtain evidence that will strengthen the validity of *M. daghestanicus* in debate and to contribute to the literature containing a limited number of molecular studies.

2. MATERIALS AND METHODS

Two *M. daghestanicus* samples were collected from Ovit Mountain region of Rize Province (Latitude: 41.02633572, Longitude: 40.51527612) in northeastern part of Turkey during the field studies in August 2017 with the permission of Ankara University Local Ethics Committee for Animal Experiments (Document no: 2016-21-184) and Republic of Turkey Ministry of Agriculture and Forestry (Document no: 72784983-488.04-117392). Besides, *M. subterraneus* (3 samples, Samsun and Giresun provinces), *M. majori* (3 samples, Ordu and Artvin provinces), *M. mystacinus* (3 samples, Erzurum and Muş provinces) and *M. arvalis* (2 samples, Ardahan Province and Hungary) sequences belong to Ankara University Mammalian Research Collection (AUMAC, http://www.mammalia.ankara.edu.tr) from previous studies. As an outgroup, one *Myodes rufocanus* sequence (Accession number: HM380211.1) acquired from GenBank (https://www.ncbi.nlm.nih.gov/genbank/) was used.

DNA samples were isolated from liver tissue using the GeneAll® ExgeneTM Tissue SVmini kit (Atlas Biotechnology, Ankara, Turkey) and 720 base pair *COI* gene region was amplified with BatL5310 and R6036R primers [23]. For this purpose, reaction mix and PCR conditions were modified from Çetintürk et al. [7]. PCR products were electrophoresed on 0.8% agarose gel for 1 hr at 70 V in 1× TAE [Tris-Acetate-EDTA (ethylenediaminetetraacetic acid)] and PCR bands were viewed in the SYNGENE Bio Imaging system (Ankara, Turkey). Forward and reverse sequencing was carried out by BM LABOSIS (Ankara, Turkey).

Sequences were displayed and controlled in Chromas Lite 2.1.1 (www.technelysium.com.au), and the 507 base-pair region was formed for analysis by aligning using the software MEGAX [24]. Mean genetic distance values (*d*) between species according to the *p*-distance Parameter [25] were calculated in MEGAX [24], and fixation index values (F_{ST}) were defined in the DnaSP 6 Programme [26]. Bayesian Inference tree was generated in MrBayes 3.2.7a [27] and displayed using FigTree 1.4 (http://tree.bio.ed.ac.uk/software/figtree). HKY+I Parameter [28] was chosen as an appropriate evolutionary model based on the Akaike Information Criterion (AIC) and the Bayesian Information Criterion (BIC) with the help of jModelTest 2.1.7 [29,30]. The Markov Chain Monte Carlo (MCMC) approach was performed for two different runs with 10.000.000 generations with 100 samples each, with a 25% burn-in. Further, Median-joining network was constructed using haplotypes in the software POPART version 1.7 [31]. Using BEAST 1.75 Programme [32], evolutionary divergence times of *Microtus* species were also found considering the mammalian mtDNA divergence rate (2% per 1 million year; [33]) the divergence times of the Asian-Anatolian and European populations of *M. arvalis* based on *COI* gene (0.298 MYA [7]) were used as a calibration point. BEAST analyses were controlled in terms of effective sample size (ESS) values in Tracer 1.5 Software (http://beast.bio.ed.ac.uk/Tracer), and effective sample size (ESS) values of 200 or higher were accepted.

3. RESULTS

According to the results which Table 1 showed that mean genetic distance values (*d*) between *M. daghestanicus* and other *Microtus* species varied between 7.6-16.0%. The *d* value between *M. daghestanicus* and *M. arvalis* (16.0%) was the highest and the *d* value between *M. daghestanicus* and *M. subterraneus* (7.6%) was the lowest. In total, the mean distance values between all *Microtus* species ranged from 5.9 to 16.3. Fixation index values

(F_{ST}) were calculated as between 0.552 (*M. daghestanicus* and *M. subterraneus*) and 0.911 (*M. majori* and *M. mystacinus*).

Bayesian Inference tree and Median-joining network gave similar results in phylogenetic approaches. In Bayesian Inference tree (Figure 1), all species were split with high posterior probability values (pp=0.96-1.00), and *M. daghestanicus* was located, separately as closer to *M. subterraneus* (pp=0.96) than *M. majori*. Similarly, *M. daghestanicus* and *M. subterraneus* haplotypes were diverged as closer to each other than other species in Median-joining network (Figure 2), and *M. majori* was also separated from *M. arvalis* and *M. mystacinus* with more mutations than from *M. daghestanicus* and *M. subterraneus*.

Evolutionary divergence times of the studied species were defined as follows: *M. daghestanicus* and *M. subterraneus*: 0.766 MYA; *M. majori* and *M. daghestanicus/M. subterraneus*: 1.583 MYA; *M. majori/M. daghestanicus/M. subterraneus* and *M. arvalis/M. mystacinus*: 1.883 MYA; *M. arvalis* and *M. mystacinus*: 0.618 MYA.

TABLE 1. Mean genetic distance values (*d*) with standard errors and Fixation index values (F_{ST}).

SPECIES	***d*** **VALUES WITH STANDARD ERRORS**	**FIXATION INDEX VALUES (F_{ST})**
M. daghestanicus-M. subterraneus	0.076±0.011	0.552
M. daghestanicus-M. majori	0.141±0.017	0.784
M. daghestanicus-M. mystacinus	0.149±0.018	0.857
M. daghestanicus-M. arvalis	0.160±0.018	0.770
M. subterraneus-M. majori	0.134±0.016	0.799
M. subterraneus-M. mystacinus	0.150±0.018	0.882
M. subterraneus-M. arvalis	0.163±0.019	0.797
M. majori-M. mystacinus	0.140±0.017	0.911
M. majori-M. arvalis	0.145±0.017	0.811
M. mystacinus-M. arvalis	0.059±0.010	0.715

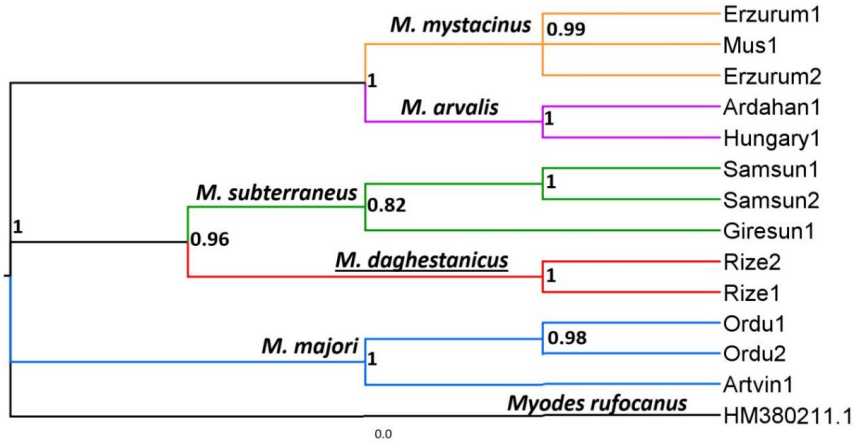

FIGURE 1. Bayesian Inference tree acquired using *COI* gene sequences. Numbers on branches indicate posterior probability (pp) values.

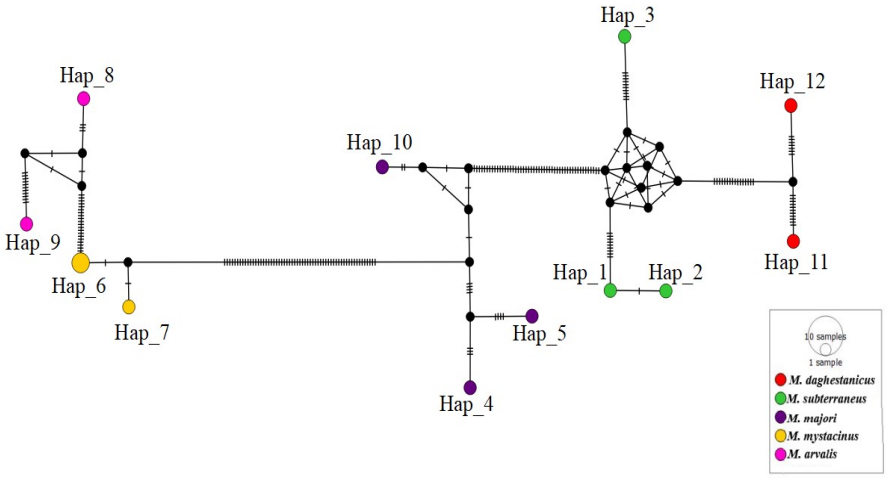

FIGURE 2. Median-joining network constructed with *COI* haplotypes. Numbers of mutations were given with black lines on branches.

4. DISCUSSION

Microtus daghestanicus Shidlovsky, 1919 has been recorded from a limited area in Caucasia, Northeastern Turkey and Northwestern Iran and is controversial regarding its taxonomic status [8,9]. Some authors [10,11]

offered that similar to *M. majori,* Daghestan pine vole is a subspecies of *M. subterraneus*. However, karyological [12] and molecular studies consisting *CYTB*, *IRBP, BRCA1* and *XIST* analyses [3,13,14] suggested that *M. daghestanicus* is a separate species as closely-related to *M. subterraneus*. According to the mitochondrial *CYTB* results in these studies, Jaarola et al. [3] found that phylogenetic trees supported the split of three pine vole species with *M. majori*'s separation from *M. daghestanicus* and *M. subterraneus.* Baskevich et al. [13] obtained results showed the divergence of *M. daghestanicus* and *M. majori* in phylogenetic dendrograms as well as 9.15% genetic distance. Likewise, Bogdanov et al. [14] analysed *CYTB*, *IRBP, BRCA1* and *XIST* gene markers and determined the similar separation in dendrograms. They also calculated the genetic distance values (7.8%, 9.65% and 9.68% between *M. daghestanicus* and *M. subterraneus, M. daghestanicus-M. majori* and *M. subterraneus-M. majori*, respectively) for *CYTB* gene. Results in this study yielded similar results with these studies regarding that *M. daghestanicus* was diverged from *M. subterraneus* and *M. majori,* and *M. majori* is the first separated taxon. Mean genetic distance values were found as 7.6% (*M. daghestanicus-M. subterraneus*), 14.1% (*M. daghestanicus-M. majori*) and 13.4% (*M. subterraneus-M. majori*). With respect to accepted intraspecies variation as <10% for *COI* [16] as well as interspecific genetic distance values (2%-11% in general [34], 0.0%–4.7% (mean 1.5%) for rodents and 0.2%–4.4% (2.0%) for genus *Microtus* [35]), obtained data implies interspecific genetic distance data. In addition, fixation index values (F_{ST}) of 0.25 and above are regarded to indicate high level of differentiation [36]. Klaus et al. [37] calculated the F_{ST} value between *Microtus richardsoni* U.S.A. populations as 0.624 and accepted this value as high. Heckel et al. [38] found the high mean F_{ST} value between *Microtus arvalis* European populations as 0.70. Sheremetyeva et al. [39] also determined the high F_{ST} value (0.624) between *Microtus maximowiczii* populations in Middle Amur River Region. Çetintürk et al. [40] analysed *Microtus mystacinus* interpopulations from Asia and Europe and F_{ST} values were found from moderate (0.195) to high (0.741). These findings are similar to high F_{ST} values in Table 1. Moreover, evolutionary divergence times coinciding with Pleistocene Period pointed that *M. majori* first diverged from *M. daghestanicus/M. subterraneus* group 1.583 MYA and *M. daghestanicus* split from *M. subterraneus* 0.766 MYA.

In conclusion, this performed study accepted the species status of *Microtus daghestanicus* Shidlovsky, 1919 and offered that mitochondrial *COI* gene is

effective to identify *Microtus* species that consist of recently diverged species.

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ÇUBUK STREAM POLLUTION AND ENVIRONMENTAL EFFECTS

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ABSTRACT. The water quality parameters of Çubuk Stream were examined in terms of physical and chemical aspects and the pollution level of Çubuk Stream was determined by comparing these parameters with the Surface Water Quality Regulation (SWQR). For this purpose; samples were taken from three different stations between August 2012 and April 2013, the water samples were compared with each other and with previous studies, the water quality of Çubuk Stream was determined according to the surface water quality regulation. In our study, the biological oxygen requirement of Çubuk Stream according to SWQR (BOD5: 22.5mg/l) IV. class, ammonium nitrogen element (NH4–N:2.34 mg/l) IV. class, nitrite nitrogen element (NO2–N: 0.035 mg/l) II. class, nitrate nitrogen element (NO3 –N:0.95 mg/l) Class I, according to phosphate value (PO4:0.075 mg/l) Class I and trace elements (Pb, Cd, Cr, Ni, Mn) values Class I. water quality. According to the ratio of NH4-N/PO4 (2.34/0.075=31.2 mg/l), the element that limits eutrophication in Çubuk Stream is P. Compared to previous studies, after the wastewater treatment plants established in the region, higher quality water is provided, especially in terms of NO3 and PO4. It has been determined that the nitrogen and phosphorus ratios are high because the region, which is dense in terms of organic matter, is close to industrial establishments.

1. INTRODUCTION

Water is one of the indispensable elements of our life. Besides being an important inorganic nutrient, water; as a good solvent and carrier, minerals and compounds play an active role in the realization of all kinds of biochemical reactions in our body [1]. Therefore, a life without water is not possible. Water concerns the living thing and everything it affects. Although the fact that 75% of our earth is covered with water shows that the world has

Keywords. Water quality, Çubuk Creek, water pollution, physical and chemical parameters
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a large water reserve, the rate of drinkable water is only below 1%. As of 1765, while the industrial revolution was in its infancy, the world population reached 1 billion, 2.5 billion in the last quarter of the 20th century, and 8.5 billion towards 2020.

The increase in the population in our world, the rapid development of industry and technology, as well as the inability to establish or spread environmental awareness sufficiently, cause the amount of potable water in the world to decrease gradually. In addition, it is inevitable that irreparable ecological problems occur due to unconscious pollution of potable water density [2]. It is predicted that a universal water crisis will emerge with the increase in the required water density and the intersection of clean water reserves in 2030 according to the estimates [3]. In recent years, studies have been carried out especially for the monitoring of freshwater resources. This is an indication that the importance of water in today's world has become very important on a global scale [4].

Excessive algae growth in water occurs when nutrient elements such as nitrogen and phosphate coming from wastewater mixed with rivers, especially from agricultural soils by drainage and rain water, reach the maximum level (0.8 mg/l N [nitrogen] and 0.1 mg/l P [phosphorus]). This is called secondary pollution or eutrophication [5]. Nitrogen in water is mostly in the form of ammonium, nitrite and nitrate. In waters with high oxygen content, ammonium is found at very low levels. Ammonium nitrogen is the waste material of living things in aquatic ecosystems and can be reabsorbed by these organisms [6]. Nitrite is an intermediate in the biological oxidation from ammonium to nitrate. It is known that nitrite rate is low in clean surface waters and high in polluted surface waters [7]. Nitrate, the most common form of nitrogen in surface waters, occurs in oxygen-rich waters. Nitrate is an important element that can limit or increase the growth of algae. Nitrogen content is low in oligotrophic waters and quite high in eutrophic waters [8]. Phosphorus, which affects the productivity of natural waters and is one of the most basic elements of eutrophication, has a limiting effect on the growth of autotrophic and heterotrophic organisms. It also gives information about the presence of phosphorus, organic matter mixed with water and the presence of household waste. Depending on the presence of detergent in household wastes and the organic metabolism in the water, changes are seen in the phosphorus ratio. Phosphorus is found in trace amounts in uncontaminated waters [9-11].

The aim of this study; to determine the extent of the pollution of Çubuk Stream, which is an important water source for Ankara, to compare the

situation before and after the establishment of treatment plants in the region according to physical and chemical parameters, to determine which element is limited by the system by looking at the nitrogen and phosphorus amounts of the eutrophication event observed in Çubuk Stream. In addition, the results of the research are important for the control of Çubuk Stream water pollution, by determining the factors causing pollution, ensuring that these factors are brought to normal standards by taking into account the interests of the country's economy, in the development of other studies and in terms of its contribution to the scientific literature.

1.1. Geographical structure of the region

Çubuk Stream is an important water source for Ankara. Located in the north of the city center of Ankara, this stream crosses the Ankara plain between 40°00' - 40°18' latitudes and 32°53' - 33°08' longitudes. The Çubuk Stream, which originates in two branches from the Aydos Mountains within the Köroğlu Mountain mass and continues to the south, is 84 km long and is located in the Sakarya River Basin. The total precipitation area of the basin is 58160 km² [12] (SHW [State Hydraulic Works], 2006). The Çubuk Stream, which passes through Çubuk district center, divides the district into two. Although there are many side streams that mix with the stream, the Çubuk Stream has an average flow of 0.5 m³/sec, since they do not have much water. The heaviest time of the Çubuk Stream is in late spring and the heaviest time is late summer and early autumn. Summers in the region are hot and dry, and winters are cold and rainy. The lowest recorded temperature is -21.5 ^{0}C, and the hottest temperature is 37.7 ^{0}C. Rain averages are 472 mm³ [13].

2. MATERIALS AND METHODS

2.1. Determination of Physical and Chemical Parameters of Water

Water samples were taken from 3 different stations on Çubuk Stream in August 2012, November 2012, January 2013 and April 2013. While determining the parameters in this study, the water quality parameters based on the SWQR [14,15] quality criteria were considered. Biological oxygen demand from oxygenation parameters; nutrient parameters such as ammonium, nitrite and nitrate nitrogen, and the amount of phosphate; among the trace elements, parameter values such as cadmium, lead, copper, nickel

and zinc were examined. With these values, quality criteria were made according to the classes of inland surface water resources.

After the Karaköy and Çubuk Wastewater Treatment plants were opened in 2009, no study was conducted on Çubuk Stream. Therefore, because of this study, the parametric values of the base stations were compared with the studies before 2009. N and P amounts were determined in the studied stations of Çubuk Stream and it was revealed which of the N and P elements that caused the eutrophication event had a limiting effect. Physical and chemical parameters such as pH, EC, BOD5, B, PO4 -P, SO4, S, CN, Zn, NH4-N, Al, Fe, NO2-N, NO3-N, Cl, F, ˚F, Cu, Pb, Cd, Cr, Ni, Mn were analyzed. Physical parameters were analyzed with Multi Parameter Water Quality Meter WQC-24, chemical parameters were analyzed with The Spectroquant NOVA 60 photometers.

2.2. Selection and Definition of Water Sample Stations

In order to analyze the physical and chemical parameters, water samples were collected from 0.5-liter unused sterile water bottles, approximately 5 cm from the surface of the water; it was taken from the widening parts of the Çubuk Stream bed where the current is low, and the water seems clear.

Station 1 is the exit of Çubuk 2 Dam. Çubuk 2 Dam was established on Çubuk Stream, 54 km north of Ankara, 5 km north of Çubuk, in a place where the valley is relatively narrow to meet the water needs of the city. There are many factories in the area after the 1st station. There are feed-food industry, flour industry, tile industry, concrete pipe and parquet, glass industry, pharmaceutical industry and agriculture areas as well as slaughterhouses.

In the region where factories are concentrated between the 1st station and the 2nd station, there is Çubuk Wastewater Treatment Plant. This facility was put into service in 2009 in order to treat the wastewater of Çubuk district and its neighborhoods.

2nd station: It was taken from an Ülker factory near the Akyurt district of Ankara, in the Pursaklar-Saray region on the Esenboğa airport road. There are many slaughterhouses in this region. There is Karaköy Wastewater Treatment Plant between the 2nd station and the 3rd station. Located in Karaköy, this facility; It treats the wastewater of a part of the Pursaklar district, Akyurt district and Esenboğa, Sirkeli, Karacaören, Altınova and

Sarayköy districts, as well as the surrounding neighborhoods and settlements. It was put into service in 2009 [13].

3rd station: it was taken from Torunoğlu concrete pipe industry zone from the lower side of Keçiören-Hasköy Bridge. Next to this area is Erdemler furniture factory. It is a residential area. The bottom of the stream is covered with mud.

3. RESULTS

Measurements of physical and chemical parameters of Çubuk Stream are given in Table 1-5.

TABLE 1. Some physical and chemical properties of Çubuk Stream according to stations in August 2012

Control Parameters	**First Station Çubuk (Dam Exit)**	**Second Station Saray-Esenboğa Road**	**Third Station Kecioren-Haskoy**
EC µS/cm	319	1247	1256
pH	7.75	7.17	7.11
BOD5	15	24	47
B (µg /L)	0.12	8.5	0.31
P04 (mg/l)	0.07	0.09	0.09
$SO4^{=}$ (mg/l)	130.2	141.1	132.22
S (mg/l)	0.035	0.285	10.05
CN^{-} (µg /L)	0.006	0.006	0.008
Zn (mg/l)	0.24	0.41	0.47
NH4 -N (mg/l)	0.07	0.27	1.14
Al (mg/l)	0.01	0.01	0.01
Fe (mg/l)	0.12	0.42	0.60
NO2 -N (mg/l)	0.03	0.01	0.01
NO3 –N (mg/l)	1.6	1	1.4
Cl^{-} (mg/l)	11	204	136
F^{-} (mg/l)	<0.1	0.11	0.07
°F (Fr)	0.01	0.01	0.01
Cu (µg/L)	0.870	0.144	0.310
Pb (µg/L)	0.250	0.250	0.873
Cd (µg/L)	0.02	0.02	0.03
Cr (µg /L)	0.12	0.38	0.44
Ni (µg/L)	0.46	0.34	0.74
Mn (mg/l)	0.74	0.34	0.46

TABLE 2. Some physical and chemical properties of Çubuk Stream according to stations in November 2012

Control Parameters	First Station Çubuk (Dam Exit)	Second Station Saray-Esenboğa Road	Third Station Keçioren-Haskoy
EC µS/cm	317	918	1166
pH	8.04	7.72	7.51
BOD5	9	29	45
B (µg/L)	0.1	0.15	0.17
P04 (mg/l)	0.075	0.08	0.09
$SO4^{=}$ (mg/l)	16.02	38.2	91.2
S(mg/l)	0.035	0.027	0.037
CN^{-} (µg/L)	0.005	0.003	0.004
Zn (mg/l)	0.39	2.55	0.87
NH4 -N (mg/l)	0.44	3.81	3.54
Al (mg/l)	0.01	0.01	0.01
Fe (mg/l)	0.33	0.10	0.14
NO2 -N (mg/l)	0.04	0.59	0.02
NO3 -N(mg/l)	0.1	3.4	0.10
Cl^{-} (mg/l)	21	98	102
F^{-} (mg/l)	0.26	0.29	0.10
°F (Fr)	20	32	30
Cu (µg/L)	0.01	0.01	0.01
Pb (µg/L)	0.040	0.026	0.144
Cd (µg/L)	<0.1	<0.1	<0.1
Cr (µg/L)	0.02	0.02	0.03
Ni (µg/L)	0.12	0.12	0.12
Mn (mg/l)	0.02	0.05	0.02

TABLE 3. Some physical and chemical properties of Çubuk Stream according to stations in January 2013

Control Parameters	First Station Çubuk (Dam Exit)	Second Station Saray-Esenboğa Road	Third Station Kecioren-Haskoy
EC µS/cm	258	916	1092
pH	7.8	7.7	7.2
BOD5	8	18	40
B (µg/L)	0.09	0.13	0.17
P04 (mg/l)	0.07	0.07	0.07
$SO4^{=}$ (mg/l)	14.02	36.3	82.4
S (mg/l)	0.035	0.027	0.035
CN^{-} (µg/L)	0.02	0.02	0.03
Zn (mg/l)	0.36	2.50	0.91
NH4 -N(mg/l)	0.56	3.9	3.63
Al (mg/l)	0.02	0.02	0.01
Fe (mg/l)	0.10	0.10	0.10
NO2-N (mg/l)	0.05	0.55	0.02
NO3 -N (mg/l)	0.1	3.1	0.9
Cl^{-} (mg/l)	10	62	94
F^{-} (mg/l)	0.25	0.25	0.10
°F (Fr)	20	32	30
Cu (µg/L)	0.01	0.01	0.01
Pb (µg/L)	0.040	0.026	0.144
Cd (µg/L)	<0.1	<0.1	<0.1
Cr (µg/L)	0.02	0.02	0.02
Ni (µg/L)	0.12	0.12	0.12
Mn (mg/l)	0.02	0.05	0.02

TABLE 4. Some physical and chemical properties of Çubuk Stream according to stations in April 2013

Control Parameters	First Station Çubuk (Dam Exit)	Second Station Saray-Esenboğa Road	Third Station Keçioren-Haskoy
EC µS/cm	280	964	1102
pH	7.9	7.7	7.3
BOD5	8	21	40
B (µg/L)	0.1	0.14	0.21
P04 (mg/l)	0.06	0.07	0.07
$SO4^{=}$ (mg/l)	73.6	81.7	110.3
S (mg/l)	0.030	0.14	3.02
CN^{-} (µg/L	0.006	0.006	0.008
Zn (mg/l)	0.30	2.45	0.62
NH4 -N (mg/l)	0.60	3.90	3.70
Al (mg/l)	0.02	0.01	0.01
Fe (mg/l)	0.22	0.10	0.12
NO2 -N (mg/l)	0.06	0.33	0.02
NO3 -N (mg/l)	0.4	2.5	0.8
Cl^{-} (mg/l)	15	83	85
F^{-} (mg/l)	<0.1	0.21	0.10
°F (Fr)	0.01	0.01	0.01
Cu (µg/L)	0.04	0.01	0.01
Pb (µg/L)	0.097	0.074	0.320
Cd (µg/L)	<0.1	<0.1	<0.1
Cr (µg/L)	0.01	0.01	0.02
Ni (µg/L)	0.10	0.11	0.11
Mn (mg/l)	0.02	0.07	0.02

TABLE 5. Çubuk Stream; comparison with water quality parameters SWQR 2015

Parameters	Water Quality Classes			Çubuk Stream (Average value)
	I	II	III	
pH	6.5-8.5	6.5-8.5	6.5-8.5	7.7
PO4 (mg/l)	0.03	0.20	0.50	0.075
NO2 (mg/l)	<0.01	0.06	0.12	0.035
NO3 (mg/l)	5	7.5	15	0.95
NH4 (mg/l)	<0.2	1	2	2.34

The comparison of the pollution parameters of Çubuk Stream taken from approximately the same location [16] from the water analyzes before and after the opening of the wastewater treatment plants is shown in Table 6.

TABLE 6. Average annual values of some physical and chemical parameters before and after the establishment of the wastewater plant.

Control Parameters	Second Station Saray-Esenboğa road	Third Station Keçiören-Hasköy	Çubuk and Karaköy Before Waste Water Treatment Plant	Çubuk and Karaköy After Waste Water Treatment Plant
pH	7.7	7.25	8.42	7.475
BOD5	21	42.5	12.60	31.75
NH4 -N (mg/l)	3.855	3.585	3.52	3.72
P04 (mg/l)	0.075	0.08	0.170	0.0775

The comparison of the pollution parameters of Ankara Stream. The continuation of Çubuk Stream with our analysis is shown in Table 7 [17].

TABLE 7. Average annual values of some physical and chemical parameters of Ankara Stream and Çubuk Stream before and after the establishment of the wastewater plant

Control Parameters	Çubuk and Karaköy Before the Waste Water Treatment Plant Opens	Çubuk and Karaköy After the Waste Water Treatment Plant Opens
pH	7.35	7.7
EC µS/cm	922	941
NH4 -N (mg/l)	8.55	3.585
Cl (µg/L)	76.65	90.5
NO2 –N (mg/l)	0.0525	0.450
BOD5	39	22.5
PO4 (mg/l)	3.65	0.0775

The average values of the physical and chemical parameters of the water samples taken in different seasons of Çubuk Stream in 2012 and 2013 are shown in Table 8.

TABLE 8. The median, minimum, maximum and standard deviation values of some physical and chemical properties according to the water samples taken from the Çubuk Stream in 2012-2013

Control Parameters	First Station Average (Min-Max. ± Sd)	Second Station Average (Min-Mak. ± Sd)	Third Station Average (Min-Mak.±Sd)	Çubuk Stream Average (Min-Mak.±Sd)
EC µS/cm	298.5 (258-319 ± 29.6)	941 (916-1247 ± 158.7)	1134 (1092-1256 ± 75.4)	941 (258-1256 ± 404.14)
pH	7.85 (7.75-8.04 ± 0.12)	7.7 (7.17-7.72 ± 0.268)	7.25 (7.11-7.51 ± 0.171)	7.7 (7.11-8.04 ± 0.309)
BOD5	8.5 (8-15 ± 3.36)	22.5 (18-29 ± 4.69)	42.5 (40-47 ± 3.55)	22.5 (8-47 ± 14.61)
B (µg/L)	0.1 (0.09-0.12 ± 0.12)	0.145 (0.13-8.5 ± 4.18)	0.19 (0.17-0.31 ± 0.066)	0.145 (0.09-8.5 ± 2.41)

Control Parameters	First Station Average (Min-Max. ± Sd)	Second Station Average (Min-Mak. ± Sd)	Third Station Average (Min-Mak.±Sd)	Çubuk Stream Average (Min-Mak.±Sd)
P04 -P (mg/l)	0.07 (0.06-0.075 ± 0.006)	0.075 (0.07-0.09 ± 0.01)	0.08 (0.07-0.09 ± 0.011)	0.075 (0.06-0.09 ± 0.009)
$SO4^{=}$ (mg/l)	44.81 (14.02-130.2 ± 55.2)	59.95 (36.3-141.1 ± 49)	100.75 (82.4-132.2 ± 22)	82.05 (14.2-141.1 ± 44.8)
S (mg/l)	0.035 (0.03-0.035 ± 0.005)	0.08 (0.02-0.28. ± 0.123)	1.525 (0.03-10.05 ± 4.7)	0.035 (0.02-10.05 ± 2.93)
CN^{-} (µg/L)	0.006 (0.005-0.02 ± 0.007)	0.006 (0.003-0.02 ± 0.1)	0.008 (0.004-0.03 ± 0.1)	0.006 (0.003-0.03 ± 0.01)
Zn (mg/l)	0.33 (0.24-0.39 ± 0.066)	2.475 (0.41-2.55 ± 1.04)	0.745 (0.47-0.91 ± 0.19)	0.545 (0.24-2.55 ± 0.924)
NH4 -N(mg/l)	0.5 (0.07-0.6 ± 0.24)	3.855 (0.27-3.9 ± 1.8)	3.585 (1.14-3.7 ± 1.24)	2.34 (0.07-1.8 ± 1.709)
Al (mg/l)	0.015 (0.01-0.02 ± 0.005)	0.01 (0.01-0.02 ± 0.005)	0.01 (0.01-0.01 ± 0)	0.01 (0.01-0.02 ± 0.004)
Fe (mg/l)	0.17 (0.1-0.33 ± 0.105)	0.1 (0.1-0.42 ± 0.16)	0.13 (0.1-0.6 ± 0.24)	0.12 (0.1-0.33 ± 0.162)
NO2 -N (mg/l)	0.045 (0.03-0.06 ± 0.012)	0.44 (0.01-0.59 ± 0.265)	0.02 (0.01-0.02 ± 0.005)	0.035 (0.01-0.59 ± 0.217)
NO3 -N(mg/l)	0.25 (0.1-1.6 ± 0.714)	2.8 (1-3.4 ± 1.06)	0.85 (0.1-1.4 ± 0.535)	0.95 (0.1-3.4 ± 1.16)
Cl^{-} (mg/l)	13 (10-21 ± 4.99)	90.5 (62-204 ± 63.24)	98 (85-136 ±22.27)	84 (10-204 ± 58.08)
F^{-} (mg/l)	0.175 (0.01-0.26 ± 0.12)	0.23 (0.11-0.29 ± 0.077)	0.1 (0.07-0.1 ± 0.015)	0.105 (0.01-0.29 ± 0.091)
°F (Fr)	10.005 (0.01-20 ± 11.5)	16.005 (0.01-32 ± 18.46)	15.005 (0.01-30 ± 17.31)	10.005 (0.01-32 ± 14.78)
Cu (µg/L)	0.025 (0.01-0.87 ± 0.425)	0.01 (0.01-0.144 ± 0.06)	0.01 (0.01-0.31 ± 0.15)	0.01 (0.01-0.87 ± 0.252)
Pb (µg/L)	0.065 (0.04-0.25 ± 0.099)	0.05 (0.026-0.25 ± 0.11)	0.232 (0.144-0.87 ± 0.3)	0.117 (0.026-0.87 ± 0.26)
Cd (µg/L)	0.1 (0.02-0.1 ± 0.04)	0.1(0.02-0.1 ± 0.04)	0.065 (0.01-0.1 ± 0.046)	0.1 (0.01-0.1 ± 0.039)
Cr (µg/L)	0.02 (0.01-0.12 ± 0.051)	0.02 (0.02-0.38 ± 0.18)	0.025 (0.02-0.44 ± 0.208)	0.02 (0.01-0.44 ± 0.151)
Ni (µg/L)	0.12 (0.1-0.46 ± 0.173)	0.12 (0.11-0.34 ± 0.111)	0.12 (0.11-0.74 ± 0.311)	0.12 (0.1-0.74 ± 0.2001)
Mn (mg/l)	0.02 (0.02-0.74 ± 0.36)	0.06 (0.05-0.34 ± 0.14)	0.02 (0.02-0.46 ± 0.22)	0.035 (0.02-0.74 ± 0.235)

The average electrical conductivity of Çubuk Stream varies according to the stations in 2012-2013 (Table 8). The first station Çubuk 2 Dam was measured at EC: 298.5 µS/cm. In terms of this feature, I. class quality water shows characteristics. In the sample taken from the 2nd station, Saray-Esenboğa Road. EC was measured as 941 µS/cm. According to this value this station III. class water quality feature. The EC was measured as 1134µS/cm in the water sample taken from the 3rd station Keçiören-Hasköy region. According to this feature, this station III. class water quality.

The average EC of Çubuk Stream is 941 µS/cm. According to the average EC, Çubuk Stream II. class water quality characteristics. It has been observed that there are agricultural areas and greenhouse cultivation around the water sample areas taken from Çubuk Stream (Figure 1). Çubuk Stream, which is used as irrigation water in these areas. is classified as salty waters according to the average value of EC (EC:941) (C3 class EC: 750-2250).

According to SWQR, surface waters with pH values between 6.5 and 8.5 do not pose a problem in pollution (Figure 2).

BOD_5, which is the parameter that gives information about the oxygen amount in water quality parameters, was measured as 8.5 mg/l at the exit of the first station. Çubuk 2 Dam, in the average values of BOD_5 of the water

samples taken from the Çubuk Stream in 2012-2013. According to this value, in the quality criteria according to SWQR II. quality class water. It was measured as 22.5 mg/l at station 2 and 42.5 mg/l at station 3. According to these values, IV. quality class water (Figure 3). The average BOD of >20 in Çubuk Stream is also (22.5) IV. shows the quality class water feature.

The average Boron value of Çubuk Stream was measured as 0.145 µg/l (Figure 4). It was determined that the boron concentration was higher especially in the second station. The second station is near a food factory where boron is widely used. Boron level less than 1 µg/l is a tolerable amount in water pollution.

In Çubuk Stream phosphate values, average phosphate values of 4 months between 2012 and 2013; 1st station is 0.07 mg/l. 2nd station is 0.075 mg/l. 3rd station is 0.080 mg/l. The average annual value of the three stations is 0.075 mg/l. In addition, according to Figure 5. water samples were taken at different seasonal temperatures and the phosphate values were highest in august. when the temperature was the highest; It was observed that the temperature was lower in January when the temperature was the lowest.

The average sulfate rate of Çubuk Stream is 82.05 mg/l. Since it is lower than 200 mg/l according to this value of sulfate. it has class I water quality (SWQR, 2015). In addition, according to Figure 6. the water samples were taken at different seasonal temperatures and the sulfate values were highest in august. when the temperature was the highest.

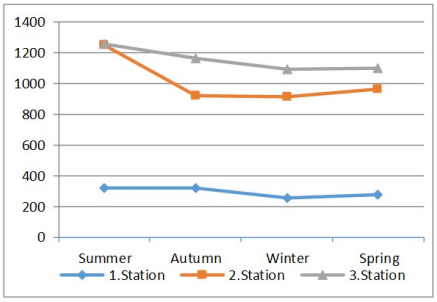

FIGURE 1. Çubuk Stream EC values

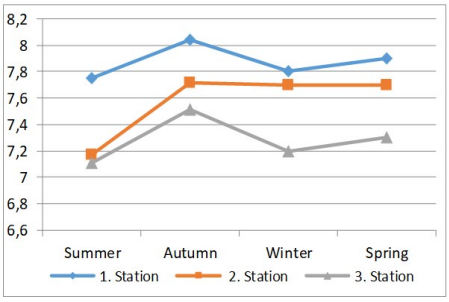

FIGURE 2. Çubuk Stream pH values

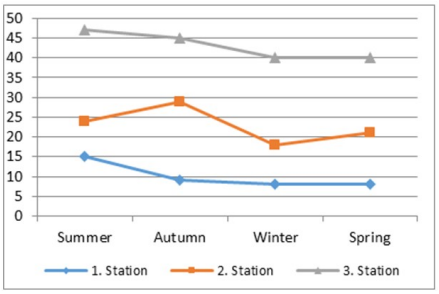

FIGURE 3. Çubuk Stream BOD5 values

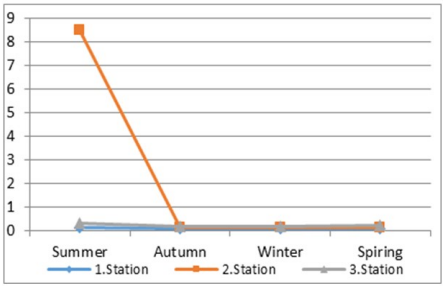

FIGURE 4. Çubuk Stream Boron values

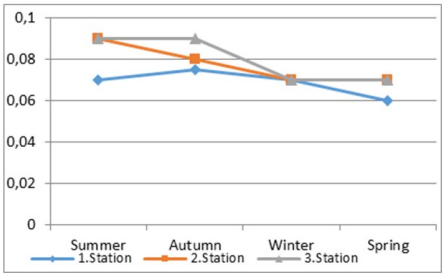

FIGURE 5. Çubuk Stream PO_4^{-3} values

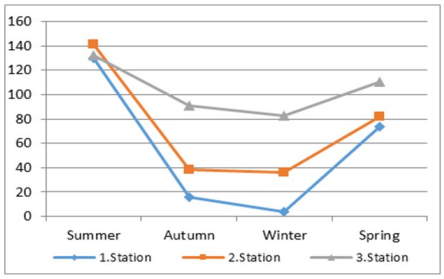

FIGURE 6. Çubuk Stream S^{-2} values

The toxicity of cyanide increases 2-3 times with every 10˚C increase in temperature [18]. According to Figure 7. the cyanide value is at a high level in the coldest winter season. The average cyanide rate in Çubuk Stream is 0.006 µg/l.

The average zinc ratio in the measurement made in Çubuk Stream is 0.545 mg/l. In terms of this parameter. Çubuk Stream is III. class water quality. According to Figure 8. it is seen that the zinc value is at a high level in the autumn and winter seasons when the air temperature is low.

The average zinc ratio in the measurement made in Çubuk Stream is 0.01 mg/l. The average pH of Çubuk Stream is close to neutral. Therefore, Çubuk Stream is not under threat in terms of aluminum value. In addition, the change in the aluminum value of Çubuk Stream depending on the seasonal temperature is shown in Figure 9.

Among the stations selected in Çubuk Stream, it was measured that the 3rd station region where the densest industrial area is located was higher than the other stations (average Fe: 0.13 mg/l at the 3rd station; average Fe: 0.1 mg/l

at the 2nd station; 1st station average Fe: 0.17 mg/l). The average Fe value of Çubuk Stream is 0.12 mg/l. Since the Fe value is less than 0.3 mg/l according to SWQR it has I. class quality water standards. Figure 10. shows the variation of iron ratio according to seasonal temperature.

Average ammonium values of 3 months taken between 2012 and 2013; 1st station is 0.5 mg/l. 2nd station is 3.855 mg/l. 3rd station is 3.585 mg/l. It is seen that the ammonium value is at the highest value according to Figure 11.

Nitrite values in this study we have done about Çubuk Stream: Average nitrite values of 4 months taken between 2012 and 2013; 1st station is 0.045 mg/l. 2nd station is 0.44 mg/l. 3rd station is 0.02 mg/l. The variation of nitrite values depending on seasonal temperature is shown in Figure 12. According to SWQR the average nitrite concentration is 0.035 mg/l. in terms of this parameter II. class water quality.

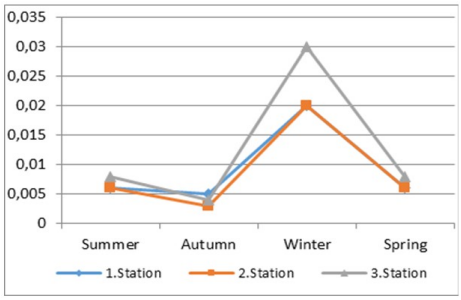

FIGURE 7. Çubuk Stream CN values

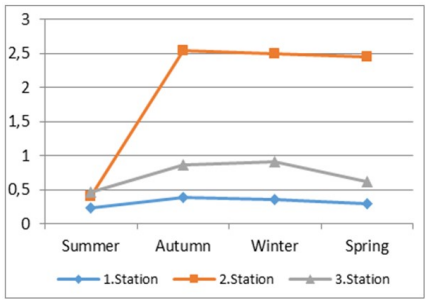

FIGURE 8. Çubuk Stream Zn values

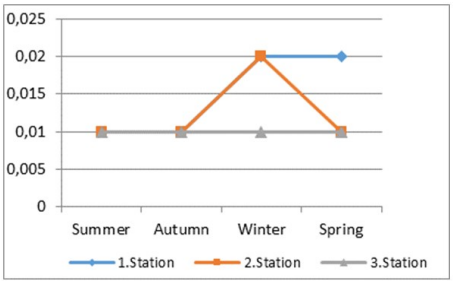

FIGURE 9. Çubuk Stream Al values

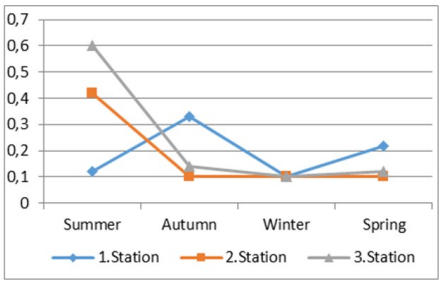

FIGURE 10. Çubuk Stream Fe values

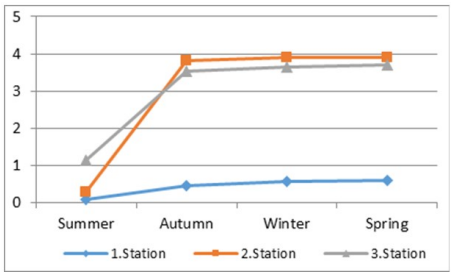

FIGURE 11. Çubuk Stream NH_4^+ values

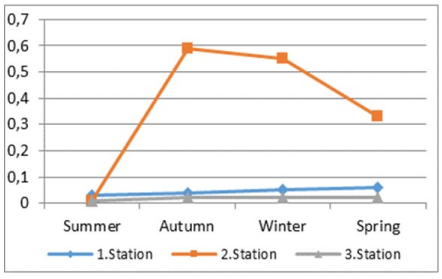

FIGURE 12. Çubuk Stream NO_2^- values

The average nitrate level in Çubuk Stream was 1.283 mg/l. Especially, the nitrate concentration in the 2nd station area is higher than the other two stations (2nd station average nitrate value: 2.5 mg/l; 1st station average nitrate value: 0.55 mg/l; 3rd station average nitrate value: 0.8 mg/l). In addition, in the measurements made during the sampling from the 2nd station region macroscopically mossy layers were observed on the water surface in this region. We can say that there is water pollution due to eutrophication in the 2nd station area.

In Çubuk Stream, average nitrate values of 4 months taken between 2012 and 2013; 1st station is 0.25 mg/l. 2nd station is 2.8 mg/l. 3rd station is 0.85 mg/l. The variation of nitrate values depending on seasonal temperature is shown in Figure 13. As shown in Table 5 the average nitrate concentration of 0.95 mg/l according to the SWQR values indicates class I water quality in terms of this parameter.

As a result of the measurements made in Çubuk Stream, the average Cl^- ions were measured as 13 mg/l at the 1st station, 90.5 mg/l at the 2nd station, and 98 mg/l at the 3rd station. These values show direct proportion with EC. According to Figure 14, it is seen that the chloride value is high in the summer season when the temperature is high and the precipitation is low. In areas used as irrigation water. The amount of chloride should be kept under control. High chloride concentration damages many trees and plants [18]. The average value of the chloride ion (84 mg/l) less than 200 mg/l shows I. class water quality according to SWQR.

The average fluoride value measured in Çubuk Stream is 0.105 mg/l. The 0.23 mg/l fluoride rate should be controlled. Especially in the industrial areas where agricultural areas are concentrated and Çubuk Stream is used as irrigation water in the second station area which is dense. The variation of fluoride values depending on seasonal temperature is shown in Figure 15.

Clay soils and areas that will be irrigated for less than 20 years should be preferred when using the analyzed regions in Çubuk Stream as irrigation water. In addition, the variation of copper values depending on seasonal temperature is shown in Figure 16.

The average lead value in Çubuk Stream is 0.117. It can be said that this environment is not conducive to the survival of sensitive macro organisms. The variation of lead values depending on the seasonal temperature is shown in Figure 17.

The fact that the cadmium values were measured as <0.1 in all three stations selected from the Çubuk Stream shows that there is a water value with acceptable standards in terms of this parameter. The variation of cadmium values depending on seasonal temperature is shown in Figure 18.

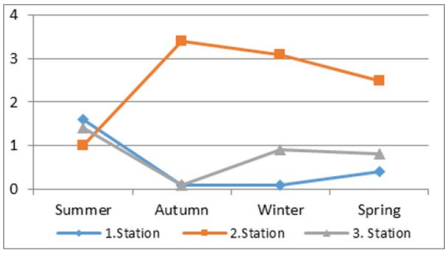

FIGURE 13. Çubuk Stream NO_3^- values

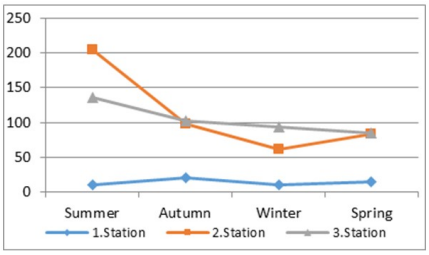

FIGURE 14. Çubuk Stream Cl^- values

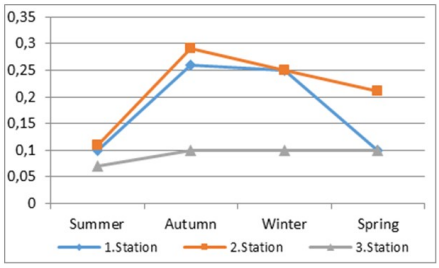

FIGURE 15. Çubuk Stream F^- values

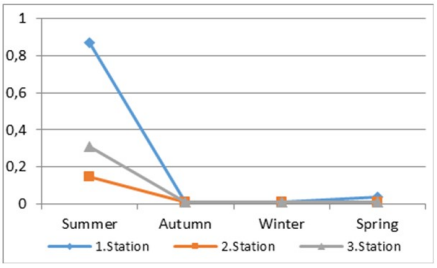

FIGURE 16. Çubuk Stream Cu values

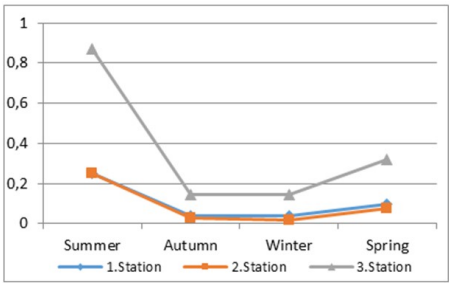

FIGURE 17. Çubuk Stream Pb values

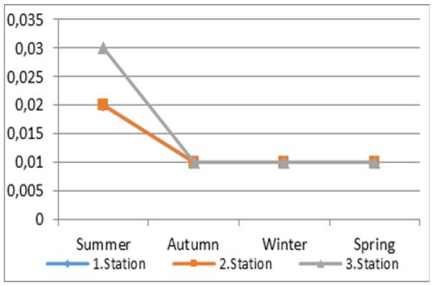

FIGURE 18. Çubuk Stream Cd values

The average chromium value of Çubuk Stream was measured as 0.1 µg/l. It can be said that this value will not adversely affect the activities of organisms in Çubuk Stream. According to Figure 19 it is seen that the chromium value is also high in the summer season when the seasonal temperature is the highest.

The average concentration of nickel in Çubuk Stream is 0.12 µg/l. This ratio shows that nickel will not harm the aquatic ecosystem. According to Figure 20 it is seen that the nickel value is high in the summer season when the seasonal temperature is the highest.

The average manganese density of Çubuk Stream is 0.035 mg/l. In terms of this ratio manganese cannot be considered to be harmful when used as irrigation water and considering the vital activities of other microorganisms in Çubuk Stream. According to Figure 21 it is seen that the manganese value is high in the summer season when the seasonal temperature is the highest.

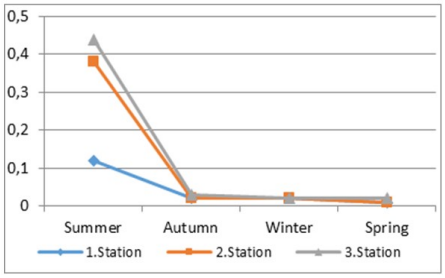

FIGURE 19. Çubuk Stream Cr values

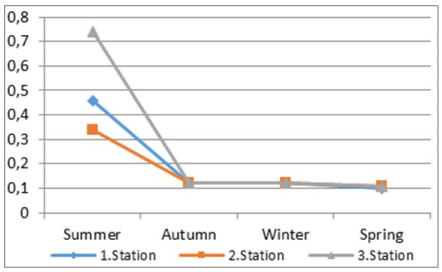

FIGURE 20. Çubuk Stream Ni values

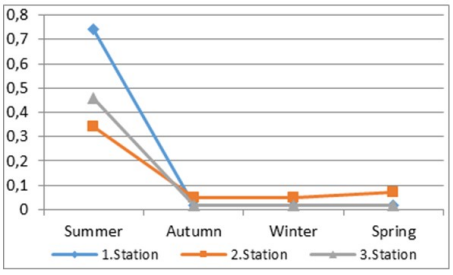

FIGURE 21. Çubuk Stream Manganese values

4. CONCLUSIONS AND DISCUSSION

According to the data obtained there are differences in the quality of Çubuk Stream during 2012-2013. These differences are mostly due to seasonal factors such as precipitation heat and temperature. In Çubuk Stream has been determined that it is polluting in terms of electrical conductivity and chlorine by looking at the average values of some physical and chemical properties. EC and Cl depend on the amount of dissolved substance in the water [20]. At the 3rd station EC and Cl parameters are in class 4 water quality according to SWQR there are greenhouse zones near this station. Compared to works [21] it is seen that there is a decrease in the sulfate rate in Çubuk Stream. The mixing of domestic and industrial wastewater into the surface waters increases the salt content of sulfate in the water. Especially when they are used as irrigation water they will adversely affect the soil and therefore the plant community in that region. Growing salt-tolerant plants in these regions provides healthier agricultural production. Water with this feature. which is used as irrigation water, is used in soils with medium and good permeability. In addition, regular soil washing programs should be followed to prevent salt accumulation in these soils.

Its pH was measured at 7.7 compared to works [16-21] with previous studies it has been determined that pH approaches neutral values over time (Table 6 and Table 7). The fact that the pH is at basic values increases the eutrophication event that causes water pollution [5]. The fact that Çubuk Stream reached a neutral value over time compared to the water samples taken in 2012-2013 suggests that eutrophication may have decreased.

According to SWQR, in terms of BOD_5 parameter it is IV class water quality. When studies in similar locations were compared according to works [16-21] an increase was found in the values for the BOD_5 parameter.

This increase may adversely affect the aerobic organisms in the water. It can also inhibit the completion of nitrification. When the previous studies are compared according to [17] a decrease in the values has been detected Ankara Stream the continuation of Çubuk Stream may have been exposed to more pollutants.

The average sulfur value measured in Çubuk Stream is 0.035 mg/l (Figure 7). Based on stations the region where the industrial zone and houses are most concentrated is the 3rd station. Considering that this station is exposed to domestic and industrial pollution at a higher rate than the other two stations it has an above-average sulfur content.

As a result of the measurements, it was determined that the ammonium concentration of the 2nd and 3rd stations was higher than the 1st station. In addition, it is seen that the ammonium values at the 2nd station are higher than the ammonium values at the 1st and 3rd stations in all 3 months of the measurement. Fertilizer, nitrocellulose, leather, food, beer, water industries and slaughterhouses are industrial establishments with a high nitrogen load [20].

Station 2 is next to a food factory, this suggests the possibility that industrial waste and fertilizers, which contain abundant nitrogenous compounds may have mixed with Çubuk Stream. Due to the large number of slaughterhouses in this location, this suggests that the wastes of these slaughterhouses may have been mixed into Çubuk Stream.

The average ammonium value of Çubuk Stream is at the 2nd and 3rd stations according to SWOR, IV. class water quality, at the 1st station II. class water quality. Çubuk Stream Average ammonium value of 3 stations (2.34 mg/l) IV. class water quality. According to the previous studies [16] water which has not changed much in the NH_4-N ratio is in terms of this parameter IV class water quality. According to works [17] the value decreased from 8.55 mg/l to 3.585. The reason for this decrease may be that Çubuk Stream merges with Hatip Stream while forming Ankara Stream and nitrogen pollutants in Hatip Stream may be high.

As a result of the measurements it is seen that the average nitrite density of the 2nd station is higher than the other two stations. This may be related to the fact that the area where the slaughterhouses and industrial factories are concentrated is the 2nd station region and the waste water is discharged to Çubuk Stream. The fact that the nitrite ratio is higher than nitrate in water indicates that life is limited in aerobic conditions [7]. At the same time, according to the 2012-2013 values of Çubuk Stream, ammonium from

nitrite; the fact that the nitrite is higher than the nitrate amount proves the incomplete nitrification event. It also allows us to predict that nitrosomanas bacteria may be more than nitrobacteria in Çubuk Stream.

It was measured that the chromium value at the 3rd station was higher than the 2nd station and the 2nd station value was higher than the 1st station value. The fact that the 2nd station and 3rd station regions of the three selected stations in Çubuk Stream are industrial zones supports the possibility of these factories to discharge their wastewater into Çubuk Stream.

The amount of zinc element, which is one of the trace elements is 0.545 mg/l in Çubuk Stream. In terms of this parameter Çubuk Stream is III. class water quality, 2nd station IV class water quality. If the zinc ratio exceeds 2.0 g/l in surface waters, it may cause chlorosis problem in plants. If Çubuk Stream will be used as irrigation water in the 2nd and 3rd station regions these environments must be clayey soils [2]. According to the result, the treatment plants established close to these regions should also provide treatment to reduce the amount of heavy metals.

The amount of phosphate decreased according to some works [16]. The increase in phosphate value in surface waters causes the reproduction of organisms, especially algae, in the water [5]. With the opening of wastewater treatment plants in the region, it can be predicted that eutrophication due to the amount of phosphate will decrease.

NH_4-N/PO_4 ratio is used to determine the limiting nitrogen and phosphate elements of eutrophication [22]. If NH_4-N/PO_4 > 20, the system is restricted by phosphorus and when NH_4-N/PO_4 < 5, the system is restricted by nitrogen. According to previous studies, NH_4-N/PO_4 = 20.7; According to after studies, NH_4-N/PO_4 = 2.34; during 2012 – 2013, Çubuk Stream was measured at NH_4-N/PO_4 = 48 values. According to the 2012-2013 NH_4-N/PO_4 values and Çubuk Stream the system is limited by nitrogen. According to Atıcı and Ahıska [17] the system is limited by phosphate.

In this study on the pollution of Çubuk Stream, higher quality water can be supplied to the region with the wastewater treatment plants established in 2009. Treatment facilities were established with the aim of reducing the nitrate nitrogen and phosphate ratio in the use of Çubuk Stream as irrigation water for the region [13]. Our study result supported the confirmation of this aim. A similar study was conducted in St. Johns River (LSJR), located in Florida and the causes of pollution in rivers were determined [23], and China's Guanzhong Basin, similar studies have been carried out to see how

the emergence of the pollution load in the groundwater with the increase in population creates the results. [24].

While the 1st station was more distant from the anthropogenic effect in the 3rd stations studied. The 3rd station was more affected by this effect. Therefore, people need to be made aware of this issue. A regional public service announcement can be created regarding this. Information can be given in local newspapers. Likewise, in the 2nd station area where industrial establishments are concentrated. Inspections should be made more frequently for establishments that cause pollution. These are important for the future and sustainability of Çubuk Stream. No unexpected pollutants from chemical and physical parameters were detected in Çubuk Stream. This study is important because it will contribute to the country's economy in determining the factors that cause pollution in matters related to water pollution and will contribute to the scientific literature in studies on wastewater treatment applications.

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FIRST RECORDS FOR BENTHIC MACROINVERTEBRATE FAUNA IN GÜZELDERE WATERFALL, TURKEY

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ABSTRACT. In this paper, the benthic macroinvertebrate fauna of the Güzeldere Stream and Waterfall were investigated. Samples were collected in October 2020 at 3 stations. A total of 41 taxa including Gastropoda, Oligochaeta (9 species), Ephemeroptera (7 species), Odonata, Coleoptera, Chironomidae (15 species), Simuliidae, Blephariceridae (2 species), Plecoptera (3 species), and Trichoptera (1 species) were identified in the area. The dominant taxon was Chironomidae at all three stations followed by Oligochaeta and Ephemeroptera, respectively. The second station had the highest individual numbers (195) and the highest species diversity (32). All of the identified taxa were the first records for the study area because there have been no studies conducted for the determination of the Güzeldere Stream and Waterfall.

1. INTRODUCTION

Benthic macroinvertebrates, one of the biotic components of aquatic ecosystems, are defined as organisms that are larger than 0.5 mm, live on the bottom of water bodies, can be seen with the naked eye, and have no internal skeletons [1,2]. Common inhabitants of lentic and lotic systems, benthic macroinvertebrates play a crucial role in the movement of energy through food webs [3]. The diversity of taxonomic groups that make up the benthic macroinvertebrate fauna is a crucial link in the energy flow from the deep compartment to the aquatic environment. This fact is related to their characteristics and ecological requirements, especially given that they are ubiquitous [4], sedentary, and have with a distribution that may be influenced by some physical or chemical disturbance [5,6], as well as by substrate characteristics [7]. Additionally, these invertebrates are a

Keywords. Biodiversity, freshwater ecosystems, macroinvertebrates
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contemporary tool frequently used for monitoring freshwater aquatic ecosystems [8]. Compared to its rapidly changing physicochemical properties, benthic macroinvertebrates are the most prevalent faunal assemblages for bio-assessment and offer a more accurate assessment of long-term ecological changes in the quality of aquatic systems. They are helpful in determining temporal and spatial changes within an aquatic ecosystem due to their numerous life stages, sedentary lifestyle, and varying levels of susceptibility to environmental stressors [3].

Turkey has 25 freshwater basins including many water regions. One of these water regions is waterfalls, which are home to many aquatic benthic macroinvertebrates. Waterfalls are rocky biotopes with a rapid flow that have a unique geomorphic structure (stream channels and channel slopes typically erode to parent material). These biotopes typically reach heights of more than 1 m and flow vertically without obstruction [9]. Waterfalls are more prevalent in high elevation areas where the stream gradient is steeper and the river channels create discrete, sequential pools and waterfalls (or cascades and riffles) [10]. More than 66 waterfalls have been reported in the Turkish literature so far [11,12]. One of these waterfalls, Güzeldere Waterfall, located in Düzce Province, has a fall height of 130 m. This waterfall is among the highest waterfalls in Turkey [13].

In Turkey, there are many studies about the benthic macroinvertebrate fauna of lakes and rivers [e.g., 14-17] but there are no studies about the benthic fauna of waterfalls. The current study aimed to determine the benthic macroinvertebrate fauna of the Güzeldere Stream and Waterfall. In the area, there have been no studies about benthic macroinvertebrate fauna, although there are a few faunistic studies about the insect fauna in the area [18-20].

2. MATERIALS AND METHODS

The Güzeldere Stream and Waterfall are located in the province of Düzce in the Western Black Sea Basin. The waterfall was declared a nature park in July 2011. It covers an area of 22.76 ha. The 130-m-high waterfall is a multiple-drop waterfall [13].

In order to determine the macroinvertebrate fauna of the Güzeldere Stream and Waterfall, samples were taken from 3 stations in October 2020. The first station among the sampling areas was the area where the waterfall flows. The second and third stations are on the Güzeldere Stream.

Benthic macroinvertebrate samples were collected with a hand net and the sieved samples were fixed with 70% alcohol. Temperature, pH, and dissolved oxygen parameters were also measured *in situ*. After the benthic samples brought to the laboratory were sorted under a stereomicroscope, preparations were made for identification at the species level. For the identification of macroinvertebrate specimens at the species level, the identification keys of Schütt (1965), Zhadin (1965), Müller-Liebenau (1969), Bilgin (1980), Şahin (1984), Eliot et al. (1988), Harker (1989), Sauter (1992), Epler (1995), Cranston (1995), Nilsson (1996), Nilsson (1997), Glöer ve Meier-Brook (1998), Glöer (2002), Bouchard (2004), Eiseier (2005), Timm (2009), Bauernfeind and Soldán (2012), and Thorp and Rogers (2019) were used [21-39].

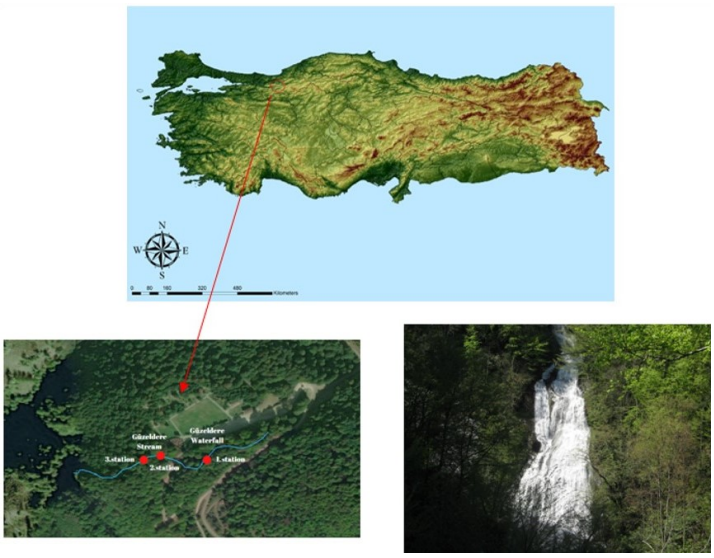

FIGURE 1. Geographical positions of sampling stations

3. RESULTS AND DISCUSSION

As a result of the examination of the samplings from three stations in the Güzeldere Stream and Waterfall in October 2020, 41 taxa were identified in the zoobenthic community structure. Table 1 shows the dominance values of these taxa and their distribution according to the stations, where it can be seen that the dominant macrozoobenthic taxon in the Güzeldere Stream and Waterfall was Chironomidae (31.25%, 42.56%, and 30.40% at stations 1, 2, and 3, respectively), followed by Oligochaeta and Ephemeroptera, respectively.

TABLE 1. Dominance values of macrozoobenthic taxa detected in the Güzeldere Stream and Waterfall and their distribution according to stations.

	Güzeldere Waterfall	Güzeldere Stream	Güzeldere Stream	Mean
Taxon/Sampling stations	**1**	**2**	**3**	
Gastropoda	1.14	4.10	8.80	4.68
Oligochaeta (in total)	29.55	29.74	32.00	-
Chaetogaster diastrophus (Gruithuisen, 1828)	2.84	3.08	3.20	3.04
Chaetogaster langi Bretscher, 1896	2.84	0.00	1.60	1.48
Nais barbata Müller, 1773	7.39	4.10	4.80	5.43
Nais bretscheri Michaelsen, 1899	14.20	11.79	4.80	10.27
Nais pardalis Piguet, 1906	0.00	4.62	9.60	4.74
Pristinella jenkinae (Stephenson, 1931)	0.00	2.56	3.20	1.92
Aulodrilus pigueti Kowalewski, 1914	1.70	2.05	0.00	1.25
Potamothrix hammoniensis (Michaelsen, 1901)	0.00	1.54	1.60	1.05
Psammoryctides albicola (Michaelsen, 1901)	0.57	0.00	3.20	1.26
Ephemeroptera (in total)	11.93	10.77	12.80	-
Caenis luctuosa (Burmeister, 1839)	2.84	1.03	0.00	1.29
Caenis macrura Stephens, 1836	3.98	0.00	1.60	1.86
Baetis vernus Curtis, 1834	1.14	5.64	6.40	4.39
Baetis muticus (Linnaeus, 1758)	0.00	3.59	4.80	2.80
Electrogena lateralis (Curtis, 1834)	1.70	0.00	0.00	0.57
Ecdyonurus picteti (Meyer-Dür, 1864)	1.14	0.00	0.00	0.38
Epeorus sp.	1.14	0.51	0.00	0.55
Odonata	1.70	2.56	5.60	3.29

	Güzeldere Waterfall	Güzeldere Stream	Güzeldere Stream	Mean
Taxon/Sampling stations	**1**	**2**	**3**	
Coleoptera	1.14	2.56	1.60	1.77
Chironomidae (in total)	31.25	42.56	30.40	-
Macropelopia nebulosa Meigen, 1804	2.27	7.18	7.20	5.55
Prodiamesa olivacea Meigen, 1818	4.55	1.54	0.00	2.03
Psectrocladius calcaratus Edwards, 1929	2.27	1.03	4.80	2.70
Cardiocladius capucinus (Zetterstedt, 1850)	0.00	2.56	0.00	0.85
Rheocricotopus fuscipes Kieffer, 1909	0.00	3.08	3.20	2.09
Thienemanniella vittata Edwards, 1924	3.98	2.56	0.80	2.45
Eukiefferiella claripennis (Lundbeck, 1898)	6.25	1.03	0.00	2.43
Eukiefferiella clypeata Kieffer, 1923	3.41	1.03	3.20	2.54
Eukiefferiella ilkleyensis Edwards, 1929	0.00	7.69	2.40	3.36
Metriocnemus cubitalis (Kieffer, 1911)	2.27	0.00	0.00	0.76
Paratrichocladius sp.	0.00	3.08	2.40	1.83
Paratrichocladius rufiventris Meigen, 1830	1.14	3.08	0.00	1.40
Orthocladius (O.) thienemanni (Kieffer & Thienemann, 1906)	0.00	4.62	3.20	2.61
Chaetocladius piger Goetghebuer, 1913	5.11	1.54	0.00	2.22
Cladotanytarsus mancus Walker, 1856	0.00	2.56	3.20	1.92
Simuliidae	6.80	5.64	3.20	5.22
Blephariceridae				
Blepharicera sp. 1	4.55	0.00	0.00	1.52
Blepharicera sp. 2	2.27	0.00	0.00	0.76
Plecoptera (in total)	4.55	0.51	4.00	-
Protonemura praecox (Morton, 1894)	1.14	0.51	2.40	1.35
Perla bipunctata Pictet, 1833	2.27	0.00	1.60	1.29
Leuctra sp.	1.14	0.00	0.00	0.38
Trichoptera				
Hydroptila occulta (Eaton, 1873)	5.11	1.54	1.60	2.75
Water parameters	**Sampling stations**			
	1	**2**	**3**	
Temperature (°C)	16.5	17	17	-
pH	6.8	6.9	6.8	-
Dissolved oxygen (mg/L)	12	11	9	-

The species with the highest abundance in the macrozoobenthic community were *Nais bretscheri* (10.27%), *Nais barbata* (5.43%), and *Nais pardalis* (4.74%) from Oligochaeta. The abundance of *Nais bretscheri* was particularly high at station 1, where the Güzeldere Waterfall flows from 130 m and forms a small pond. It is noteworthy that *Nais pardalis* was not found at this station, but was abundant in the second and third stations located on the stream. *N. bretscheri,* unlike the other two dominant *Nais* species, is typical of fast-flowing streams and prefers a sediment structure consisting mainly of hard material such as stones, sand, and gravel [37, 40]. It is known that *Nais barbata* and *Nais pardalis*, the other two dominant species, can also be found in fast-flowing waters, but their population densities increase in softer (mud + sand) sediments when compared to *N. bretscheri* [41]. The fact that this station has a harder sediment structure than the other two stations and the population abundance and dominance of the species in question support the literature information. All three Naidin species are widely distributed both in Turkey and around the world [42,43].

The genus *Aulodrilus* in the Tubificidae family is not very rich in terms of the number of species [42]. Defined as a cosmopolitan species [42], *Aulodrilus pigueti*, which was detected only in stations 1 and 2, was recorded for the first time in Turkey by Arslan and Şahin (2003) in the Upper Sakarya River System [44], The fact that these areas are high in dissolved oxygen (11–12 mg/L) and the water temperature is 16.5–17 °C (Table 1) supports the habitat preferences of the species. It has been detected in aquatic systems in different parts of Turkey, especially in high-altitude mountain streams and lakes, although in small numbers [43]. It is known that the species prefers cool waters with relatively high dissolved oxygen as its distribution area [40,44]. The stations where the species was detected were station 1, which was just below the waterfall, and station 2, which was the stream section.

The species with the highest abundance among Chironomidae individuals were Tanypodin *Macropelopia nebulosa* (5.55%), Orthocladiin *Eukiefferiella ilkleyensis* (3.36%) and *Psectrocladius calcaratus* (2.70%). As seen in Table 1, although two Tanypodin species were dominant in the Chironomidae fauna, it was seen that Orthocladiin individuals constituted the majority of the Chironomidae fauna. It was reported that Orthocladiin individuals are generally found in coarse sediments (stones, gravel), but their population densities can also increase in fine-grained and sandy substrates [45,46], their tolerance to organic pollution is lower than that of other chironomid members [47], they can also be found in fast flowing sections of

rivers due to high oxygenation and in the littoral sections of lakes with a constant but high dissolved oxygen regime [48] and it has been reported that the population densities of *Orthocladius* and *Eukiefferiella* species in this group may increase and that they are distributed in gravelly, stony sediments, epirhitron and metarhitron areas of rivers [30,49]. The fact that the stations where Orthocladiin individuals were detected generally had cool waters, and flowing and hard substrate structure was in parallel with this information.

According to the average abundance values determined in the three stations in the study area, *Baetis vernus* (4.39%) and *Baetis muticus* (2.8%) were found to be the dominant species among the Ephemeroptera fauna, while *Ecdyonurus picteti* and *Electrogena lateralis* were found only at station 1, which was just below the waterfall. It is known that *Baetis vernus* nymphs are found in the rhitral and potamal parts of rivers, *Baetis muticus* nymphs are found in the krenal and rhitral parts of rivers, and both species generally prefer oligosaprobic, xenosaprobic and beta mesosaprobic areas [50]. This information is consistent with our results. It has been reported that *Ecdyonurus picteti* nymphs are found in sediments in rocky and stony places in fast-flowing areas from hyporhithron to hypocrenon sections of rivers, mostly oligosaprobic environments, but also xenosaprobic and beta-mesosaprobic environments [51]. The fact that this species was found among the stones at 1st station, which was the region where the waterfall flowed, supports the information in the literature.

The only other taxon detected in the study area is Blephariceridae members from the lower part of the waterfall, among the mosses and on the stones. It is known that the members of Blephariceridae (Table 1, *Blepharicera* sp. 1 and *Blepharicera* sp. 2), which can be identified up to the genus level, are the best-adapted Diptera group living in the waterfalls and cascades of mountain rivers, and are adapted to living in very fast flowing water thanks to their ventral discs [52]. Since blephariceridsprefer clean, cool, well-oxygenated river sections, the presence of members of this group in aquatic systems has been reported to be used as a positive bioindicator for water quality assessment [53]. Although detailed measurements to determine the water quality were not carried out in this study, the dissolved oxygen value measured in situ in the study area (12 mg/L at station 1 where the taxon was detected) indicates a water class I. The high abundance of both taxa (*Blepharicera* sp. 1 4.55% and *Blepharicera* sp. 2 2.27%) is consistent with this information. There are very few studies on the members of the family Blephariceridae in Turkey. In a study conducted by Koç and Zwick (2006), it was reported that *Blepharicera fasciata* (Westvood, 1842) is widespread in

the Mediterranean region, from Portugal to Lebanon and Iran, and in Turkey except for arid regions, it has a wide distribution [54].

Shannon index (H'), Evenness, and Margalef index values were calculated for the taxa identified in the research area and given in Table 2. The highest number of individuals (195) and the highest species diversity (32) were found at station 2. The highest Shannon index value was found at station 2 (3.20), where the highest species diversity was detected. Shannon and Margalef index values and taxa numbers detected in the Güzeldere Stream and Waterfall were higher than expected, despite the fact that the study area was a small aquatic system. This suggests that this was due to the high diversity of microhabitats formed by stream and waterfall systems that can host different taxonomic groups.

TABLE 2. Index values of the stations according to the distribution and abundance of macrozoobenthic taxa detected in the Güzeldere Stream and Waterfall.

	Sampling stations		
Indices	**1**	**2**	**3**
Taxa number	31	32	27
Individuals	174	195	125
Shannon index (H')	3.17	3.20	3.13
Evenness (e^H/S)	0.77	0.79	0.85
Margalef index	5.82	5.69	5.39

4. CONCLUSIONS

As a result of the examination of the samplings from three stations in the Güzeldere Stream and Waterfall, 41 taxa were identified in the zoobenthic community structure. The macroinvertebrate fauna of waterfalls, which are usually composed of streams or rivers flowing more or less at a certain height, has been a subject of little interest in hydrobiological studies. However, in waterfall systems, the gradual and (or) sudden decrease in current velocity over short distances, the change in the river bed and sediment structure, the distribution and density of aquatic plants, and the change in water parameters provide different habitat opportunities for invertebrates with very different ecological preferences over short distances, which increases taxonomic diversity [55,56]. The results obtained in this

study support this information. Since there are no previous studies to determine the macroinvertebrate fauna of the Güzeldere Stream and Waterfall, all of the taxa identified were the first records.

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