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(ONUNCU YIL/TENTH YEAR)

Biyolojik Çeşitlilik ve Koruma Üzerine Yayın Yapan Hakemli Uluslararası Bir Dergidir
An International Journal is About Biological Diversity and Conservation With Refree

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Research article/Araştırma makalesi

The Herpetofauna of Karçal Mountains (Artvin/Turkey)

Halit EKSİLMEZ [1], Abdullah ALTUNIŞIK [*1], Nurhayat ÖZDEMİR 1

[1] Department of Biology, Faculty of Arts and Sciences, Recep Tayyip Erdoğan University, Rize, Turkey

Abstract

Karçal Mountains are located in Artvin province (Turkey) that contains various types of ecosystems. Based on the results of herpetological surveys conducted between August 2014 and July 2015, totally 18 species were recorded from 11 different stations around Karçal Mountains of which 7 were amphibians (2 salamanders and 5 frogs) and 11 were reptiles (5 lizards and 6 snakes). Specimens were investigated in terms of some morphological characters like color and pattern features, number of scales and plates and body measurements. In addition, a zoogeographical assessment was also carried out by the chorotype classification of Karçal Mountains.

Key words: amphibia, reptilia, distribution, Herpetofauna, chorotype, Karçal Mountains

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Karçal dağları (Artvin/Türkiye)'nın Herpetofaunası

Özet

Temmuz 2015 tarihleri arasında Karçal dağları ve çevresinde yapılan herpetolojik araştırmalar neticesinde 11 istasyondan 7'si çift yaşar (2 semender ve 5 kurbağa) 11'i sürüngen (5 kertenkele ve 6 yılan) olmak üzere toplam 18 tür tespit edilmiştir. Örnekler renk-desen özellikleri, pul ve plak sayıları ve vücut ölçüleri gibi bazı morfolojik karakterler bakımından incelenmiştir. Buna ek olarak, Karçal Dağlarının korotip sınıflandırması ile zoocoğrafik bir değerlendirme yapılmıştır.

Anahtar kelimeler: amfibiler, Sürüngenler, dağılım, Herpetofauna, korotip, Karçal dağları

1. Introduction

Biodiversity is the basic of life on Earth, and all organisms play roles in ecosystems processes as part of the food web or otherwise (Steinke, 2016). Thanks to the different topographic and climatic properties resulting from its location and having almost entirely by the three of the world's 34 biodiversity hotspots (Caucasus, Irano-Anatolian and Mediterranean), Turkey has a rich biodiversity. According to online databases, 37 amphibian (Amphibiaweb, 2016) and 150 reptile (The reptile database, 2016) species have been recorded in Turkey until now. Therefore, the herpetofaunal biodiversity of Turkey is richer than many countries in Europe and the number of species is close to that of whole European continent.

In this species richness, mountains play important roles in Turkey: The Taurus mountains range in the south, the Northern Anatolian Mountains in the north, the Western Anatolian Mountains in the west, the Anatolian Diagonal and lots of smaller mountains from the northeast to the Mediterranean (Şekercioğlu et al., 2011). Particularly while glacial and postglacial times, these mountain ranges acted as barriers and induced notable population differentiations and/or limited distributions of the populations (Kosswig, 1955; Demirsoy, 1999).

Karçal Mountains (3431 m a.s.l.) range are in the border of Artvin province in the northeast of Turkey (Figure 1). Karçal Mountains with the Pleistocene glacial valleys that prove the glaciations and various glacial shapes and glacial lakes have partly debris covered glaciations which are still recognizable (Gürgen and Yeşilyurt, 2012). These

[*] *Corresponding author* / Haberleşmeden sorumlu yazar: Tel.: + 904642236126; Fax.: +904642235376; E-mail: abdullah.altunisik@erdogan.edu.tr
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mountains extends throughout the Çoruh and Berta valleys to Georgia border and separate the Şavşat and Borçka towns of Artvin province. Georgia and Artvin are located in the east and west of Karçal Mountains, respectively.

Karçal Mountains are one of the most important areas by means of biodiversity and show the best examples of mixed deciduous forests of the temperate zone, high endemism rate and sudden altitude changes brought about by the ecosystem diversity. There are few streams in the Karçal Mountains. As is in Black Sea Region, climate of this mountain region is an oceanic climate with high and evenly distributed rainfall the year round (Url-1).

In recent years, the studies which investigate herpetofaunal biodiversity of a certain region have increased and they contribute to species distributions in Turkey (Baran et al., 1997; Özdemir and Baran, 2002; Kumlutaş et al., 2004; Afşar and Tok, 2011; Özcan and Üzüm, 2014; Cihan and Tok 2014; Ege et al., 2015). Although previous studies exist in the literature which deal with amphibians and reptiles of Artvin and its towns (Kutrup, 2001; Baran et al., 2002; Afşar et al., 2012), no data available on the herpetofauna of Karçal Mountains. This case encouraged us to investigate amphibian and reptile species of Karçal Mountains (Artvin) and contribute to the literature on the herpetofaunal biodiversity of Turkey.

Figure 1. Stations in which specimens were collected 9 village (1. Balcı, 2.Kaynarca, 4. Efeler, 5. Maral, 6. Uğur, 7. Mısırlı, 8. Maden, 9. Yağlı, 10. Oba) and 2 lakes (3. Karagöl (Borçka), 11. Karagöl (Şavşat))

2. Materials and methods

As a result of herpetological surveys, a total of 65 specimens (36 ♂♂, 23 ♀♀, 6 juveniles) were examined within the guidelines of the local ethics committee for animal experiments (Recep Tayyip Erdoğan University, approval reference number: 2014/33) between August 2014 and July 2015 in Karçal Mountains. For each individual, sex was determined by the examination of external secondary sexual characters, and their body measurements were measured using a digital caliper with an accuracy of 0.01 mm. In addition to this, specimens were investigated in terms of some morphological characters like color and pattern features including the number of scales and plates of reptiles. The photographs of all observed specimens were taken by a digital photo camera. After these processes, specimens were released at the place of capture.

The diagnosis of the detected specimens were performed by the present literatures (Baran and Atatür, 1998; Budak and Göçmen, 2005; Baran et al., 2013) and zoogeographical evaluations were done by classifying them into main chorotypes as determined by Vigna Taglianti et al. (1999) and Sindaco et al. (2000).

3. Results

The eleven stations where the specimens were captured and observed in the studied area are shown in the Figure 1. In Figure 2, number of the species with regard to the stations is demonstrated. Karagöl (Station 6) which has eight species is the richest station in terms of species diversity. The other stations have four (Stations 4 and 10), three (Stations 3, 5 and 11), two (Station 9) and one species (Stations 1, 2, 7, 8; Figure 2). In addition to this, distribution of species richness along elevation gradient in the studied area was given in Figure 3. As it can be seen from the graph (Figure 3), species richness is higher between 1200 and 1800 m a.s.l. compared to the other elevations.

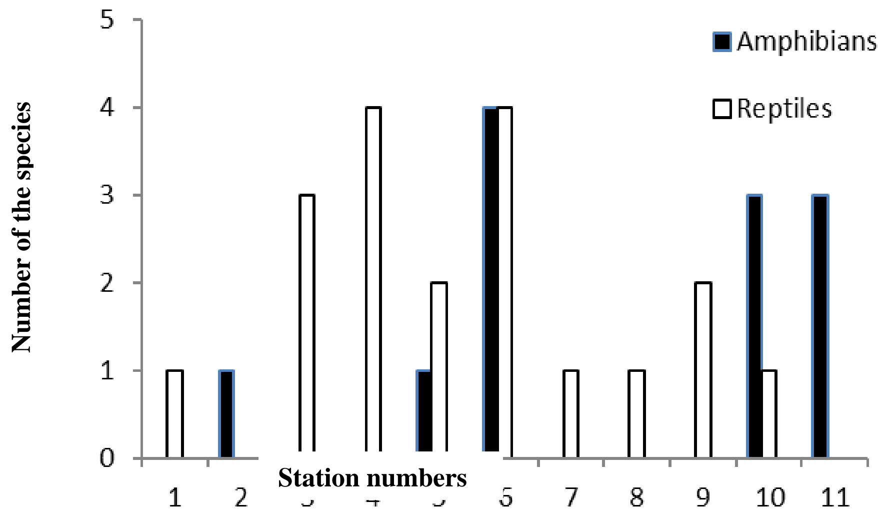

Figure 2. Number of the species according to the stations

According to the results of the herpetological survey in the study area, 18 species were observed. Of these, 7 were amphibians (*Mertensiella caucasica, Ommatotriton ophryticus, Pelophylax ridibundus*, *Bufo verrucossissimus*, *Rana macrocnemis*, *Hyla orientalis*, *Pelodytes caucasicus*) and 11 were reptiles (*Darevskia derjugini*, *Darevskia parvula, Darevskia rudis, Darevskia clarkorum, Anguis fragilis, Natrix natrix, Natrix tessellata, Eirenis modestus, Coronella austriaca, Vipera kaznakovi, Zamenis longissimus*). Although the taxonomic status of *Bufo verrucossissimus* has remained controversial, we have identified it as a valid species in our study, as supposed by Recuero et al. (2012) and IUCN (Url-2).

According to their distributions, the species found on Karçal Mountains were grouped into 12 chorotype categories in accordance with the checklist of Vigna Taglianti et al. (1999) and Sindaco et al. (2000). The most widespread chorotype in the area is Ponto-Caucasian Endemic (three species, 16.6%), followed by European (two species, 11.11%), Kolkhido-Caucasian endemic (two species, 11.11%), SW Asiatic (two species, 11.11%), Kolkhidian endemic (1 species, 5.11%), Kolkhido-Armenian endemic (1 species, 5.11%), S-European (1 species, 5.11%), Central Asiatic-Europeo-Mediterranean (1 species, 5.11%), Central Asiatic-European (1 species, 5.11%), Caucasian endemic (1 species, 5.11%), Turano-Europeo-Mediterranean (1 species, 5.11%), Europeo-Mediterranean (1 species, 5.11%) and Turano- European (1 species, 5.11%) (Table 1).

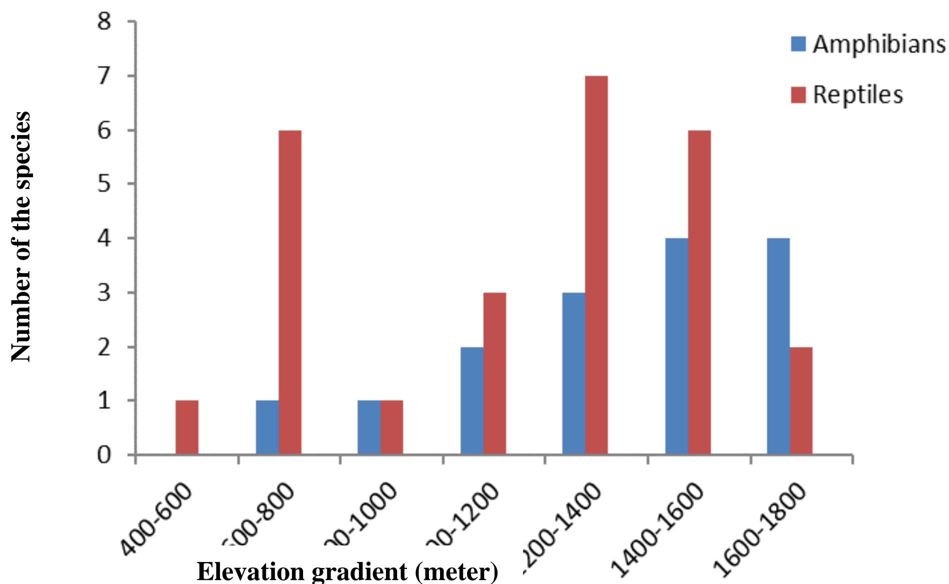

Figure 3. Number of the species according to elevation gradient

Table 1. The chorotype classification of the amphibian and reptile species in Karçal Mountains

Chorotype	Amphibia	Reptilia	Percentage	Species
European		2	11.11%	*Anguis fragilis* *Coronella austriaca*
Kolkhidian endemic		1	5.55%	*Darevskia clarkorum*
Kolkhido-Caucasian endemic	1	1	11.11%	*Bufo verrucosissimus* *Darevskia derjugini*
Kolkhido-Armenian endemic		1	5.55%	*Darevskia parvula*
Ponto-Caucasian endemic	2	1	16.66%	*Mertensiella caucasica* *Pelodtytes caucasicus Darevskia rudis*
S- European		1	5.55%	*Zamenis longissimus*
Central Asiatic-Europeo-Mediterranean		1	5.55%	*Natrix natrix*
Central Asiatic-European		1	5.55%	*Natrix tessellata*
Caucasian endemic		1	5.55%	*Vipera kaznakovi*
SW Asiatic	1	1	11.11%	*Rana macrocnemis* *Eirenis modestus*
Turano-Europeo-Mediterranean	1		5.55%	*Pelophylax ridibundus*
Europeo-Mediterranean	1		5.55%	*Hyla orientalis*
Turano- European	1		5.55%	*Ommatotriton ophyrticus*
Total species	7	11	100%	

4. Conclusions and discussion

There are few herpetofaunal studies in the East Black Sea Region. In 1997, 14 species were recorded from Çamlıhemşin (Rize) (Baran et al., 1997). In Ordu-Giresun region, Kumlutaş et al. (1998) detected 17 species. In another study, Kutrup (2001) reported that 20 species were living in Murgul (Artvin). In addition to this, Baran et al. (2002), considering the previous records in the region, reported that 38 species are living in Rize, Artvin and Ardahan provinces and their environments. More recently, Afşar et al. (2012) notified that 15 species inhabiting at Camili Biosphere Reserve, known as the first and only biosphere site of Turkey. In the present study, 18 species of amphibians and reptiles were recorded on Karçal Mountains which occupies an area of about and covers Şavşat, Borçka and Camili regions, too.

N. cf. megalocephala is the only species which was reported previously (Afşar et al., 2012) for Camili Biosphere Reserve area but not encountered in this study. However, according to Hille (1997), there was no genetic difference between *Natrix n. natrix* and *N. megalocephala.* The validity of *N. megalocephala* is still debate according to Böhme (1999). Differently from Afşar et al. (2012)'s study, four species (*Darevskia clarkorum*, *Vipera kaznakovi*, *Eirenis modestus* and *Natrix tessellata*) were firstly detected in this study area.

Of the 18 species found on the Karçal Mountains, *Darevskia clarkorum* and *Vipera kaznakovi* are in the 'Endangered (EN)' category, *Mertensiella caucasica* is in the 'Vulnerable (V)' category and *Ommatotriton ophryticus, Bufo verrucosissimus*is and *Darevskia derjugini* are in the 'Near threatened (NT)' category with regard to the IUCN Red List data, while the others are in the 'Least Concern (LC)' category.

Degradation and destruction of the habitats due to road construction works, disturbance by people especially in touristic areas (Borçka Karagöl and Şavşat Karagöl) and decrease in the available water resources depending on the ongoing hydroelectric power plants in the region are the main factors that threaten the amphibian and reptile species of Karçal Mountains. In addition to these factors, we also observed that some fish species were introduced by local people into several lakes on the mountains. Therefore, local authorities must ensure that the local people be informed of the conservation of the biological assets that they possess.

Since the study area is home to many venomous snake species under protection, present study demonstrates the importance of Karçal Mountains as a herpetological area. Moreover, that no detailed study was conducted on the herpetofauna of Karçal Mountains to date, underlines the necessity of this study. We consider that herpetological information obtained from this study will provide essential data for possible future conservation biology studies..

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Research article/Araştırma makalesi

Flora of alpine grasslands of the Eğribel pass in the Giresun mountains (Turkey)

Rena HÜSEYİNOĞLU [*1], Erkan YALÇIN [2], Oksal MACAR [3]

[1] Şebinkarahisar School of Applied Sciences, Giresun University, Giresun, Turkey
[2] Department of Biology, Faculty of Arts and Science, Ondokuz Mayıs University, Samsun, Turkey

Abstract

In this study it was aimed to determine floristic composition of alpine grasslands of Eğribel Pass in the Giresun Mountains in North-Eastern part of Turkey. The study area belongs to the Colchic Province of the Euro-Siberian phytogeographical region. The vascular flora of Eğribel Pass was studied between 2014 and 2016. According to the results, in the study area 230 taxa belonging to 138 genera and 44 families were identified. The richest 3 families are Asteraceae with 39 taxa (17%), Poaceae with 28 taxa (12.2%) and Fabaceae with 18 taxa (7.9%). The richest genera are *Trifolium* L. and *Ranunculus* L. (6 taxa), followed by *Poa* L. (5 taxa). With 32.6% of the plants belonging to the Euro-Siberian Region, 18.3% are of the Irano-Turanian, 3.5% are of the Mediterranean and with 45.6% of the pluriregional or unknown. The life-form ratio of the taxa was as follows: hemicryptophytes 54.4%, cryptophytes 21.3%, chamaephytes 13.5%, therophytes 10.4% and phanerophytes 0.4%. The endemism ratio is 12.2% (28 taxa).

Key words: alpine grasslands, Eğribel pass, flora, Giresun mountains, Turkey

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Giresun dağları Eğribel geçidi alpin çayırlarının florası (Türkiye)

Özet

Bu çalışmada Türkiye'nin kuzey-doğu bölgesinde yer alan Giresun Dağları Eğribel Geçidi alpin çayırların florasının tespit edilmesi amaçlanmıştır. Araştırma alanı Avrupa-Sibirya fitocoğrafik bölgesinin Kolşik kısmında yer almaktadır. Sonuçlara göre araştırma alanında; 44 familya ve 138 cinse ait toplam 230 takson tespit edilmiştir. Araştırma alanında en fazla takson sayısına sahip üç familya sırasıyla Asteraceae 39 takson (% 17), Poaceae 28 takson (%12.2) ve Fabaceae 18 takson (%7.9)'dir. En zengin cinsler *Trifolium* L. ve *Ranunculus* L. (6 takson), üçüncüsü ise *Poa* L. (5 takson)' dır. Fitocoğrafik bölgelere göre dağılımlara bakılırsa, taksonların %32.6' sı Avrupa-Sibirya, %18.3'ü İran-Turan, %3.5'i Akdeniz bölgelerine ait olup, %45.6 'sı ise coğrafi bölgesi bilinmeyen veya birden fazla bölgede yayılış gösterenlerdir. Taksonların hayat formlarına göre oranları sırasıyla şöyledir: hemikriptofit %54.4, kriptofit %21.3, kamefit %13.5, terofit %10.4 ve fanerofit %0.4. Endemizm oranı %12.2 (28 takson)'dur.

Anahtar kelimeler: alpin çayırlar, Eğribel geçidi, flora, Giresun dağları, Türkiye

1. Introduction

Biodiversity contains the differences in genes, species and ecosystems and is the most important natural richness of a country (Deveci, 2012). By reason of great diversity in geology, geomorphology, topography and climate Turkey has the reachest flora in the temperate zone. It has nearly 10,000 vascular plants and is especially well known for endemic plants. Besides with its rich flora, Turkey is very rich in habitat and landscape diversity (Kandemir, 2009; Kılınç et al. 2010).

Alpine grasslands is one of the areas that have unique and valuable habitats and high endemism ratio due to presence of microhabitats, climate changes, long-term changes in floristic composition, geographical isolation, and speciation of new ones in these areas. Almost all of the floristic and vegetation studies in Turkey especially focused on

[*] *Corresponding author* / Haberleşmeden sorumlu yazar: Tel.: +904543101000; Fax.: +904543101016; E-mail: rena.huseyinoglu@giresun.edu.tr
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the forest and steppic vegetation whereas there are only a few studies about the alpine and subalpine graasland vegetation which have a broad geographical distribution (Tatlı, 1987; Karakaya and Kılınç, 1996; Vural, 1996; Uysal et al. 2011).

The main objective of this study is to identify the flora of alpine grasslands in Giresun Mountains in North-Eastern part of Turkey. In this context, this study could be helpful for further studies on supporting of alpine landscapes and protection efforts of alpine plant species in Turkey.

2. Materials and methods

2.1 Study area

This study was performed in the alpine belt of the Giresun Mountains of Giresun Province, in the North-Eastern part of Turkey. Giresun Mountains are a system of mountains that extend up to the peaks on Karadağ mountain at 3391 m in the East and on the Karagöl plateauat 3095 m in the West.

The study area is surrounded by high mountains where subalpine *Abies nordmanniana* subsp. *nordmanniana* (Steven) Spach forests do not develop because of climatic limitations. Geographyical map of the study area is illustrated in Fig.1. In the study area the alpine belt extends from 1800 m (timberline) to 2600 m upwards on south-facing slopes. These altitudinal boundaries run about 100 m lower in the northern part because of being subject to a more maritime climate.

The nearest province to the study area (Şebinkarahisar) has a Mediterranean type climate with 525 mm mean annual precipitation (P) and a drought period that is observed in July with 0.5 mm precipitation. Mean annual temperature is 11.3℃. Summer rainfall (PE) is 37 mm. Mean maximum for the hottest month (M) and mean minimum for the coldest month (m) are 30.3 and -16.1℃, respectively. Index of xericity (S=PE/ME) is 1.8. Pluviometric quotient (Q= 2000P/M + m + 546.4[M-m]) is 40.7 and the precipitation regime is SubMediterranean (Spring, Autumn, Winter, Summer; Sp, Au, Wi, Su).

Alpine grasslands in the study area were characterized by *Festuca pinifolia* (Hackel ex Boiss.), Bornm. var. *pinifolia, Festuca amethystina* L. subsp. *orientalis* Krajina var. *turcica* Markgr.-Dann., *Sibbaldia parviflora* Willd. var. *parviflora, Minuartia umbellulifera* (Boiss.) McNeill subsp. *umbellulifera* var. *umbellulifera, Thymus sipyleus* Boiss., *Vaccinium myrtillus* L., *Potentilla crantzii* (Crantz) Beck & Fritsch and many other species. Plant species in the study area are fragile with its species and populations being directly and indirectly influenced by changes in land-use practice, especially abandonment of small-scale agriculture, and fragmentation of habitats.

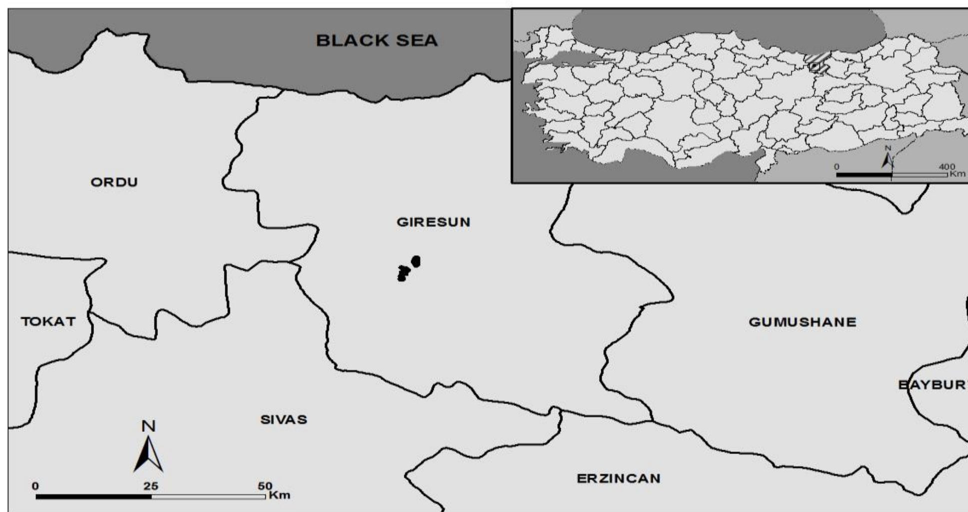

Figure 1. The geographical map of the study area

Generally, the study site is exposed to low but frequent disturbance factors. As an important disturbance factr, grazing reduces the dominance of competitive species and by trampling creates germination niches in the bare soil. It therefore has a direct effect on the structure and organization of grasslands. Today, many of the pastures in the study area are still in use (i.e. mowing, grazing or both); fallow farmland of pastures can be found in different successional stages. The numbers of grazing cows and sheep reach 50.000 individuals during the year.

2.2 Vegetation sampling and identification

The materials of this study includes some vascular plant specimens collected between August 2014 to June 2016. Nonvascular plant specimens were generally omitted. At least 1 sample for each taxon was prepared by

herbarium techniques (Erik et al. 1996) and kept at Herbarium of the Faculty of the Arts and Sciences of the Ondokuz Mayıs University (OMUB). Plant specimens were mainly identified and listed according to the "Flora of Turkey and East Aegean Islands" (Davis, 1965-1988; Davis et al. 1988; Güner et al. 2000; Güner et al. 2012) and the other related literatures (Tutin et al. 1964-1980). The floristic elements are listed in the Results. All taxa in the floristic list are given according to the order in the Flora of Turkey (Davis, 1965-1985). In the Results, each species is represented with the following details: family and taxon name, authors of the species, altitudes, collection dates, and collectors' names and numbers. Author abbrevations of the species names are given according to Brummit and Powell (1992). Additionally, endemism, IUCN threat categories, their phytogeographical regions, and the life-forms (Raunkiaer, 1934) are given. Threatened categories are proposed for the endemic and some nonendemic taxa according to IUCN risk categories (Ekim et al. 2000; IUCN, 2010 Version 8.1). The phytogeographical regions of the taxa were determined according to Davis (1965-85, 1988). The abbreviations used in the floristic list are as follows: Cosm: Cosmopolitan; Euro-Sib: Euro-Siberian element; Eux: Euxine element; Hyrc-Eux: Hyrcano-Euxine element; Ir-Tur: Irano-Turanian element; Medit: Mediterranean element; E: East; mt: mountain; EN: Endangered; DD: Data Deficient; LR: Lower Risk; cd: Conservation Dependent; lc: Least Concern; nt: Near Threatened; VU: Vulnerable; RH: Rena Hüseyinoğlu; EY: Erkan Yalçın; Hcrp: Hemicryptophytes; Crp: Cryptophytes; Chp: Chamaephytes; Thp: Therophytes; Php: Phanerophytes. Hyrc-Eux, Hyrc-Eux (mt), Eux and Eux (mt) species were evaluated as subcategories of Euro-Sib, whereas E. Medit species were evaluated as subcategories of the Mediterranean phytogeographical region..

3. Results

The Floristic List

PTERIDOPHYTA

Polypodiaceae
Polypodium vulgare L. subsp. *vulgare* L.
2450 m, 23.07.2015, EY 270
Crp.

SPERMATOPHYTA

GYMNOSPERMAE (CONIFEROPHYTA)
Cupressaceae
Juniperus communis L. var. *saxatilis* Pall.
2308 m, 14.08.2014, RH 11
Php.

ANGIOSPERMAE (MAGNOLIOPHYTA)

DICOTYLEDONAE (MAGNOLIOPSIDA)
Ranunculaceae
Anemone narcissiflora L. subsp. *willdenowii* (Boiss.) Greuter & Burdet
2500 m, 13.06.2015, EY 100
Hcrp.
Pulsatilla albana (Stev.) Bercht. & J.Presl. subsp. *albana*
2500m, 13.06.2015, EY 101
Ir-Tur., Hcrp.
Ranunculus sericeus Banks & Sol.
2005 m, 11.05.2015, RH 163
Ir-Tur., Hcrp.
R. cappadocicus Willd.
2263 m, 14.08.2014, RH 79
Eux., Crp.
R. repens L.
2450 m, 13.06.2015, EY 103
Hcrp.
R. polyanthemos L.
2500 m, 13.06.2015, EY 102
Chp.
R. grandiflorus L.
2200 m, 14.06.2015, EY 145
Hcrp.
R. ficaria L. subsp. *ficariiformis* Rouy & Foucaud
2005 m, 11.05.2015, RH 158
Crp.
Papaveraceae
Corydalis conorhiza Ledeb.
2260 m, 11.05.2015, RH 162
Eux.-Crp.
Brassicaceae (Cruciferae)
Crambe orientalis L. subsp. *orientalis* var. *orientalis*
2080 m, 14.06.2015, EY 113
Ir-Tur., Hcrp.
Aethionema iberideum (Boiss.) Boiss.
2100 m, 14.06.2015, EY 114
Chp.
Thlaspi huetii Boiss.
2250 m, 05.06.2016, RH 279
Thp.
Noccaea ochroleuca (Boiss. & Heldr.) F.K.Mey.
2500 m, 13.06.2015, EY 88
Chp.
Capsella bursa-pastoris (L.) Medik.
2005 m, 17.08.2014, RH 32
Cosm., Thp.
Alyssum minutum Schlecht.
2250 m, 05.06.2016, RH 280
Widespread, Thp.
A. pseudo-mouradicum Hausskn. & Bornm.
2260 m, 05.06.2016, EY 276
Endemic, LR (lc), Hcrp.
A. armenum Boiss.
2080 m, 14.06.2015, EY 116
Thp.
Draba bruniifolia Stev. subsp. *bruniifolia*
2500 m, 13.06.2015, EY 89
Chp.
D. siliquosa M.Bieb.
2500 m, 13.06.2015, EY 90
Hyrc-Eux. (mt), Chp.
Arabis alpina L. subsp. *alpina*
2550 m, 13.06.2015, EY 91
Widespread, Hcrp.
Cardamine impatiens L. subsp. *impatiens*
2300 m, 14.06.2015, RH 115
Euro-Sib., Crp.
Erysimum pulchellum (Willd.) J. Gay subsp. *pulchellum*
2280 m, 05.06.2016, RH 277
Hcrp.

Sisymbrium elatum K.Koch
2200 m, 14.06.2015, EY 112
Hcrp.

Cistaceae
Helianthemum tomentosum Gray
2260 m, 21.07.2015, EY 211
Hcrp.
Violaceae
Viola altaica Ker.-Gawl. subsp. *oreades* (M.Bieb.) Becker
2250 m, 05.06.2016, EY, 289
Hcrp.
Polygalaceae
Polygala pruinosa Boiss. subsp. *pruinosa*
2260 m, 21.07.2015, EY 269
Hcrp.
Caryophyllaceae
Minuartia hirsuta (M.Bieb.) Hand.-Mazz. subsp. *falcata*
2260 m, 21.07.2015, RH 222
Hcrp.
M. recurva (All.) Schinz. &Thell. subsp. *carica* McNeill
2300 m, 21.07.2015 EY, 290
Endemic, VU, Medit., Hcrp.
M. juniperina (L.) Maire & Petitm.
2200 m, 21.07.2015 EY, 291
Chp.
M. umbellulifera (Boiss.) McNeill subsp. *umbellulifera* var. *umbellulifera*
2100 m, 14.06.2015, RH 124
Endemic, LR (lc), Chp.
Stellaria media (L.) Vill.
2005 m,17.08.2014, RH 30
Thp.
S. holostea L.
2250 m, 15.06.2015, EY 146
Euro-Sib., Hcrp.
S. persica Boiss.
2200 m, 14.06.2015, RH 126
Thp.
Spergularia rubra (L.) J.Presl & C.Presl
2411 m, 16.08.2014, RH 67
Thp.
Dianthus zederbaueri Vierh.
2100 m, 21.07.2015, RH 196
Endemic, LR (cd), Ir-Tur., Hcrp.
Petrorhagia alpina (Habl.) P.W.Ball. & Heywood subsp. *alpina*
2100 m, 21.07.2015, RH 195
Thp.
Silene italica (L.) Pers. subsp. *italica*
2005 m, 17.08.2014, RH 33
Medit., Hcrp.
S. spergulifolia (Desf.) M.Bieb.
2200 m, 14.06.2015, RH 125
Ir-Tur., Hcrp.
Polygonaceae
Polygonum alpinum All.
2450 m, 13.06.2015, EY 111
Euro-Sib., Hcrp.
P. bistorta L. subsp. *bistorta*
2550 m, 16.08.2014, RH 27
Eux. (mt), Hcrp.
P. setosum Jacq. subsp. *setosum*
2550 m, 16.08.2014, RH 62
Ir-Tur., Hcrp.
P. arenastrum Boreau
2410 m, 16.08.2014, RH 61
Thp.
Rumex acetosella L.
2005 m, 17.08.2014, RH 63
Cosm., Hcrp.
R. scutatus L.
2005 m, 17.08.2014, RH 31
Chp.
R. alpinus L.
2100 m, 21.07.2015, EY 205
Crp.
Amaranthaceae
Chenopodium foliosum Asch.
2400 m, 16.08.2014, RH 57
Widespread, Thp.
Hypericaceae (Guttiferae)
Hypericum elongatum Ledeb ex Rchb. var. *elongatum*
2411 m, 14.08.2014, RH 68
Ir-Tur., Hcrp.
H. scabrum L.
2100 m, 14.06.2015, EY 130
Ir-Tur., Hcrp.
H. orientale L.
2306 m, 14.08.2014, RH 69
Hcrp.
Geraniaceae
Geranium ibericum Cav. subsp. *jubatum* (Hand.-Mazz.) P.H.Davis
2550 m, 16.08. 2014, RH 28
Endemic, LR (lc), Eux.(mt), Hcrp.
Celastraceae
Parnassia palustris L.
2440 m, 16.08.2014, RH 51
Hcrp.
Fabaceae (Leguminosae)
Cytisus pygmaeus Willd.
2100 m, 14.06.2015, RH 135
Euro-Sib., Chp.
Genista januensis Viv. subsp. *lydia* (Boiss.) Kit Tan & Ziel.
2450 m, 23.07.2015, EY 267
Medit., Chp.
Astragalus barba-jovis DC.
2070 m, 14.06.2015, EY 147
Ir-Tur., Chp.
A. anthylloides Lam.
2350 m, 22.07.2015, EY 247
Ir-Tur., Hcrp.
A.alyssoides Lam.
2100 m, 14.06.2015, RH 136
Ir-Tur., Hcrp.
A. angustifolius Lam. subsp. *angustifolius*
2070 m, 14.06.2015, EY 288
Chp.
Oxytropis lazica Boiss.
2550 m, 13.06.2015, EY 94
Euro-Sib. (mt), Chp.
Trifolium repens L.var. *repens*
2265 m, 23.07.2015, EY 250
Hcrp.
T. hybridum L. var. *anatolicum* (Boiss.) Boiss.
2380 m, 22.07.2015, RH 232
Hcrp.
T. sintenisii Freyn
2282 m, 15.08.2014, RH 53
Euro-Sib., Thp.
T. badium Schreb. subsp. *rytidosemium* (Boiss & Hohen.) Hossain var. *rytidosemium*
2282 m, 14.08.2014, RH 52

Hyrc. (mt), Hcrp.
T. pratense L. var. *pratense*
2411 m, 16.08.2014, RH 25
Hcrp.
T. ochroleucum Huds.
2411 m, 16.08.2014, RH 223
Hcrp.
Melilotus officinalis (L.) Desr.
2400 m, 16.08.2014, RH 70
Hcrp.
Medicago papillosa Boiss. subsp. *macrocarpa* (Boiss.) Urb.
2450 m, 22.07.2015, EY 228
Hcrp.
Lotus corniculatus L. var. *alpinus* Ser.
2300 m, 14.08.2014, RH 71
Hcrp.
L. corniculatus L. var. *corniculatus*
2450 m, 22.07.2015, EY 229
Hcrp.
Onobrychis montana DC. subsp. *cadmea* (Boiss.) P.W.Ball.
2470 m, 22.07. 2015, EY 227
Hcrp.
Rosaceae
Rubus idaeus L. subsp. *idaeus*
2300 m, 14.08.2014, RH 72
Euro-Sib., Chp.
Potentilla inclinata Vill.
2410 m, 16.08.2014, RH 60
Hcrp.
P. crantzii (Crantz) Fritsch
2400 m, 16.08.2014, RH 59
Euro-Sib., Chp.
P.cappadocica Boiss.
2450 m, 13.06.2015, EY 87
Endemic, LR (nt), Euro-Sib. (mt), Hcrp.
P. erecta (L.) Räusch.
2270 m, 14.08.2014, RH 64
Crp.
Sibbaldia parviflora Willd. var. *parviflora*
2207 m, 14.08.2014, RH 22
Chp.
Alchemilla caucasica Buser
2308 m, 14.08.2014, RH 76
Eux. (mt), Crp.
A. erythropoda Juz.
2450 m, 22.07.2015, RH 238
Euro-Sib., Crp.
A. orthotricha Rothm.
2411 m, 16.08.2014, RH 75
DD, Eux., Crp.
A. orduensis B.Pawl.
2050 m, 11.05.2015, EY 168
Endemic, EN, Euro-Sib., Hcrp.
Onagraceae
Epilobium angustifolium L.
2300 m, 14.08.2014, RH 65
Crp.
E. gemmascens C.A.Mey.
2300 m, 14.08.2014, RH 56
Eux. (mt), Crp.
Crassulaceae
Phedimus spurius (M.Bieb.)'t Hart
2308 m, 14.08.2014, RH 49
Hyrc-Eux., Hcrp.
Sedum pallidum M.Bieb.
2550 m, 16.08.2014, RH 50
Eux., Crp.
Sempervivum armenum Boiss. & A.Huet subsp. *insigne* (Muirhead) Karaer
2250 m, 05.06.2016, RH 286
Endemic, LR (cd), Hcrp.
Apiaceae (Umbelliferae)
Bunium microcarpum (Boiss.) Freyn & Bornm. ex Freyn subsp. *bourgaei* (Boiss.) Hedge & Lamond.
2100 m, 14.06.2015, RH 118
Ir-Tur., Crp.
Carum meifolium (M.Bieb). Boiss.
2350 m, 22.07.2015, RH 245
Chp.
Geocaryum cynapioides (Guss.) Engstrand subsp. *macrocarpum* (Boiss. & Spruner)Menemen
2100 m, 14.06.2015, EY 292
Crp.
Asteraceae (Compositae)
Helichrysum graveolens (M.Bieb.) Sweet
2550 m, 16.08.2014, RH 42
Hcrp.
H. arenarium (L.) Moench subsp. *aucheri* (Boiss.) P.H.Davis & Kupicha
2500 m, 23.07.2015, EY 254
Endemic, LR (lc), Ir-Tur., Hcrp.
Filago arvensis L.
2450 m, 22.07.2015, RH 187
Cosm.,Thp.
Solidago virgaurea L. subsp. *virgaurea*
2450 m, 23.07.2015, EY 265
Euro-Sib., Hcrp.
Aster alpinus L.
2400 m, 23.07.2015, RH 268
Crp.
Bellis perennis L.
2308 m, 14.08.2014, RH 12
Euro-Sib., Hcrp.
Turanecio taraxacifolius (M.Bieb.) Hamzaoğlu var. *taraxacifolius*
2310 m, 14.08.2014, RH 34
Hcrp.
Senecio recemosus (M. Bieb.)DC.
2500 m, 23.07. 2015, EY 253
Ir-Tur., Hcrp.
S. pseudo-orientalis Schischk.
2100 m, 21.07.2015, EY 201
Ir-Tur., Hcrp.
S. viscosus L.
2150 m, 21.07.2015, RH 202
Thp.
Tussilago farfara L.
2005 m, 11.05.2015, RH 157
Euro-Sib., Crp.
Archanthemis marschalliana (Willd.) Lo Presti & Oberpr. subsp. *pectinata* (Boiss.) Lo Presti & Oberpr.
2050 m, 21.07.2015, EY 206
Eux., Hcrp.
Cota tinctoria (L.) J.Gay ex Guss. var. *tinctoria*
2411 m, 17.08.2014, RH 40
Hcrp.
C. melanoloma (Trautv.) Holub subsp. *melanoloma*
2500 m, 23.07.2015, RH 257
Endemic, LR (lc), Hcrp.
Achillea millefolium L. subsp. *millefolium* var. *millefolium*
2410 m, 17.08.2014, RH 37
Euro-Sib., Hcrp.
Tanacetum parthenium (L.) Sch.Bip.
2300 m, 14.06.2015, EY 293

Widespread, Hcrp.
T. albipannosum Hub.-Mor. & Grierson
2050 m, 21.07.2015, EY 191
Endemic, LR (cd), Ir-Tur., Hcrp.
T. armenum (DC.) Sch.Bip.
2150 m, 21.07.2015, RH 132
Hcrp.
Tripleurospermum sevanense (Manden.) Pobed.
2411 m, 17.08.2014, RH 36
Hcrp.
Cirsium tomentosum C.A. Mey.
2212 m, 15.08.2014, RH 43
Ir-Tur., Hcrp.
C. rhizocephalum C.A.Mey. subsp. *sinuatum* (Boiss.) P.H.Davis & Parris
2411 m, 17.08.2014, RH 35
Hcrp.
Carduus lanuginosus Willd.
2350 m, 14.08.2014, RH 44
Endemic, LR (lc), Hcrp.
Jurinella moschus (Hablitz) Bobrov subsp. *pinnatisecta*
2300 m, 14.06.2015, RH 48
Ir-Tur., Hcrp.
Centaurea salicifolia M.Bieb. ex Willd. subsp. *abbreviata* K.Koch
2500 m, 23.07.2015, RH 252
Eux., Hcrp.
C. armena Boiss.
2500 m, 23.07.2015, EY 263
Endemic, LR (lc), Ir-Tur., Hcrp.
Psephellus mucronifer (DC.) Wagenitz
2100 m, 14.06.2015, EY 139
Endemic, LR (lc), Ir-Tur., Hcrp.
Cyanus reuterianus (Boiss.) Holub var. *reuterianus*
2050 m, 21.07.2015, RH 189
Endemic, LR (lc), E. Medit., Hcrp.
Scorzonera cana (C.A. Mey.) Griseb. var. *jacquiniana* (W.Koch.) D.F.Chamb.
2350 m, 22.07.2015, EY 244
Hcrp.
S. cana (C.A.Mey.) Griseb. var. *radicosa* (Boiss.) D.F.Chamb.
2150 m, 22.07.2015, RH 248
Hcrp.
Leontodon hispidus L. subsp. *hispidus*
2450 m, 23.07.2015, EY 192
Euro-Sib., Crp.
L. crispus Vill. subsp. *asper* (Waldst. & Kit.) Röhl. var. *asper*
2550 m, 16.08.2014, RH 46
Widespread, Hcrp.
Hieracium pollichiae Sch. Bip.
2411 m, 16.08.2014, RH 47
Euro-Sib., Hcrp.
H. cyaneum Arvet-Touvet
2411 m, 17.08.2014, RH 38
Euro-Sib., Hcrp.
H. giresunense Hub.-Mor.2050 m, 21.07.2015, EY 193
Endemic, EN, Euro-Sib.**,** Hcrp.
Pilosella hoppeana (Schultz) F.W.Schultz & Sch. Bip. subsp. *testimonialis* (Naegli ex Peter) P.D.Sell & C.West
2410 m, 16.08.2014, RH 41
Euro-Sib., Hcrp.
Lapsana communis L. subsp. *intermedia* (M.Bieb.)Hayek var. *intermedia*
2411 m, 17.08.2014 RH, 39
Hcrp.
Taraxacum microcephaloides van Soest
2490 m, 16.08.2014, RH 45
Hcrp.
T. bithynicum DC.
2005 m, 11.05.2015, RH 164
Hcrp.
T. stevenii DC.
2550 m, 13.06.2015, EY 106
Ir-Tur., Hcrp.
Campanulaceae
Campanula tridentata Schreb.
2450 m, 22.07.2015, EY 241
Eux. (mt), Crp.
C. stevenii M.Bieb. subsp. *beauverdiana* (Fomin) Rech.f. & Schiman-Czeika
2100 m, 14.06.2015, EY 138
Ir-Tur., Hcrp.
Asyneuma amplexicaule (Willd.) Hand.-Mazz. subsp. *amplexicaule* var. *amplexicaule*
2550 m, 16.08.2014, RH 78
Widespread, Hcrp.
Jasione supina Sieber ex Spreng. subsp. *pontica* (Boiss.) Damboldt
2270 m, 14.08.2014, RH 77
Endemic, LR (lc), Euro-Sib., Hcrp.
Ericaceae
Vaccinium myrtillus L.
2308 m, 14.08.2014, RH 21
Euro-Sib., Chp.
Primulaceae
Primula longipes Freyn & Sint.
2300 m, 13.06.2015, EY 294
Endemic, NT, Eux., Hcrp.
P. auriculata Lam.
2300 m, 13.06.2015, RH 84
Ir-Tur., Hcrp.
P.algida Adams
2550 m, 13.06.2015, EY 295
Hcrp.
P. acaulis (L.) L. subsp. *rubra* (Sm.) Greuter & Burdet
2000 m, 11.05.2015, RH 148
Eux., Hcrp.
Androsace albana Steven
2550 m, 13.06.2015, RH 93
Eux. (mt), Thp.
Cyclamen parviflorum Pobed.
2300 m, 14.06.2015, EY 296
Endemic, LR (lc), Eux. (mt), Crp.
Gentianaceae
Gentianella ciliata (L.) Borkh. subsp. *blepharophora* (Bordz.) N.M.Pritch
2500 m, 16.08.2014, RH 1
Hyrc-Eux.(mt), Hcrp.
Boraginaceae
Myosotis sylvatica Hoffm. subsp. *cyanea* (Hayek)Vestergren
2400 m, 16.08.2014, RH 58
Hcrp.
M. alpestris F.W.Schmidt subsp. *alpestris*
2500 m, 13.06.2015, RH 85
Crp.
M. olympica Boiss.
2005 m, 11.05.2015, RH 160
Eux. (mt), Crp.
M. lithospermifolia Hornem.
2300 m, 14.06.2015, EY 131
Hcrp.
M. propinqua Fisch. & C.A.Mey.
2240 m, 05.06.2016, RH 283

Eux-Hyrc. (mt), Thp.
Echium italicum L.
2148 m, 17.08.2014, RH 29
Medit., Hcrp.
Solanaceae
Hyoscyamus niger L.
2050 m, 16.06.2015, RH 287
Hcrp.
Scrophulariaceae
Verbascum armenum Boiss. & Kotschy ex Boiss. var. *tempskyanum* (Freyn & Sint.)Murb.
2050 m, 21.07.2015, RH 180
Endemic, LR (lc), Ir-Tur., Hcrp.
V. froedinii Murb.
2300 m, 14.06.2015, EY 121
Ir-Tur., Hcrp.
Orobanchaceae
Pedicularis caucasica M.Bieb.
2500 m, 13.06.2015, EY 99
Hyrc-Eux. (mt), Hcrp.
Euphrasia pectinata Ten.
2550 m, 14.08.2014, RH 297
Euro-Sib., Widespread, Thp.
Lamiaceae (Labiatae)
Ajuga orientalis L.
2550 m, 13.06.2015, RH 92
Crp.
Teucrium chamaedrys L. subsp. *chamaedrys*
2050 m, 21.07.2015, EY 194
Euro-Sib., Chp.
Lamium macrodon Boiss. & Huet
2105 m, 14.06.2015, EY 128
Ir-Tur., Thp.
L. album L. subsp. *album*
2411 m, 16.08.2014, RH 26
Euro-Sib., Hcrp.
L. tomentosum Willd.
2450 m, 23.07.2015, RH 266
Ir-Tur., Hcrp.
L. galactophyllum Boiss. & Reuter
2020 m, 14.06.2015, EY 127
Endemic, LR (lc), Ir-Tur., Thp.
Marrubium astracanicum Jacq. subsp. *astracanicum*
2050 m, 21.07.2015, RH 185
Hcrp.
Nepeta italica L.
2450 m, 23.07.2015, RH 271
Medit., Chp.
N.nuda L. subsp. *nuda*
2080 m, 14.06.2015, EY 129
Euro-Sib., Hcrp.
Thymus sipyleus Boiss.
2300 m, 14.08.2014, RH 81
Chp.
Mentha longifolia (L.) L. subsp. *longifolia*
2005 m, 17.08.2014, RH 55
Eux., Crp.
Plumbaginaceae
Acantholimon bracteatum (Girard) Boiss.
2050 m, 21.07.2015, EY182
Ir-Tur.**,** Chp.
Plantaginaceae
Plantago lanceolata L.
2411 m, 16.08.2014, RH 24
Hcrp.
P. argentea Chaix.
2450 m, 13.06.2015, EY 105
DD, Euro-Sib., Hcrp.
Linaria genistifolia (L.) Mill. subsp. *linifolia* (Boiss.) P.H.Davis
2050 m, 21.07.2015, EY 181
Hcrp.
Digitalis lamarckii Ivanina
2050 m, 21.07.2015, EY 170
Endemic, LR (lc), Ir-Tur., Hcrp.
Veronica gentianoides Vahl. subsp. *gentionoides* var. *alpina*
2080 m, 11.05.2015, RH 149
Endemic, EN, Crp.
V. hispidula Boiss. & A.Huet subsp. *hispidula*
2050 m, 11.05.2015, RH 166
Ir-Tur., Thp.
V. anagallis-aquatica L.
2005 m, 17.08.2014, RH 73
Widespread, Hcrp.
V. peduncularis M.Bieb.
2300 m, 14.06.2015, EY 122
Eux., Crp.
Euphorbiaceae
Euphorbia herniariifolia Willd. var. *glaberrima* Halácsy
2400 m, 13.06.2015, EY 120
Hcrp.
E. rigida M.Bieb.
2100 m, 14.06.2015, EY 137
Medit., Hcrp.
Urticaceae
Urtica dioica L. subsp. *dioica*
2306 m, 14.08.2014, RH 23
Euro-Sib., Hcrp.
Rubiaceae
Crucianella gilanica Trin. subsp. *pontica* (Ehrend.) Ehrend.
2260 m, 21.07.2015, RH 221
Eux., Chp.
Asperula prostrata (Adams) K.Koch
2200 m, 14.06.2015, RH 134
Eux. (mt), Chp.
A. nitida Sm. subsp. *subcapitellata* Ehrend.
2260 m, 21.07.2015, EY 210
Endemic, LR (nt), Ir-Tur., Chp.
A.suavis Fisch. & Mey.
2080 m, 21.07.2015, RH 197
Endemic, LR (lc), Ir-Tur., Chp.
Galium humifusum M.Bieb.
2050 m, 14.06.2015, EY 133
Thp.
G. verum L. subsp. *verum*
2005 m, 17.08.2014, RH 80
Euro-Sib., Hcrp.
G.incanum Sm. subsp. *elatius* (Boiss.) Ehrend.
2450 m, 13.06.2015, EY 104
Ir-Tur., Hcrp.
Cruciata taurica (Pall. ex Willd.) Ehrend.
2250 m, 05.06.2016, RH 284
Widespread, Ir-Tur., Hcrp.

MONOCOTYLEDONAE (LILIOPSIDA)

Asparagaceae
Scilla siberica Haw. subsp. *armena* (Grossh.) Mordak
2270 m, 11.05.2015, RH 155
Ir-Tur., Crp.
Ornithogalum oligophyllum E.D.Clarke
2270 m, 11.05.2015, RH 96
Crp.
Muscari aucheri (Boiss.) Baker

2500 m, 13.06.2015, RH 97
Endemic, LR (lc), Crp.
M. armeniacum Leichtlin ex Baker
2260 m, 11.05.2015, RH 156
Widespread, Crp.
Colchicaceae
Colchicum szovitsii Fisch.& C.A.Mey. subsp. *szovitsii*
2500 m, 13.06.2015, RH 98
Ir-Tur., Crp.
Liliaceae
Fritillaria latifolia Willd.
2550 m, 13.06.2015, EY 95
Eux. (mt), Crp.
Gagea glacialis K.Koch
2080 m, 11.05.2015, RH 150
Ir-Tur., Crp.
Melanthiaceae
Veratrum album L.
2100 m, 14.06.2015, RH 298
Euro-Sib., Crp.
Amaryllidaceae
Allium aucheri Boiss.
2080 m, 21.07.2015, RH 198
Ir-Tur., Crp.
Iridaceae
Crocus kotschyanus K.Koch subsp. *suworowianus* (K.Koch) B.Mathew
2500 m, 16.08.2014, RH 2
Crp.
C. speciosus M.Bieb. subsp. *speciosus*
2350 m, 21.09.2014, RH 9
Crp.
Orchidaceae
Gymnadenia conopsea (L.) R.Br.
2470 m, 22.07.2015, EY 226
Euro-Sib., Crp.
Juncaceae
Juncus effusus L. subsp. *effusus*
2280 m, 14.09.2014, RH 82
Cosm., Hcrp.
Luzula spicata (L.) DC. subsp. *italica* (Parl.) Areang.
2400 m, 22.07.2015, RH 299
Hcrp.
L. stenophylla Steud.
2450 m, 22.07.2015, RH 236
Eux. (mt), Crp.
L. campestris (L.) DC.
2050 m, 11.05.2015, RH 167
Euro-Sib., Hcrp.
Cyperaceae
Kobresia simpliciuscula (Wahlenb.) Mackenzie subsp. *simpliciuscula*
2306 m, 14.08.2014, RH 14
Hcrp.
Carex oreophila C.A.Mey.
2450 m, 22.07. 2015, EY 233
Ir-Tur., Crp.
C. davalliana Sm.
2306 m, 14.08.2014, RH 16
Euro-Sib., Crp.
C. nigra (L.) Reichard subsp. *dacica* (Heuffel) Soó
2500 m, 13.06.2015, EY 110
Eux., Crp.
Poaceae (Gramineae)
Elymus repens (L.) Gould
2400 m, 17.08.2014, RH 6
Crp.
Bromus commutatus Schrad.
2050 m, 21.07.2015, RH 207
Thp.
B. lanceolatus Roth.
2100 m, 14.06.2015, EY 142
Thp.
B. danthoniae Trin. subsp. *danthoniae*
2100 m, 14.06.2015, EY 141
Thp.
B. tectorum L.
2270 m, 14.08.2014, RH 18
Widespread, Thp.
Helictotrichon argaeum (Boiss.) Parsa
2450 m, 13.06.2015, EY 300
Endemic, LR (lc), Ir-Tur., Chp.
H. pubescens (Huds.) Schult. & Schult. subsp. *pubescens*
2450 m, 13.06.2015, RH 107
Euro-Sib., Hcrp.
Trisetum flavescens (L.) P.Beauv.
2306 m, 14.08.2014, RH 4
Euro-Sib., Hcrp.
Koeleria eriostachya Pančič
2400 m, 23.07.2015, RH 144
Hcrp.
Calamagrostis arundinacea (L).Roth.
2308 m, 14.08.2014, RH 13
Euro-Sib., Crp.
Agrostis canina L.
2263 m, 14.08.2014, RH 17
Euro-Sib., Hcrp.
A. capillaris L. var. *capillaris*
2450 m, 13.06.2015, EY 199
Euro-Sib., Crp.
A. capillaris L. var. *aristata* (Parnell) Druce
2450 m, 13.06.2015, EY 108
Euro-Sib., Hcrp.
A. stolonifera L.
2282 m, 15.08.2014, RH 20
Euro-Sib., Hcrp.
Alopecurus pratensis L.
2400 m, 17.08.2014, RH 7
Euro-Sib., Hcrp.
Phleum alpinum L.
2200 m, 14.06.2015, EY 234
Euro-Sib., Crp.
P. pratense L.
2050 m, 21.07.2015, RH 143
Euro-Sib., Widespread, Chp.
Rhizocephalus cristata (L.) Tzvelev var. *cristata*
2300 m, 14.06.2015, EY 301
Hcrp.
Festuca amethystina L. subsp. *orientalis* Krajina var. *turcica* Markgr.-Dann.
2350 m, 22.07.2015, EY 235
Endemic, LR (cd), Eux. (mt), Hcrp.
F. pinifolia (Hackel ex Boiss.) Bornm. var. *pinifolia*
2600 m, 16.08.2014, RH 3
E. Medit., Hcrp.
Poa pratensis L.
2400 m, 23.07.2015, RH 274
Widespread, Crp.
P. longifolia Trin.
2400 m, 23.07.2015, RH 272
Eux., Crp.
P. chaixii Vill.
2100 m, 14.06.2015, EY 140
Euro-Sib., Hcrp.

P. alpina L. subsp. *fallax* F.Herm.
2270 m, 21.07.2015, RH 225
Chp.
P. bulbosa L.
2200 m, 14.06.2015, EY 117
Chp.
Dactylis glomerata L. subsp. *hispanica* (Roth) Nyman
2400 m, 17.08.2014, RH 5
Crp.
Nardus stricta L.
2450 m, 22.07.2015, RH 240
Euro-Sib., Hcrp.
Stipa ehrenbergiana Trin. & Rupr.
2100 m, 21.07.2015, EY 200
Ir-Tur., Chp.

4. Conclusions and discussion

The vascular plant flora of Eğribel Pass grasslands is represented by 230 taxa belonging to 138 genera and 44 families. Almost all of them belong to Spermatophyta (229 taxa) and only a taxon belongs to Pteridophyta. Gymnosperms and Angiosperms included 1 and 228 taxa, respectively. Of the Angiosperms, 180 taxa are Dicotyledonae and 48 taxa are Monocotyledonae.

The taxa in the study area, classified with respect to phytogeographical regions may be listed as follows: Euro-Siberian elements 75 (32.6%), Irano-Turanian elements 42 (18.3%), and Mediterranean elements 8 (3.5%). The remaining 105 (45.6%) taxa are pluriregional or unknown phytogeographic region. The high composition rate of Euro-Siberian element is not unexpecting and showed that the study area is a part of this floral element. Similar results were obtained by former studies which have been done in the East Black Sea region of Turkey. Irano-Turanian elements come in the second rank in the study area. The comparison of the distribution of the phytogeographic elements and endemism ratio in the study area and nearby areas is given in Table 1. The results in Table 1 demonstrated that studies by Karakaya and Kılınç (1996), Eminağaoğlu et al. (2008), Deveci (2012) and Şenel et al. (2014) have similar results in respect of three floral elements.

Table 1. Comparison (%) of floristic results between the present study and previous studies performed in nearby areas with respect to number of taxa, the phytogeographical elements, and endemism

Studies	Number of taxa	Euro-Sib.	Ir.-Tur.	Medit.	Endemism
Present study	230	32.60	18.30	3.50	12.2
Karakaya & Kılınç (1996)	323	46.74	4.03	0.93	8.7
Eminağaoğlu et al. (2008)	990	48.20	3.50	1.90	2.30
Deveci (2012)	540	40.56	7.78	2.96	11.50
Şenel et al. (2014)	482	36.93	7.90	3.52	6.4

Twenty-eight of the identified taxa are endemic with total 12.2% endemism rate. Endemic taxa are listed as Euro-Siberian 8 (3.48%), Irano-Turanian 11(4.78%), Mediterranean 2 (0.87%) and unknown or with more than one origin 7 (3.04%). Totally 28 taxa, all endemic, and 2 nonendemic taxa were assessed according to IUCN risk categories (Ekim et al. 2000; IUCN, 2010 Version 8.1). The results are shown in Table 2. The threat categories are as follows: 3 EN, 1 VU, 1 NT, 4 LR (cd), 17 LR (lc), and 2 LR (nt) were determined in endemic taxa, and 2 DD categories were determined in nonendemic taxa. Endemism ratio in the study area was higher compared to former studies performed in nearby areas. Uysal et al. (2011) have pointed out that existence of microhabitats, geographical isolation, climate changes, historical changes in floral composition, and speciation of new ones at higher altitudes give rise to greater endemism ratio in these areas. However, this ratio is low with the mean endemism ratio (34.5%) in the Flora of Turkey (Güner et al. 2000) and the endemism ratio of the Black Sea region (16%) (Ansin et al. 2002).

Table 2. Dispersial rates of the phytogeographic elements, endemic, nonendemic, and threat categories in the present study

Phytogeographical region	Endemic		Nonendemic		Total	
	Number	Percent (%)	Number	Percent (%)	Number	Percent (%)
Euro-Sib.	8	3.48	67	29.13	75	32.60
Ir.-Tur.	11	4.78	31	13.48	42	18.30
Medit.	2	0.87	6	2.61	8	3.50
P. Reg. Or Unk.	7	3.04	98	42.61	105	45.60
Total	28	12.2			230	100.00
EN	3	1.30			3	1.30
VU	1	0.43			1	0.43
NT	1	0.43			1	0.43
LR (cd)	4	1.74			4	1.74
LR (lc)	17	7.39			17	7.39
LR (nt)	2	0.87			2	0.87
DD			2	0.87	2	0.87
Total	28		2		30	

The largest families with regard to number of genera were Asteraceae (25), Poaceae (15), Brassicaceae (11), Fabaceae (9), Lamiaceae (7), Caryophyllaceae (6), Rosaceae (4), Plantaginaceae (4), Rubiaceae (4) and Ranunculaceae (3). The major families with regard to number of taxa were Asteraceae (39), Poaceae (28), Fabaceae (18), Brassicaceae (14), Caryophyllaceae (12), Lamiaceae (11), Rosaceae (10), Plantaginaceae (8), Ranunculaceae (8) and Rubiaceae (8) (Table 3). The total rate of the major families is 67.83%, with the remaining families consisting of 32.17%. It has been indicated that the major three families in Turkey are Asteraceae, Fabaceae and Lamiaceae (Davis, 1965-1985, 1988). In this study, Poaceae was determined in the second order because alpine belt in the North-Eastern part of Turkey is commonly represented by plants with a grass form. Some differences in major family ranks might be explained by the dissimilarities in climate and habitats. A comparison of families in terms of the largest number of species found in this study and in previous studies conducted in nearby regions is given in Table 4. In general, the results of this study complied with those of other similar studies (Eminağaoğlu et al. 2008; Korkmaz et al. 2008; Deveci, 2012; Şenel et al. 2014). Asteraceae (the largest family in our list) is the largest family in the Flora of Turkey (Güner et al. 2000).

Table 3. Numerical and dispersial rates of major families including the most taxa identified in the present study

Family	**Number of genera**	**Number of taxa**	**Rates**
Asteraceae	25	39	16.95
Poaceae	15	28	12.20
Fabaceae	9	18	7.82
Brassicaceae	11	14	6.08
Caryophyllaceae	6	12	5.22
Lamiaceae	7	11	4.77
Rosaceae	4	10	4.35
Plantaginaceae	4	8	3.48
Ranunculaceae	3	8	3.48
Rubiaceae	4	8	3.48
Other Families	50	74	32.17
Total	138	230	100.00

Table 4. Comparison (%) of similar studies with respect to the major families

Family	**Present study**	**Eminagaoglu et al. (2008)**	**Korkmaz et al. (2008)**	**Deveci (2012)**	**Şenel et al. (2014)**
Asteraceae	16.9	11.5	9.9	11.9	14.5
Poaceae	12.2	7.0	9.0	8.7	4.9
Fabaceae	7.8	6.0	8.2	10.0	8.1
Brassicaceae	6.1	4.7	2.2	2.6	5.4
Caryophyllaceae	5.2	3.3	3.1	-	4.4
Lamiaceae	4.8	4.3	6.5	6.1	6.6
Rosaceae	4.4	6.0	5.4	4.1	3.9
Plantaginaceae	3.5	-	-	-	2.7
Ranunculaceae	3.5	3.5	-	-	-
Rubiaceae	3.5	-	-	-	-

The largest genera with regard to the number of taxa were *Trifolium* L. (6), *Ranunculus* L. (6), *Poa* L. (5), *Astragalus* L. (4) and *Polygonum* L. (4) (Table 5). *Astragalus* L., *Verbascum* L. and *Centaurea* L. are the major three genera in Turkey (Davis, 1965-1985, 1988). However, *Astragalus* is the fourth major genus in the present study. Also, *Verbascum* and *Centaurea* are not observed in the first five in the floristic list. There are some differences in the ranks of genera between Flora of Turkey and the study area. These differences might be explained by the dissimilarities in climatic, geomorphologic, phytogeographic and edaphic features. Alpine grasslands in the study area were characterized mainly by grass species whereas *Astragalus* and *Verbascum* are distributed in steppic vegetation in the central and eastern parts of Anatolia. In the study area these genera were found only in south-facing slopes, where demonstrate more steppic features and dry climate. On the other hand, Turkey is very rich Mediterranean country for *Trifolium* L. genus with over 100 species in its natural flora (Deveci, 2012). *Trifolium* and *Astragalus* genera are present in all studies conducted in the Eastern Black Sea Region of Turkey (Davis 1965, 1985; Eminağaoğlu and Ansin, 2003, 2004; Uzun and Terzioğlu, 2008; Severoğlu et al. 2011; Deveci, 2012).

Table 5. Numerical and dispersial rates of major genera including the most taxa identified in the present study

Genera	**Number of Taxa**	**Rates**
Trifolium L.	6	2.61
Ranunculus L.	6	2.61
Poa L.	5	2.17
Astragalus L.	4	1.74
Polygonum L.	4	1.74

The biological spectrum of the taxa comprised of hemicryptophytes 125 (54.4%), cryptophytes 49 (21.3%), chamaephytes 31(13.5%), therophytes 24 (10.4%) and phanerophytes 1(0.43%) (Figure 2). Hemicryptophytes were the best observed lfe-form in the study area. High ratio of hemicryptophytes might be thougt of as an adaptation to grazing (Yalçın et al. 2011). Since traditional land management (e.g. grazing by cattle and sheep) prevails at moderate intensity in the study area, hemicryptophyte species with rosette leaves have defensive and effective allocation mechanism againist grazing. This distribution is specific for grassland ecosystems. Cryptophytes were in the second order with renewing buds under soil.

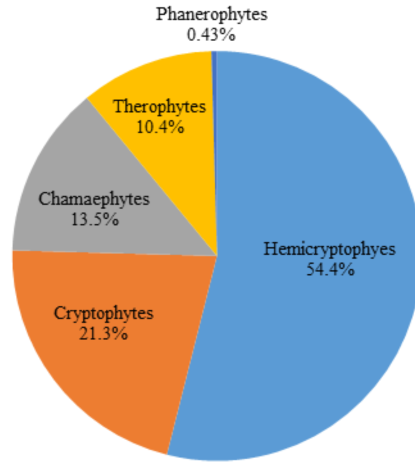

Figure 2. Biological spectrum of the plant species in the present study

Finally, considerable results were found in this study, which was carried out to draw attention to and improve understanding of the plant diversity in the alpine belt of Colchic province in the North-Eastern Black Sea Region. In this context, this study could be helpful for further studies on supporting of alpine landscapes and protection efforts of alpine plant species in Turkey..

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Research article/Araştırma makalesi

LC-MS/MS and GC-MS analyses of three endemic *Astragalus* species from Anatolia towards their total phenolic-flavonoid contents and biological activities

Nesrin HAŞİMİ [1], Abdulselam ERTAŞ [*1], Mustafa Abdullah YILMAZ [3], Mehmet BOĞA [4], Hamdi TEMEL [5], Serpil DEMİRCİ [6], Tuğba Yılmaz-ÖZDEN [7], İsmail YENER [3] and Ufuk KOLAK[8]

[1] Department of Nutrition and Dietetics, School of Health, Batman University, 72060 Batman, Turkey
[2] Department of Pharmacognosy, Faculty of Pharmacy, Dicle University, 21280 Diyarbakir, Turkey
[3] Dicle University Science and Technology Research and Application Center (DUBTAM), Dicle University, 21280 Diyarbakir, Turkey
[4] Department of Pharmaceutical Technology, Faculty of Pharmacy, Dicle University, 21280 Diyarbakir, Turkey
[5] Department of Pharmaceutical Chemistry, Faculty of Pharmacy, Dicle University, 21280 Diyarbakir, Turkey
[6] Department of Pharmaceutical Botany, Faculty of Pharmacy, Cukurova University, 01330 Adana, Turkey
[7] Department of Biochemistry, Faculty of Pharmacy, Istanbul University, 34116 Istanbul, Turkey
[8] Department of General and Analytical Chemistry, Faculty of Pharmacy, Istanbul University, 34116 Istanbul, Turkey

Abstract

Present work aims to determine the chemical profile and some biological activity of three endemic *Astragalus* species from Anatolia. The chemical contents of *Astragalus leporinus* var. *hirsutus*, *Astragalus distinctissimus* and *Astragalus schizopterus* were characterised by LC-MS/MS and GC-MS. In terms of biological activity; the antioxidant, anticholinesterase, antimicrobial and cytotoxic activities were determined. Additionally, the antioxidant properties of the major components were also determined and compared to the antioxidant capacities of these extracts. The most abundant flavonoids in these *Astragalus* species were determined as rutin (1028.276-13351.76 µg/g extract) and hesperidin (1604.348-9695.435 µg/g extract). A high amount of quinic acid (111302.774 µg/g extract) was detected in *A. schizopterus* methanol extract. Palmitic acid (C16:0) was found to be the major compound in *A. leporinus* var. *hirsutus* (32.9%), *A. distinctissimus* (32.5%), and *A.schizopterus* (23.4%). *A. schizopterus* methanol extract exhibited the highest antioxidant effect in lipid peroxidation (19.62±0.29), DPPH free (54.61±0.38) and ABTS cation radicals scavenging activity (22.01±0.07), and CUPRAC assays. Among all of the extracts, only *A. leporinus* var. *hirsutus* petroleum ether extract showed moderate inhibitory activity against acetyl- and butyryl-cholinesterase enzymes. The methanol extracts of the plants exhibited moderate activity against *C. albicans*. *A. leporinus* var. *hirsutus* methanol extract indicated the most viability against L929 fibroblast cells, and the highest cytotoxic effect against A549 cells. In consideration of our findings , these *Astragalus* species used as animal feed could be a source of naturally, biologically active agents that can be used in food and pharmaceutical industry.

Key words: *Astragalus*, LC-MS/MS, antioxidant, anticholinesterase, antimicrobial

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Anadolu'daki üç endemik Astragalus türünün toplam fenolik-flavonoid içerikleri ve biyolojik aktivitelerine yönelik LC-MS / MS ve GC-MS analizleri

Özet

Bu çalışmada, Anadolu'da yetişen üç endemik *Astragalus* türünün kimyasal içeriği ile bazı biyolojik aktivitelerinin belirlenmesi hedeflenmiştir. *Astragalus leporinus* var. *hirsutus*, *Astragalus distinctissimus* ve *Astragalus schizopterus* türlerinin kimyasal içeriği LC-MS/MS ve GC-MS ile karakterize edilmiştir. Biyolojik aktivite açısından; ekstrelerin antioksidan, antikolinesteraz, antimikrobiyal ve sitotoksik aktiviteleri tespit edilmiştir. Ek olarak, ekstrelerin ana bileşenlerinin de antioksidan özellikleri belirlenerek elde edilen sonuçlar ekstrelerin antioksidan kapasiteleri ile

[*] *Corresponding author* / Haberleşmeden sorumlu yazar: Tel.: +904122488030; Fax.: +904122488030; E-mail: abdulselamertas@hotmail.com
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karşılaştırılmıştır. Çalışılan *Astragalus* türlerinde en bol bulunan flavonoidin rutin (1028,276-13351,76 µg/g ekstre) ve hesperidin (1604,348-9695,435 µg/g ekstre) olduğu, ayrıca *A. schizopterus* metanol ekstresinde de yüksek miktarda kuinik asit (111302,774 µg/g ekstre) bulunduğu belirlenmiştir. Palmitik asitin (C16:0), *A. leporinus* var. *hirsutus* (%32,9), *A. distinctissimus* (%32,5) ve *A.schizopterus* (%23,4)'da başlıca bileşik olduğu saptanmıştır. *A. schizopterus* metanol ekstresi lipit peroksidasyon (19,62±0,29), DPPH serbest radikal (54,61±0,38) , ABTS katyon radikali süpürücü aktivitesi (22,01±0,07), ve CUPRAC yönteminde en yüksek aktiviteyi göstermiştir. Çalışılan tüm ekstrelerden sadece *A. leporinus* var. *hirsutus* petrol eteri ekstresi orta derecede asetil- ve bütiril- kolinesteraz enzim inhibisyonu sergilemiştir.. Bitkilerin metanol ekstreleri *C. albicans*'a karşı orta derecede antimikrobiyal aktivite ortaya koymuşlardır. *A. leporinus* var. *hirsutus* metanol ekstresi, L929 fibroblast hücrelerine karşı en fazla canlılığı ve A549 hücrelerine karşı en yüksek sitotoksik etkiyi göstermiştir. Çalışmdan elde ettiğimiz bulgular ışığında, hayvan yemi olarak kullanılan bu *Astragalus* türleri, gıda ve ilaç endüstrisinde kullanılabilen doğal, biyolojik olarak aktif maddeler kaynağı olabilir.

Anahtar kelimeler: *Astragalus*, LC-MS/MS, antioksidan, antikolinesteraz, antimikrobiyal

1. Introduction

The genus *Astragalus* L. (Fabaceae), with about 2500–3000 species, is the largest genus of flowering plants in the world. It comprises about 439 taxa, 309 of them are endemic, and it is classified into 63 sections in Turkey (Chamberlain and Matthews 1970; Ozhatay and Kultur 2006). *Astragalus* species have been used in the pharmaceutical, food, loom, paper and cosmetic industries (Golmohammadi, 2013). Some of them has been used to obtain the gum tragacanth (Calis and Sticher, 1996; Yılmaz et al., 2013). In Anatolia, some Turkish *Astragalus* species are known as "geven" and they are generally consumed as animal feed in Turkey (Baytop, 1984).

Phytochemical investigations have shown up the main component of Turkish *Astragalus* species as cycloartane-type triterpene glycosides, a series of oleanane- and triterpenoidal saponins. (Horo et al. 2012; Savran et al. 2012). These compounds have potent pharmacological traits such as being cytotoxic (Krasteva et al. 2008), anti-protozoal (Ozipek et al., 2005), antiviral (Gariboldi et al., 1995), wound healing (Sevimli-Gur et al., 2011), immunostimulating and adjuvant effects (Nalbantsoy et al., 2011).

Astragalosides I-VII obtained from the roots of *Astragalus* species (Calis et al., 1997) have been commonly used as dietary supplement to enhance the immune system, additive in foods and beverages in European, American and Asian countries (Chu et al., 2010). Because of these features, *Astragalus* species have an important potential in the field of health care and food industry (Verotta and Sebakhy, 2001).

Recently, there have been quite a lot of studies related to the biological properties and quantitation of natural phenolic compounds (Ertas et al. 2014a; Awad et al. 2014; Abdelhady et al., 2015). The facts that there isn't any detailed study on phenolic constituents of Turkish A*stragalus* species. The lack of the research on endemic *Astragalus leporinus* Boiss. var. *hirsutus* (Post) Chamberlain, *A. distinctissimus* Eig and *A. schizopterus* Boiss. species have led us to this investigation. Thus, in this study, we tried to determine their total phenolic and flavonoid contents together with their fatty acid compositions. The antioxidant, anticholinesterase, antimicrobial and cytotoxic activities of these species were also investigated. Twenty-four phenolic compounds (flavonoids, flavonoid glycosides, phenolic acids, phenolic aldehyde, coumarin) and three non-phenolic organic acids in the methanol extracts of these *Astragalus* species were analyzed by LC-MS/MS, and chemical composition-activity relationships were evaluated together with chemotaxonomical aspects.

2. Materials and methods

1.1. Plant material

The aerial parts and roots (whole plants) of *Astragalus leporinus* Boiss. var. *hirsutus* (Post) Chamberlain, *A. distinctissimus* Eig and *A. schizopterus* Boiss. were collected from southeastern Turkey (Kahramanmaras) in May, August and May 2012, respectively, and identified by S. Demirci. Voucher specimens were deposited in the Herbarium of Istanbul University, Faculty of Pharmacy (*Astragalus leporinus* var. *hirsutus* ISTE 97142, *A. distinctissimus* ISTE 98035, *A. schizopterus* ISTE 97141).

1.2. Preparation of plant extracts for LC-MS/MS, biological activities and GC-MS

Plant marerials were dried at shadow and powdered.Ten grams of each plant materials were extracted three times with Methanol (50 mL each) at room temperature for 24 hours. Afterwards, the extracts obtained were combined, filtered and evaporated under low pressure. Dry filtrates were reconstituted in methanol at a concentration of 250 mg/L and passed through the 0.2 µm PTFE filter for LC-MS/MS.

Then,100 g plant materials macerated three times with petroleum ether (250 mL), acetone (250 mL), methanol (250 mL) and water (250 mL) at 25 ºC for 24 hours. After filtration, the solvent was evaporated under vacuum. The

yields of the petroleum ether extracts were calculated as *A. leporinus* var. *hirsutus* petroleum ether extract (ALP) 0.8%, *A. distinctissimus* petroleum ether extract (ADP) 0.6%, *A. schizopterus* petroleum ether extract (ASP) 0.60%, the acetone extracts as ALA 1.3%, ADA 0.7%, ASA 0.80%, the methanol extracts as ALM 9.1%, ADM 5.2%, ASM 7.2%, and the water extracts as ALW 2.8%, ADW 2.3%, ASW 3.1% (w/w).

Esterification of the petroleum ether extracts, and GC-MS procedure described by Ertas et al. (2014a; 2014b) were applied. Thermo Scientific Polaris Q GC-MS/MS was used.

1.3. *Identification and quantitation of phenolic compounds*

LC-MS/MS analyses of the phenolic compounds were performed by using a Nexera model Shimadzu UHPLC coupled to a tandem MS instrument. The liquid chromatograph was equipped with LC-30AD binary pumps, DGU-20A3R degasser, CTO-10ASvp column oven and SIL-30AC autosampler. The chromatographic seperation was performed on a C18 reversed-phase Inertsil ODS-4 (150 mm × 4.6 mm, 3µm) analytical column. The column temperature was fixed at 40°C. The elution gradient consisted of mobile phase A (water, 5mM ammonium formate and 0.1% formic acid) and mobil phase B (methanol, 5mM ammonium formate and 0.1% formic acid). The gradient program with the following proportions of solvent B was applied t (min), %B: (0, 40), (20, 90), (23.99, 90), (24, 40), (29, 40). The solvent flow rate was maintained at 0.5 mL/min and injection volume was settled as 4 µL.

Subsequent to several combinations of trials, a gradient of methanol (5mM ammonium formate and 0.1% formic acid) and water (5mM ammonium formate and 0.1% formic acid) system was concluded to be the best mobile phase solution for LC-MS/MS analyses. For rich ionization and the seperation of the molecules, the mentioned mobile phase was proved to be the best of all. ESI source was chosen instead of APCI (Atmospheric Pressure Chemical Ionization) and APPI (Atmospheric Pressure Photoionization) sources as the phenolic compounds were small and relatively polar molecules. Tandem mass spectrometry was decided to be used for the current study since this system is commonly used for its fragmented ion stability (Ertas et al. 2014a; Ertas et al. 2015). The working conditions were determined as interface temperature; 350°C, DL temperature; 250°C, heat block temperature; 400°C, nebulizing gas flow (Nitrogen); 3 L/min and drying gas flow (Nitrogen); 15 L/min.

Detailed information on method validation parameters and Estimation of uncertainty can be provided from previous manuscripts of our study group (Ertas et al. 2014a; Ertas et al. 2015).

1.4. Biological activities of the extracts

In terms of biological activity, the total phenolic and flavonoid contents, antioxidant, anticholinesterase, antimicrobial and cytotoxic activity were determined. The total amount of phenolic and flavonoid contents of extracts was calculated according to Slinkard and Singleton (1977) and Moreno et al. (2000) using following equations and stated as pyrocatechol and quercetin equivalents, respectively.

Absorbance = 0.0123 pyrocatechol (µg) + 0.0349 (R2 = 0.9910)

Absorbance = 0.1701 quercetin (µg) – 0.7078 (R2 = 0.9939)

Antioxidant activity was determined according to the relevant literature by using four different methods, including β-Carotene linoleic acid test system, DPPH free radical scavenging activity, ABTS cation radical decolonization and cupric reducing antioxidant capacity (CUPRAC) assay (Blois, 1958; Miller, 1971; Re et al., 1999; Apak et al., 2004).

Anticholinesterase activity of the extracts was assessed according to the literature (Ellman et al. 1961) based on acetyl- and butyryl-cholinesterase enzyme inhibitory effect detected spectrophotometrically.

Streptococcus pyogenes ATCC19615, *Staphylococcus aureus* ATCC 25923, *Pseudomonas aeruginosa* ATCC 27853, *Escherichia coli* ATCC 25922 and *Candida albicans* ATCC10231 were used for evaluating the antimicrobial activity. The disc diffusion method (NCCLS, 1997) was established for this purpose. The minimum inhibitory concentration (MIC) of active extracts was also determined (NCCLS, 2009).

Cytotoxic activity was determined by MTT assay performed in accordance with ISO 10993-5 standards. A549 and L929 fibroblast cells, which were stored in liquid nitrogen tank were centrifuged after dissolution. Later, these cells were placed on a 96-well plate after addition of 3 mL (DMEM 10% + fetal bovine serum 100%+ containing 1% antibiotics), and incubated under 5% CO2 and at 37°C. When the cells reached a sufficient growth, they were discharged with trypsin-EDTA solution, and passaging process was continued. The MTT assay is sensitive for cell proliferation measurement that 3-[4,5-dimethylthiazol-2-yl] -2,5-diphenyltetrazolium bromide (MTT) tetrazolium salt is used. MTT is reduced to insoluble formazan dye in water by mitochondrial enzymes associated with metabolic activity. MTT reduction is primarily associated with the glycolytic activity in the cells and depends on the presence of NADH (nicotine amide adenine dinucleotide) and NADPH (nicotinamide adenine dinucleotide phosphate). In the reactions of mitochondria of healthy cells or early stages of apoptotic cells, colored formazan crystals constitute with the degradation of the tetrazolium ring which is found in MTT solution by dehydrogenase enzymes in cell mitochondria. The color change which observed in living cells gives the absorbance values in Elisa reader.

L929 fibroblast and A549 (human lung cancer) cells were cultivated on 96-well plate (10x103 cells/well). Cells were incubated for 24 hours. Later, previously prepared plant extracts at different concentrations (0-12.5µg/mL - 25µg/mL - 50µg/mL - 100µg/mL) was applied onto the cells and then incubated for 24 hours. The samples were studied 5 times. As a positive control, the medium was only applied onto cells. After 24 hours, waste in each well was discarded and 100 µL of medium and 20 µL of MTT solution were added. After 3.5 h incubation at 37 °C, 150 µL of MTT solvent was added to the wells, and cells were incubated for extra 15 minutes. In order to determine cell viability, absorbance values of plates were recorded by ELISA reader at 570 nm. According to the absorbance values of control group, percentage cell viability was calculated.

1.5. Statistical analysis

The results of the antioxidant and anticholinesterase activity assays were represented as means ± SD. The results were evaluated using an unpaired *t*-test and ANOVA variance analysis with the NCSS statistical computer package. The differences were considered statistically significant at $p < 0.05$.

3. Results

1.1. Quantitative analysis of phenolic and flavonoid compounds by LC-MS/MS

Since the research of fruits and vegetables have revealed the protective effect of phenolic acids on oxidative damage diseases such as coronary heart disease, stroke, cancers, cardiovascular diseases and inflammation, phenolic acids have been a remarkable research topic for researchers. Because of their powerful antioxidant properties not only the researchers but also the food manufacturers deal with the phenolic compounds. (Awad et al., 2014; Ertas et al., 2014a). There are some studies about the use of liquid chromatography electrospray ionization tandem mass spectrometry for determining the phenolic compounds quantitatively (Yunfei et al., 2008; Ertas et al., 2014a). In this way, a valid method was developed for the analyses of twenty-four phenolic compounds and three non-phenolic organic acids in the methanol extracts of *A. leporinus* var. *hirsutus, A. distinctissimus* and *A. schizopterus*. The specific MRM (Multiple reaction monitoring) fragmentation reactions was selected in order to monitor the aforesaid twenty-seven compounds. Twenty-four phenolic compounds and three non-phenolic organic acids were monitored by the transition from the specific deprotonated molecular ions to the corresponding fragment ions. Molecular ions, fragments observed in MS/MS, related collision energies for these fragments and the quantified results for three *Astragalus* species were presented in Table 1.

Our results showed that the most abundant flavonoids in the three studied *Astragalus* species were rutin (1028.276-13351.76 µg/g extract), hesperidin (1604.348-9695.435 µg/g extract) and hyperoside (228.3135-1992.697 µg/g extract) (Table 1 and Figure 1). ASM extract was found to be the richest extract in terms of myricetin (110.769 µg/g extract), quercetin (75.94 µg/g extract), naringenin (76.0115 µg/g extract), luteolin (86.6995 µg/g extract), kaempferol (123.662 µg/g extract) and apigenin (30.5195 µg/g extract) as well as rutin (13351.76 µg/g extract), hesperedin (9695.435 µg/g extract) and hyperoside (1992.697 µg/g extract) (Table 1 and Figure 1). Furthermore, the highest amount of phenolic acids such as chlorogenic (400.3195 µg/g extract), protocatechuic (1607.988 µg/g extract), p-coumaric (475.859 µg/g extract) and rosmarinic acids (751.17 µg/g extract) was determined in ASM extract. On the other hand, ADM and ALM extracts possessed the highest quantity of 4-OH benzoic acid (1799.27 µg/g extract) and salicylic acid (1696.10 µg/g extract), and tr-caffeic acid (144.7665 µg/g extract), respectively.

When the analysis results were investigated in terms of the non-phenolic organic acids, ALM extract was found to be the richest extract in terms of malic (7802.659 µg/g extract) and tr-aconitic acids (972.159 µg/g extract). Indeed, quinic acid (111302.774 µg/g extract) content of ASM extract was probably the most distinctive result of this study. Because quinic acid is known to be a versatile chiral starting material for the synthesis of new pharmaceutical compounds. To treat influenza A and B strains, a medicine called "Tamiflu" has been developed via this way (Kim et al. 1997). In the previous studies, HPLC-DAD and HPLC-MS/MS techniques were used to detect several phenolic and flavonoid compounds such as rutin, quercetin, rosmarinic acid, kaempferol, calycosin, ononin, formononetin and some derivatives of them in *Astragalus* species (Qi et al., 2008; Yunfei et al., 2008; Montoro et al., 2012; Zhang et al., 2013). As far as we know, this research is the first on detecting naringenin, vanillin, hesperidin, myricetin and protocatechuic, quinic, tr-aconitic and 4-OH benzoic acids in *Astragalus* species.

In the flora of Turkey, the genus *Astragalus* is classified into 63 sections (Chamberlain and Matthews, 1970). *A. leporinus* var. *hirsutus* is grouped into the *Myobroma* Bunge, *A. distinctissimus* into the *Dasyphyllium* Bunge, and *A. schizopterus* into the *Proselius* Bunge sections. Our results indicated that the *Myobroma*, *Dasyphyllium* and *Proselius* sections contain different amount of phenolic compounds and non-phenolic organic acids. According to our analysis results, quinic acid content might be used as a chemotaxonomical marker for *A. schizopterus*. In addition, quercetin, naringenin, luteolin, and kaempferol is encountered in *A. schizopterus* methanol extract.

Table 1. Analytical parameters of LC-MS/MS method, and identification and quantification of phenolic compounds in ALM, ADM and ASM

No	Analyte	Parent ion (m/z)[a]	MS²(CE)[b]	Ionization Mode	RT[c]	$R^{2,d}$	RSD%[e]	Linearity Range (mg/L)	LOD/LOQ (µg/L)[f]	Recovery (%)	U[g]	Quantification (µg analyte/g extract)[h]		
												ALM	ADM	ASM
1	Quinic acid	190,95	85 (22),93 (22)	Neg	3.32	0.9927	0.0388	250-10000	22.3 / 74.5	103.3	4.8	**4814.49±23.23**	**5094.77±244.51**	**111302.77±5342.49**
2	Malic acid	133,05	115 (14),71 (17)	Neg	3.54	0.9975	0.1214	250-10000	19.2 / 64.1	101.4	5.3	**7802.65±413.50**	**7531.38±399.14**	**5286.27±280.16**
3	tr-Aconitic acid	172,85	85 (12),129 (9)	Neg	4.13	0.9933	0.3908	250-10000	15.6 / 51.9	102.8	4.9	972.15±47.62	461.00±22.58	400.83±19.60
4	Gallic acid	169,05	125 (14),79 (25)	Neg	4.29	0.9901	0.4734	25-1000	4.8 / 15.9	102.3	5.1	24.25±1.22	28.18±1.43	290.63±14.79
5	Chlorogenic acid	353	191 (17)	Neg	5.43	0.9932	0.1882	250-10000	7.3 / 24.3	99.7	4.9	173.95±8.47	306.45±14.99	400.31±19.60
6	Protocatechuic acid	152,95	109 (16),108 (26)	Neg	5.63	0.9991	0.5958	100-4000	25.8 / 85.9	100.2	5.1	267.26±13.61	122.04±6.22	**1607.98±81.95**
7	Tannic acid	182,95	124 (22),78 (34)	Neg	6.46	0.9955	0.9075	100-4000	10.2 / 34.2	97.8	5.1	115.50±5.86	50.38±2.55	104.79±5.30
8	tr-Caffeic acid	178,95	135 (15),134 (24),89 (31)	Neg	7.37	0.9942	1.0080	25-1000	4.4 / 14.7	98.6	5.2	144.76±7.48	25.67±1.30	94.80±4.88
9	Vanillin	151,05	136 (17),92 (21)	Neg	8.77	0.9995	0.4094	250-10000	10.1 / 33.7	99.2	4.9	57.34±2.79	77.06±3.77	132.21±6.46
10	p-Coumaric acid	162,95	119 (15),93 (31)	Neg	9.53	0.9909	1.1358	100-4000	15.2 / 50.8	98.4	5.1	460.36±23.46	392.87±19.99	475.85±24.22
11	Rosmarinic acid	358,9	161 (17),133 (42)	Neg	9.57	0.9992	0.5220	250-10000	10.4 / 34.8	101.7	4.9	392.09±19.20	235.44±11.51	751.17±36.79
12	Rutin	609,1	300 (37), 271 (51), 301 (38)	Neg	10.18	0.9971	0.8146	250-10000	17.0 / 56.6	102.2	5.0	**7389.81±369.45**	**1028.27±51.40**	**13351.76±667.55**
13	Hesperidin	611,1	303 (24),465 (12)	Poz	9.69	0.9973	0.1363	250-10000	21.6 / 71.9	100.2	4.9	**9441.78±462.61**	**1604.34±78.59**	**9695.43±475.06**
14	Hyperoside	463,1	300 (27),301 (26)	Neg	10.43	0.9949	0.2135	100-4000	12.4 / 41.4	98.5	4.9	932.24±45.66	228.31±11.17	**1992.69±97.60**
15	4-OH Benzoic acid	136,95	93 (17),65 (27)	Neg	11.72	0.9925	1.4013	25-1000	3.0 / 10.0	106.2	5.2	184.86±9.56	**1799.27±93.54**	**1033.00±53.71**
16	Salicylic acid	136,95	93 (16),65 (31),75 (30)	Neg	11.72	0.9904	0.6619	25-1000	4 / 13.3	106.2	5.0	170.09±8.50	**1696.10±84.80**	**1003.78±50.15**
17	Myricetin	317	179 (19),151(23),137 (26)	Neg	11.94	0.9991	2.8247	100-4000	9.9 / 32.9	106.0	5.9	N.D.[i]	D.[j]	110.76±6.49
18	Fisetin	284,95	135 (22),121 (27)	Neg	12.61	0.9988	2.4262	100-4000	10.7 / 35.6	96.9	5.5	D.	N.D.	N.D.
19	Coumarin	146,95	103 (17),91 (26),77 (27)	Poz	12.52	0.9924	0.4203	100-4000	9.1 / 30.4	104.4	4.9	D	D.	D.
20	Quercetin	300,9	179 (19),151 (21),121 (28)	Neg	14.48	0.9995	4.3149	25-1000	2.0 / 6.8	98.9	7.1	N.D.	N.D.	75.94±5.32
21	Naringenin	270,95	151 (18),119 (24),107 (26)	Neg	14.66	0.9956	2.0200	25-1000	2.6 / 8.8	97.0	5.5	17.92±0.93	D.	76.01±4.18
22	Hesperetin	300,95	164 (25),136 (33),108 (42)	Neg	15.29	0.9961	1.0164	25-1000	3.3/ 11.0	102.4	5.3	N.D.	N.D.	N.D.
23	Luteolin	284,95	217 (25),199 (28),175 (29)	Neg	15.43	0.9992	3.9487	25-1000	5.8 / 19.4	105.4	6.9	39.37±2.69	N.D.	86.69±5.93
24	Kaempferol	284,95	217 (29),133 (32),151 (23)	Neg	15.43	0.9917	0.5885	25-1000	2.0 / 6.6	99.1	5.2	N.D.	N.D.	123.66±6.39
25	Apigenin	268,95	151 (25),117 (35)	Neg	17.31	0.9954	0.6782	25-1000	0.1 / 0.3	98.9	5.3	22.89±1.16	N.D.	30.51±1.59
26	Rhamnetin	314,95	165 (23),121 (28),300 (22)	Neg	18.94	0.9994	2.5678	25-1000	0.2 / 0.7	100.8	6.1	16.40±0.97	N.D.	N.D.
27	Chrysin	253	143 (29),119 (32),107 (26)	Neg	21.18	0.9965	1.5530	25-1000	0.05 / 0.17	102.2	5.3	5.60±0.29	8.02±0.42	6.18±0.31

[a]Parent ion (*m/z):* Molecular ions of the standard compounds (mass to charge ratio), [b]MS²(CE): MRM fragments for the related molecular ions (CE refers to related collision energies of the fragment ions), [c]RT: Retention time, [d]R^2: coefficient of determination, [e]R^2: coefficient of determination, [f]LOD/LOQ (µg/L): Limit of deteection/Limit of quantification, [g]U (%): Percent relative uncertainty at 95% confidence level (k=2), [h]Values in µg/g (w/w) of plant extract, [i]N.D: not detected, [j]D: peak observed, concentration is lower than the LOQ but higher than the LOD, ALM: *Astragalus leporinus* var. *hirsutus* methanol extract , ADM: *A. distinctissimus* methanol extract , ASM: *A. schizopterus* methanol extract

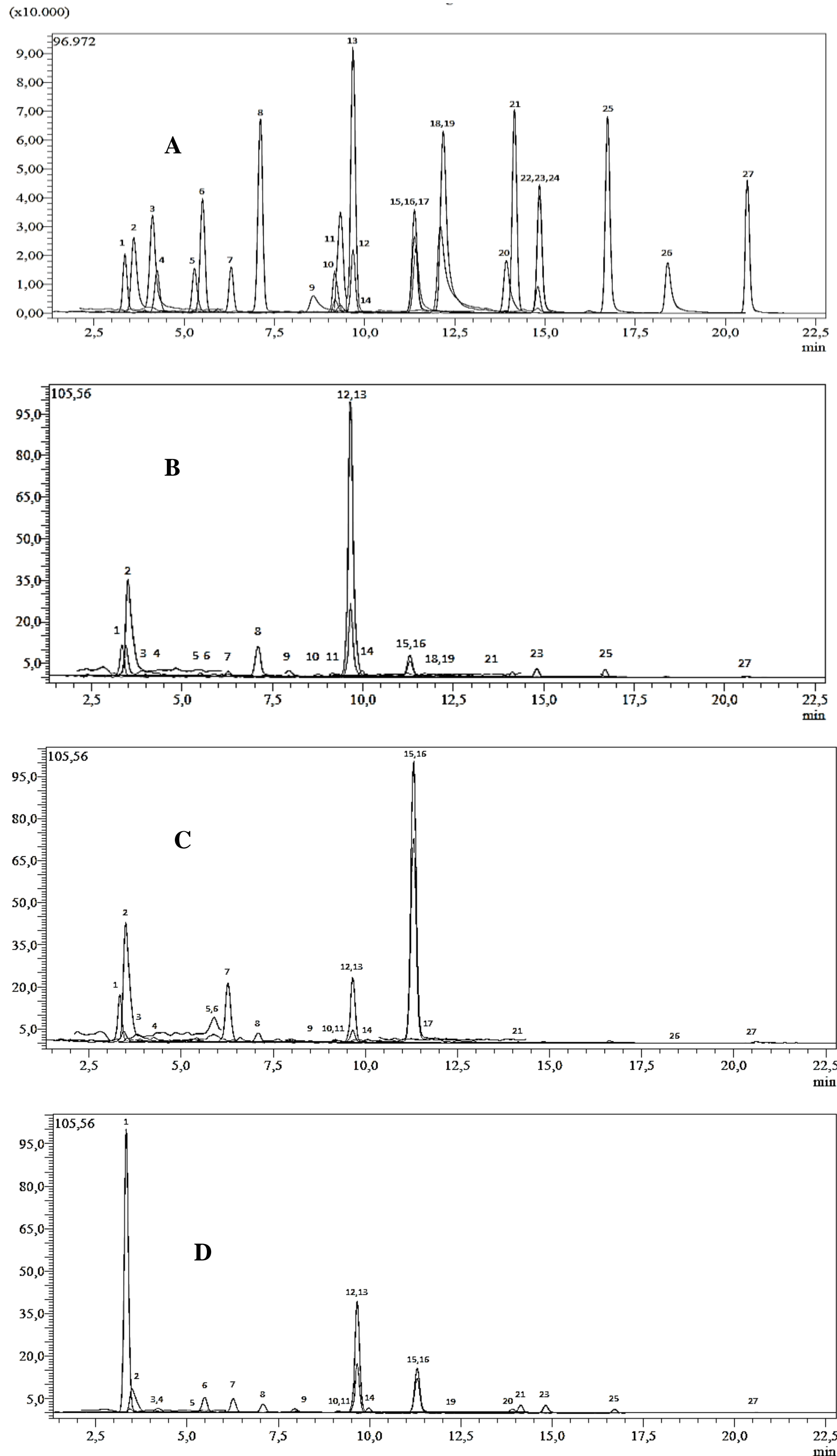

Figure 1. LC-MS/MS chromatograms of A: 250 µg/L of standard mix, B: AL methanol extract, C: AD methanol extract, D: AS methanol extract. 1: Quinic acid, 2: Malic acid, 3: tr-Aconitic acid, 4: Gallic acid, 5: Chlorogenic acid, 6: Protocatechuic acid, 7: Tannic acid, 8: tr-Caffeic acid, 9: Vanillin, 10: p-Coumaric acid, 11: Rosmarinic acid, 12: Rutin, 13: Hesperidin, 14: Hyperoside, 15: 4-OH Benzoic acid, 16: Salicylic acid, 17: Myricetin, 18: Fisetin,19: Coumarin, 20: Quercetin, 21: Naringenin, 22: Hesperetin, 23: Luteolin, 24: Kaempferol, 25: Apigenin, 26: Rhamnetin, 27: Chrysin.

1.1. Fatty acid compositions by GC-MS

The fatty acid compositions of the petroleum ether extracts were determined by GC-MS analysis. As shown in Table 2, eleven components were identified, constituting 99.7% of the petroleum ether extract of *A. leporinus* var. *hirsutus*, and the main constituents were identified as palmitic acid (C16:0) (32.9%), arachidic acid (C20:0) (13.6%) and linoleic acid (C18:2 omega-6) (12.7%). Eleven components were identified, constituting 99.8% of *A. distinctissimus* petroleum ether extract in which palmitic acid (32.5%), linolenic acid (C18:3 omega-3) (17.5%) and linoleic acid (15.9%) were found as major components. Fourteen components were identified, constituting 99.8% of the petroleum ether extract of *A. schizopterus,* and the main constituents were behenic acid (C22:0) (24.5%), palmitic acid (23.4%), arachidic acid (14.0%), stearic acid (C18:0) (8.9%), and oleic acid (C 18:1 omega-9) (8.8%). This is the first report on the fatty acid compositions of these three endemic *Astragalus* species. Palmitic, linoleic and arachidic acids could be chemotaxonomically important for the *Myobroma* section, palmitic, linoleic and linolenic acids for the *Dasyphyllium* section, arachidic and behenic acids for *Proselius* section.

Table 2. GC-MS analysis of ALP, ADP and ASP

Rt (min)[a]	**Constituents**[b]	**Composition (%)**		
		ALP	**ADP**	**ASP**
12.00	Lauric acid	-	2.5	0.3
12.75	Nonanedioic acid	-	-	0.3
14.39	10-Undecenoic acid	2.5	-	1.4
18.60	Myristic acid	2.1	2.7	-
24.94	Palmitoleic acid	2.1	-	0.4
25.27	Palmitic acid	32.9	32.5	23.4
28.47	Margaric acid	-	-	0.3
29.75	Phytol	-	-	1.5
30.64	Linoleic acid	12.7	15.9	6.7
30.77	Oleic acid	9.2	7.8	8.8
30.86	Linolenic acid	5.7	17.5	8.3
31.54	Stearic acid	8.9	7.6	8.9
36.23	Nonacosanol	-	6.7	-
37.38	Arachidic acid	13.6	1.3	14.0
38.19	6-Hexadecenoic acid	-	3.8	-
39.36	Docosane	0.8	-	1.0
43.82	Behenic acid	9.2	1.5	24.5
	Total	99.7	99.8	99.8

[a]Retention time (as minute), [b]A nonpolar Phenomenex DB-5 fused silica colum, ALP: *Astragalus leporinus* var. *hirsutus* petroleum ether extract, ADP: *A. distinctissimus* petroleum ether extract, ASP: *A. schizopterus* petroleum ether extract.

There are some studies on fatty acid compositions of *Astragalus* species from Turkey in literature. Bagci (2006) reported that the main constituents of the fatty acids in *A. echinops* Aucher ex. Boiss., *A. subrobustos* Boiss., *A. jodostachys* Boiss. & Buhse., *A. falcatus* Lam., *A. fraxinifolius* DC. were linolenic acid (23–41%), linoleic acid (23–37%), and oleic acid (8–19%). Adiguzel et al. (2006) reported that the major constituents of the fatty acids were identified as *cis*-9-octadecenoic acid (39.23%) for *A. coadunatus*, 25:0 N alcohol for *A. kurdicus* Boiss., palmitic acid (24.66%) for *A. lagurus* Willd, palmitic acid (21.62%) for *A. christianus* L., 1-docosanol (57.85%) for *A. cicer* L. and palmitic acid (32.60%) for *A. atrocorpus* Champ & Matthews. In Bagci's report (2006), the amount of unsaturated fatty acids was found to be higher than the amount of saturated fatty acids in studied *Astragalus* species. However, our results were in accordance with those of Adiguzel et al. (2006); the amount of saturated fatty acids was found to be higher than that of unsaturated fatty acids.

1.2. Antioxidant activity and total phenolic-flavonoid content

The antioxidant activity of the petroleum ether (ALP, ADP and ASP), acetone (ALA, ADA and ASA), methanol (ALM, ADM and ASM) and water (ALW, ADW and ASW) extracts prepared from the whole plants of *A. leporinus* var. *hirsutus*, *A. distinctissimus* and *A. schizopterus*, respectively, was carried out using β-carotene bleaching, DPPH free radical scavenging, cupric reducing antioxidant capacity and ABTS cation radical decolorisation assays (Table 3). The total phenolic and flavonoid contents of these extracts were also investigated. Total phenolic and flavonoid amounts in the crude extracts were expressed as pyrocatechol and quercetin equivalents, respectively ($y = 0.0123$ pyrocatechol (µg) + 0.0349 ($R^2 = 0.9910$), and $y = 0.1701$ quercetin (µg) – 0.7078 ($R^2 = 0.9939$)). The phenolic and flavonoid components of the ASM extract were identified to be the richest. The amount of total flavonoid from ASM was about 50 µg/mg according to the quercetin standard substance. The phenolic components were found to be higher than flavonoid components. The results were shown in Table 3.

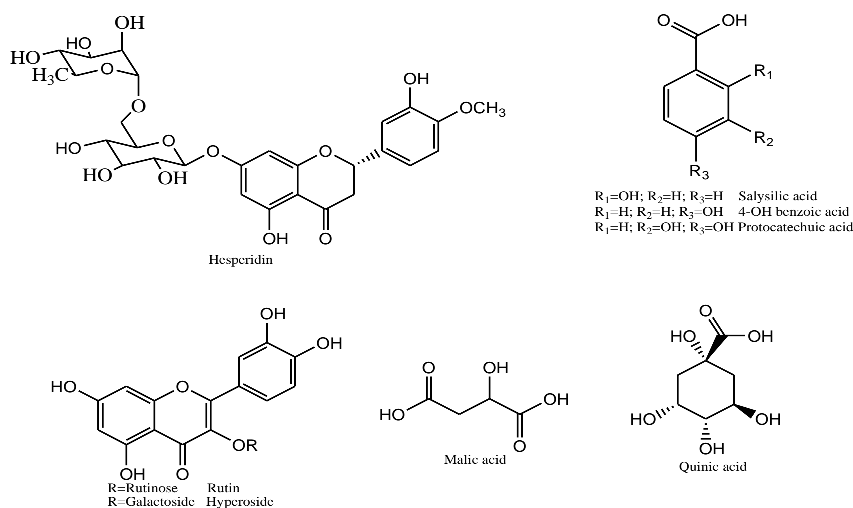

Figure 2. Quantified major compounds from *Astragalus* species

In this study, antioxidant and radical scavenging activities of various extracts of three endemic *Astragalus* species were compared to BHT and α-tocopherol. These comparisons were performed by using various in vitro bioanalytical methods such as; *β*-carotene bleaching, DPPH free radical scavenging, cupric reducing antioxidant capacity and ABTS cation radical decolorisation assays. As it is known from several studies, the abilities of antioxidants that affect diseases are relative to their power to lower DNA damage, mutation, carcinogenesis and bacterial growth. Antioxidant property is a marker for biologicaly active compounds (Roginsky and Lissi, 2005).

Lipid peroxidation includes a series of chain reactions caused free radicals and related to cellular damages. Antioxidants serve a function in inhibition of lipid peroxidation or defence against biological damages caused by free radicals (Dargel, 1992). Besides, the electron donating capacity of bioactive components reflect their reduction power and related to their antioxidant activity. It is a widespread phenomenon that free radical chain reaction is the common mechanism for lipid peroxidation. Radical scavenging substances can directly react with and dispel peroxide radicals in order to terminate chain reactions and develop the quality and stability of food products (Soares et al., 1997). To determine the antioxidant chromogens assays attributed to the use of $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ radicals are amongst the most widely used spectrophotometric methods and free radicals can directly react with antioxidants. Also, $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ scavenging methods are commonly used to determine the antioxidant activities of compounds as they are simple, fast, sensitive and repeatable (Ozcelik et al., 2003). Furthermore, antioxidants could be reductants and inactive oxidants. . On the other hand, another low cost, fast and selective method applied in this study is Cuprac method being founded on the reduction of Cu^{2+} to $Cu^{+.}$ Also, this method can apply to various antioxidants without making a distinction of structure or hyrophilicity (Apak et al., 2004).

While ASA and ASM extracts showed high inhibition of lipid peroxidation (IC_{50}: 26.38±0.74 and 19.62±0.29 µg/mL, respectively) in β-carotene bleaching method, ALA, ADA and ASP extracts exhibited moderate inhibition (IC_{50}: 98.32±0.31, 88.21±1.18 and 61.93±0.82 µg/mL, respectively) (Table 3). ASA and ASM extracts indicated moderate activity in DPPH free radical scavenging activity (IC_{50}: 65.89±0.21 and 54.61±0.38 µg/mL, respectively) (Table 3).

Our results were found to be parallel to the literature. According to Adiguzel et al. (2009), the methanol extracts of the aerial parts of the *Astragalus* species showed slight free radical sweeping effect with 50% inhibition between the concentrations 68.8 and 400.4 µg/mL. However, hexane/dichloromethane extracts of the aerial parts of the *Astragalus* species showed no free radical sweeping effect. While methanolic extracts of the roots of *A. microcephalus* Willd.*, A. macrocephalus* Willd.*, A. erinaceus* Fisch. & Mey. ex Fischer*, A. psoraloides* Lam. ve *A. argyroides* Becker ex Stapf. were slight antioxidants, their non-polar extracts were highly active in DPPH test. Their IC_{50} values were detected as 35.2 µg/mL, 21.0 µg/mL, 22.0 µg/mL, 20.3 µg/mL ve 38.0 µg/mL respectively. Among the studied *Astragalus* species, *A. psoraloides* Lam. extracts showed the strongest inhibitory effect in β-carotene-linoleic acid system.

ALM, ALW, ADM, ADW and ASA extracts indicated moderate activity (IC_{50}: 65.37±0.44, 48.82±0.35, 54.71±0.09, 64.01±0.17 and 71.09±0.63 µg/mL, respectively) in ABTS cation radical scavenging assay (Table 3). However, ASM and ASW extracts exhibited good effects in ABTS cation radical scavenging assay (IC_{50}: 22.01±0.07 and 32.91±0.80 µg/mL, respectively). ASM extract exhibited higher activity (91.22% inhibition) at 100 µg/mL than α-tocopherol (89.14%) and BHT (88.34%), which were used as standards in the ABTS cation radical scavenging assay. The other tested five extracts showed weak or no activity in ABTS cation radical scavenging assay. Our results of ABTS cation radical scavenging assay were parallel to the literature. According to Luo and Fan's (2011) report, the

polar methanolic extracts of the four studied *Astragalus* species showed good cation radical scavenging activity. ASM extract and α-tocopherol exhibited the same activity (22.35±0.12 and 22.94±0.17 $A_{0.5}$ value, respectively) in CUPRAC (Table 3). The other tested extracts showed weak or no activity in CUPRAC. According to the literature search, this is the first study of the cupric reducing antioxidant capacity of the *Astragalus* species.

Table 3. Antioxidant and anticholinesterase activities[a], and total phenolic-flavonoid contents[a] of AL, AD and AS extracts, BHT, α-TOC and galanthamine

Sample	Inhibition % against AChE	Inhibition % against BChE	Phenolic content (μg PEs/mg extract)[d]	Flavonoid content (μg QEs/mg extract)[e]	IC_{50} (μg/mL)			$A_{0.5}$ (μg/mL)
					Lipid Peroxidation	DPPH Free Radical	ABTS Cation Radical	CUPRAC
ALP	46.96± 4.06[a]	66.15± 4.08[a]	-	-	113.21± 0.94[a]	>200[a]	156.82± 1.92[a]	NA[c]
ALA	NA	37.50± 2.46[b]	-	-	98.32± 0.31[b]	127.39± 0.49[b]	109.62±1.28[b]	NA
ALM	NA	19.78± 1.24[c]	82.68± 0.12[a]	15.88±0.33[a]	127.43±0.82[c]	102.62±0.97[c]	65.37±0.44[c]	50.06±0.21
ALW	NA	NA	-	-	>200[d]	>200[a]	48.82±0.35[d]	NA
ADP	17.79± 0.81[b]	41.36± 3.31[b]	-	-	112.63±0.11[a]	>200[a]	183.61±1.39[e]	NA
ADA	NA	19.71± 1.15[c]	-	-	88.21±1.18[e]	142.82±1.93[d]	89.81±0.43[f]	NA
ADM	NA	NA	83.73± 2.81[a]	16.51±0.10[b]	138.41±1.03[f]	101.21±0.27[c]	54.71±0.09[g]	NA
ADW	NA	NA	-	-	>200[d]	>200[a]	64.01±0.17[h]	NA
ASP	13.21± 0.21[c]	46.01± 0.45[d]	-	-	61.93±0.82[g]	129.89±0.17[b]	119.02±0.77[i]	44.22±0.36
ASA	12.11± 0.34[c]	32.13± 0.32[e]	-	-	26.38±0.74[h]	65.89±0.21[e]	71.09±0.63[j]	NA
ASM	NA	17.12± 0.87[c]	89.12± 2.13[b]	50.12 ± 0.07[c]	19.62±0.29[i]	54.61±0.38[f]	22.01±0.07[k]	22.35±0.12
ASW	NA	NA	-	-	111.61±0.49[a]	113.92±0.72[g]	32.91±0.80[l]	NA
Galanth.[b]	85.11 ± 0.69[d]	82.51±0.48[f]	-	-	-	-	-	-
α-TOC[b]	-	-	-	-	15.21±0.09[j]	17.41±0.39[h]	9.54±0.09[m]	22.94±0.17
BHT[b]	-	-	-	-	9.48±0.12[k]	49.91±0.17[i]	10.31±0.14[n]	7.81±0.28

[a]Values expressed are means ± SEM of three parallel measurements ($p<0.05$), [b]Standard drug, [c]NA: Not active, [d]PEs, pyrocatechol equivalents (y = 0.0123 x + 0.0349 $R^2 = 0.9910$), [e]QEs, quercetin equivalents (y = 0.1701 x – 0.7078 $R^2 = 0.9939$)

In general, the result of antioxidant activity tests showed that among the twelve different extract ASM exhibited strongest activity followed by ADM and ALM. High antioxidant activity is mainly due to the phenolic compounds in the extracts. Significant correlations were observed between the total phenolic contents and antioxidant activities of the methanolic extracts (Table 5). The high antioxidant activity of ASM may mainly stem from gallic, chlorogenic, protocatechuic and rosmarinic acid contents in this extract. These compounds had potent antioxidant activity (Table 4) and the highest amounts were found in ASM (Table 1). The results showed that there was a strong negative correlation between antioxidant activity and these phenolic acids in the extracts (Table 6). Moreover, some flavonoids such as rutin, hesperidin, myricetin, hyperoside, quercetin, luteolin, kaempferol and apigenin found in ASM may contributed to its high antioxidant activity (Table 4 and Table 6).

1.3. Anticholinesterase activity

The extracts exhibited enzyme inhibitory activity at various values indicating weak activity except ALP extract which recorded 46.96± 4.06 and 66.15± 4.08 inhibition ratio against acetyl- and butyryl-cholinesterase, respectively. Also, ADP and ASP extracts showed moderate enzyme inhibitory activity against butyryl-cholinesterase with 41.36± 3.31 and 46.01± 0.45 inhibion ratio, respectively. (Table 3). So,we can say that petroleum ether extract more active than other extract in term of cholinesterase enzyme inhibitory activity. Previously, Zengin et. al. (2016) have investigated the enzyme inhibitory effects of ethyl acetate, methanol, and aqueous extracts from *Astragalus lagurus* against cholinesterase, tyrosinase, α-amylase and α-glucosidase and observed the higher enzyme inhibitory effects of ethyl acetate compared to methanol and aqueous extracts. According to Teyeb et. al.(2011), among the *Astragalus gombiformis* extracts the ethyl acetate aerial part extract was found to be the most active extract in terms of anticholinesterase activity with an IC_{50} of 110μg/ml.

Table 4. Antioxidant activity of the main compounds in the methanol extracts and standard compounds

Samples	IC$_{50}$ (µg/mL)		
	Lipid Peroxidation	**DPPH Free Radical**	**ABTS Cation Radical**
Quinic acid	>200	>200	>200
Malic acid	>200	>200	>200
tr-Aconitic acid	>200	>200	>200
Gallic acid	48.41±0.12	7.52±0.28	<<1
Chlorogenic acid	>200	6.33±0.09	3.58±0.05
Protocatechuic acid	48.02±0.21	10.23±1.07	1.82±0.04
Tannic acid	9.14±0.06	5.61±0.30	<<1
Caffeic acid	26.15±1.29	16.82±0.58	2.41±0.08
p-coumaric acid	>200	>200	<1
Rosmarinic acid	12.12±0.02	1.21±0.06	1.70±0.07
Rutin	18.54±0.03	24.18±0.86	2.48±0.06
Hesperidin	>200	>200	5.02±0.03
Hyperoside	>200	5.12±0.31	2.81±0.12
4-Hydroxy benzoic acid	>200	>200	24.38±0.12
Salycilic acid	105.62±0.72	>200	>200
Myricetin	8.92±0.35	9.47±0.12	<1
Quercetin	5.17±0.71	2.79±0.14	1.03±0.09
Luteolin	9.63±0.03	11.83±0.09	3.03±0.03
Kaempferol	8.04±0.06	82.09±0.82	2.82±0.02
Apigenin	5.63±0.08	>200	3.03±0.08
α-TOC	15.54±0.12	18.76±0.41	8.06±0.08
BHT	10.35±0.67	48.86±0.50	10.67±0.11

Values are means ± S.D., n = 3, $p < 0.05$, significantly different with Student's *t*-test

Table 5. Correlation coefficients (r^2) for relationships between phenolic contents and antioxidant activities (IC$_{50}$ value) of methanol extracts

	Phenolic content	Lipid Peroxidation	DPPH Free Radical
Lipid Peroxidation	-0.97222		
DPPH Free Radical	-0.99195	0.994033	
ABTS Cation Radical	-0.99634	0.948644	0.97749

Table 6. Correlation coefficients (r^2) for relationships between quantification of the compounds in methanol extracts and antioxidant activities (IC$_{50}$ value) of methanol extracts

Quantification	Lipid Peroxidation	DPPH Free Radical	ABTS Cation Radical
Gallic acid	-0.99534	-0.99992	-0.97473
Chlorogenic acid	-0.76124	-0.82744	-0.92729
Protocatechuic acid	-0.99999	-0.99347	-0.94704
Tannic acid	-0.43799	-0.33732	-0.13111
Rosmarinic acid	-0.97654	-0.94723	-0.85828
Rutin	-0.89668	-0.84304	-0.71059
Hesperidin	-0.5931	-0.50174	-0.30794
Hyperoside	-0.94806	-0.9077	-0.79874

1.4. Antimicrobial activity

The antimicrobial activity of *A. leporinus* var. *hirsutus*, *A. distinctissimus* and *A. schizopterus* extracts against different microorganisms were assessed according to the inhibition zone diameter. The results of the active extracts were given in Table 7. The methanol extracts showed weak activity (inhibition zone < 12 mm) against bacteria, and moderate activity (inhibition zone < 20-12 mm) against yeast. ADA extract was active against *S. aureus*, *S. pyogenes* and *C. albicans*. On the other hand, ADA extract was found to be not active against gram negative bacteria. The other tested extracts showed no antimicrobial activity. The highest activities were recorded by methanol extracts against *C. albicans* with 15 mm inhibition zone diameter, and the lowest MIC value was recorded by ALM extract against *C. albicans* (20 µg/mL). Adiguzel *et al*. (2009) reported that the methanol and hexane extracts of thirteen *Astragalus* species have no antimicrobial activity against 40 microorganisms including 24 bacteria, 15 fungi and a yeast species, in the current study the antimicrobial screening revealed that the methanol and acetone extracts had inhibitory effect towards bacteria and yeast. According to Pistelli et al. (2002), several extracts of *A. verrucosus* (ethyl acetate, butanol, ethanol) had strong antimicrobial activity against *Aspergillus* and *Botrytis* species and hexane extract had inhibitory effect against *S. aureus*.

1.5. Cytotoxicity activity

MTT assay was used to determine cell viability. Only the medium was applied for control group. Figure 3 shows viability of L929 fibroblast cells and Figure 4 cell viability of A549. In the control group there were no toxicity. At low concentration (12.5 µg/mL), the highest viability was found in ALM extract applied L929 fibroblast cells; however, in the same concentration (12.5 µg/mL), ALM extract applied to A549 cells were found to possess more cytotoxic effect than the other methanol extracts. Briefly, *Astragalus* species contain oleanane- and cycloartane-type glycosides and triterpenoidal saponins as principal constituents besides phenolic compounds. There are few studies on their phenolic contents (Zhang et al. 2013; Qi et al. 2008; Yunfei et al. 2008; Montoro et al. 2012). Thus, the phenolic contents of three endemic *Astragalus* species might be a valuable data in this field.

Table 7. Zones of growth inhibition (mm) and MIC values of AL, AD, and AS extracts compared to positive controls

		Microorganisms				
		Gram positive		*Gram negative*		*Yeast*
		S. aureus	*S.pyogenes*	*E.coli*	*P. aeruginosa*	*C. albicans*
ALA	**[a]DD**	9±0.4	11±0.3	10±0.1	10±0.3	9±0.3
	MIC	250±0.1	1000±0.4	1250±0.2	80±0.2	1000±0.5
ALM	**[a]DD**	10±0.6	10±0.3	11±0.3	10±0.4	15±0.2
	MIC	45±0.5	75±0.5	50±0.1	40±0.7	20±0.1
ADA	**[a]DD**	9±0.2	11±0.2	-	-	12±0.3
	MIC	50±0.2	250±0.3	-	-	100±0.2
ADM	**[a]DD**	10±0.5	11±0.3	11±0.2	10±0.4	15±0.1
	MIC	55±0.3	260±0.2	265±0.7	80±0.3	30±0.4
ASM	**[a]DD**	10±0.5	11±0.2	10±0.4	9±0.5	15±0.2
	MIC	65±0.6	300±0.4	280±0.3	95±0.4	30±0.5
Positive controls	**[b]DD**	35±0.2	19±0.2	20±0.1	-	30±0.3
	MIC	1.95±0.3	7.815±0.1	7.815±0.4	-	3.125±0.2

-: Not active, [a]DD: Inhibition zone in diameter (mm) around the discs (6 mm) impregnated with 30 mg/mL of plant extracts, [b]DD: Inhibition zone in diameter (mm) of positive controls that are ampicillin for bacteria and fluconazole for yeast. Minimum inhibitory concentration (MIC) values are given as µg/mL

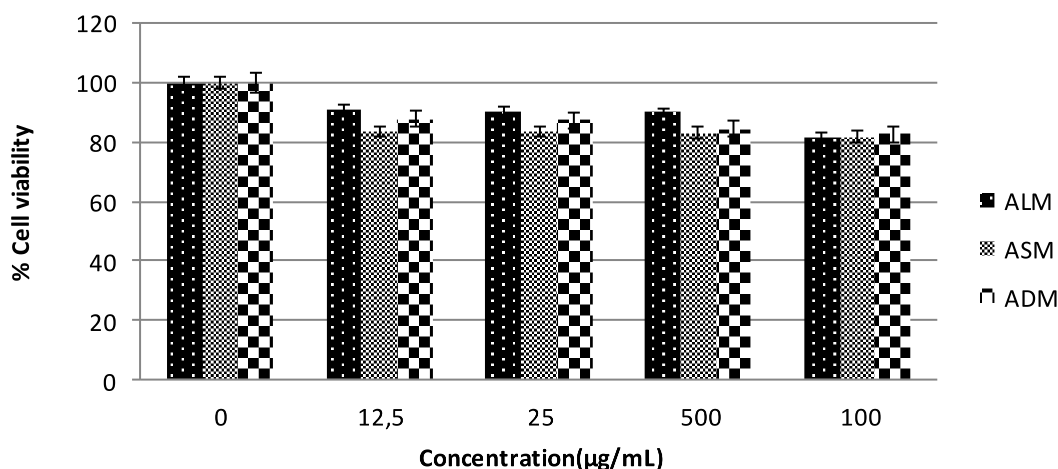

Figure 3. L929 Cytotoxic activity of AL, AD and AS methanol extracts

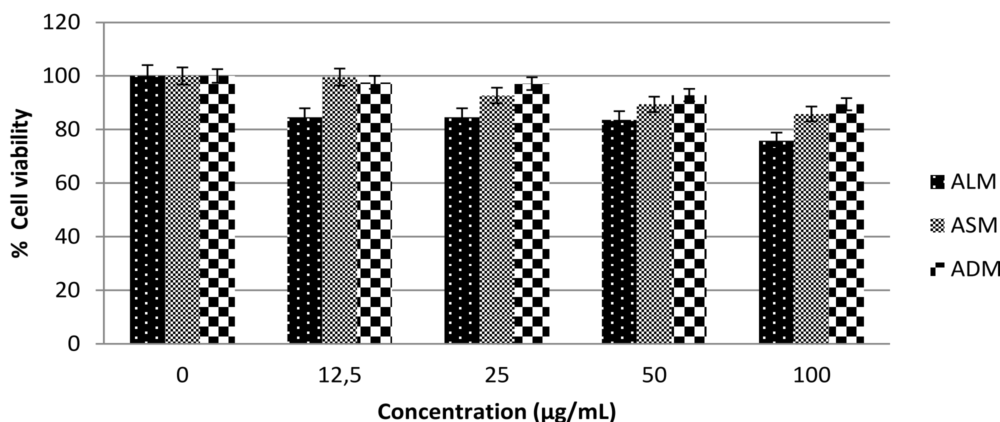

Figure 4. A549 Cytotoxic activity of AL, AD and AS methanol extracts

This study indicates that the different type and quantity of phenolic compounds in three endemic *Astragalus* species grouped into different sections could be of chemotaxonomic importance, along with their fatty acid profiles. Further phytochemical studies on other *Astragalus* species grown in Turkey are needed to confirm this assumption.

The present study showed that *A. schizopterus* methanol extract had strong antioxidant activity in *β*-carotene-linoleic acid test system, DPPH free radical and ABTS cation radical scavenging activities. The antioxidant capacity of *A. schizopterus* methanol extract was the highest among three *Astragalus* species, and that result was in accordance with the total phenolic and flavonoid contents. The reason why the methanolic extract of *A. schizopterus* was the most active of all the twelve extracts tested for four antioxidants methods used, could be related to its high phenolic acids and flavonoid contents. According to our study, rutin and hesperidin were found to be the most abundant flavonoid in three endemic *Astragalus* species. Moreover, naringenin, vanillin, hesperidin, myricetin and protocatechuic, quinic, tr-aconitic and 4-OH benzoic acids were detected for the first time in *Astragalus* species. The high total phenolic and flavonoid content of the methanol extract of *A. schizopterus* showed parallelism to the LC-MS/MS results. In addition, the methanol extract of *A. schizopterus* contained a high level of quinic acid.

The high antioxidant capacity of *A.schizopterus* methanol extract being quite rich with respect to flavonoid content might be related to its high total flavonoid content.

As a consequence, *A. schizopterus* methanol extract which possessed high amounts of quinic acid and total flavonoid content with strong antioxidant capacity could be phytochemically investigated to find their active secondary metabolites.

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Research article/Araştırma makalesi

The bryophyte flora of Baskil district (Elazığ/Turkey)

Mevlüt ALATAŞ [*1], Nevzat BATAN [2]

[1] Munzur Üniversitesi, Mühendislik Fakültesi, Biyomühendislik Bölümü, Tunceli, Turkey
[2] Karadeniz Teknik Üniversitesi, Maçka Meslek Yüksekokulu, Trabzon, Turkey

Abstract

In this study, the bryophyte flora of the Baskil District (Elazığ) was investigated between April and October 2015. In total 54 taxa (1 liverwort, 53 mosses), belonging to 13 families and 29 genera were determined by identifying bryophyte specimens collecting the study area. From these taxa, twenty four taxa are new records for Elazığ province, according to the grid-square system of Henderson (1961) fifteen taxa are new records for B9 grid square. *Pellia epiphylla* has been reported for the first time in Elazığ province with the present paper. While the largest families in terms of number of taxa are Pottiaceae (14), Brachytheciaceae (9), Bryaceae and Grimmiaceae (8), the largest genera are *Syntrichia* (6), *Grimmia* (6) and *Ptychostomum* (4). In addition, the life forms of the taxa which were examined in terms of ecological and floristic have been analyzed. From life forms; while Turf life form ranks the first, Cu life form ranks the second. Most of the taxa within the floristic list in terms of some ecological characteristics are xerophyt, photophyt and subneutrophyt.

Key words: briyofit, flora, Baskil, Elazığ, Türkiye

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Baskil ilçesi briyofit florası

Özet

In this study, the bryophyte flora of the Baskil District (Elazığ) was investigated between April and October 2015. In total 54 taxa (1 liverwort, 53 mosses), belonging to 13 families and 29 genera were determined by identifying bryophyte specimens collecting the study area. From these taxa, twenty four taxa are new records for Elazığ province, according to the grid-square system of Henderson (1961) fifteen taxa are new records for B9 grid square. *Pellia epiphylla* has been reported for the first time in Elazığ province with the present paper. While the largest families in terms of number of taxa are Pottiaceae (14), Brachytheciaceae (9), Bryaceae and Grimmiaceae (8), the largest genera are *Syntrichia* (6), *Grimmia* (6) and *Ptychostomum* (4). In addition, the life forms of the taxa which were examined in terms of ecological and floristic have been analyzed. From life forms; while Turf life form ranks the first, Cu life form ranks the second. Most of the taxa within the floristic list in terms of some ecological characteristics are xerophyt, photophyt and subneutrophyt.

Anahtar kelimeler: bryophyte, flora, Baskil, Elazığ, Turkey

1. Giriş

Türkiye florası; sahip olduğu farklı iklim tipleri (karasal, osiyanik ve Akdeniz), jeolojik ve jeomorfolojik çeşitlilik, zengin su kaynakları (deniz, göl ve akarsu), büyük yükselti farklılıkları (deniz seviyesi- 5000 m), çok çeşitli habitat tipleri ve üç fitocoğrafik bölgenin (Avrupa-Sibirya, Akdeniz ve İran-Turan) buluştuğu konumdan dolayı olağanüstü zenginlik ve çeşitliliğe sahiptir (Özhatay vd., 2005). Bu zenginlik diğer bitki gruplarında olduğu gibi briyofitler üzerinde de etkisini göstermiştir. Son yıllarda Türkiye'de yapılan briyofloristik çalışmalar sonucunda çok sayıda önemli ve kayda değer kayıtlar tespit edilmiş (Kırmacı et al., 2012; Batan and Özdemir, 2013; Abay and Keçeli,

[*] *Corresponding author* / Haberleşmeden sorumlu yazar: Tel.: +904282131794; Fax.: +904282131794; E-mail: mevlutalatas@hotmail.com
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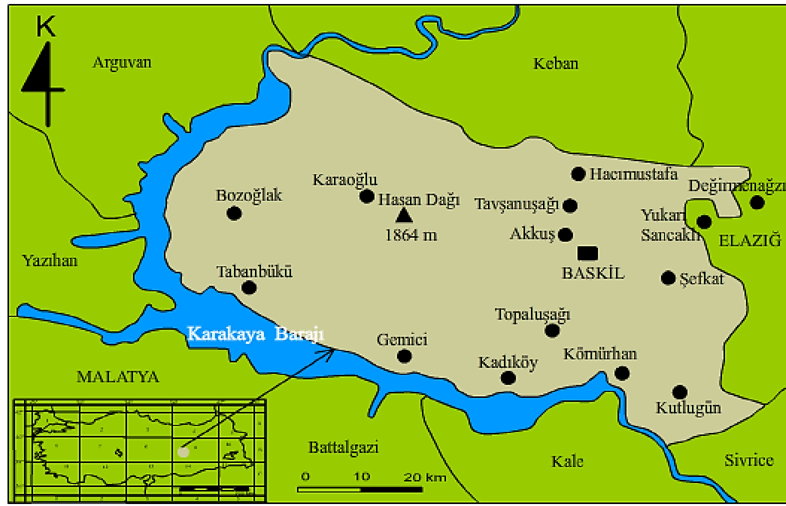
2014; Ören and Keçeli, 2014; Batan et al., 2014; Ezer et al., 2015; Özçelik et al., 2015; Kesim and Ursavaş, 2015; Batan et al., 2016) olmasına rağmen Türkiye briyofit florasının ortaya çıkarılabilmesi için daha çok çalışmaya ihtiyaç vardır. Bu çalışılacak alanların başında yok denecek kadar az çalışmaya sahip Doğu ve Güneydoğu Anadolu Bölgeleri gelmektedir (Alataş vd., 2014; Alataş ve Batan, 2015, 2016). Doğu Anadolu Bölgesinde bulunan Elazığ ilinin, Baskil ilçesinde yapılan bu çalışma ile alanın briyofit florası belirlenerek, Türkiye briyofit florasına bir katkı sağlamak amaçlanmıştır.

1.1 Çalışma Alanı

Doğu Torosların başlangıcında bulunan ve Doğu Anadolu Bölgesinin, Yukarı Fırat Bölümünde kalan Elazığ'ın Baskil ilçesi, doğuda Elazığ ili ve Hazar Gölü, batı ve güneyde Karakaya Barajı ile kuzeyinde ise Keban ve Keban Baraj Gölü ile çevrilidir (Şekil 1). Keban dağ silsilesinin güneyinde kalan Baskil ilçe merkezinin rakımı 1220 metre olup il merkezine olan uzaklığı 38 km'dir. Henderson (1961)'un Türkiye kareleme sistemine göre ise B9 karesi içerisinde bulunan Baskil, İran-Turan fitocoğrafik bölgesinde yer almaktadır (Anşin, 1983).

Yüzey şekilleri bakımından Fırat'a doğru giderek alçalan dağ silsileleri yer yer küçük düzlükleri içine almaktadır. Bunların yanı sıra özellikle Baskil'in batı yönüne doğru oldukça büyük düzlükler de görülmektedir. Doğu Torosların başladığı yerde en yüksek nokta 1864 m yükseltili Hasan Dağı'dır. Doğuya geldikçe uzanan dağ sırası, Baskil demiryolunun geçtiği çöküntü hattından sonra, ilçe dışında kalan Bulutlu'da 2004 m ve Karga dağında ise 1925 m 'dir.



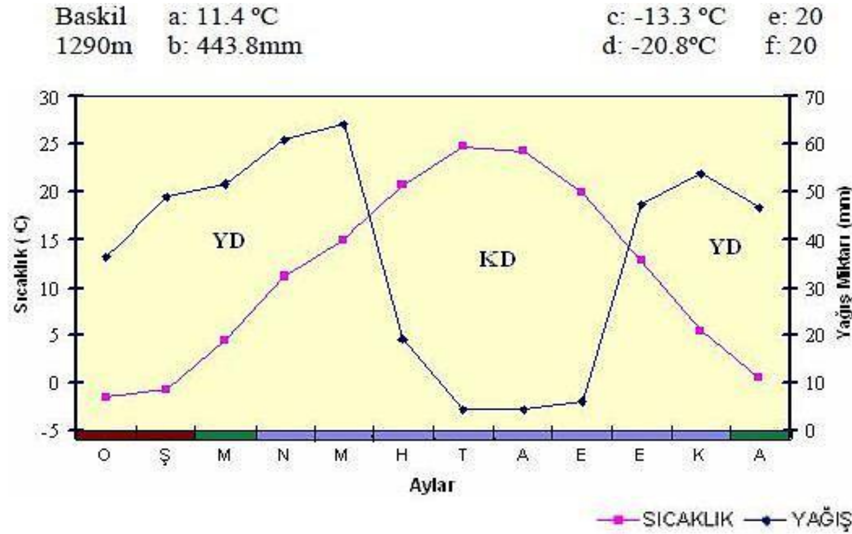
Şekil 1. Henderson (1961)'un Türkiye kareleme sistemi ve çalışma alanının haritası.

Jeolojik yapı olarak, Baskil ilçe merkezinin yer aldığı ova ile ilçenin kuzeyinden başlayıp (Hasan Dağını batıdan bir yay gibi kuşatan yapı) Şelil Dağına kadar devam eden kısım ''Baskil Mağmatitleri'' olarak tanımlanmıştır. (Asutay, 1988). Üst kretase yaşlı, genelde tonalit ve volkanik (andezit bazalt) karakterli yapılardan meydana gelmiştir. İlçenin kuzeyindeki Hasan dağı gri beyaz renkli kireç taşlarından meydana gelmiştir.

Çalışma alanının iklimi, Devlet Meteoroloji İşleri Genel Müdürlüğü'nden Baskil Meteoroloji istasyonuna ait 20 yıllık (e: Sıcaklık, f: Yağış ölçme yılı) verilerin yorumlanması ile elde edilmiştir. Alanda; yıllık ortalama sıcaklık (a) 11,4 ºC, yıllık ortalama yağış (b) 443,8 mm, en soğuk ayın minimum sıcaklık ortalaması -13,3 ºC ve en düşük sıcaklık ise -20,8 ºC'dir. Yağışlı Devrede (YD), Ocak ve Şubat ayları mutlak donlu, Aralık ve Mart ayları ise don olması muhtemel olduğu aylardır. Geriye kalan aylar ise yaz kuraklığının yaşandığı kurak devre (KD) aylarıdır (Şekil 2). Çalışma alanına ait S (Kurak devre) değerinin 0,76 olması alanın Akdeniz ikliminin etkisinde, Yarı Kurak Doğu Akdeniz Biyoiklim katında olduğunu göstermektedir (Akman, 1990; Kurşat vd., 2005). Bununla birlikte Doğu Anadolu Bölgesi'nin karasal iklim özelliklerinden de ayrı düşünemeyeceğimiz çalışma alanımızın etrafında bulunan barajların etkisiyle son yıllarda ikliminde nemlenmeye doğru bir değişme olduğu da görülmektedir.

Baskil ve çevresinde vejetasyon Mart ayı sonu ile Nisan aylarında uyanır. Araştırma alanındaki ormanlar genellikle meşe ve ardıçlardan oluşmuş ormanlardır. Doğal iklim koşulları ve diğer faktörler yüksek dağlık alanların doruk kısımlarında orman yetişmesine imkan vermediğinden, buralar özellikle kurak bir karaktere sahip daha çok dikenli ve yer yer nemli sub-alpin çayırlardan meydana gelen bir bitki örtüsü ile kaplanmıştır. Vadi tabanlarında ılgın, söğüt, kavak ve benzeri gibi nemcil bitki türlerinden oluşan bir topluluk meydana gelmiştir. Araştırma alanında yer alan orman vejetasyonu, kışın yapraklarını döken ağaçlar ile her dem yeşil olan ağaçlardan oluşan meşe-ardıç ormanları yer almaktadır. Alanda genel olarak baskın saf bir orman vejetasyonu hemen hemen yoktur. Seyrek ve pak görünümünde kalıntı orman niteliği taşımaktadır. Genel olarak; Quercus petraea (Mattuschka) Liebl. subsp. pinnatiloba (C. Koch) Menitsky, Pinus sylvestris L., Juniperus oxycedrus L., Prunus domestica L., Cerasus incana (Pallas) Spach

var. incana (Pallas) Spach, Cerasus mahaleb (L.) Miller, Rosa canina L., Fraxinus angustifolia Vahl., Crataegus szovitsii Pojark., Lonicera caprifolium L. ve Cotoneaster nummularia Fısch. Et Mey. Orman vejetasyonunu oluşturan ana elementler iken Astragalus sp.'ler step vejetasyonunu, Centaurium pulchellum (Swartz) Druce., Draba bruniifolia Stev. ve Trifolium arvense L., yüksek dağ vejetasyonunu ve dere boylarındaki Populus nigra L., Salix fragilis L. ve Platanus orientalis L.' ler ise sucul vejetasyonu oluşturan elementlerdir. Bunların yansıra tarım alanlarında yıllık 68000 bin ton üretimi ile başta Prunus armeniaca L. olmak üzere Malus sylvestris (L.) Mill., Pyrus communis L., Amygdalus communis L., ve Prunus avium L. gibi meyve ağaçlarıda bulunmaktadır.



Şekil 2. Baskil meteoroloji istasyonuna ait ombro-termik iklim diyagramı (Kurşat vd., 2005)

2. Materyal ve yöntem

Araştırma materyalini, 2015 yılının farklı vejetasyon dönemlerinde yapılan arazi çalışmalarında, 14 örnekleme noktasından toplanan briyofit örnekleri oluşturmaktadır (Tablo 1). Toplanan örnekler çeşitli flora ve revizyon eserleri kullanılarak teşhis edilmiştir (Hedenäs, 1992; Lewinsky, 1993; Zander, 1993; Smith, 1996, 2004; Kürschner and Frey, 2011). Bitki listesinin hazırlanışı ile geçerli isim ve sinonimlik durumlarının tespitinde Ros et al. (2007), Ros et al. (2013) ve Lara et al. (2016) dikkate alınmıştır. Örneklerin toplandığı habitata ait nemlilik durumu, ışık ve asidite gibi ekolojik özellikler Dierβen (2001), hayat formları ise Hill et al. (2007)'ye göre düzenlenmiştir. Teşhis edilen taksonların Türkiye'deki durumları Uyar and Çetin (2004), Kürschner and Erdağ (2005) ve Ros et al. (2007, 2013) tarafından yayınlanan son kontrol listelerine göre değerlendirilmiştir. Teşhis edilen taksonlar Munzur Üniversitesi, Biyomühendislik Bölümünde araştırmacının kişisel koleksiyonunda muhafaza edilmektedir.

Tablo1. Lokalitelere ait veriler.

Lokalite No	Yükseklik (m)	Tarih	GPS Kaydı	Lokalite
1	1450	23.04.2015	N 38º 35'45. 17", E 038º 55'31. 45"	Baskil Girişi, Değirmenağzı
2	1416	23.04.2015	N 38º 35'18. 63", E 038º 55'21. 07"	Sancaklı
3	1617	24.04.2015	N 38º 37'33. 60", E 038º 50'33. 78"	Tavşanuşağı-Hacımustafa arası
4	1444	24.04.2015	N 38º 36'02. 86", E 038º 49'26. 51"	Akkuş Yolu
5	1266	20.08.2015	N 38º 33'57. 99", E 038º 51'37. 98"	Şefkat
6	929	20.08.2015	N 38º 28'37. 34", E 038º 50'20. 55"	Topaluşağı
7	747	21.08.2015	N 38º 26'33. 15", E 038º 49'11. 84"	Kömürham Köprü Mevkii
8	790	21.08.2015	N 38º 27'21. 43", E 038º 51'15. 21"	Kömürham Kavurma Karşısı
9	752	23.09.2015	N 38º 26'24. 27", E 038º 41'23. 85"	Kadıköy
10	733	23.09.2015	N 38º 28'17. 01", E 038º 35'25. 28"	Gemici Mevkii
11	1039	28.10.2015	N 38º 37'00. 77", E 038º 29'25. 77"	Bozoğlak
12	1178	28.10.2015	N 38º 37'46. 39", E 038º 38'10. 40"	Karaoğlu
13	1472	28.10.2015	N 38º 36'16. 21", E 038º 45'36. 47"	Hasan Dağı Etekleri
14	1660	28.10.2015	N 38º 36'36. 29", E 038º 44'41. 62"	Hasan Dağı

3. Bulgular

Araştırma alanının farklı lokalite ve habitatlarındaki çeşitli substratlardan toplanmış briyofit örneklerinin teşhis edilmesi sonucunda, 13 familya ve 29 cinse ait 54 takson (1 ciğerotu, 53 karayosunu) tespit edilmiştir. Bu taksonlardan 24 tanesi Elazığ ili için, 15 tanesi ise Henderson (1961) kareleme sistemine göre B9 karesi için yenidir. (Tablo 2). Alanda *Pellia epiphylla* (L.) Corda. ciğerotu türünün tespit edilmesiyle Elazığ ilinden ilk kez bir ciğerotu kaydının verilmesi önem arz etmektedir.

Tablo 2. Floristik liste ve taksonların ekolojik özellikleri (LN: lokalite numarası, substrat (A: ağaç, T: toprak, K: kaya, DİT: dere içi taş), N: nemlilik (m: mezofit, h: higrofit, k: kserofit, r: reofit), I: ışıklanma (S: sciofit, f: fotofit), A: asidite (a: asidofit, s: subnötrofit, b: bazifit), HF: hayat formu, Mr (Mat rough, pürüzlü halı), Tf (Turf), Cu (Cushion, yastık), We (Weft, saçak), Tuft (öbek), Ms (Mat smooth, düz halı), Mt (Mat thalloid, talluslu halı), (*): B9 karesi, (+): Elazığ ili için yeni).

Famiyalar	LN	Takson	HF	A	N	I	Substrat				B9	E
							A	T	K	DİT		
		MARCHANTIOPSIDA										
Pelliaceae	1	*Pellia epiphylla* (L.) Corda.	Mt	s	h	S		+		+	*	*
		BRYOPSIDA										
Amblystegiaceae	1,2,9	*Amblystegium serpens* (Hedw.) Schimp.	Mr	a	h	S	+	+				
Brachytheciaceae	2	*Brachytheciastrum velutinum* (Hedw.) Ignatov & Huttunen.	Mr	a	m	S	+					
	5	*Brachythecium albicans* (Hedw.) Schimp.	We	a	m	f		+			*	*
	1	*Brachythecium rivulare* Schimp.	Mr	a	h	S		+				
Bryaceae	1,3,4,8	*Bryum argenteum* Hedw.	Tf	s	k	f		+	+			
	5	*Bryum canariense* Brid.	Tf	s	k	f		+			*	*
	1	*Bryum funkii* Schwägr.	Tf	s	m	f		+			*	*
Hypnaceae	1	*Calliergonella cuspidata* (Hedw.) Loeske.	We	s	h	f		+				*
Amblystegiaceae	2	*Campyliadelphus chrysophyllus* (Brid.) R.S.Chopra	We	s	m	f	+				*	*
Amblystegiaceae	1,2	*Cratoneuron filicinum* (Hedw.) Spruce.	We	b	h	f	+	+				
Pottiaceae	4	*Didymodon acutus* (Brid.) K.Saito.	Tf	b	m	f			+			
	4,8	*Didymodon luridus* Hornsch.	Tf	b	k	f			+			
	4,5,6,8	*Didymodon vinealis* (Brid.) R. H. Zander.	Tf	b	k	f		+	+			
Encalyptaceae	3	*Encalypta ciliata* Hedw.	Tuft	s	k	f			+			
	3,4,8	*Encalypta streptocarpa* Hedw.	Tuft	s	k	f		+	+		*	*
Funariaceae	1,4,8	*Funaria hygrometrica* Hedw.	Tuft	s	m	f		+	+			*
Grimmiaceae	3,4,6	*Grimmia orbicularis* Bruch ex Wilson.	Cu	s	k	f			+			*
	4,6,7,8	*Grimmia ovalis* (Hedw.) Lindb.	Cu	s	k	f			+			*
	3,4,6,8,12	*Grimmia pulvinata* (Hedw.) Sm.	Cu	a	k	f			+			
	6,7	*Grimmia tergestina* Tomm. ex Bruch & Schimp.	Cu	b	k	f			+			*
	3	*Grimmia trichophylla* Grev.	Cu	a	h	S			+		*	*
	6	*Grimmia unicolor* Hook.	Cu	a	m	f			+		*	*
Pottiaceae	4	*Gymnostomum viridulum* Brid.	Tf	b	k	f		+			*	*
Brachytheciaceae	2,4	*Homalothecium philippeanum* (Spruce) Schimp.	Mr	b	k	f	+		+			
Brachytheciaceae	4	*Homalothecium sericeum* (Hedw.) Schimp.	Mr	b	k	f	+					
Bryaceae	1	*Imbribryum mildeanum* (Jur.) J.R. Spence.	Cu	s	m	f		+			*	*
Brachytheciaceae	2	*Kindbergia praelonga* (Hedw.) Ochyra.	We	a	h	S			+			
Orthotrichaceae	2	*Lewinskya affinis* (Schrad. ex Brid.) F.Lara, Garilleti & Goffinet.	Cu	a	m	f	+					
	4	*Orthotrichum cupulatum* var. *riparium* Huebener.	Cu	s	k	S			+		*	*

Tablo 2 (Devam ediyor)

Orthotrichaceae	2	*Orthotrichum pumilum* Sw. ex anon.	Cu	s	k	f	+					
Brachytheciaceae	4	*Oxyrrhynchium hians* (Hedw.) Loeske.	Mr	a	m	f		+				
Amblystegiaceae	2	*Palustriella commutata* (Hedw.) Ochyra.	We	b	h	f		+				
Bartramiaceae	1	*Philonotis calcarea* (Bruch & Schimp.) Schimp.	Tf	b	h	S		+				
Mniaceae	1	*Pohlia wahlenbergii* (F.Weber & D.Mohr) A.L.Andrews.	Tf	a	h	f		+			*	*
Leskeaceae	2	*Pseudoleskeella rupestris* (Berggr.) Hedenäs & L.Söderstr.	Mr	b	k	S		+			*	*
Bryaceae	5,6	*Ptychostomum capillare* (Hedw.) Holyoak & N. Pedersen.	Tf	s	m	f		+				
	8	*Ptychostomum donianum (Grev.) Holyoak & N.Pedersen*	Tf	s	k	S			+		*	*
	1,2,3,5,6,8,9	*Ptychostomum imbricatulum* (Müll. Hal.) Holyoak & N. Pedersen.	Tf	s	m	f	+	+				
	1,2,5	*Ptychostomum moravicum* (Podp.) Ros & Mazimpaka.	Tf	s	m	S	+	+				
Brachytheciaceae	2	*Rhynchostegiella tenella* (Dicks.) Limpr.	Mr	s	m	S	+					
Brachytheciaceae	1	*Rhynchostegium riparioides* (Hedw.) Cardot.	Ms	a	h	S				+		
Grimmiaceae	4,8,11	*Schistidium apocarpum* (Hedw.) Bruch & Schimp.	Tuft	a	k	f			+			
	4,8	*Schistidium confertum* (Funck) Bruch & Schimp.	Cu	a	k	f			+			
Pottiaceae	3,6	*Syntrichia laevipila* Brid.	Tf	s	k	f		+				
	4	*Syntrichia latifolia* (Bruch ex Hartm.) Huebener.	Tf	s	m	S			+			*
	2	*Syntrichia montana* Nees.	Tuft	b	k	f		+				*
	2,3,4,5,6,8,9,11,12,13,14	*Syntrichia ruralis* (Hedw.) F.Weber & D.Mohr.	Tf	b	k	f	+	+	+			
	3,6	*Syntrichia ruralis* var. *ruraliformis* (Besch.) Delogne.	Tf	b	k	f		+	+			
	2,3,4,7,8	*Syntrichia virescens* (De Not.) Ochyra.	Tf	s	k	f	+	+	+			
	4	*Tortella tortuosa* (Hedw.) Limpr.	Tuft	b	k	f			+			*
	1,5,8	*Tortula muralis* Hedw.	Tf	s	m	f		+	+			*
	1	*Tortula muralis* var. *aestiva* Brid. ex Hedw.	Tf	s	m	f		+			*	*
	3,4,5,8,10	*Tortula subulata* Hedw.	Tuft	s	k	f	+	+	+			

4. Sonuçlar ve tartışma

Alanda belirlenen karayosunlarının % 70'i akrokarp, % 30'u ise pleurokarptır. Çoğunluğu kserofit karakterli akrokarp türlerin fazlalığı; alanın Iran-Turan fitocoğrafik bölgesi içerisinde yer alması, alanda kurak bir karaktere sahip daha çok dikenli sub-alpin çayır ile seyrek ve pak görünümünde kalıntı orman vejetasyonunun hakim olması ve Yarı Kurak Doğu Akdeniz Biyoiklim katının etkisi altında kalmasından kaynaklanmaktadır. Alanda bulunan en yaygın türler; *G. pulvinata, B. argenteum*, *D. vinealis, P. imbricatulum, F. hygrometrica* ve *S. ruralis'*dir.

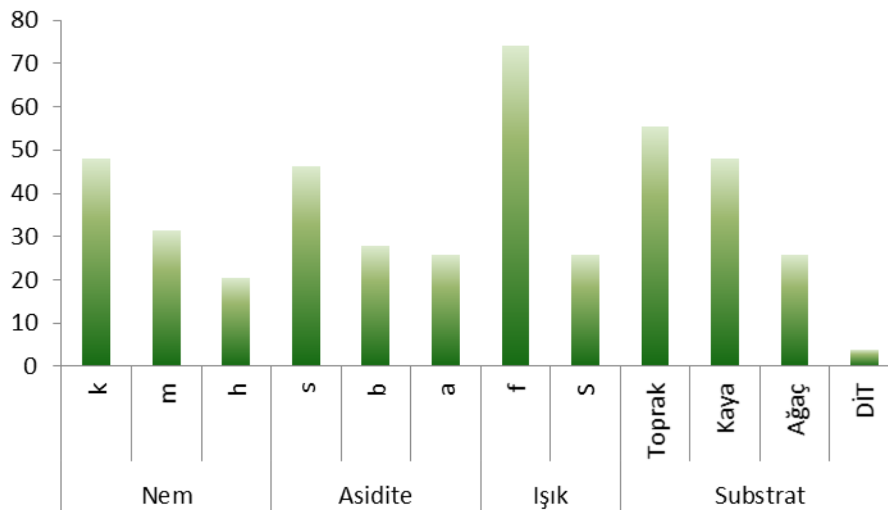
Takson sayısı bakımından en kalabalık familyalar Pottiaceae (14), Brachytheciaceae (9) ve Bryaceae ile Grimmiaceae (8) olup belirlenen taksonların % 71'ini oluşturmaktadırlar. Takson sayısı bakımından en zengin cinsler ise *Syntrichia* ve *Grimmia* (6), *Ptychostomum* (4*), Bryum, Didymodon, Orthotrichum* ve *Tortula* (3)'dür. Bu sonuçlar, alana yakın yapılan Alataş vd., (2014) ve Alataş ve Batan (2015; 2016) çalışmaları ile karşılaştırıldığında, gerek familya ve gerek cins düzeyinde sonuçların benzer olduğu görülmektedir. Bu çalışma, Alataş vd., 2014 çalışmasından (23 ortak takson) ziyade Alataş ve Batan (2015; 2016) çalışmalarına daha benzerdir (Tablo 3). Alataş ve Batan (2016) ile olan benzerliği (27 ortak takson), çalışmaların yapıldığı alanların farklı illerde olmasına rağmen bitişik komşu

olması, Alataş ve Batan (2015) ile daha fazla yakınlık (30 ortak takson) göstermesini ise aynı ilde ve komşu ilçeler olmasının yanı sıra, her iki alanda da görülen aynı iklim çeşitliliği ve benzer karakterli habitatların varlığı ile açıklanabilir. Alanda tespit edilen fakat diğer çalışmalarda olmayan türler ise; *P. epiphylla, B. albicans, B. canariense, B. funkii, C. chrysophyllus, E. streptocarpa, G. trichophylla, G. unicolor, G. viridulum, I. mildeanum, O. cupulatum* var. *riparium, P. wahlenbergii, P. rupestris, P. donianum* ve *T. muralis* var. *aestiva*'dır.

Tablo 3. B9 karesinde yapılan diğer çalışmalar ile bu çalışmamızın karşılaştırılması (ortak taksonlar açısından).

Alataş ve Batan, 2016	Alataş vd., 2014	Alataş ve Batan, 2015
Amblystegium serpens, Brachytheciastrum velutinum, Bryum argenteum, Cratoneuron filicinum, Didymodon vinealis, Encalypta ciliata, Funaria hygrometrica, Grimmia pulvinata, Homalothecium philippeanum, Homalothecium sericeum, Kindbergia praelonga, Lewinskya affinis, Orthotrichum pumilum, Oxyrrhynchium hians, Palustriella commutata, Philonotis calcarea, Ptychostomum imbricatulum, Ptychostomum moravicum, Rhynchostegiella tenella, Rhynchostegium riparioides, Schistidium apocarpum, Syntrichia laevipila, Syntrichia latifolia, Syntrichia virescens, Tortella tortuosa, Tortula muralis	*Amblystegium serpens, Brachytheciastrum velutinum, Brachythecium rivulare, Cratoneuron filicinum, Didymodon acutus, Didymodon vinealis, Grimmia pulvinata, Homalothecium philippeanum, Kindbergia praelonga, Lewinskya affinis, Orthotrichum pumilum, Oxyrrhynchium hians, Palustriella commutata, Philonotis calcarea, Ptychostomum capillare, Ptychostomum imbricatulum, Ptychostomum moravicum, Rhynchostegiella tenella, Rhynchostegium riparioides, Schistidium apocarpum, Schistidium confertum, Tortula subulata*	*Amblystegium serpens, Brachytheciastrum velutinum, Brachythecium rivulare, Bryum argenteum, Cratoneuron filicinum, Didymodon acutus, Didymodon luridus, Didymodon vinealis, Encalypta ciliata, Grimmia pulvinata, Homalothecium philippeanum, Homalothecium sericeum, Kindbergia praelonga, Lewinskya affinis, Orthotrichum pumilum, Oxyrrhynchium hians, Palustriella commutata, Philonotis calcarea, Ptychostomum capillare, Ptychostomum imbricatulum, Ptychostomum moravicum, Rhynchostegiella tenella, Rhynchostegium riparioides, Schistidium apocarpum, Schistidium confertum, Syntrichia laevipila, Syntrichia ruralis* var. *ruraliformis, Syntrichia virescens, Tortula subulata*

Taksonların ekolojik özellikleri ile hayat formları ve yaşam stratejileri değerlendirilirken literatür bilgilerinin yanı sıra arazi gözlemleri de dikkate alınmıştır. Taksonların asiditesine bakıldığında, % 46'sının subnötrofit (pH= 5,7-7), % 28'nin bazifit (pH > 7) ve % 26'sının asidofit (pH < 5,7) karakterde olduğu görülmektedir (Şekil 3).

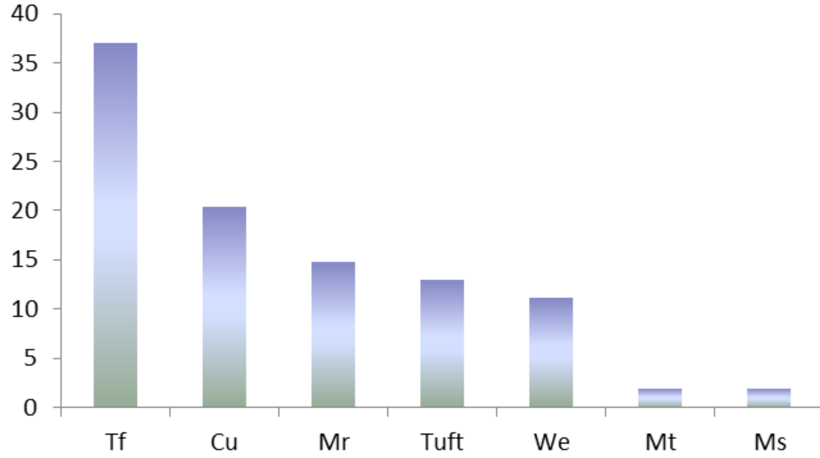


Şekil 3. Taksonların ekolojik tercihleri

Tespit edilen taksonların nem istekleri değerlendirildiğinde ise; % 48'inin kserofit, % 31'inin mezofit, % 20'sinin ise higrofit karakterde olduğu görülmektedir (Şekil 3). Bu sonuçlar alanda görülen iklimle uyumlu olarak çoğunluğunun kurak olduğu, nemli ve yarı kurak mikrohabitatların çeşitliliğini de göstermektedir. Işık isteklerine göre taksonların; % 74'ü fotofit karakterde olup yarı gölgelik ve açık alanlarda yayılış gösterirken, % 26'sı skafit karakterde olup gölgelik alanları tercih etmektedirler (Şekil 3).

Taksonların substrat tercihleri göz önüne alındığında bazı taksonların birden fazla substratda bulunabildiği görülmektedir (Tablo 2). En çok tercih edilen substrat 30 takson ile toprak üzeri olurken 26 takson ile kaya üzeri ikinci sırada yer almaktadır. Bunları 14 taksonla ağaç gövdesi ve 2 taksonla da dere içi taş üzerinden alınan örnekler takip etmektedir (Şekil 3).Toprak ve kaya üzerinden alınan örneklerin çokluğunu, alanın orman varlığının azlığı ve step alanlarının çokluğu ile ifade edilebilir.

Teşhis edilen taksonlar hayat formları açısından değerlendirildiğinde, özellikle akrokarp karayosunlarında dik gametofitlerin birbirine yakın olarak düzenlendiği Tf hayat formu % 37'lik oranla ilk sırada yer alırken, yastık biçiminde koloniler oluşturan Cu % 20'lik oranı ile ikinci, dalların dik, gövdelerin sürünücü olduğu Mr hayat formu % 15'lik oranı ile üçüncü sırada yer almaktadırlar. Bunları; akrokarp karayosunlarında gevşek yastıklar oluşturan Tuft, fazlaca dallanan gevşek örtüler oluşturan We, dalların dik, gövdelerin sürünücü olduğu Ms ve substrat üzerinde sürünen sürgünleri ile bir tallus tabakasından oluşan Mt hayat formları takip etmektedir (Şekil 4). Bu verilere dayanarak, taksonlara ait hayat formları oranlarının, alanda görülen iklim, habitat ve mikrohabitatların çeşitliliği ve özellikleri ile oldukça uyumlu olduğu söylenebilir.



Şekil 4. Taksonların hayat formları

Sonuç olarak, briyofit florası bilinmeyen Baskil ilçesinin listesi çıkarılarak; B9 karesi için 15, Elazığ ili için ise 24 yeni takson belirlenmiş olup, Türkiye briyofit florasına oldukça önemli katkılar sağlanmıştır.

Teşekkür

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Research article/Araştırma makalesi

Morpho-anatomical investigations on *Ajuga postii* Briq and *Ajuga relicta* P.H.Davis

Emel SÖNMEZ *[1], Yavuz Bülent KÖSE [1]

[1] Anadolu University, Faculty of Pharmacy, Department of Pharmaceutical Botany, 26470 Eskişehir, Turkey

Abstract

In the present study morphological and anatomical features of *Ajuga postii* and *Ajuga relicta* (Lamiaceae) which are local endemic for Turkey have been carried out. Corolla of *A. postii* is dark purple-pink (drying purplish) and upper lip obsolescent or lost; corolla of *A. relicta* is cream colored when dried yellowish-beige and upper lip 1 mm, bidentate to the base. Anatomical verities have been represented in cross sections of roots, stems, and leaves of the species. *A. relicta* has been observed without trichomes and *A. postii* with sparsely non-glandular trichomes on the stems. In root cross sections of the species, sclerenchyma cells have been observed in the cortexes. In the leaf cross-sections, glandular trichomes have not been observed, only non-glandular trichomes have been determined.

Key words: *Ajuga,* morphology, anatomy, local-endemic, Turkey

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***Ajuga postii* Briq ve *Ajuga relicta* P.H.Davis üzerine morfolojik-anatomik bir araştırma**

Özet

Yapılan çalışma ile Türkiye'de lokal endemik türler olan *Ajuga postii* ve *Ajuga relicta* (Lamiaceae)'nın morfolojik ve anatomik özellikleri belirlenmiştir. Buna göre *A. postii*'nin korollası koyu pembe-mor (kuruyunca morumsu) renkli, üst dudak eksilmiş veya kaybolmuş; *A. relicta'*nın korollası krem rengi kuruduğunda ise sarımsı-bej rengi, üst dudak 1 mm, tabanda iki dişlidir. Anatomik özellikler çalışılan örneklerin kök, gövde ve yaprak anatomik kesitleri üzerinden verilmiştir. *A. relicta'*nın gövdesi üzerinde herhangi bir tüy oluşumu görülmezken, *A. postii'*nin gövdesi üzerinde seyrek olarak örtü tüyleri gözlenmiştir. Çalışılan taksonların kök anatomik kesitlerinde sklerenkima hücreleri korteks tabakasında belirgindir. Yaprak enine kesitlerde salgı tüyleri gözlenmezken, örtü tüyleri gözlenmiştir.

Anahtar kelimeler: *Ajuga*, morfoloji, anatomi, lokal-endemik, Turkey

1. Introduction

Lamiaceae is represented by 236 genera and about 7,200 species in the worldwide (Heywood et al., 2007). In Turkey, Lamiaceae is the third largest plant family, is represented by 46 genera and 580 species, 260 species are endemic, and endemism rate of the family is about 44% (Davis, 1982; Davis et al., 1988; Güner et al., 2000; Dirmenci, 2012).

Ajuga L. is located in the subfamily Ajugoideae of Lamiaceae. Ajugoideae has four-lobed drupa and nonpermanent stylus (Judd, 2008). Members of the *Ajuga* are grown in Europe, Asia, Africa, Australia and North America. *Ajuga* species are used in traditional medicine; in Uganda tea of *A. alba* (Gürke) Robyns is used for cold and tiredness (Hamill et al., 2003); in Morocco *A. iva* (L.) Schreber is used as anthelminthic and for gastrointestinal disorders and breast cancer (Ziyyat et al., 1997; Ouhaddou et al., 2014); in Kenya *A. remota* Benth. is used against to malaria and diabetes (Keter and Mutiso, 2012). In Turkey, *A. orientalis* L. is used for hemorrhoids (Güneş and Özhatay, 2011) and skin diseases (Koyuncu et al., 2010); *A. chamaepitys* (L.) Schreber is used for wound healing (Tümen et al., 2006), hemorrhoids, as diuretic and antivenom (Sarac and Ugur, 2007). *A. chamaepitys* (L.) Schreber subsp. *chia*

* *Corresponding author* / Haberleşmeden sorumlu yazar: Tel.: +902223350580/3703; Fax.: +902223350750; E-mail: emels@anadolu.edu.tr
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(Schreber) Arcangeli var. *chia* (Schreber) Arcangeli is used for hemorrhoids and wound healing (Kılıç and Bagci, 2013).

Ajuga is represented with 13 species and 10 subspecies in Turkey (Güner et al. 2012). *Ajuga postii* Briq and *Ajuga relicta* P.H. Davis are endemic species of Turkey. The studied species distribute between 1350-1500 m.

In the Flora of Turkey (Davis, 1982), localities of *A. postii* are shown as Hatay and Mersin provinces. However, the species was not found in Hatay. On the other hand, type locality of *A. relicta* is demonstrated on Kahramanmaraş - Ahir Mountain, but like *A. postii* the species was not found determined locality, however, *A. relicta* was rediscovered close to the stated locality (Kahramanmaraş - Çimen Mountain) (Varol et al., 1998). Restricted localities and endemic features of the species make the present study crucial for the future perspective. The aim of the study is to determine morphological and anatomical features of *A. postii* and *A. relicta* which are local endemics for Turkey flora.

2. Materials and methods

2.1. Plant materials

A. postii was collected from İçel: Çamlıyayla, Namrun Castle, 37° 16.8’ 54.5’’ N 34° 60.1’ 16.5’’ E, 1350 m, 10.07.2015 and *A. relicta* was collected from Kahramanmaraş: Çimen Mountain, Yavşan Hill, Pekmezpınarı, , 37° 28’ 42’’ N 36° 42’ 21’’ E, 1500 m, 03.06.2015. Localities of the species have been shown in Figure 1. Collected samples are stored at Anadolu University Faculty of Pharmacy Herbarium (ESSE no 15083-15084).

Figure 1. Localities of collected Ajuga species.

2.2. Morpho-Anatomical studies

Morphological features of the herbs were determined from different plant parts. At least 10 plant samples were used for average measurements. Anatomical structures were studied on the roots, stems, and leaves. Plant materials were maintained in the 70% alcohol, were stained with Sartur reagent (60 ml lactic acid, 45 ml Sudan III, 2 gr aniline, 0.2 gr I, 1 gr KI, 10 ml alcohol (95 %) and 80 ml distilled water), and chloral hydrate. The permanent preparates were prepared with glycerine-gelatine. Photographs were captured with Zeiss AXIO Lab. A1 binocular light microscope and Axiocam ERc 5s camera.

3. Results

Strongly perennial herb, 77-84 cm with a short woody rhizome. Stems virgate, simple or branched above, pilose on angles. Cauline and floral leaves shortly petiolate, leaves ovate-lanceolate, 3.5-10 × 1-4 cm, entire or irregularly serrate or dentate with sparsely villose, base attenuate, apex acute. Flower 2 per verticillaster, large, c. ½ as long as floral leaves. Calyx 8-12 mm, divided to ½ into lanceolate 5 teeth, glandular-villous teeth or glabrous. Corolla dark purple-pink (drying purplish), 24-50 mm, upper lip obsolescent or lost, emarginate; lower lip 3 x tube, resupinate, densely pubescent (Figure 2 and 3). Stamen 4, 13-18 mm, filament simple, trichomes lanate-villose. Stylus 22-24 mm, glabrous, not gynobasic, inserted well above the base of ovary lobes, bifid stigma. Fruit baccate, obscurely 4-lobed, c.3-6 mm with a fleshy purplish-black pericarp (Table 1).

Figure 2**.** Habit of *A. postii*

Figure 3. Flowers of *A. postii*

Table 1. Morphological results comparison of A. postii in present study and the study of Flora of Turkey (Davis, 1982).

Characteristic		**Flora of Turkey (Davis, 1982)**	**Present study**
Habit		Perennial	Perennial
Plant length		c. 1 m	77-84 cm
Stem	Branching	simple or branched above	simple or branched above
	Indumentum	with long trichomes on the corners	with long trichomes on the corners
Cauline leaves	Size (cm)	1.5-4 x 4-10	3.5-10 x 1-4
	Base	-	attenuate
	Apex	-	acute
	Margin	entire or irregularly serrate or dentate	entire or irregularly serrate or dentate
	Indumentum	with rare long trichomes	with rare long trichomes
Verticillasters	Flowers	2	2
Bracts	Size (cm)	1.5-4 x 4-10	3.5-10 x 1-4
	Shape	ovate- lanceolate	ovate- lanceolate
	Margin	entire or irregularly serrate or dentate	entire or irregularly serrate or dentate
	Apex	-	acute
Calyx	Length (mm)	8-11	8-12
	The shape of teeth	-	lanceolate
	The apex of teeth	-	acute
	Indumentum	teeth with glandular hairs	with glandular hairs or glabrous
Corolla	Color	pink, when dried purple	dark purple, pink
	Length (mm)	30-50	24-50
	The shape of lobe	reduced upper lip	reduced or lost upper, resupinate lower lip
	Indumentum	-	inner side with lanate-villous trichomes
Stamen	Length (mm)	-	13-18
Filament	Indumentum	-	lanate-villous
Style	Length (mm)	-	22-24
	Indumentum	-	glabrous
Stigma	Type	-	bifid
Nutlet	Width (mm)	c.6	3-4

Distribution and ecology: *A. postii* is endemic to Turkey and grows only in *Fagus* L. forest, *Quercus* L. macchie and rocky mountain slopes elevations from 60-1600 m, Eastern Mediterranean element (Davis, 1982).
Phenology: Flowering from June to July.

3.2. Anatomy of *Ajuga postii*

Root: In the cross sections of the root, in the outer layer of the root, periderm tissue has been observed with 2-3 layers of flattened cells. Beneath the periderm, the cortex is multi-layered with parenchymatous and polygon-shaped cells. Sclerenchymatous cells take a large place in the cortex. The cambium which is generally located between phloem

and xylem cells is indistinguishable. Regular trachea and tracheid cells are located in xylem tissue. The pith of the root is located with 1 to 2 layers of primary pith rays between the secondary xylem cells (Figure 4).

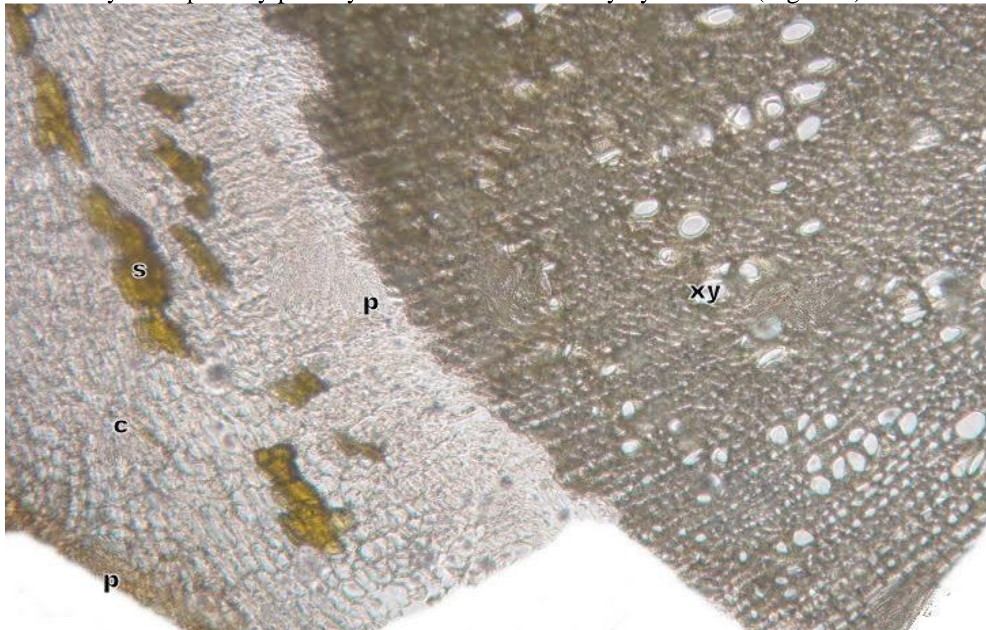


Figure 4. The cross section of the root of *A. postii* p: Periderm, c: Cortex, s: Sclerenchyma, p: Phloem, xy: Xylem

Stem: *A. postii* has the typical square stem of Lamiaceae. The stem epidermis is located monolayered, and the outer layer is covered with the cuticle. Beneath the epidermis, 5-6 layered of collenchyma is observed on the corners, and with 1-2 layers between the corners. Cortex has been observed with oval and parenchymatous shaped cells. In the cross section of the stem, endodermis and cambium layers were not observed. Although phloem is located with 2 to 3 layered cells, and xylem is taken larger place than phloem. The pith region is placed with large polygon shaped and parenchymatous cells (Figure 5).

Figure 5. The cross section of the stem of *A. postii* e: Epidermis, c: Cortex, p: Phloem, xy: Xylem

Leaf: In the leaf cross section, the epidermis is located uniseriate on the adaxial and abaxial sides. Epidermis is covered with the thin layered cuticle. The trichomes are non-glandular and are located with 1 to 5 cells (Figure 7). Palisade parenchyma and spongy parenchyma cells are not distinguished to each other. The collateral vascular system is located in the midrib region, and the outer of the phloem cells sclerenchyma is observed (Figure 6). The leaf is bifacial and the stoma is diasitic (Figure 8). The stoma is only observed on the lower surface of the leaf.

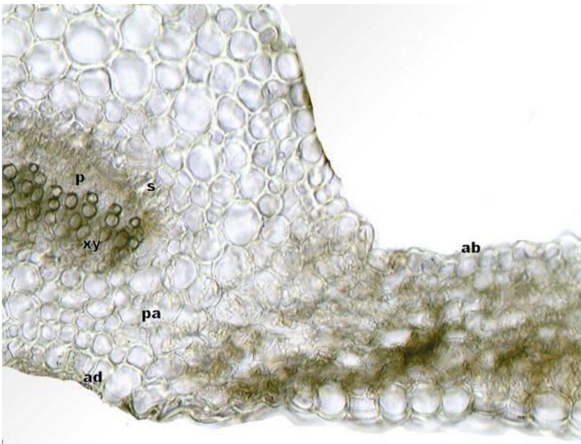


Figure 6. The cross section of the leaf of *A. postii* ad: Adaxial epidermis, ab: Abaxial epidermis, pa: Parenchyma, s: Sclerenchyma, p: Phloem, xy: Xylem

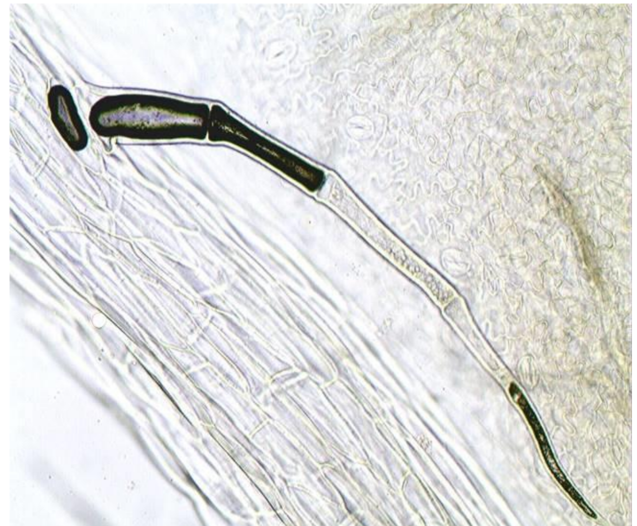
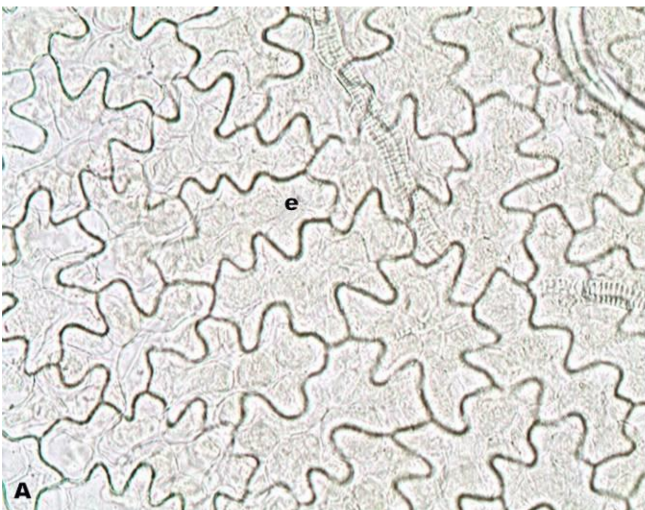


Figure 7. Non-glandular hair of the leaf of *A. postii*



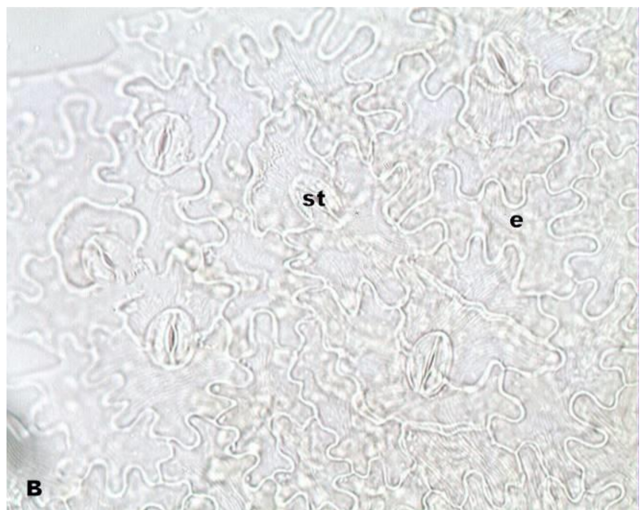


Figure 8. The surface sections of the leaf of *A. postii* (A) Adaxial surface (B) Abaxial surface e: Epidermis , st: stoma

3.3. Morphology of Ajuga relicta

Perennial herb, 23-49 cm. Stem strong, erect, shortly branched in the upper part. Leaves lanceolate (at least on axillary

shoots), glabrous, base leaves lanceolate, attenuate, edges entire or rarely toothed, apex acute, glabrous, 3-4.6 × 0.9-1.2 cm. Those on the main stem with petioles triangular and amplexicaul at the base (lamina withered); leaves on axillary shoots narrower, entire or rarely toothed, apex acute, glabrous 4-8.5 × 0.8-3.3 cm, attenuate at the base and subsessile. Inflorescence terminal, spike-like 3-14 cm, axis glandular-villous, verticillasters densely 6-14 (16) flowered, at least lower ones subtended by leaf-like bracts, upper shorter. Bracts lanceolate, base semi-amplexicaul, edges entire, apex acute, glabrous, 1.7-5.0 × 1.0-1.2 cm. Calyx lanceolate, 7-11 mm, divided to ½ into glandular-villous narrowly lanceolate 5 teeth; apex acute-cuminate, gray lanate-villose. Corolla 11-19 mm, cream colored, yellowish-beige when dried, tube slender and twice as long as 3-lobed lower lip; upper lip 1 mm, bidentate to base, gray lanate-villose (Figure 9 and 10). Stamen 4, filament lanate-villose, 4-7 mm. Stylus 10-14 mm, not gynobasic, stigma bifid. Fruit nutlet, reticulate-rugulose, 1-1.5 mm, dark yellow (Table 2).

Figure 9. *A. relicta*

Figure 10. *A. relicta* flowers

Table 2. Compared data of *A. relicta* with description of Flora of Turkey

Characteristic		Flora of Turkey (Davis, 1982)	Present study
Habit		Perennial	Perennial
Plant length (cm)		more 30	23-49
Stem	Branching	shortly branched above	shortly branched above
	Indumentum	glabrous	glabrous
Base leaves	Size (cm)	-	3-4.6 x 0.9-1.2
	Shape	lanceolate	lanceolate
	Base	attenuate	attenuate
	Margin	-	entire or rarely dentate
	Apex	-	acute
	Indumentum	glabrous	glabrous
Cauline leaves	Size (cm)	2.5-5	4-8.5 x 0.8-3.3
	Base	amplexicaul	amplexicaul
	Apex	-	acute
	Margin	-	entire or rarely dentate
	Indumentum	glabrous	glabrous
Verticillasters	Flowers	6-12	6-14(16)
	Length (cm)	-	3-14
Bracts	Size (cm)	-	1.7-5 x 1-1.2
	Shape	-	lanceolate
	Margin	-	entire
	Apex	-	acute
	Base	-	semi-amplexicaul
Calyx	Length (mm)	8-11	7-11
	The shape of teeth	lanceolate	lanceolate
	The apex of teeth	-	acute-cuminate
	Indumentum	-	grey lanate-villous
Corolla	Color	-	cream, when dried yellowish-beige
	Length (mm)	c. 16	11-19
	Number of lobe	3-lobed lower lip	3-lobed lower lip, reduced upper lip (2 dentate)
	Indumentum	-	grey lanate-villous
Filament	Length (mm)	-	4-7
	Indumentum	-	lanate-villous
Style	Length (mm)	-	10-14
	Indumentum	-	glabrous
Stigma	Type	-	bifid
Nutlet	Shape	reticulate-rugulose	reticulate-rugulose
	Size (mm)	3.2-1.5	1-1.5

Distribution and ecology: *A. relicta* is endemic to Turkey and grows only in open areas of *Cedrus libani* A. Rich. and *Abies cilicica* Ant. & Kotschy Carriere an elevation 1500 m. It is Eastern Mediterranean element (Davis, 1982).

Phenology: Flowering from June to July.

3.4. Anatomy of *Ajuga relicta*

Root: The outermost layer of the root is located with disrupted cells of the phellogen. Under the phellogen, several layered phelloderm is observed. Beneath the periderm, the multi-layered cortex stays with oval and parenchymatous cells. Sclerenchyma cells are observed in the cortex layer. Similarly *A. postii* between phloem and xylem cells, cambium has not been observed. Beneath the phloem, xylem is composed of the ordinate trachea and tracheid elements and xylem cells are extended to the pith. Pith region is located with 1 to 2 layers of primary pith rays between the secondary xylem cells (Figure 11).

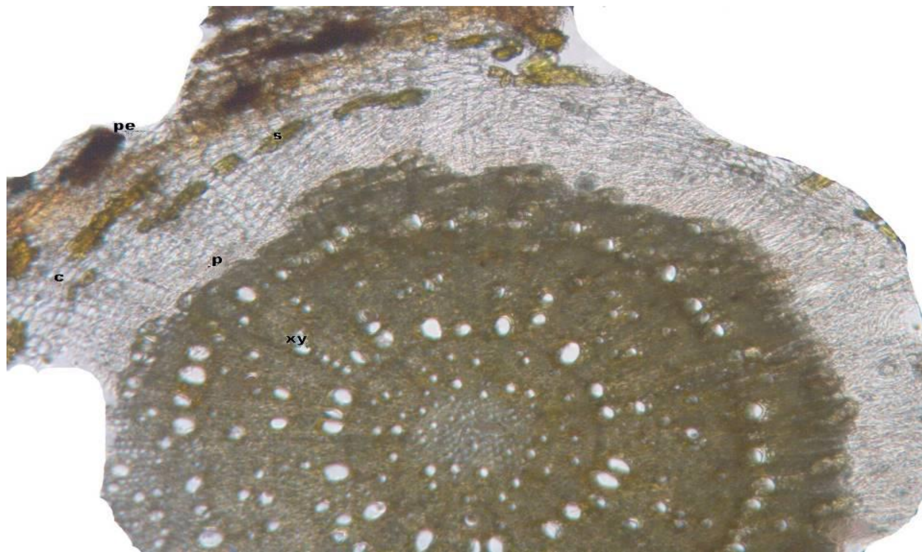


Figure 11. The cross section of the root of *A. relicta* pe: Periderm, c: Cortex, s: Sclerenchyma, p: Phloem, xy: Xylem

Stem: *A. relicta* has been observed with the quadrangular stem of Lamiaceae. Transverse section of the stem, epiderma is located with 1 to 2 layered cells with a thin layered cuticle. Beneath the epiderma, collenchyma cells are multilayered on the corners and are monolayered between the corners. The cortex is stated with parenchymatous cells, and sclerenchyma cells are observed on the phloem. In stem cross section of *A. relicta*, the vascular system is well developed, phloem consists of amorphous and oval-shaped cells, and xylem is located with round, oval and polygonal shaped cells. Cambium layer is not definite between phloem and xylem cells. Pith region occurs large, polygon shaped, rounded parenchymatous cells. (Figure 12).

Figure 12. The cross sections of the stem of *A. relicta* e: Epidermis, c: Cortex, s: Sclerenchyma, p: Phloem, xy: Xylem

Leaf: In the crosscut section of the leaf, adaxial and abaxial sides of the epidermis are located with 1-2 layered of flattened epidermal cells. Non-glandular hair has been observed on the edge of the leaf with 4-5 cells (Figure 14). The vascular system is the collateral type and is located in parenchymatous cells. Xylem is located in the upper epidermis side and phloem stays in the lower epidermis side. Sclerenchyma cells are located above the phloem (Figure 13). The stoma is diasitic and the leaf is bifacial. The stoma is presented on upper and lower surfaces of the leaf (Figure 15).

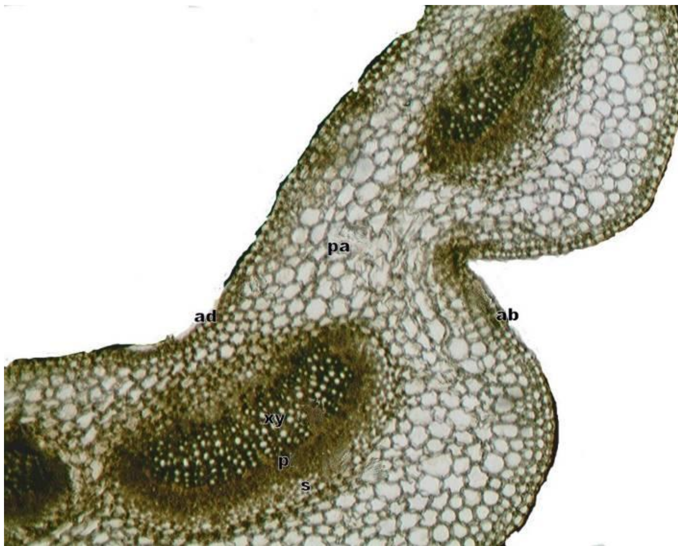


Figure 13. The cross section of leaf of *A. relicta* ad: Adaxial epidermis , ab: Abaxial epidermis , pa: Parenchyma, s: Sclerenchyma, p: Phloem, xy: Xylem

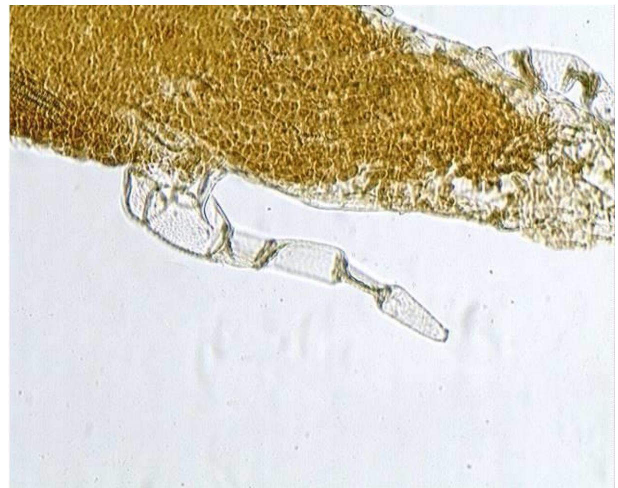
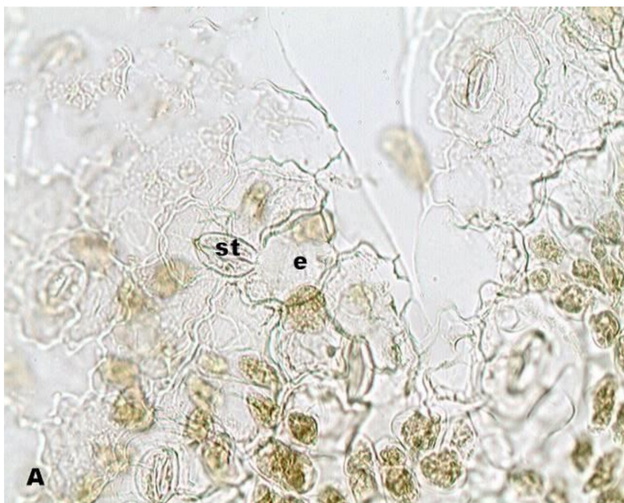


Figure 14. Non-glandular hair of *A. relicta* on the edge of the leaf



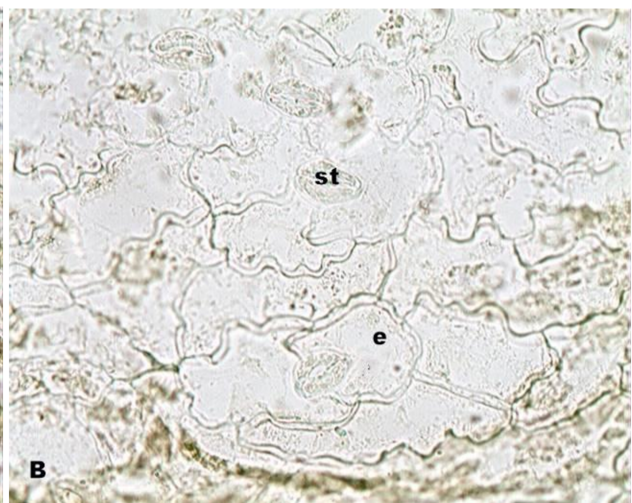


Figure 15. The surface sections of the leaf of *A. relicta* (A) Adaxial surface (B) Abaxial surface. e: Epidermis cell, st: stoma

4. Conclusions and discussion

In the present study morphologies of *A. postii* and *A. relicta* have been compared with Flora of Turkey (Davis, 1982), and anatomical structures of the two species have been studied for the first time.

In the present study, *A. postii* plant length has been found between 77-84 cm, however, in the Flora of Turkey (Davis, 1982), the length of the plant was shown about 1 m. In the present study, lengths and widths of the cauline leaves have been determined as 3.5-10 x 1-4 cm, on the other hand in the description of Flora of Turkey (Davis, 1982) measures were given as 4-10 x1.5-4 cm. The base of the leaves has been determined to attenuate, and the apex of the leaves is acute in the present study, the specified features were not determined in the Flora of Turkey (Davis, 1982). Bract sizes are as similar as cauline leaves in the both present study and Flora of Turkey (Davis, 1982). The apex of bracts has been determined as acute in the present study, the feature was not determined in the Flora of Turkey (Davis, 1982). Calyx teeth shape and apex have been determined respectively as lanceolate and acute in the present study for

the first time. Corolla length has been found between 24-50 mm in the present study, however, in the Flora of Turkey (Davis, 1982) the length was determined between 30-50 mm. In the present study, the indumentum of corolla has been determined as the lanate-villous inner side of the corolla, in the Flora of Turkey (Davis, 1982) the feature was not found. In the present study, stamen length, indumentum of the filaments, the length of style, and indumentum feature have been determined for the first time. Stamen length is between 13-18 mm, indumentum of the filaments is the lanate-villous, the length of style is between 22-24 mm, and indumentum of the style is glabrous.

Morphological features of *A. relicta* have been compared with Flora of Turkey (Davis, 1982). In the present study, the plant length has been determined between 23-49 cm, however, the length was given more than 30 cm in Flora of Turkey (Davis, 1982). The size of the base leaves is found 3-4.6 x 0.9-1.2 cm, the margin is entire or rarely dentate, and the apex is acute in the present study, however, the measures, margin and the apex of the base leaves were not determined in Flora of Turkey (Davis, 1982). The size of the cauline leaves has been determined to 4-8.5 x 0.8-3.3 cm, on the other hand in the Flora of Turkey (Davis, 1982) only the length of the leaves was given and was determined as 2.5-5 cm. The margin and the apex of the cauline leaves have been determined respectively entire or rarely dentate, and acute in the present study. The number of the verticillaster was shown as 6-12 in the Flora of Turkey (Davis, 1982), however, in the present study, the number is changed from 6 to 16, the length of the verticillaster has been found 3-14 cm. Features of the bracts were not determined in the Flora of Turkey (Davis, 1982), however in the present study size, shape, margin, apex and base of the bracts have been determined. The other missing features of *A. relicta* have been determined in the present study, the apex of calyx teeth is acute-cuminate, indumentum of calyx is grey lanate-villous, corolla color is cream, indumentum of corolla is grey lanate-villous, length of filament is 4-7 mm, indumentum is lanate-villous, length of style is 10-14 mm, and indumentum is glabrous. In the Flora of Turkey (Davis, 1982), the length of corolla was determined as 16 mm, however, in the present study, the length has been found between 11-19 mm.

The investigated plant species are thought to be relict endemic, with regards to climatic diversity and sheltered sides of Turkey during the glacial periods "Şekercioğlu et al., 2011". Therefore, the study is important to identify the species. Studied two species are located in Lamiaceae, and the family is specific with trichomes (Navarro and El Oualie, 2000), and glandular hairs (Metcalfe and Chalk, 1950), however in the present study *A. relicta* has been observed without trichomes on the stem, base-cauline leaves, and bracts. *A. postii* has sparsely non-glandular trichomes on the stem, cauline leaves, and bracts. *A. relicta* has been investigated with trichomes on the calyx, corolla, and filaments, and *A. postii* has been observed with trichomes on the calyx teeth, and the inner side of the filaments and corolla (Table 1 and 2). Anatomical features of *A. postii* and *A. relicta* have been studied for the first time in the present study. *A. postii* and *A. relicta* are perennial herbs and both species have been observed with periderm layers on the outer side of the cross sections of the roots, the same result was determined on the root cross section of *A. reptans* L. (Erkara and Koyuncu, 2009). In the present paper, the both roots of the species have been observed with sclerenchymatous cells in the cortexes, and indefinite cambium layers between phloem and xylem.

Anatomical features of Lamiaceae were determined by Metcalfe and Chalk (1950), according to the study, Lamiaceae members are specific with square shape of the stem, and collenchyma cells on the corners. The leaf of Lamiaceae was detected as dorsiventral (Metcalfe and Chalk, 1950), in the present study, leaves of the studied species have been determined dorsiventral like the previous study, however palisade and spongy parenchyma cells of the mesophyll of *A. postii* are not differentiated, the same result was observed on *A. reptans* (Erkara and Koyuncu, 2009). Stomata of Lamiaceae members were determined on one or both two sides of the leaves (Metcalfe and Chalk, 1950), in the present study, the similar results have been observed. Stoma structure of *A. postii* has been only observed on the abaxial side of the leaf; on the other hand, *A. relicta* are located with the stomata on both two leaf sides like *A. orientalis* (Çalı, 2014), *A. chamaepitys* subsp. *chia var. chia* "Akçın et al., 2006", and *A. reptans* (Erkara and Koyuncu, 2009). Stomata of Lamiaceae are specific with diasitic types (Metcalfe and Chalk, 1950), the same results have been determined in the present study, and *A. orientalis* (Çalı, 2014), *A. chamaepitys* subsp. *chia var. chia,* and *A. reptans* "Akçın et al., 2006". Xylem and phloem were specified as collateral bundles of the stems in some Lamiaceae members (Metcalfe and Chalk, 1950), in the present study, studied species have shown the same result, however, in the stem cross section of *A. orientalis,* between phloem and xylem vascular cambium was observed and the property was collateral open (Çalı, 2014). Sclerenchyma was observed on the phloem in the stem cross sections (Metcalfe and Chalk, 1950; Çalı, 2014), for the present paper the same result has been observed on *A. relicta*, however, in the stem crosscut section of *A. postii,* the structure has not been determined. Pith region of the stem was determined as homogeneous (Metcalfe and Chalk, 1950), the same result has been observed in the present study for both two species. Hair of *Ajuga* genus was determined with the long handle (Metcalfe and Chalk, 1950), in the present study, two species have the same features. In the present paper, studied plant species have been observed with non-glandular leaf hairs. Trichomes of *A. postii* are located on the leaf surface (Fig. 7), and non-glandular hair structure of *A. relicta* has been observed on the edge of the leaf (Fig. 14), however, in the morphological findings of the leaf of *A. relicta* has been determined as glabrous on the leaf surfaces. In the literature studies, glandular hairs were observed on the stem, petiole, leaf, calyx and corolla of *A. orientalis* (Çalı, 2014); glandular hairs and trichomes on the stem of *A. reptans,* and on the stem and leaves of *A. chamaepitys* subsp. *chia* var. *chia* "Akçın et al., 2006". Vascular bundles of the leaves of the xerophytic plants

were observed with sclerenchymatous cells (Metcalfe and Chalk, 1950), in the present study, the same result has been determined for the two species.

As a conclusion, in the present paper, morphological and anatomical features of the local endemic species *A. postii* and *A. relicta* have been determined. Anatomical characters of the taxa have been studied for the first time. Thus, the present study is a resource to identify the species, and to use for medicinal purposes.

Acknowledgements

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Research article/Araştırma makalesi

Some geophyte plants determined in Bartın/Turkey

Burçin EKİCİ [*1]

[1] Namık Kemal University, Faculty of Fine Arts, Department of Landscape Architecture, Tekirdağ, Turkey

Abstract

Geophyte plants, the subject of our research, are quite important in terms of ecology, economy, and ethnobotany. The discovery and promotion of these plants are important for conservation and sustainability of biodiversity.In this study, the province of Bartın and its surroundings were examined in terms of geophyte plant. Within this context, field works were made between the years 2012 and 2014 and geophytic species were collected. Collected plants were dried and preserved according to herbarium standards and their diagnostics were made utilizing some taxonomic and floristic references. 36 taxa from 4 families were identified as a result of this study.1 plant is Iranian-Turanian (2.8%), 7 plants are European-Siberian (19.4%), and 13 plants are Mediterranean elements (36.1%), whereas 15 plants (41.7%) are from the group, the phytogeographical region of which are unknown. *Galanthus plicatus* Bieb. ssp. *byzantinus* (Baker.) D. A. Webb. and *Crocus ancyrensis* (Herbert) Maw are endemic and the rate of endemism is 5.6%. With this study, one of the key components of biodiversity, the geophyte plants, that spread in the province of Bartın are identified, their current conditions and the constraints on them are explained and improvement opportunities are specified. Besides this, it is intended to contribute to the identification and conservation of biological resources.

Key words: geophyte plant, flora, biodiversity, Bartın, Turkey

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Bartın kentinde tespit edilen bazı geofit bitkiler

Özet

Araştırma konumuzu oluşturan geofit bitkiler, ekolojik, ekonomik ve etnobotanik açıdan oldukça önemlidir. Bu bitkilerin keşfi ve tanıtımı, biyoçeşitliliğin korunması ve sürdürülebilirliği açısından önem taşımaktadır. Bu çalışmada, Bartın kenti ve yakın çevresi geofit bitki yönünden incelenmiştir. Bu kapsamda 2012- 2014 yılları arasında arazi çalışmaları yapılmış ve geofit bitki türleri toplanmıştır. Toplanan bitkiler herbaryum standart ve ölçülerinde kurutularak saklanmış, teşhisleri bazı taksonomik ve floristik referanslardan yararlanılarak yapılmıştır. Çalışma sonucunda 4 familyaya ait 36 takson saptanmıştır. Bitkilerin 1'i İran- Turan elementi, (% 2.8), 7'si Avrupa- Sibirya (% 19.4), 13'ü Akdeniz elementi (% 36.1) olup 15'i fitocoğrafik bölgesi bilinmeyenler (% 41.7) grubundandır. *Galanthus plicatus* Bieb. subsp. *byzantinus* (Baker.) D. A. Webb. ve *Crocus ancyrensis* (Herbert) Maw taksonları endemik olup, endemizm oranı % 5.6'dır. Bu çalışma ile Bartın kentinde yayılış yapan ve biyoçeşitliliğin önemli bileşenlerinden biri olan geofit bitkiler tespit edilmiş, mevcut durumları ve maruz kaldıkları baskılar açıklanarak koruma ve iyileştirme olanakları belirtilmiştir. Bununla, biyolojik kaynakların belirlenmesi ve korunmasına katkı sağlanması amaçlanmıştır.

Anahtar kelimeler: geofit bitki, flora, biyoçeşitlilik, Bartın, Türkiye

1. Introduction

Ecosystem is naturally occurring unit of organisms such as plants and animals, and their relationships with the environment (Demiröz et al., 2014). With its topography including different altitudes, soil diversity, geologic and geomorphologic properties, and its location on the junction of three different phytogeographical regions, Turkey has a rich ecosystem and flora. Geophytes, which are the subject of our research, have an important place within the floral

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[*] *Corresponding author* / Haberleşmeden sorumlu yazar: Tel.: +90282502926; Fax.: +902822509926; E-mail: bekici@nku.edu.tr
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diversity of Turkey (Kandemir and Yakupoğlu, 2016). "Geophytes" is the name given to herbs with underground organs such as bulbs, corms, rhizomes, and tubers, which gained food- storing specialty by mutation (Mammadov and Sahranç, 2003; Özuslu and İskender, 2009; Sargın et al., 2013). In our country, approximately 900 geophytic species grow naturally and almost all of them are economical and medical plants. As a result of floristic studies, the number of geophytes and other group plants increase year by year (Koyuncu and Alp, 2014).

Due to their properties, geophytes have been the focus of people's attention for many years and they were initially used in folk medicine and are currently used in modern medicine. Part of the geophytic plants is used as aromatic herbs due to their fragrances. Also, as these plants, which stay underground during large period of the year and blossom beautiful and glossy flowers at the beginning of spring and in autumn, have high ecological tolerances and they are easily cultivated, and blossom very shortly after planted. They are outstanding for landscape planning. Besides, the endemism rate of geophytic plants is quite high. The discovery, promotion, and if necessary, cultivation of these plants are needed for conservation and sustainability of biodiversity (Özel and Erden, 2010).

Several studies have been made in order to determine the richness of our country in terms of geophytic floral diversity. As a result of studies, the ecological, ethnobotanical, economical, and ecotourism potentials of species in different regions are assessed and anthropogenic negative impacts on taxa are identified (Demiröz et al., 2014; Özuslu and İskender, 2009; Sargın et al., 2013; İpek et al., 2013; Eker et al., 2008; Sandal and Söğüt, 2010; Kayıkçı et al., 2012; Özhatay et al., 2013). Floristic researches had been made earlier in Bartın, which is the subject of our research. Kaya and Başaran (2006) provided detailed information about the flora of the province and its surroundings. Moreover, Ekici (2010) evaluated the use of natural flora of the cities in landscape planning. Ekici and Kaya (2014) made contribution to identification of natural flora of the vicinity.

In this study, the province of Bartin and its vicinity was examined in terms of geophytes and these plants were evaluated according to IUCN Red List (Ekim et al., 2000). Turkey is a country that signs agreements on protecting the biological diversity (Özyavuz et al., 2006). IUCN brings the countries under the same roof in terms of protection standards, as national protection regulations. However, some of these countries are quite different, and they undertake the maintenance and the sustainability of biological diversity by legal or several other means. Thus, accurate data on local to global protection status of geophytes will be provided as well as the making their promotions.

2. Materials and methods

The study is based on the geophytes naturally grown around the province of Bartın and its surroundings. In this study, field works were made between years of the 2012 and 2014, geophytic species were collected, photographed, and notes were taken about their habitats and cultivation conditions. Collected plants were dried and preserved according to herbarium standards and their diagnostics were made utilizing Davis (1984).

3. Results

In this research, geophytes spreading in the Bartın and surroundings are determined. 36 taxa from 4 families are identified. Photos of some plants are stated below (Figure 1). 1 plant is Iranian-Turanian (2.8%), 7 plants are European-Siberian (19.4%), and 13 plants are Mediterranean elements (36.1%), whereas 15 plants (41.7%) are from the group, the phytogeographical region of which are unknown. *Galanthus plicatus* ssp. *byzantinus* and *Crocus ancyrensis* taxa are endemic and their endemism rate is 5.6%.

The study offers an insight into nature conservation studies, identifies endemic plants as well as rare and threatened non-endemic species, and provides support for their conservation. In this study, the identified plants are also evaluated according to the IUCN Red List. From the plants in this context, *Crocus ancyrensis* is in "VU"(Vulnerable) and *Crocus speciosus* Bieb. ssp. *speciosus* is in "LC" (Least concern) categories.

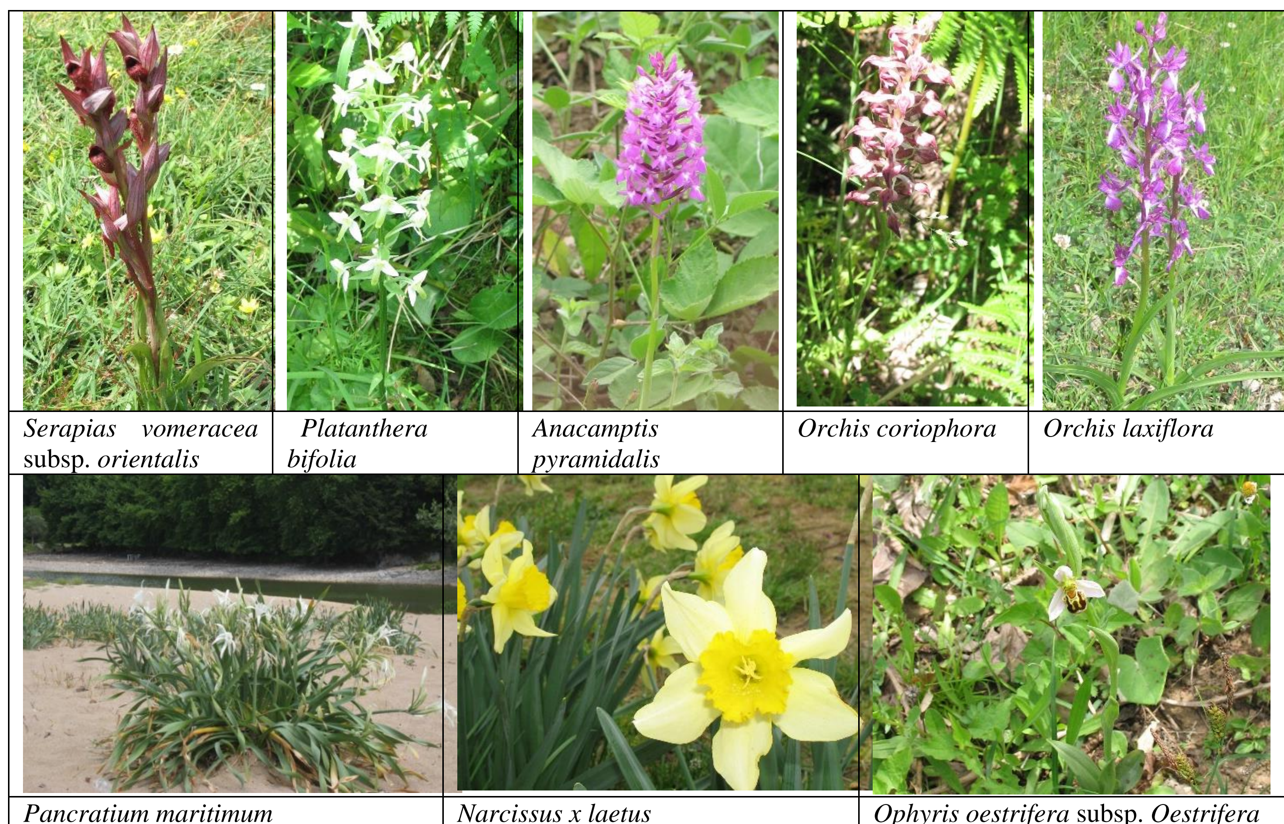
The statuses of collected plants according to their families, scientific names, place and time of collection, phytogeographical regions, endemism, and IUCN categories are stated below.

AMARYLLIDACEAE

1) ***Galanthus plicatus*** Bieb. ssp. ***byzantinus*** (Baker.) D. A. Webb., Zoni Plateau (Arıt), 475 m, 07.03.2014, Endemic, Euro-Siberian element.
2) ***Leucojum aestivum*** L., Gecen village, in the wetlands, 50 m, 13.05.2014, Euro-Siberian element.
3) ***Narcissus x laetus*** Salisb., Karaköy, in the agricultural fields, 110 m, 08.03.2014.
4) ***Narcissus pseudonarcissus*** L., Kozcağız, in the *Carpinus* sp. forests, 120 m, 22.04.2013.
5) ***Pancratium maritimum*** L., İnkumu coast, dune fields, 1 m, 29.06.2014, Mediterranean element.

IRIDACEAE

6) ***Crocus ancyrensis*** (Herbert) Maw, Başköy (Kurucaşile), 260 m, 18.04.2012, Endemic, Iranian-Turanian element, LC.
7) ***C. speciosus*** Bieb. ssp. ***speciosus***, İnkumu, in the wooded area that behind the coast, 55 m, 03.09.2013, VU.
8) ***Iris germanica*** L., Amasra, Ağlayan ağaç sites, 120 m, 13.05.2014.



Serapias vomeracea subsp. *orientalis* | *Platanthera bifolia* | *Anacamptis pyramidalis* | *Orchis coriophora* | *Orchis laxiflora*

Pancratium maritimum | *Narcissus x laetus* | *Ophyris oestrifera* subsp. *Oestrifera*

Figure 1. The some geophyte plants identified in the research area

9) ***I. pseudacorus*** L., Amasra, Ağlayan ağaç sites, in the humid areas, 364 m, 04.05.2014.

LILIACEAE

10) ***Allium flavum*** L. ssp. ***tauricum*** (Besser ex Reichb.) K. Richter var. ***tauricum*** Beser ex Reichb., Kaynarca, in the agricultural fields, 52 m, 13.05.2014, Mediterranean element.
11) ***Asparagus acutifolius*** L., Ağdacı village, in the *Carpinus* sp.-*Quercus* sp. forest areas, 114 m, 12.08.2013, Mediterranean element.
12) ***A. aphyllus*** L. ssp. ***orientalis*** (Baker) P. H. Davis, Amasra, Ağlayan ağaç sites, 120 m, 11.07.2013, Mediterranean element.
13) ***Muscari armeniacum*** Leichtlin. ex Baker., İnkumu coast, in the dune fields, 5 m, 10.04.2013.
14) ***M. comosum*** (L.) Mill., Ağdacı village, in the *Carpinus* sp.- *Quercus* sp. forest areas, 114 m, 12.06.2013, Mediterranean element.
15) ***M. neglectum*** Guss., Kanlıırmak sites, in the humid areas, 65 m, 03.04.2013.
16) ***Ornithogalum armeniacum*** Baker, Ağdacı village, in the *Carpinus* sp.-*Quercus* sp. forest areas, 114 m, 13.04.2014, Mediterranean element.
17) ***O. fimbriatum*** Willd., İnkumu, in the wooded area that behind the coast, 55 m, 03.03.2013, Mediterranean element.
18) ***O. narbonense*** L.,Ağdacı village, in the agricultural areas, 120 m, 15.04.2014, Mediterranean element.
19) ***O. umbellatum*** L., Kanlıırmak surroundings, 54 m, 10.04.2014.
20) ***O. wiedemannii*** Boiss., Gecen village, in the damp meadows, 110 m, 12.04.2013.
21) ***Polygonatum hirtum*** (Bosc ex Poiret.) Pursh., Gecen village, in the damp meadows, 110 m, 12.04.2013, Euro-Siberian element.
22) ***P. multiflorum*** (L.) All., Bartın-Amasra road sites, in the mixed coniferous forests, 274 m, 13.05.2013.
23) ***Ruscus aculeatus*** L. var. ***acuelatus***, Ağdacı village, in the *Carpinus* sp.- *Quercus* sp. forest areas, 114 m, 12.06.2014.
24) ***R. hypoglossum*** L., Bartın-Amasra road sites, in the mixed coniferous forests, 274 m, 13.05.2013, Euro-Siberian element.
25) ***Scilla bifolia*** L., Ağdacı village, in the *Carpinus* sp.- *Quercus* sp. forest areas, 114 m, 12.03.2013, Mediterranean element.
26) ***S. bithynica*** Boiss., Bartın-Amasra road sites, 310 m, 13.05.2013, Euro-Siberian element.
27) ***Smilax excelsa*** L., Ağdacı village, in the *Carpinus* sp.-*Quercus* sp. forest areas, 114 m, 12.06.2014, Mediterranean element.

ORCHIDACEAE

28) ***Anacamptis pyramidalis*** (L.) L. C. M. Richard., Ağdacı village, in the *Carpinus* sp.- *Quercus* sp. forest areas, 114 m, 12.06.2014.
29) ***Dactylorhiza incarnata*** (L.) Soó., Kozcağız surroundings, in the wetlands, 02.06.2013.
30) ***D. romana*** (Seb.) Soó. ssp. ***romana***, Bartın-Amasra road sites, in the mixed coniferous forests, 274 m, 13.05.2013, Mediterranean element.
31) ***Ophyris oestrifera*** Bieb. ssp. ***oestrifera***, Orduyeri Quarter, in the meadows, 286 m, 20.06.2014.
32) ***Orchis coriophora*** L., Mugada coast, in the dune areas, 5 m, 23.05.2013.
33) ***O. laxiflora*** Lam., Campus of Bartın University (Ağdacı), 110 m, 11.05.2014, Mediterranean element.
34) ***O. purpurea*** Huds., Bartın-Amasra road sites, in the mixed coniferous forests, 310 m, 13.05.2013, Euro-Siberian element.daw
35) ***Platanthera bifolia*** (L.) L.C.M. Richard, Ağdacı village, in the *Carpinus* sp.-*Quercus* sp. forest areas, 130 m, 23.05.2013, Euro-Siberian element.

Serapias vomeracea (Burm. Fil.) Briq. ssp. ***orientalis*** Greuter, Orduyeri Quarter, in the meadows, 250 m, 20.06.2014, Mediterranean element.

4. Conclusions and discussion

With this study, several geophytic plants spread around the province of Bartın are determined. Within this scope, field studies were made between 2012 and 2014 and 36 taxa are identified. When the distribution by phytogeographical regions of these plants is examined, it is seen that they spread in 13 taxa and Mediterranean flora (Table 1). When the distribution of geophytic plants identified in the last 10 years in Turkey is examined, it is seen that Antalya, Muğla, and Konya has the most species. They are neighboring provinces located in the Mediterranean belt. This information supports the fact that the Mediterranean belt is a center in terms of geophytes (Koyuncu and Alp, 2014).

Table 1. The distribution of geophytes identified in the research area, by phytogeographical regions

Phytogeographical region	The number of taxa	Rate (%)
Iranian-Turanian	1	2.8
Euro-Siberian	7	19.4
Mediterranean	13	36.1
Unknowns as phytogeographically	15	41.7

The identified plants in the research area, mostly belong to the *Liliaceae* family (50%). *Liliaceae* is followed by *Orchidaceae* family. The family had the least species in the research area is *Iridaceae*. (11%) (Table 2, Figure 2).

Table 2. The situation of families identified in the research area, by the number of taxa

Family	The number of taxa	Rate (%)
Amaryllidaceae	5	14
Iridaceae	4	11
Liliaceae	18	50
Orchidaceae	9	20

Two taxa of identified plants, *Galanthus plicatus* ssp. *byzantinus* and *Crocus ancyrensis* are endemic and their endemism rate is 5.6%. These plants are assessed according to the IUCN categories, and it is observed that *Crocus ancyrensis* is in “VU” and *Crocus speciosus* ssp. *speciosus* is in “LC” categories. Necessary regulations must be made and human-induced negative impacts on these endemic species and geophytes which are sensitive according to IUCN, must be reduced in order to maintain the conservation of biodiversity and nature.

As they have glossy structures and are used for economical purposes, they are excessively collected in the areas they spread. Especially the *Orchis* species are under intensive anthropogenic pressure because of high demand and errant harvests. In Turkey, *Orchis* sp. “Salep” is obtained from the tubers of this species for ages and it is sold both in the domestic market and in the foreign markets. Our Salep exports continued until 1996, however as of 2001 exports of its tubers, powder, tablets and drugs in any form was prohibited. Exporting any product with Salep symptoms is also prohibited. However, the domestic consumption still continues (Sezik, 1984). Apart from that, in field study observation, it is clearly seen that *Narcissus x laetus, Pancratium maritimum, Iris germanica* and *Iris pseudacorus* which stand out with their flamboyant blossoms and *Ruscus aculeatus* var. *acuelatus*, *Asparagus* sp. which is used in cut flowers, are collected excessively. The likewise collection of these plants, creates the danger of extinction for these taxa. Also geophytes such as *Allium flavum* ssp. *tauricum* var. *tauricum*, *Leucojum aestivum*, *Polygonatum hirtum* and *Smilax excelsa* identified near the agricultural areas, are exposed to anthropogenic pressure. Therefore, cultural production and reproduction of these plants became compulsory in order to protect the species and maintain its use for different purposes.

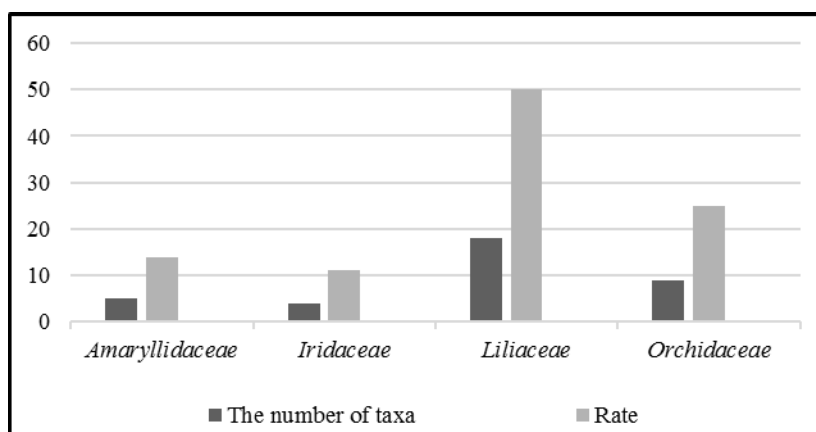


Figure 2. The distribution of families identified in the research area

Conservation of nature and the future of these plants which have ecological, economical, and visual importance, depend on the measures. Although the current regulations and prohibitions made to prevent the gene sources of geophytic plants reduce the amount of ecocide, it is still insufficient. Knowledge of biodiversity is required for conservation. The real solution can be obtained by making the promotion of these plants, determining growing techniques, and cultivation.

Geophytes identified in the research area such as *Leucojum aestivum*, *Anacamptis pyramidalis*, *Ophyris oestrifera* ssp. *oestrifera*, *Platanthera bifolia*, *Serapias vomeracea* ssp. *orientalis* and species of *Muscari* sp., *Ornithogalum* sp., *Scilla* sp., *Dactylorhiza* sp. provide visual impacts on the landscape. Rare species, these geophytic plants, stand out with their visual effects and provide opportunities for an alternative tourism type; flora tourism. It will provide the opportunity to make trekking, to see these plants on site and to learn the properties of the neighborhood. By this way, the local community will be supported economically and the awareness of learning the neighborhood and protecting the nature will be raised.

With this study, the geophytic plants spreading around the province of Bartın are identified, dangers are explained, reasons of pressure are set forth, and improvement opportunities are specified. This study aims to make contribution to future studies, nature conservation, social progress, and to determine the biological resources..

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Research article/Araştırma makalesi

Mycorrhizal diversity in some species of *Dactylorhiza* genus (Orchidaceae)

Yasemin OZDENER KOMPE *[1], Vildan AKIN MUTLU [1]

[1] University of Ondokuzmayıs, Faculty of Arts and Sciences, Department of Biology, Atakum-SAMSUN, Turkey.

Abstract

The research was designed to identify the mycorrhizal fungi of four of eleven species of *Dactylorhiza* in Turkey. To isolate and identify of mycorrhizal fungi of *Dactylorhiza euxina* var *euxina, D. osmanica* var. *osmanica* (endemic), *D. romana* subsp. *romana* and *D. urvilleana,* the roots of them were collected from three habitats of Black sea region. In additon to, to determine the relationship between color polymorphysim flowers of *D. romana* subsp. *romana* and mycorrhizal diversity, the isolations were done from the roots of the pink and yellow flowering individuals of *D. romana* subsp. *romana* separately. The isolates were characterized via morphological and molecular methods. For molecular identification, the fungal DNA were extracted from purified fungal culture and fungal ITS regions were PCR amplified using the primer pairs ITS1 and ITS4 and sequenced. Phylogenetic analysis revealed that the majority of *Dactylorhiza* mycorrhizal fungi are Tulasnellaceae and Ceratobasidiaceae belonging to Basidiomycota. Interestingly, *Verpa conica verpa*, a member of Morchellaceae, in individual pink flowering plants of *D. romana* subsp. *romana* was also found. *Tulasnella bifrons* were shared by plants of *D.euxina* var. *euxina, D. osmanica* var. *osmanica* and *D. urvilleana.* Fungal partners of *Dactylorhiza* species in wet meadows belong to Tulasnellaceae, those of under *Quercus* scrubs and on the wet forest margins are Ceratobasidiaceae, Morchellaceae and Tulasnellaceae, respectively. These results may indicate that fungal partner(s) of orchids may vary depending on habitat.

Key words: *Orchidaceae, Dactylorhiza, fungal diversity, mycorrhiza*

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***Dactylorhiza* cinsine(Orchidaceae) dahil bazı türlerin mikorizal çeşitliliği**

Özet

Bu araştırma, Türkiye'deki onbir *Dactylorhiza* türünden dördünün mikorizal çeşitliliğini belirlemek için yapılmıştır. *Dactylorhiza euxina* var *euxina, D. osmanica* var. *osmanica* (endemic), *D. romana* subsp. *romana* ve *D. urvilleana* türlerinin mikorizal funguslarını izole etmek ve tanımlamak için, bu türlerin kökleri Karadeniz Bölgesinde üç farklı habitatdan toplanmıştır. Buna ilave olarak, *D. romana* subsp. *romana*'nın çiçeklerindeki renk polimorfizmi ve fungal çeşitlilik arasındaki ilişkiyi belirlemek için sarı ve pembe çiçekli bireylerin köklerinden ayrı ayrı izolasyonlar yapılmıştır. İzolatlar morfolojik ve moleküler yöntemlerle tanımlanmıştır. Moleküler tanımlama için, saf fungus kültüründen DNA ekstrakte edildi ve fungal ITS bölgeleri, ITS1 ve ITS 4 primer zincirleri kullanılarak PCR ile çoğaltılıp sekansları yapılmıştır. Filogenetik analizler, *Dactylorhiza* türlerinin mikorizal funguslarının çoğunluğunun Basidiomycota'ya ait Tulasnellaceae ve Ceratobasidiaceae familyalarından olduğunu ortaya koydu. İlginç olarak, *D. romana* subsp. *romana*'nın pembe çiçekli bireylerinin köklerinde Morchellaceae familyasına ait *Verpa conica verpa* bulundu. *Tulasnella bifrons, D.euxina* var. *euxina, D. osmanica* var. *osmanica* ve *D. urvilleana* türlerinin köklerinde ortak olarak bulunmuştur. Bu araştırmanın sonuçlarına göre, nemli çayırlardaki *Dactylorhiza* türlerinin fungal partnerlerinin *Tulasnella*'ya, meşe ormanı altındaki ve nemli orman kenarlarındakilerin *Ceratobasidiaceae, Morchellaceae ve Tulasnellaceae* 'ye ait oldukları belirlendi. Bu sonuçlar, orkidelerin fungal partnerlerinin habitata bağlı olarak değişebileceğini işaret etmektedir.

Anahtar kelimeler: *: Orchidaceae, Dactylorhiza, fungus çeşitliliği, mikoriza*

* *Corresponding author* / Haberleşmeden sorumlu yazar: Tel.: +903623121919; Fax.: +903624576081; E-mail: yasemino@omu.edu.tr
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1. Introduction

The Orchidaceae is one of the largest flowering plant families and they have very different life strategies such as epiphytic, saprophytic, terrestrial (Dressler,1981; McCormick et al., 2004). It is known that mycorrhizal colonization is very important for both the life cycle of adult plants and understanding of the evolutionary history of orchids and seed germination (Rasmussen,1995; Rasmussen and Whigham, 2002; Rasmussen and Rasmussen 2009; Rasmussen and Rasmussen 2014). Little is known about what role fungal diversity plays in affecting an orchid's distribution, population size, and genetic diversity of the fungal association (McCormick et al.,2004). The fungi of orchid mycorrhiza have been largely included teleomorph genus such as *Ceratobasidium*, *Sebacina*, *Tulasnella* and *Thanatephorus* belonging to Basidiomycetes (Currah et al., 1997; Rasmussen,2002; Zettler et al., 2004; Dearnaley,2007). Mycorrhizal preference of orchids is variable (Xing et al.,2015); nonphotosynthetic orchids have very specific associations including saprophytic or ectomycorrhizal fungi (Dearnaley et al., 2012) while in photosynthetic orchids, the level of specificity varies considerably (McCormick et al., 2004; Waud et al., 2016). Therefore, the available of appropriate fungal partner (or partners) may determine habitat and geographic distribution of orchid plants (McCormick et al., 2009; Illyeset al., 2010; Jacquemyn et al.,2011; Jacqumyn et al., 2012b; Jacquemyn et al.,2014; Xing et al.,2015). The appropriate mycorrhizal fungus is a requirement for the orchid seed germination, as the seeds do not have the endosperm. The mycorrhizal fungi support nutrition for the seed germination and early development (Rasmussen,1995). More knowledge about orchid-fungal diversity in the same or different habitats will be very important and useful for orchid seed germination and propagation (Xing et al.,2015).

Turkey has a high species richness of Orchidaceae. There are about 200 species that belong to 24 genera in Turkey. According to the most recent data, there are 11 species of *Dactylorhiza* in Turkey (Guner, 2012). Their tubers are used for ice-cream and "salep" (as a Turkish drink) and as medicinal materials. Therefore, millions of tubers arecollected every year. All orchid species of Turkey are under serious threat of extinction due to over-collection and environmental destruction (Türkiş and Ertürk, 2015). To conserve surviving of wild orchid populations (*Dactylorhiza* and the others) and reintroduce the plants into their habitat require understanding mycorrhizal partner of each orchid species. But no research has been done related to the identification of orchid mycorrhizal fungi by using molecular techniques and diversity and distribution of the fungi in the same or different ecosystems of Turkey. We considered that the relationship of mycorrhizal diversity of orchids in the different habitats (wet meadow, *Quercus* shrubs or wet forest marjins) of Turkey should be well-known to produce the orchids in natural conditions from the seeds. For this reason, we designed this research. The orchids of the research are four *Dactylorhiza* species. Some of *Dactylorhiza* species and the others grow in forest and meadow ecosystems in Turkey, respectively. The roots of *Dactylorhiza romana* subsp. *romana* (habitat1) and *D. urvilleana* (habitat2) were collected at an altitude of 50m from Quercus scrub and at an altitude of 900m from wet forest margins, respectively. *D. euxina var euxina* and *D. osmanica var. osmanica* (habitat3) were collected at an altitude of 2350m from the meadow of the plateau of Black Sea Region of Turkey.

The distance between habitat 2 and 3 is 40km, habitat 1 is about 400-450km away from both of 2 and 3. With the completion of this research, the diversity of mycorrhizal fungi of some *Dactylorhiza* species in Turkey and the relationship between habitats of *Dactylorhiza* species and their mycorrhizal fungi will be well-known.

2. Materials and methods

1.1. Habitats and Plant Collection

The roots of *Dactylorhiza urvilleana (habitat2), D. euxina, D. osmanica* var. *osmanica* (endemic) *(habitat3)* were collected from East Black Sea Region in Turkey, the roots of *D. romana* subsp *romana* at flowering stage were from middle Black sea. *Dactylorhiza romana* subsp. *romana* have both purple and white flowers (habitat1). The roots of white and purple flowering plants were collected separately to determine the relationship between color polymorphism and mycorrhizal diversity. To avoid damaging the plants, we carefully hollowed around the individuals and one or two root parts collected from per individual plant.

Habitat1: Samsun/Turkey: ***D. romana* subsp. *romana***

Habitat2: Köprübaşı-Trabzon/Turkey: ***D. urvilleana*** (950m)

Habitat3: Ismail Aga Plato-Köprübaşı/Trabzon/Turkey: ***D. euxina* and *D. osmanica* var. *osmanica*** (2300m)

1.2. Microscopical observations for fungal pelotons, fungus isolation and morphological analysis

2.2.1 Microscopical observation

The roots were washed with tap water and then the cross sections were taken from the roots with a razor. The fungal colonizations were confirmed with a light microscope (Leica).

2.2.2 Fungus isolation

After microscopical observations, the roots parts containing fungal pelotons were surface sterilized with 1.5% NaOCl for 2-3 minutes and then washed with sterile distilled water for three times. The roots parts were sectioned in

aseptic conditions and the sections were placed in fungus isolation medium in the Petri dishes Clements et al., 1985). Fungus isolation medium was prepared according to Clements et al., 1985. The Petri dishes were covered with aluminum foil and incubated at 25 ºC (±2 ºC) in the dark in an incubator for 2 weeks.

2.2.3. Morphological analysi

The fungi growing on Potato Dextrose Agar (PDA, Merck) were examined for colony type and color, sclerotium color, nucleus number and hyphe diameter with Leica microscope. For preidentification, hyphal features and nuclear condition of the isolates were examined as explained in Karaca et al., 2002 after stained with Safranin O and 3% KOH (Bandoni, 1979). The colony and sclerotium colours were determined according to Royal Horticultural Society (RHS) Colour Chart (1995).

2.3. Molecular phylogeny

2.3.1 DNA extraction, amplification, sequencing

Genomic DNA (gDNA) was extracted from each piece of root according to Doyle (1991) protocol with some modifications. Firstly, 50 mg of fungal tissue was grounded to powder under liquid nitrogen using a mortar and pestle. The powder mycelium was transferred into a 1.5-µL centrifugation tube containing cetyltrimethyl ammonium bromide (CTAB) buffer (500 µL, 100 mM Tris-HCl, pH 8,0, 20 mM EDTA, 2% CTAB, 1.4 M NaCl, and 0.1% b-mercaptoethanol). The lysis mixture was incubated at 65°C for 60 min and the content of the tubes was mixed by turning the tubes every 10 min. At room temperature, the solution was extracted twice with chloroform: isoamyl alcohol (24: 1) by centrifugation at 12.000rpm. The supernatant was precipitated with 0.6 volumes of ice–cold ethanol. The precipitate was then collected by centrifugation at 12000rpm. The pellet was washed with 70% ethanol and air dried. Finally, the DNA pellet was dissolved in 25 µL TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) and stored at –20◦C prior to use. Amplification of the rDNA-ITS region was done with the primers ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) / ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) (White et al., 1990). The PCR procedure of Pascual et al.,(2000) was used to amplify rDNA-ITS intergenic spacer loci with an MWG Primus thermal cycler. PCR amplifications were carried out in a total reaction volume of 50 μL, containing approx. 50 ng gDNA as the template, 50 pmol of each primer, 2.5 mM dNTP mix (Ambresco USA), 1U Tag polymerase (Promega, USA), 1.5 $MgCl_2$, 1X PCR buffer(Promega, USA) and dd H_2O. An MWG Primus thermocycling profile consisted of an initial denaturation step at 94°C for 3 min, followed by 30 cycles of 94°C at 1mins, 2 min at 49°C, 72°C for 3 min and the final extension at 72°C for 7 min. The PCR product were electrophoresed on 1% agarose gel (Amresco, USA) prepared in 1X TBE (Tris-Borate- EDTA) buffer. And finally the PCR product was stained with Gel Red(Biotium), gels were visualized with the GeneGenius Bio imaging system (Syngene, Synoptics Group, Cambridge, UK).

2.3.2. Phylogenetic analysis

The rDNA-ITS regions of the isolates were sequenced in both directions by Macrogen (Korea) with using ABI 3730 XL sequencer. The sequences were checked and edited using the program SeqMan II module of the LASERGENE 99 system (Applied Biosystem) and aligned with CLUSTAL X(Thomson etal.,1997) then optimized by hand. Firstly, the identity of isolates was determined by making BLAST search. Later, data sets were analyzed using the package program MrBayes version 3.2 (Ronquist et al.,2012). Bayesian analyses were run for 10 million generations, a sampling frequency of 1000 and a burn-in of 20%.

3. Results

1.1. Fungus isolation and morphological characterization

Mycorrhizal colonization was observed in cortical cells in the cross sections of the roots of all of *Dactylorhiza* species in the research (Figure 1).

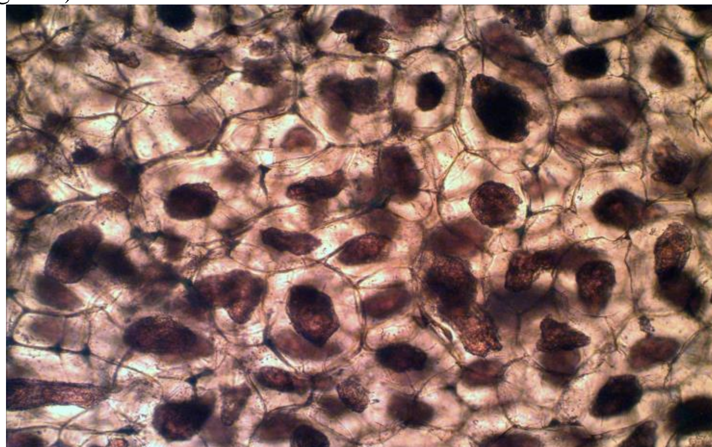

Figure 1: Mycorrhizal colonization in the cortical cells of *Dactylorhiza* roots

The fungal hyphae growing in the medium was purified by being transferred to the fresh fungus isolation medium. Then the purified fungi were grown on the PDA for morphological analysis (hyphae diameter, grow rate, colony features) and stocked on the PDA at 4ºC.

Eight fungi were isolated from the roots of three adult plants of each *Dactylorhiza* species, totally.

In all the *Dactylorhiza* species investigated, eight *Rhizoctonia*-like isolates and one *Verpa* spp. were obtained and morphological characteristics of them were given in Table 1 and Figure 2.

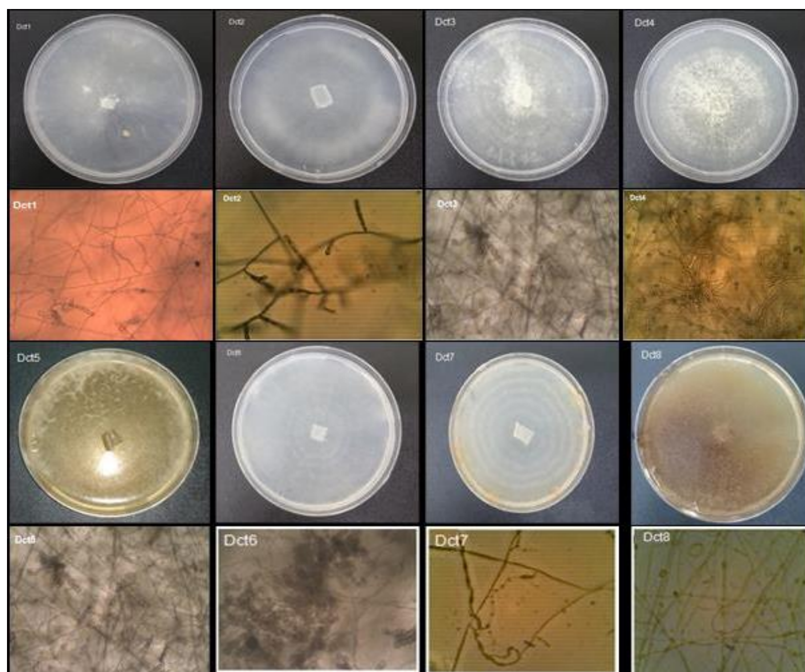

Figure 2: Colony and hyphal features of the isolated fungi

Dct1 isolate (from *D. osmanica* subsp. *osmanica*) formed the colony with aerial hyphae and colony white, hyphal diameters was 3.86±1,02µm. Colony colors of Dct2 (from *D.euxina* var. *euxina*) and Dct3 (*D. urvilleana*) isolates were white with submerged hyphae. The hyphal diameters of them were 4,3±1,26µm and 4,91±0,59µm, respectively. Dct4 formed the colony with aerial hyphae and sclerotium. Colony and sclerotium colors were gray-white, (colony and sclerotium colors were the same) and hyphae diameter 5,12±0,60 µm. Colony type, colony, and sclerotium colors, hyphae diameter of Dct5 (from *D. romana* subsp. *romana*, pink flowers) were with aerial hyphae, gray orange, brown and 4,68±0,47µm, respectively. Dct6 (from *D. romana* subsp. *romana* white flowers) was grey-white with submerged hyphae, no sclerotium, and 5,01±0,34µm in diameter. Dct7 (from *D. romana* subsp. *romana,* white flowers) formed grey-white colony and sclerotium, hyphal colony and hyphal diameter were aerial and 5,43±0,35µm, respectively. Finally, Dct8 was were isolated from *D. romana* subsp. *romana* (pink flower). The fungus had a gray-orange colony with aerial hyphae and sclerotium, hyphae diameter was 9,21± 2,24µm, nucleus number was 20.

1.2. Molecular characterization

DNA was extracted from fungal culturesand PCR amplification was carried out using the standard set of primers ITS1-ITS4. Amplification of the corresponding internal transcribed spacer region gave rise to amplicons whose electrophoretic profiles displayed bands from 500 to 800 bp. Representative cases were sequenced and the results were examined by BLAST against the GenBank database. Seven of the obtained sequences corresponded to Basidiomycetes and one of them Ascomycetes sequences. We found that three of the isolates, five isolates, and one isolate belonged to Tulasnellaceae, Ceratobasidiaceae, and Morchellaceae, respectively. Results of the BLAST analysis were shown in Table 2. The sequences were compared with known sequences in the Gen Bank using BLASTN. Based on a 95% cutt off value of similarity between sequences. Phylogenetic relationships based on the individual datasets were inferred from Bayesian inference (BI) analyses. BI analyses of individual marker and datasets were performed in MrBayes v.3.1.2.. The best fitting model of evolution was chosen for ITS marker using ModelTest v.3.7 The evolutionary models selected by the Akaike information criteria (AIC) estimator were GTR+I+G for ITS. On the tree, Tulasnellaceae family species grouped in three main clades. Tulasnellaceae members constituted the ITS-5.8S rDNA sequence similarities between 93% and 99%. Sequences of the isolates Dct1,Dct2, Dct3 shared high homology with those of uncultured Tulasnellaceae (Table 2). *Additionally,* data sets were formed for *Tulasnella* spp sequences using phylogenetic algorithms such as Mr. Bayesian. The ITS sequences of the several strains exhibited 100% identity. The group consists

of 36 sequences, shown by sequence similarity analysis to be related to the *Tulasnellaceae* family. In the Mr. Bayesian tree of *Tulasnellaceae*, there were three major lineages supported with a bootstrap value of 91 %. Dct 1, Dct 2, Dct 3 sequences placed in the clade with *Tulasnella bifrons.* Dct 1, Dct 2, Dct 3 do not associate closely with *Tulasnella bifrons* but they were closer to this species than the other species presented in the database (Figure 3).

The sequences of Dct4, Dct5 and Dct 6 showed high sequence identity (99, 100, 99%, respectively) to uncultured Ceratobasidiaceae. Sequences of the isolates Dct7 showed high sequence identity (100 %) uncultured Ceratobasidiaceae. The biggest group consists of 38 sequences, shown by sequence similarity analysis to be related to the *Ceratobasidiaceae* family. Mr. Bayesian analyses generated three lineages with a bootstrap value of 100 %. Uncultured Ceratobasidiaceae (Dct4, Dct5) were clustered in clade II, and also Dct6 were in clade III with *Ceratobasidium albasitensis* (Figure 4).

Finally, The sequences of Dct8 revealed high identity 100% of those of *Verpa conica verpa* (Morchellaceae-Ascomycota) in GenBank (accession numbers AF008230). The smallest group consists of 13 sequences, shown by sequence similarity analysis to be related to the *Morchellaceae* family. There were two major linages supported with a bootstrap value of 100%.The 712 bp Dct 8 sequence placed in a clade with *Verpa conica*. On the tree, Dct 8 from *Dactylorhiza romana* subsp. *romana* (pink flower) showed the close relation with *Verpa conica* (GenBank accession AF008230) with 100% nucleotide sequence similarity (Figure 5).

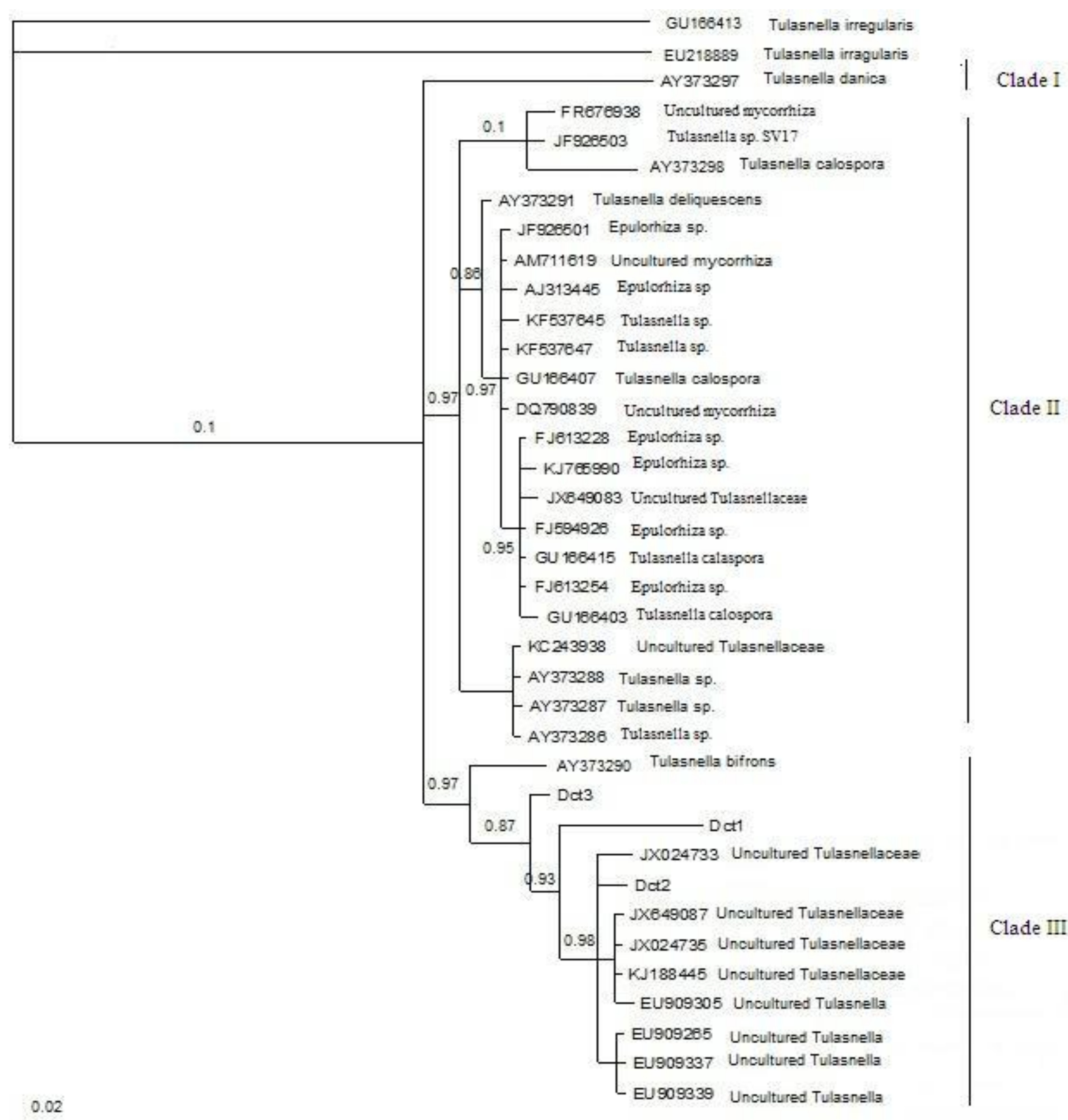

Figure 3: Phylogenetic relationship of fungal ITS sequences from *Dactylorhiza.* Basidiomycota (Tulasnellaceae) ITS sequences

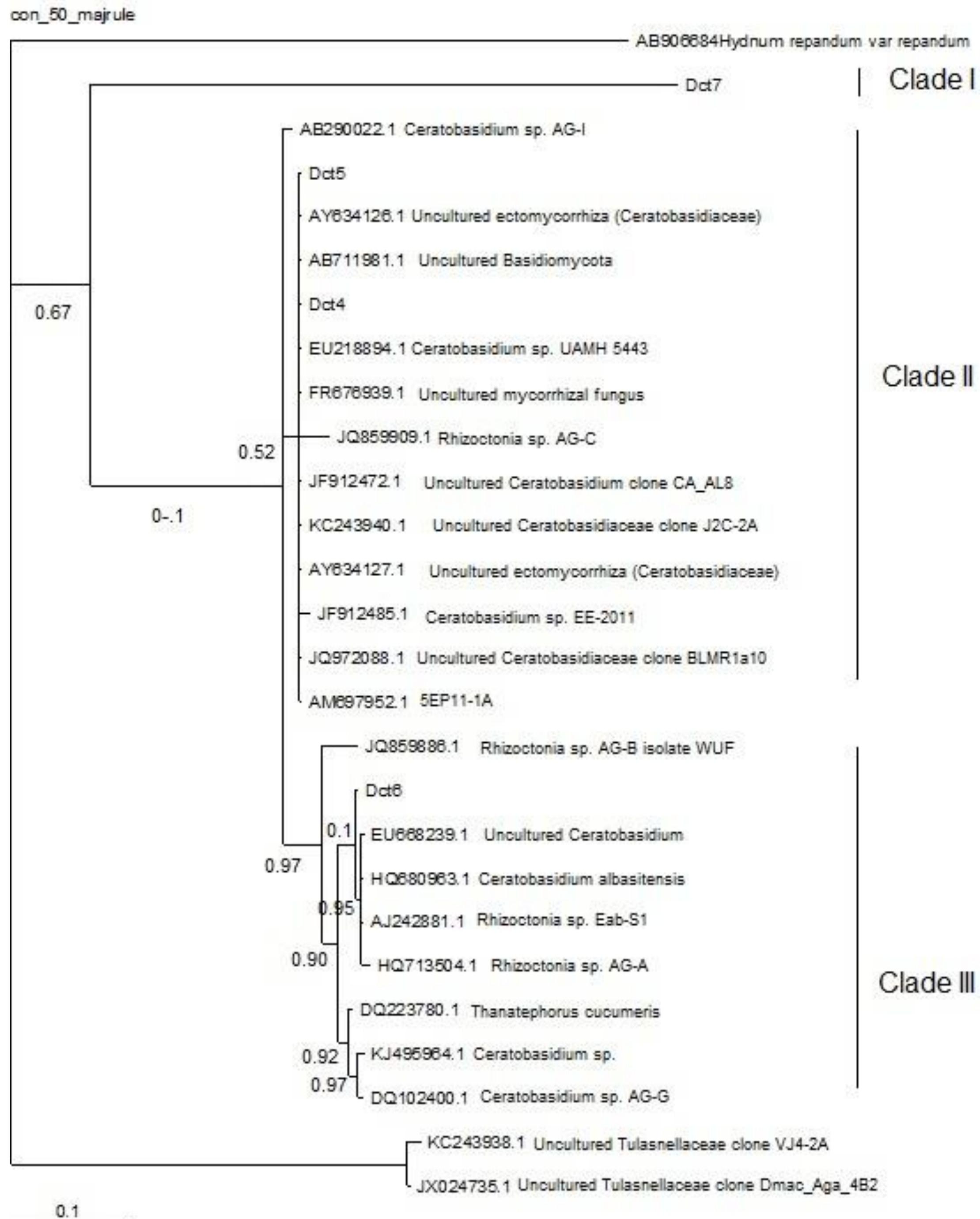

Figure 4: Phylogenetic relationship of fungal ITS sequences from *Dactylorhiza.* Basidiomycota (Ceratobasidiaceae) ITS sequences

Table1: Summary of cultural, morphological and molecular characteristics of the fungal isolates

Isolate	Host	Colony type	Hyphal diameter (µm)	Colony color	Sclerotium color	Number of nucleus	GenBank Accession code	Length(bp)	Sequence Identity	Phylogenetic relationship[a] Closest match in GenBank
Dct 1	*D.osmanica* var. *osmanica*	Aerial	3,86±1,02	White	Absent	2	(JX024735)	543	93	Uncultured Tulasnellaceae clone Dmac_Aga_4B2 from *Dactylorhiza* sp(*D. fuchsii, D. incarnata, D. maculata, D. majalis and D. praetermissa*)
Dct 2	*D.euxina* var. *euxina*	Submerged	4,3±1,26	White	Absent	2	(KC243938)	640	99	Uncultured Tulasnellaceae clone VJ4-2A from Gymnadenia conopsea
Dct 3	*D. urvilleana*	Submerged	4,91±0,59	White	Absent	2	(EU909346)	644	99	Uncultured Tulasnella mycobiont of Aneura pinguis
Dct 4	*D.urvilleana*	Aerial	5,12±0,60	Gray-white	Gray-white	2	(KC243940)	661	99	Uncultured Ceratobasidiaceae from Gymnadenia conopsea
Dct5	*D.romana* subsp *romana*(pink flower)	Aerial	4,68±0,47	Gray-orange	Brown	2	(KC243940)	554	100	Uncultured Ceratobasidiaceae from Gymnadenia conopsea
Dct 6	*D.romana* subsp *romana* (white flower)	Submerged	5,01±0,34	Gray-white	Absent	2	(JX649076)	649	99	Uncultured Ceratobasidiaceae clone OTUC1_OgaAQ01) from *Anacamptis morio* and *Dactylorhiza fuchsii*
Dct 7	*D.romana* subsp *romana* (white flower)	Aerial	5,43±0,35	Gray-white	Gray-White	2	(KC243940)	663	100	Uncultured Ceratobasidiaceae
Dct 8	*D.romana* subsp *romana* (pink flower)	Aerial	9,21±2,24	Gray orange	Absent	20	(AF008230)	719	100	*Verpa conica verpa*

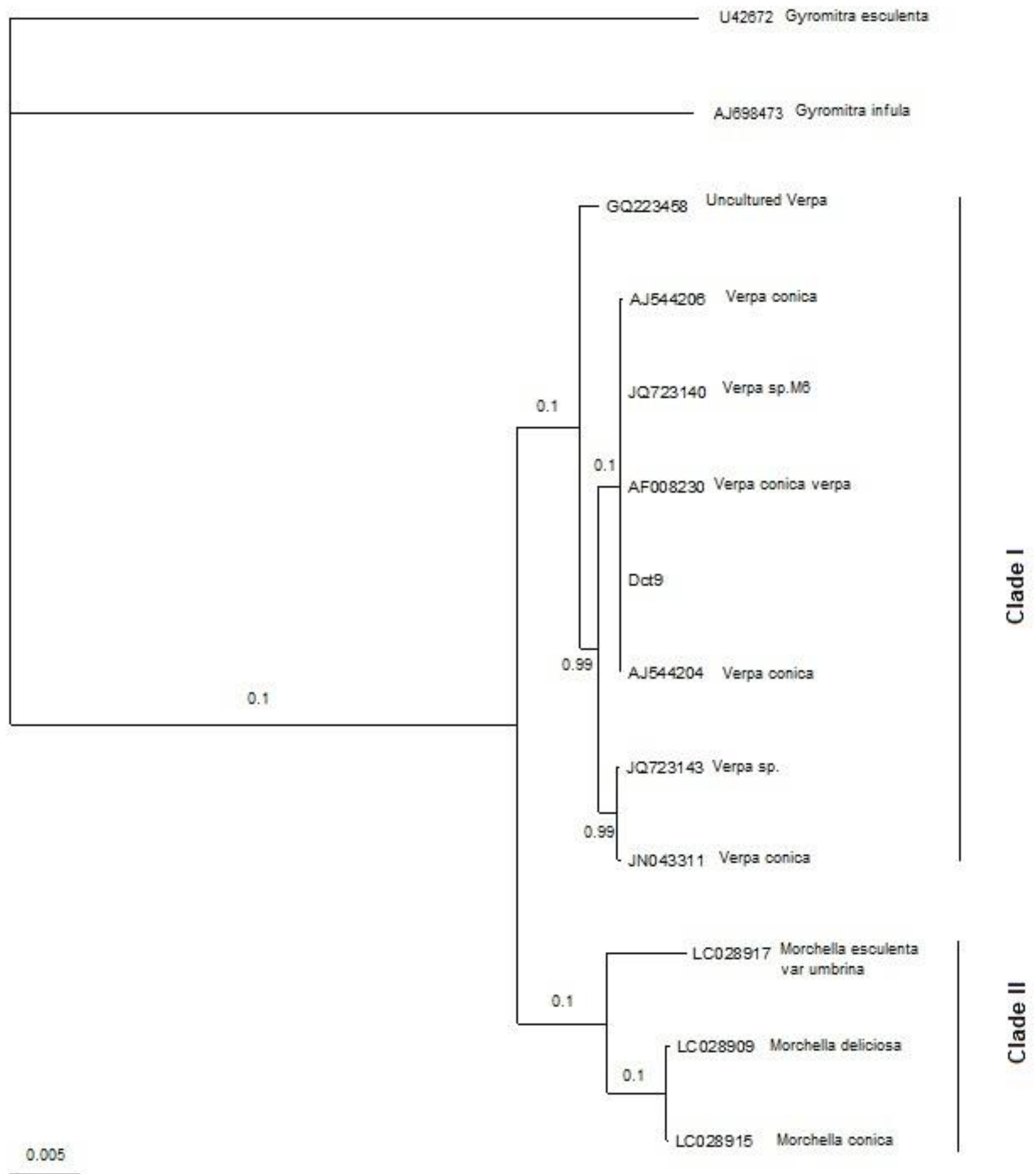

Figure 5: Phylogenetic relationship of fungal ITS sequences from *Dactylorhiza.* Ascomycota (Morchellaceae) ITS sequences.

4. Conclusions and discussion

This study represents the first investigation of phylogenetic relationship mycorrhizal fungi associated with some *Dactylorhiza* species in Turkey. Researchers revealed that only hyphal characteristics for the identification of *Rhizoctonia*-like fungi may not be sufficient. Recently, a molecular analysis in addition to the morphological characterizations is used for identification of *Rhizoctonia*- like fungi and the other fungi (Rasmuusen, 2002; Taylor et al.,2008).We also used the both morphological and molecular analysis methods for identification of *Rhizoctonia*-like fungi and the others.

Fungi isolated from the roots of four *Dactylorhiza* species have been characterized using morphological and molecular analysis techniques. All of the isolated fungal partners from *Dactylorhiza* were identified as Tulasnellaceae

and Ceratobasidiaceae, which are the main mycorrhizal partners for terrestrial orchids (Rasmussen, 2002; Dearnaley,2007; Yukawa et al,2009) except Dct 8, the fungus was identified as Pezizalean Genus, *Verpa* (Morchellaceae). The few studies published so far also found that species of the genera *Dactylorhiza* commonly associate with fungal taxa related to *Tulasnella.* In *D. majalis*, ITS sequences of symbionts found in roots fell into two main clades: one of the genera *Tulasnella* and the second one of distantly related *Laccaria* (Shefferson et al.,2008). In the analysis of mycorrhizal associations in four *Dactylorhiza* species associated with a wide range of Tulasnella and Ceratobasidiaceae fungi. Phylogenetic analysis of fungal ITS sequences related that unique fungal partner of *D. euxina* var. *euxina*, *D. osmanica* var. *osmanic*a (endemic) and was *Tulasnella bifrons*, those of *D. urvilleana* were *T. bifrons* and *Tulasnella* spp. Fungal partners of *D. romana* subsp. *romana* (Pink and White flower) were different according to flower color polymorphism. Fungal partners of the pink flowering were identified as uncultured Ceratobasidiaceae (Dct5) and *Verpa conica verpa* (Dct8). Those of the white flowers were identified as uncultured Ceratobasidiaceae (Dct 6 and Dct 7).On the other hand, Dct6 associated exclusively with *Ceratobasidium albasitensis* from *Dactylorhiza purpurella* in Hungary. Color polymorphism of flowers of *Dactylorhiza sambucina* did not affect fungal diversity in the roots. Although little data are available on the relationship between the mycorrhizal partner and floral color variation, Taylor et al. (2003) showed that floral variation correlates with single *Sebacina* taxa and *Hexalectris spicat*a. In addition to, Pellegrino and Bellusci (2009) suggested that there is no relationship between mycorrhizal partner and flower colors of orchid. Additional researches must be done to reveal the relationship between the color polmorphism and mycorrhizal diversity of *D. romana* subsp. r*omana.* All the *Rhizoctonia*-like fungi, except *Verpa*, are well-known the partners of orchid mycorrhiza in all over the World (Kristiansen et al., 2001; Rasmussen, 2002; Pellegrino and Bellusci, 2009). Although fungal specificity is doubtful in photosynthetic orchids, fully mycoheterotrophy orchids have high fungal specificity (Girlanda et al., 2006). The results of the research revealed that there is no specific relation between four *Dactylorhiza* species and their mycorrhizal partners. *Verpa conica verpa* was isolated from orchid roots for the first time (Pezizales/Ascomycota) in Turkey. Similarly, the roots from *Gymnadenia conopsea* was isolated five Pezizalean genera (Stark et al., 2009) and they suggested that mycorrhizal taxa within Pezizales may be a potential fungal partner of orchids. Even so, most of the mycorrhizal fungi in the roots of orchids are related to Basidiomycetes fungi, some members of Ascomycetes have been isolated from the roots of some *Dactylorhiza* species and the other orchids (Bidartondo et al. 2004; Selosse et al., 2004). Mycorrhizal status of *Verpa conica verpa* isolated from the roots of *D. romana* subsp. *romana* should be investigated in detail in future researches.

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Research article/Araştırma makalesi

Antimycobacterial activity of some lichen extracts

Nalan YILMAZ SARIÖZLÜ [1], Meral YILMAZ CANKILIÇ [*1]

[1] Anadolu University, Faculty of Sciences, Department of Biology, 26470, Eskişehir, Turkey

Abstract

The acetone, methanol and chloroform extracts of lichens *Cetraria aculeata*, *Cladonia foliacea*, *Parmelia sulcata*, *Pseudevernia furfuracea* var. *furfuracea*, *Ramalina farinacea* and *Tornabea scutellifera* were investigated for antimycobacteral properties against *Mycobacterium tuberculosis* H37Rv strain using Microplate Alamar Blue Assay (MABA). All of the lichen extracts showed considerable antimycobacterial effect with minimal inhibitory concentration (MIC) values ranging from 1250 to 156.25 µg/ml. Among the tested lichens, the chloroform extract of the lichen *Pseudevernia furfuracea* var. *furfuracea* exhibited the strongest activity with an MIC value of 156.25 µg/ml.

Key words: antimycobacterial activity, lichen, lichen extracts, MABA, *Mycobacterium tuberculosis*

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Bazı liken ekstraktlarının antimikobakteriyal aktivitesi

Özet

Cetraria aculeata, *Cladonia foliacea*, *Parmelia sulcata*, *Pseudevernia furfuracea* var. *furfuracea*, *Ramalina farinacea* ve *Tornabea scutellifera* likenlerinin aseton, metanol ve kloroform ekstraktlarının *Mycobacterium tuberculosis* H37Rv suşuna karşı antimikobakteriyal özellikleri Microplate Alamar Blue Assay (MABA) kullanılarak incelenmiştir. Liken ekstraktlarının tümü 1250-156.25 µg/ml minimal inhibisyon konsantrasyonu (MİK) aralığındaki değerler ile önemli ölçüde antimikobakteriyal etki göstermiştir. Test edilen likenler arasında *Pseudevernia furfuracea* var. *furfuracea* likeninin kloroform ekstraktı 156.25 µg/ml'lik MİK değeri ile en güçlü aktiviteyi göstermiştir.

Anahtar kelimeler: antimikobakteriyal aktivite, liken, liken ekstraktları, MABA, *Mycobacterium tuberculosis*

1. Introduction

Tuberculosis (TB) is one of the most important infectious diseases caused by mycobacteria belonging to the *Mycobacterium tuberculosis* complex that are a major cause of morbidity and mortality worldwide. According to the latest global tuberculosis report of World Health Organization (WHO), there were an estimated 10.4 million new TB cases (including 1.2 million among HIV-positive people) worldwide in 2015, of which 5.9 million were among men, 3.5 million among women and 1.0 million among children and 1.4 million TB deaths and also TB was one of the top 10 causes of death (WHO, 2016). Moreover in this report, there were an estimated 480 000 new cases of multidrug-resistant TB (MDR-TB) and an additional 100 000 people with rifampicin-resistant TB (RR-TB) who were also newly eligible for MDR-TB treatment in 2015 (WHO, 2016). The discovery and development of new antituberculosis compounds are needed because number of multi-drug resistant isolates of *M. tuberculosis* increases (Cantrell et al., 2001).

Natural products and their derivatives obtained from plants, bacteria, fungi, lichens, marine organisms and associated with their microorganisms etc. have showed antimycobacterial activity (König et al., 2000; Okunade et al., 2004; Pauli et al., 2005; Honda et al., 2010; Cheng et al., 2012; Nguta et al., 2016). These natural compounds have great importance in antibiotic drug discovery.

[*] *Corresponding author* / Haberleşmeden sorumlu yazar: Tel.: +902223350580; Fax.: +902222304910; E-mail: meralyilmaz@anadolu.edu.tr
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Lichens are symbiotic associations between fungi and algae or cyanobacteria. These organisms for centuries have been used in many countries as a treatment for diseases of humans (Kosanić et al., 2012) such as *Cetraria islandica* (Iceland moss), *Lobaria pulmonaria* and *Cladonia* species were known as effective in the treatment of pulmonary tuberculosis (Vartia, 1973).

There are many investigations related to antimicrobial activities of lichens but there are limited studies in literature about the antimycobacterial activity of extracts and of pure substances isolated from lichens (Ingólfsdóttir et al., 1998; Honda et al., 2010; Lucarini et al., 2012). Therefore, the aim of the present study is to determine the activity of extracts of the lichens *Cetraria aculeata*, *Cladonia foliacea*, *Parmelia sulcata*, *Pseudevernia furfuracea* var. *furfuracea*, *Ramalina farinacea* and *Tornabea scutellifera* against *M. tuberculosis*.

2. Materials and methods

2.1. Lichen samples

The lichen species were collected from as follows; *Cetraria aculeata* (Schreb.) Fr., Bozdağ-Eskişehir; *Cladonia foliacea* (Huds.) Willd., Bozdağ-Eskişehir; *Parmelia sulcata* Taylor, Bozdağ-Eskişehir; *Pseudevernia furfuracea* var. *furfuracea* (L.) Zopf., Mihalıççık-Eskişehir; *Ramalina farinacea* (L.) Ach., Bozdağ-Eskişehir and *Tornabea scutellifera* (With.) J.R. Laundon, Çevrepınar mountain-Kahramanmaraş. The samples were identified by Dr. Mehmet Candan using standard keys (Smith et al., 2009; Wirth et al., 2013). The lichen samples were deposited in the Department of Biology, Herbarium of Anadolu University (ANES), Eskişehir, Turkey.

2.2. Preparation of the lichen extracts

Air-dried thalli of the lichen samples were powdered and 10 g portions were extracted in 100 ml of acetone, methanol and chloroform in ultrasonic bath for 30 min, then left at room temperature overnight. The lichen extracts were filtered using Whatman No. 1 filter paper and then concentrated under reduced pressure in a rotary evaporator. The dry extracts were stored at 4 °C until they were used for the analysis.

2.3. Antimycobacterial assay

Microplate Alamar Blue Assay (MABA) was used to determine antimycobacterial activities of the lichen extracts against *Mycobacterium tuberculosis* H37Rv ATCC 27294 (American Type Culture Collection). The dried lichen extracts were dissolved in 20% dimethylsulfoxide (DMSO) and then prepared dilution series from 10 mg/ml to 19.53 μg/ml using sterile distilled water. Rifampicin was used as standard at 0.8 mg/ml-1.5625 μg/ml concentration range. *M. tuberculosis* H37Rv was grown in ATCC® Medium 1395: Middlebrook 7H9 broth with ADC enrichment at 37 °C for 30 days. The turbidity of the cultures was adjusted to McFarland standard no 1. The incubation of all black, clear-bottomed, 96-well microtiter plates (Corning 3340, USA) were performed at the temperature of 37 °C for the period of 7 days. Then, a freshly prepared 1 : 1 mixture of Alamar Blue reagent (1 : 10 dilution, Invitrogen, 1025, USA) and 10% Tween 80 were added to each well on the 7th day of the incubation. The further incubation of the plates was performed at the temperature of 37 °C for the 24 h period. A color change from blue to pink indicated mycobacterial growth. The minimal inhibitory concentration (MIC) values were defined as the lowest concentration of the extracts that showed no color change. All experiments were carried out in triplicate.

3. Results

The potential antimycobacterial activity against *M. tuberculosis* H37Rv of acetone, methanol and chloroform extracts of the lichen species were shown in Table 1. The MIC values for the tested lichens extracts were determined within the range 1250-156.25 μg/ml. The chloroform extract of the lichen *Pseudevernia furfuracea* var. *furfuracea* showed the strongest antimycobacterial activity with 156.25 μg/ml MIC value among the screening lichens. The same MIC value (312.5 μg/ml) was obtained in all extract of lichen *Cladonia foliacea*. The chloroform extracts of the lichen *Cetraria aculeata* and *Pseudevernia furfuracea* var. *furfuracea* were showed stronger antimycobacterial activity than acetone and methanol extracts.

4. Conclusions and discussion

Previous studies were reported about antimicrobial activity of the lichens *Cetraria aculeata*, *Cladonia foliacea*, *Parmelia sulcata*, *Pseudevernia furfuracea* and *Ramalina farinacea* extracts and their constituents against different bacteria and yeasts (Tay et al., 2004; Yılmaz et al., 2004; Candan et al., 2007; Mitrović et al., 2011; Güvenç et al., 2012; Kosanić et al., 2013), but there are few studies related to antimycobacterial activity of these lichens extracts. Also, antimicrobial activity of the lichen *Tornabea scutellifera* has not been reported in literature.

Table 1. Minimal inhibitory concentration (MIC) values of the extracts of *C. aculeata*, *C. foliacea*, *P. sulcata*, *P. furfuracea*, *R. farinacea* and *T. scutellifera* against *M. tuberculosis* H37Rv

Lichens	Lichen extracts	MIC (µg/ml)
	Acetone	1250
Cetraria aculeata	Methanol	1250
	Chloroform	625
	Acetone	312.5
Cladonia foliacea	Methanol	312.5
	Chloroform	312.5
	Acetone	625
Parmelia sulcata	Methanol	625
	Chloroform	1250
	Acetone	625
Pseudevernia furfuracea var. *furfuracea*	Methanol	625
	Chloroform	156.25
	Acetone	312.5
Ramalina farinacea	Methanol	625
	Chloroform	312.5
	Acetone	625
Tornabea scutellifera	Methanol	1250
	Chloroform	625
Antibiotic (standard)		
Rifampicin		25

Ingólfsdóttir et al. (1998) screened the antimycobacterial activity of lichen metabolites usnic acid from *Cladonia arbuscula*, atranorin and lobaric acid from *Stereocaulon alpinum*, salazinic acid from *Parmelia saxatilis*, protolichesterinic acid from *Cetraria islandica* against *Mycobacterium aurum*, a non-pathogenic organism with a similar sensitivity profile to *M. tuberculosis*. They reported that usnic acid had the highest activity the with an MIC value of 32 µg/ml, while the atranorin, lobaric acid, salazinic acid and protolichesterinic acid had the activity with an MIC values of ≥125 µg/ml. A similar study was reported by Honda et al. (2010). They investigated of the activity against *M. tuberculosis* of twenty-six compounds from the lichens *Parmotrema dilatatum*, *Parmotrema tinctorum*, *Pseudoparmelia sphaerospora* and *Usnea subcavata*: depsides, depsidones and xanthones, usnic acid, derivatives from salazinic and lecanoric acids and lichexanthone. They determined diffractaic acid the most active compound among them with an MIC value 15.6 µg/ml followed by norstictic acid (MIC value 62.5 µg/ml) and usnic acid (MIC value 62.5 µg/ml). In that study, hypostictic acid with an MIC value 94.0 µg/ml and protocetraric acid with an MIC value 125 µg/ml showed moderate inhibitory activity while the other compounds showed lower inhibitory activity against *M. tuberculosis*, varying from 250 µM to1370 µM (MIC values ≥250 µg/ml). The activity of usnic acid against *M. tuberculosis* H37Rv as a 12.25 µg/ml was recorded by Ramos and Silva (2010). The acetone extract of lichen *Usnea steineri* was showed MIC values of 32 µg/ml against *M. tuberculosis* H37Rv while isolated compound usnic acid was even more effective against the same strain with MIC value of 8 µg/ml (Lucarini et al., 2012).

According to our results, the considerable antimycobacterial activity was observed with an MIC value of 156.25 µg/ml among the screening lichens extracts in our study**.** Further investigations to do with isolated compounds of the lichens in present study will be conducted more effective results against *M. tuberculosis*.

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Research article/Araştırma makalesi

Nineteen new records for Turkish freshwater algal flora from Lake Taşkısığı and Lake Little Akgöl

Tuğba Ongun SEVİNDİK *[1], Arif GÖNÜLOL [2], Hatice TUNCA [1], Nisa YILDIRIM GÜRSOY [1], Şeyma Nur KÜÇÜKKAYA [1], Zuhal DURGUT KINALI [1]

[1] Sakarya University, Faculty of Arts and Science, Department of Biology, Sakarya, Turkey
[2] Ondokuz Mayıs University, Faculty of Arts and Science, Department of Biology, Samsun, Turkey

Abstract

Nineteen new records for the freshwater algal flora of Turkey were determined in studies conducted from January 2013 and December 2013 in Lake Taşkısığı and Lake Little Akgöl, Sakarya, Turkey. Sampling for chemical analyses and measurement of physical variables were carried out monthly in conjunction with algae collection at the two monitoring stations in each lake. Among these new records, 2 were Cyanobacteria, 9 were Chlorophyta, 1 was Charophyta, 1 was Ochrophyta, 3 were Cryptophyta, and 3were Euglenophyta.

Key words: Lake taşkısığı, Lake little Akgöl, new record, algae, Turkey

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Taşkısığı ve Küçük Akgöl gölleri'nden Türkiye tatlı su alg florası için on dokuz yeni kayıt

Özet

Taşkısığı Gölü'nde ve Küçük Akgöl Gölü'nde (Sakarya) Ocak 2013 ve Aralık 2013 tarihleri arasında yapılan çalışmada Türkiye tatlı su alg florası için on dokuz yeni kayıt teşhis edilmiştir. Kimyasal ve fiziksel parametrelerin ölçümü, alg örneklemesiyle eş zamanlı olarak her gölde belirlenen iki istasyondan aylık olarak yapılmıştır. Tespit edilen taksonların 2 tanesi Cyanobacteria, 9 tanesi Chlorophyta, 1 tanesi Charophyta, 1 tanesi Ochrophyta, 3 tanesi Cryptophyta ve 3 tanesi Euglenophyta gruplarına aittir.

Anahtar kelimeler: Taşkısığı gölü, Küçük Akgöl gölü, yeni kayıt, alg, Türkiye

1. Introduction

Inland waters with different morphometry and hydrology may support distinct algal diversity. Moreover, substantial differences were recognizable when considering the effects of altitude and climate on algal composition (Pollingher, 1990). Since Turkey has possessed three different types of climates and exhibited noticeable altitude differences, number of new records was expected to increase in the future. Until now, many studies were done about the new records for the algal flora in Turkey (e.g., Atıcı, 2002; Baykal et al., 2009, 2012; Sevindik et. al., 2010, 2011, 2015; Baytut and Gönülol, 2016). The aim of this study was to contribute algal flora of Turkey with determined new records.

2. Materials and methods

2.1. Study areas
2.1.1. Lake Taşkısığı

Lake Taşkısığı (LT) is located at 40° 52' 18'' N, 30° 24' 14''E, 13 km north of Sakarya, Turkey (Figure 1). It lies at 12 m above the sea level and has a surface area of 0.9 km^2, a length of 1.2 km, a maximum depth of 5 m and a mean depth of 1.5 m. The lake was formed in the old Sakarya River bed. It is mainly fed by underground water sources

* *Corresponding author* / Haberleşmeden sorumlu yazar: Tel.: +902642955536; Fax.: + 902642955536; E-mail: tsevindik@sakarya.edu.tr
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located at various places and rainfalls. 20% of the lake's shore is covered with macrophtes (*Phragmites* sp., *Nymphaea alba* L. and *Ceratophyllum demersum* L.). Lake Taşkısığı is a shallow eutrophic freshwater lake (the mean annual chlorophyll-*a* concentration is 0.005 mgL^{-1}, total phosphorus is 0.036 mgL^{-1} and Secchi disk depth is 68.91 cm) according to Carlson (1977) and OECD (1982) criteria. Formerly, the influence of some physiochemical parameters on phytoplankton abundance and species composition have been reported in Lake Taşkısığı (Aykulu et al., 1999; Temel and Yardımcı, 2004).

2.1.2. Lake Little Akgöl

Lake Little Akgöl (LLA) is located at 40° 52' 38''N, 30° 25' 56''E, 20 km north of Sakarya, Turkey (Figure 1). It lies at 12.3 m above the sea level and has a surface area of 0.16 km^2, a length of 0.58 km, a maximum depth of 1.3 m and a mean depth of 0.5 m. The lake was formed in the old Sakarya River bed. The sole outlet located northern edge that connected with Çark Stream which is formerly connected with Sakarya River. Dense macrophyte (40% mean coverage) (*Phragmites* sp., *Nymphaea alba* L. and *Ceratophyllum demersum* L.) development was seen on the coasts of the lake. Surroundings of the lake are comprised of forest. In 2001, 30 hectares of the area was declared a "Wildlife Protection Area". Lake Little Akgöl is a shallow eutrophic freshwater lake (the mean annual chlorophyll-*a* concentration is 0.011 mgL^{-1}, total phosphorus is 0.167 mgL^{-1} and Secchi disk depth is 55.3 cm) according to Carlson (1977) and OECD (1982) criteria.

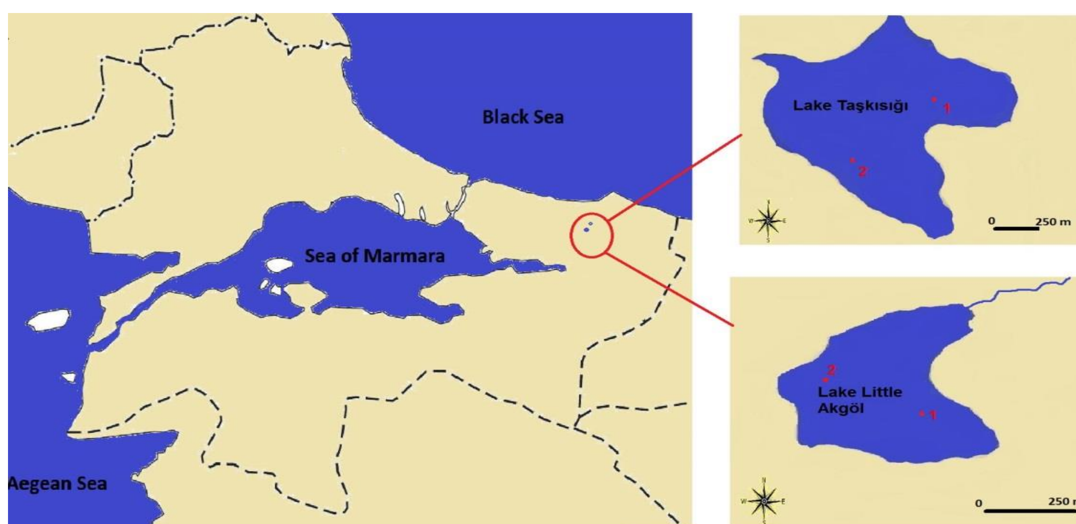

Figure 1. The map of Lake Taşkısığı and Lake Little Akgöl and the location of sampling stations

2.2. Sampling and identification

Sampling was carried out monthly at the two monitoring stations in each lake between January 2013 and December 2013. The whole water column was sampled with integrated sampling tube. In the field, samples were placed in dark bottles. In the laboratory, the samples were first agitated, then poured into 50 mL graduated cylinders and were allowed to settle for 24 hours. At the end of the settling period, 45 mL of water was aspirated from each graduated cylinder and the remaining 5 mL of water was poured into a small glass vial for microscopic analysis. (Utermöhl, 1958). Identification of samples was performed on a compound microscope, equipped with water immersion lenses and a phase contrast attachment. Algal species were identified according to Huber–Pestalozzi (1962, 1969, 1974, 1976, 1983); John et al. (2003); Philipose (1967); Komarek and Anagnostidis (1999). Taxa were photographed with a camera attached to an Olympus BX 51 microscope. Identified taxa were checked with the checklist of Gönülol et al. (1996); Aysel (2005), Şahin (2002, 2005) and Gönülol (2016) web site, determined as new taxa for Turkish algal flora. Taxonomy of algae was controlled for current accepted status of the species from Guiry and Guiry (2017) web site.

2.3. Environmental variables

Sampling for chemical analyses and measurement of physical variables were carried out in conjunction with algae collection. Specific conductance (EC), pH, dissolved oxygen (DO) and water temperature (T) were measured at 10 cm below the surface using a YSI ProPlus water quality instrument. Water transparency was measured on each sampling day using a Secchi disk. For the analysis of chemical variables, samples were collected by a tube sampler, integrating the whole water column. Concentrations of nitrate-nitrogen (NO_3-N), nitrite-nitrogen (NO_2-N), total phosphorus (TP), orthophosphate (PO_4-P), soluble silica (Si) and sulfate (SO_4) were determined spectrophotometrically

according to Strickland and Parsons (1972) and Technicon Industrial Methods (1977 a, b). Chlorophyll-*a* (Chl-*a*) was determined via extraction with 90% methanol spectrophotometrically (Youngman, 1978).

3. Results

Environmental variables of LT and LLA waters are given in Table 1. A total number of new records for freshwater algal flora of Turkey are 19 (Cyanobacteria 2, Chlorophyta 9, Charophyta 1, Ochrophyta 1, Cryptophyta 3, and Euglenozoa 3). These taxa are listed below.

Table 1. The mean and standard deviation (SD) of environmental variables measured at the sampling sites in Lake Taşkısığı and Lake Little Akgöl. (T: water temperature, EC: specific conductance, DO: dissolved oxygen, NO_3-N: nitrate-nitrogen, NO_2-N: nitrite-nitrogen, PO_4-P: orthophosphate, TP: total phosphorus, SO_4: sulphate, Si: soluble silica, Chl-*a*: chlorophyll-*a*)

	Lake Taşkısığı		**Lake Little Akgöl**	
Variable	**Station 1**	**Station 2**	**Station 1**	**Station 2**
	Mean±SD	**Mean±SD**	**Mean±SD**	**Mean±SD**
T(ºC)	18.33±7.62	18.05±7.47	18.73±8.32	19.1±8.39
pH	8.52±0.97	8.55±0.17	8.42±0.28	8.35±0.29
EC (µScm^{-1})	534.10±28.61	537.82±28.11	567.9±152.03	547.02±167.81
DO (mgL^{-1})	7.10±4.18	8.91±7.20	5.58±2.15	5.24±1.87
Secchi disk (cm)	69.91±13.31	68.37±16.61	56.25±9.79	53.83±13.78
NO_3–N (mgL^{-1})	0.27±0.18	0.35±0.24	0.17±0.13	0.18±0.22
NO_2–N (mgL^{-1})	0.009±0.005	0.009±0.004	0.017±0.09	0.018±0.02
PO_4–P (mgL^{-1})	0.020±0.033	0.019±0.021	0.15±0.01	0.17±0.14
TP (mgL^{-1})	0.040±0.024	0.034±0.022	0.17±0.08	0.18±0.10
SO_4 (mgL^{-1})	20.55±33.45	19.89±25.69	5.89±7.83	5.68±7.93
Si (mgL^{-1})	8.80±6.07	10.55±9.39	6.86±3.56	6.73±3.41
Chl-*a* (mgL^{-1})	0.002±0.001	0.006±0.005	0.011±0.01	0.011±0.01

Division: Cyanobacteria
Class: Cyanophyceae
Order: Chroococcales
Family: Microcystaceae
Genus: *Microcystis* Lemmermann, 1907
Microcystis firma (Kützing) Schmidle, 1902 (Figure 2a)
Basionym: *Micraloa firma* Kützing, 1846
Synonyms: *Micraloa firma* Kützing, 1846; *Polycystis firma* (Kützing) Rabenhorst, 1865; *Anacystis firma* (Kützing) Drouet and Daily, 1948
Reference: (Komarek and Anagnostidis, 1999; information pg. 226, figure 296)
Remarks: Colonies spherical, with densely packed cells, with distinct mucilaginous envelopes. Cells spherical, with gas vesicles, 6 µm in diameter. Found in LT.

Family: Chroococcaceae
Genus: *Chroococcus* Nageli, 1849
Chroococcus aphanocapsoides Skuja, 1964 (Figure 2b)
Reference: (Komarek and Anagnostidis, 1999; information pg. 284, figure 368 a-b)
Remarks: Colonies gelatinous, in outline spherical, 30 µm in diameter. Cells usually arranged in small aggregates (with 4-8 cells) within homogeneous, colorless, hyaline, structureless and diffluent mucilage, forming wide margin around cells. Cells spherical, 3 µm in diameter, without individual mucilaginous envelopes, with homogeneous, pale blue green content, without aerotopes. Found in LLA.

Division: Chlorophyta
Class: Chlorophyceae
Order: Sphaeropleales
Family: Selenastraceae
Genus: *Ankistrodesmus* Corda, 1838
Ankistrodesmus bernardii Komárek, 1983 (Figure 2c)
Reference: (Huber-Pestalozzi, 1983; information pg. 687, figure 193/3 a-d)
Remarks: Colonies form like bundle, with 8 cells, curled and connected together on middle. Cells long and very narrow, 45 µm long, 2 µm wide. Found in LT.

Genus: *Monoraphidium* Komárková-Legnerová, 1969
Monoraphidium subclavatum Nygaard, 1977 (Figure 2d)
Reference: (Huber- Pestalozzi, 1983; information pg. 640, figure 179/3 a-b)
Remarks: Cells fusiform, sickle-shaped, 28 µm long, 8 µm wide. The ends gradually tapered. Chloroplast parietal almost all the cell wall, without significant turning point in the middle, without pyrenoid. Found in LT.

Family: Hydrodictyaceae
Genus: *Tetraëdron* Kutzing, 1845
Tetraëdron pusillum (Wallich) West and G.S.West, 1897 (Figure 2e)
Basionym: *Micrasterias pusilla* G.C.Wallich
Synonym: *Micrasterias pusilla* G.C.Wallich
Reference: (Philipose, 1967; information pg. 159, figure 74 a-c)
Remarks: Cells cruciform with four processes, each ending in two recurved spines. In side view, elongate- ellipsoid with attenuate ends. Cells 20 µm long, 15 µm wide. Found in LT.

Family: Scenedesmaceae
Genus: *Desmodesmus* (Chodat) An, Friedl and Hegewald, 1999
Desmodesmus microspina (Chodat) Tsarenko, 2000 (Figure 2f)
Basionym: *Scenedesmus microspina* Chodat, 1926
Synonyms: *Scenedesmus microspina* Chodat, 1926; *Scenedesmus quadricauda* var. *microspina* (Chodat) Philipose 1967
Reference: (Huber-Pestalozzi, 1983; information pg. 930, figure 249/7. John et al., 2003; information pg. 395, figure 391/P)
Remarks: Coenobia of 2 linearly arranged cells; cells 8 µm long, 4 µm wide, ellipsoidal with rounded apices, cells whose outermost wall is convex, bear short and oblique spines, cell walls smooth, lacking a mucilaginous envelope. Found in LT.

Genus: *Scenedesmus* Meyen, 1829
Scenedesmus heteracanthus González, 1940 (Figure 2g)
Reference: (Huber- Pestalozzi, 1983; information pg. 890, figure 240/9)
Remarks: Coenobia of 4 linearly arranged cells; cells 10 µm long, 5 µm wide, elongate ellipsoid and outer side are slightly convex. 2 long, straight spines obliquely set in 2 diagonal corners of the coenobia. Accessory teeth often present at the poles of all cells. Found in LT.

Order: Chlamydomonadales
Family: Scotiellocystoidaceae
Genus: *Diplochloris* Korschikov, 1939
Diplochloris lunata (Fott) Fott, 1979 (Figure 2h)
Basionym: *Dichotomococcus lunatus* Fott, 1948
Synonym: *Dichotomococcus lunatus* Fott, 1948
Reference: (Huber- Pestalozzi, 1983; information pg. 675, figure 189/2)
Remarks: Colonies with two cells, cells touching with their convex longitudinal sides. Cells sickle-shaped, not twisted, rounded at the ends, 8 µm long, 3 µm wide. Found in LT.

Family: Chlamydomonadaceae
Genus: *Chlamydomonas* Ehrenberg, 1833
Chlamydomonas ampulla Skvortzov (Figure 2i)
Reference: (Huber- Pestalozzi, 1974; information pg. 432, figure 577)
Remarks: Cells spherical, 15 µm in diameter. Membrane without papilla. Chloroplast cup-shaped; pyrenoid narrow kidney-shaped, basal; stigma anterior. Found in LLA.

Chlamydomonas convexa Pascher, 1932 (Figure 2j)
Reference: (Huber- Pestalozzi, 1974; information pg. 256, figure 292)
Remarks: Cells ellipsoidal, 12.5 µm long, 10 µm wide. Membrane delicate, without particular papilla. Chloroplast cup-shaped; pyrenoid irregular; no stigma; two contractile vacuoles anterior. Found in LLA.

Class: Trebouxiophyceae
Order: Chlorellales
Family: Oocystaceae
Genus: *Scotiella* Fritsch, 1912
Scotiella tatrae Kol, 1965 (Figure 2k)
Reference: (Huber- Pestalozzi, 1983; information pg. 717, figure 200/1)
Remarks: Cells broad oval, 13 µm long, 9 µm wide. Anterior and posterior end sharply narrowing, cell wall spirally coiled. Found in LLA.

Division: Charophyta
Class: Conjugatophyceae

Order: Desmidiales
Family: Desmidiaceae
Genus: *Staurastrum* Meyen ex Ralfs, 1848
Staurastrum anatinum Cooke and Wills, 1881 (Figure 2l)
Reference: (John et al., 2003; information pg. 565, figure 139/J)
Remarks: Cells (2-)3(-4) radiate, 35 µm wide excluding processes, 65 µm wide with processes, 45 µm long excluding processes, 55 µm long with processes, deeply constricted with a wide open internally acute sinus, isthmus 15 µm wide, semicells mostly broadly cup-shaped; lateral margins moderately convex, apex slightly convex; angles usually considerably extended to form long, hollow, gradually attenuating, subparallel to divergent process terminating in 3 short, stout, divergent spines; processes with concentric rings of denticulations of variable prominence along length, continuing onto semicell body; denticulations on apex and each side of body often much enlarged, becoming short emarginate spines. Found in LT.

Division: Ochrophyta
Class: Chrysophyceae
Order: Chromulinales
Family: Dinobryaceae
Genus: *Pseudokephyrion* Pascher, 1913
Pseudokephyrion conicum Schiller, 1929 (Figure 3a, 3b)
Reference: (Huber- Pestalozzi, 1962; information pg. 200, figure 267)
Remarks: Lorica conical, yellow-brown in colour, 13 µm long, 12 µm wide. Posterior end slightly rounded. Flagella 2, unequal, the shorter one just over the edge of the lorica. Found in LT.

Division: Cryptophyta
Class: Cryptophyceae
Order: Pyrenomonadales
Family: Chroomonadaceae
Genus: *Chroomonas* Hansgirg, 1885
Chroomonas reflexa Kisselev, 1931 (Figure 3c)
Reference: (Huber- Pestalozzi, 1976; information pg. 34, figure 21)
Remarks: Cells ovate, sharpened and curved back of the posterior end, 23 µm long, 12 µm wide. Chloroplast, 2; inner part of the cell possessed starch grains. Found in LT.

Order: Cryptomonadales
Family: Cryptomonadaceae
Genus: *Cryptomonas* Ehrenberg, 1831
Cryptomonas tetrapyrenoidosa Skuja, 1948 (Figure 3d)
Reference: (Huber- Pestalozzi, 1976; information pg. 62, figure 45)
Remarks: Cells oval, 25 µm long, 15 µm wide. Anterior end obliquely rounded with a mostly shallow constriction on the ventral side. Posterior end wide, rounded. Periplast thin, colorless, smooth; flagella slightly unequal. Cytopharynx deep; trichocysts organized in longitudinal row; pyrenoid 4; nucleus posterior; chloroplast 2, brownish-olive, bowl-shaped. Found in LLA.

Cryptomonas paramaecium (Ehrenberg) Hoef-Emden and Melkonian, 2003 (Figure 3e)
Basionym: *Chilomonas paramaecium* Ehrenberg, 1831
Synonym: *Chilomonas paramaecium* Ehrenberg, 1831; *Trichoda paramaecium* Ehrenberg, 1830
Reference: (Huber-Pestalozzi, 1976; information pg. 70, figure 54)
Remarks: Cells elongated cylindrical, 35 µm long, 16 µm wide. Posterior end slightly narrowed and rounded; trichocysts organized as 8 long row in cytopharynx; flagella equal, about one-half cell length; nucleus slightly posterior. Found in LT.

Division: Euglenophyta
Class: Euglenophyceae
Order: Euglenales
Family: Phacaceae
Genus: *Phacus* Dujardin, 1841
Phacus polytrophos Pochmann, 1942 (Figure 3f)
Reference: (Huber-Pestalozzi, 1969; information pg. 186, figure 208)
Remarks: Cells elongated ovoid, 22 µm long, 10 µm wide. Anterior end slightly narrowing, posterior end narrowing to a sharp, straight tail-piece. Stigma large and mostly clear. Chloroplasts numerous, elliptical; paramylon bodies 2, elongated, large and thick. Found in LT.

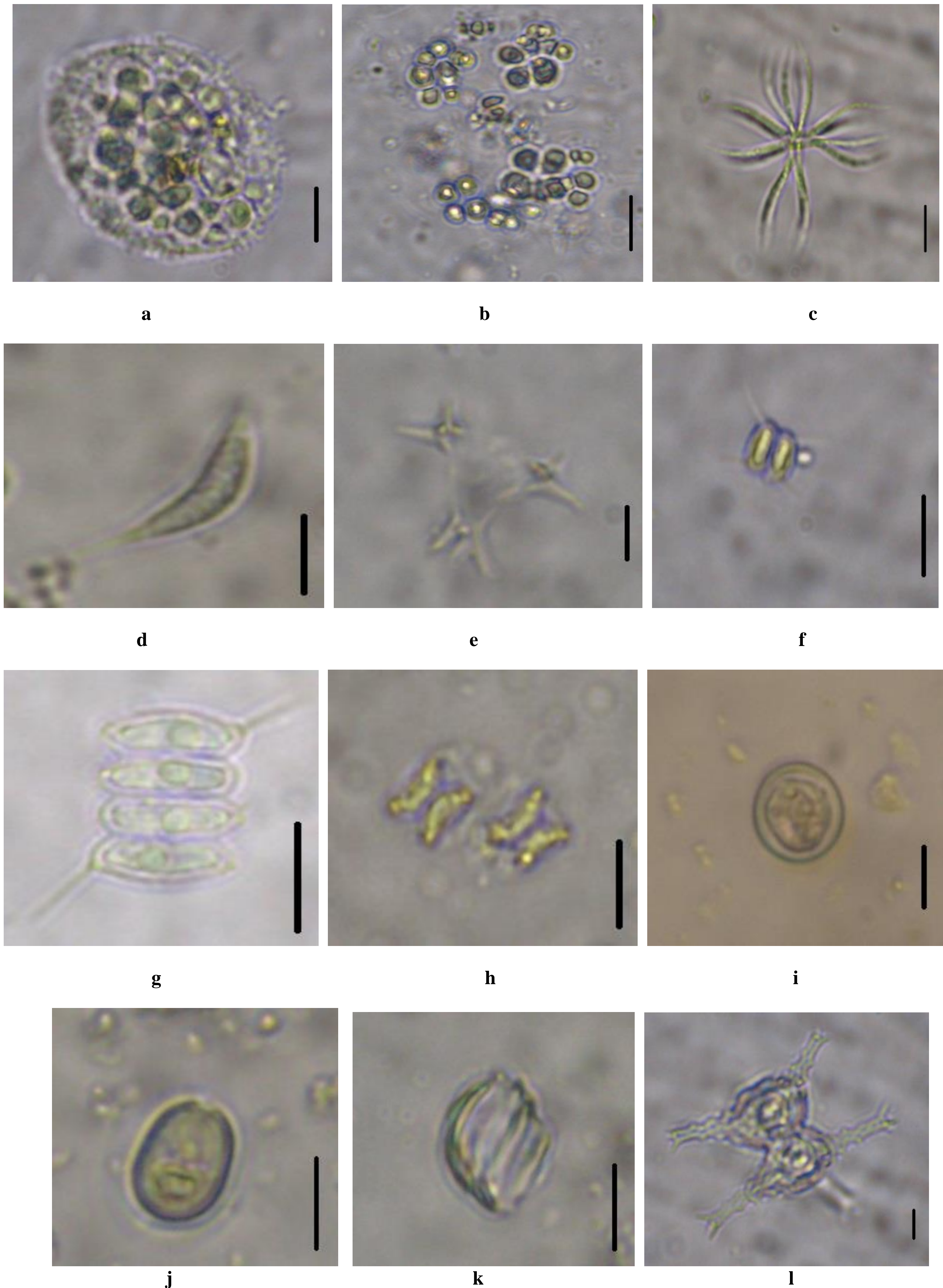

Figure 2. a. *Microcystis firma,* **b.** *Chroococcus aphanocapsoides,* **c.** *Ankistrodesmus bernardii,* **d.** *Monoraphidium subclavatum,* **e.** *Tetraëdron pusillum,* **f.** *Desmodesmus microspina,* **g.** *Scenedesmus heteracanthus,* **h.** *Diplochloris lunata*, **i.** *Chlamydomonas ampulla,* **j.** *Chlamydomonas convexa*, **k.** *Scotiella tatrae*, **l.** *Staurastrum anatinum* (Scale 10 µm)

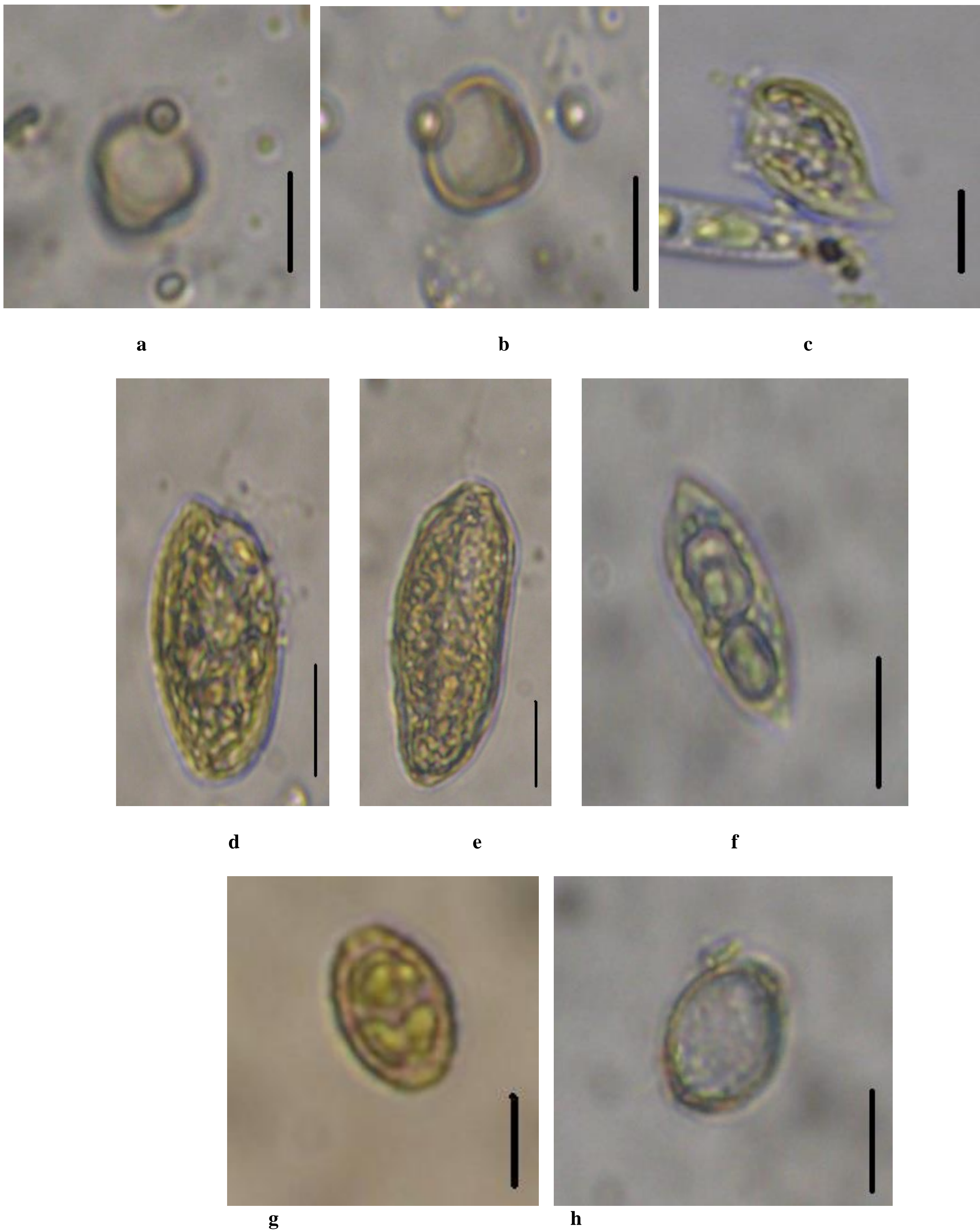

Figure 3. a.-b. *Pseudokephyrion conicum*, **c.** *Chroomonas reflexa*, **d.** *Cryptomonas tetrapyrenoidosa*, **e.** *Cryptomonas paramaecium*, **f.** *Phacus polytrophos,* **g.** *Trachelomonas eurystoma* var. *minuta*, **h.** *Trachelomonas hispida* var. *acuminata* (Scale 10 µm)

Family: Euglenaceae
Genus: *Trachelomonas,* Ehrenberg, 1835
Trachelomonas eurystoma Stein var. *minuta* Van Oye, 1927 (Figure 3g)
Reference: (Huber-Pestalozzi, 1969; information pg. 316, figure 614)

Remarks: Lorica 17 µm long, 10 µm wide, ellipsoidal. Wall serrate, yellowish brown. Anterior end with very short collar. Found in LT.

Trachelomonas hispida var. *acuminata* Deflandre, 1926 (Figure 3h)
Reference: (Huber-Pestalozzi, 1969; information pg. 296, figure 523)
Remarks: Lorica 15 µm long, 10 µm wide, ovoid, posterior end with conical bumps. Wall serrate, orange-brown. Found in LT.

4. Conclusions and discussion

A total of 19 taxa were determined as new records for Turkish freshwater algae in the divisions of Cyanobacteria, Chlorophyta, Charophyta, Ochrophyta, Cryptophyta and Euglenophyta. The division Chlorophyta contained the highest records with 9 taxa. These taxa are dispersed into genus *Ankistrodesmus*, *Monoraphidium*, *Tetraëdron*, *Desmodesmus*, *Scenedesmus*, *Diplochloris*, *Chlamydomonas* (2), which were widespread worldwide (John et al., 2003; Wehr and Sheath, 2003). It has been reported that the species belonging to these genera were cosmopolitan in lakes, ponds, reservoirs, and stagnant and slow flowing running waters in Turkey (Gonulol et al., 1996; Aysel, 2005; Celekli et al., 2007a, b; Celik and Ongun, 2008; Sevindik, 2010; Sevindik and Çelik, 2012; Sevindik et al., 2015). Only one species in genus *Scotiella* was rare in distribution (Huber- Pestalozzi, 1983). All new records of genus *Chlamydomonas* were found in LLA which were more eutrophic. *Chlamydomonas* species are abundant in small, very or extremely nutrient rich waters, particularly in the spring and early summer (John et al., 2003). It was reported that *Ankistrodesmus bernardii* was in association with aquatic macrophytes, however we found it in plankton. It was reported in Romania, Spain, Brazil, Cuba, and Southeast Asia (Huber- Pestalozzi, 1983; Guiry and Guiry, 2017). *Monoraphidium subclavatum* was reported as planktonic and found in Germany, Romania, Brazil and Denmark (Huber-Pestalozzi, 1983; Guiry and Guiry, 2017). *Tetraëdron pusillum* was reported in North America, Pakistan, China and India (Philipose, 1967; Guiry and Guiry, 2017). *D. microspina* was probably cosmopolitan and widely distributed in Europe and also reported in Iceland, Cuba, Argentina (John et al., 2003; Guiry and Guiry, 2017). *Diplochloris lunata* distributed in Britain, Germany, Spain, Taiwan, while, *Scenedesmus heteracanthus* in Europe, South America, Africa and India (Huber- Pestalozzi, 1983; Guiry and Guiry, 2017).

Microcystis firma was reported as planktic in stagnant waters of Europe and Australia; and widespread especially in North Europe (Komarek and Anagnostidis, 1999; Guiry and Guiry, 2017). *Chroococcus aphanocapsoides* was found in oligo - mesotrophic lakes and distributed in Baltic Sea, Lithuania, Spain, Australia (Komarek and Anagnostidis, 1999; Guiry and Guiry, 2017).

Staurastrum anatinum was reported as cosmopolitan, and distributed in the plankton of poor to nutrient-enriched lakes in Europe, America, Asia and Australia. It preferred alkaline waters (John et al., 2003; Guiry and Guiry, 2017). We found this species in LT, and pH ranged between 8.27 and 9.17. It was known that members of Desmidiales were common in eutrophic and mesotrophic alkaline lakes in Turkey (Gonulol and Comak, 1993).

The genus *Pseudokephyrion* (Ochrophyta) and its species *Pseudokephyrion conicum* were both reported as a new record for the first time for algal flora of Turkey. *Pseudokephyrion conicum* was recorded in Germany, Spain, Austria and New Zealand (Guiry and Guiry, 2017). It observed only during the flood in various fresh waters (Huber-Pestalozzi, 1962) especially in spring (John et al., 2003). We found this species in early spring (March).

Members of Cryptophyta occurred in very different kinds of freshwater environments, some were favored by waters rich in organic substances and several were more common during the colder months of the year (John et al., 2003). Cryptophytes were reported in Çaygören and İkizcetepeler reservoirs, Ladik, Akgöl and Mollaköy lakes and Danamandıra Ponds (Maraşlıoğlu et al., 2005; Ersanlı et al., 2006; Sevindik, 2010; Sevindik et al., 2011, 2015). *Chroomonas reflexa* was described as characteristic species of some typical ponds in Central Asia (Huber- Pestalozzi, 1976). *Cryptomonas tetrapyrenoidosa* was recorded in Europe, North America and Japan, while, *C. paramaecium* in Europe, America, Asia, Australia (Guiry and Guiry, 2017).

The division Euglenophyta contains three new records in the genus of *Trachelomonas* and *Phacus*; and all of them were found in LT. *Phacus* and *Trachelomonas* mostly occur in stagnant waters of puddles, ponds, swamps, ditches and lakes, especially in waters with high levels of organic nutrients (Prescott, 1962; Say and Whitton 1980; John et al., 2003). *Phacus polytrophos* was found in Europe, America, Africa, Asia while, *Trachelomonas eurystoma* var. *minuta* was reported in Belgium and Congo. *Trachelomonas hispida* var. *acuminata* was recorded in Brazil and France (Huber-Pestalozzi, 1969; Guiry and Guiry, 2017). *Trachelomonas* species were found widespread in both shallow lakes and reservoirs of Turkey (Gonulol et al., 1996; Aysel, 2005; Ersanlı et al., 2006; Soylu et al., 2007; Celekli et al., 2007a, b; Sevindik et al., 2015).

In conclusion, 19 new records were added to freshwater algal flora of Turkey with this study. It was observed that these taxa were distributed different regions in the world. Number of new records for algal flora of Turkey is expected to increase in the future.

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Research article/Araştırma makalesi

Erysiphe azaleae, a new powdery mildew fungus introduced into Turkey

Vasyl P. HELUTA1, Ilgaz AKATA [*1], Şanlı KABAKTEPE [3]

[1] M.G. Kholodny Institute of Botany, National Academy of Sciences of Ukraine, 2 Tereshchenkivska Str., Kiev, 01004
[2]Ankara University, Faculty of Science, Department of Biology, 06100 Ankara, Turkey
[3]Inonu University, Battalgazi Vocat Sch, TR-44210 Battalgazi, Malatya, Turkey

Abstract

In the current study, *Erysiphe azaleae* (U. Braun) U. Braun & S. Takam. (*Erysiphales*, *Ascomycota*) is reported for the first time from Turkey. A short description, host plant, locality and illustrations related to macro and micromorphologies of the species are provided. *Erysiphe azaleae* has been found only once on *Rhododendron ponticum* L. (*Ericaceae*) in Artvin province in the Black Sea region. This is the first report of this species in Asia, because the fungus known from the Russian Far East as *E. azaleae* is actually *E. izuensis.* The fungus formed both conidia and fruiting bodies. Thus, *E. azaleae* should be considered as a potentially dangerous powdery mildew pathogen for introduced and native rhododendrons in Turkey.

Key words: *Erysiphales*, new record, *Rhododendron*

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Erysiphe azaleae, Türkiye'ye tanıtılan yeni bir külleme mantarı

Özet

Mevcut çalışmada, *Erysiphe azaleae* (U. Braun) U. Braun & S. Takam. (*Erysiphales*, *Ascomycota*) Türkiye'den ilk kez rapor edilmiştir. Türün kısa deskripsiyonu, konukçu bitkisi, lokalitesi ve makro ve mikro morfolojilerine ait fotoğrafları verilmiştir. *Erysiphe azaleae* Karadeniz Bölgesinde yer alan Artvin yöresinde sadece *Rhododendron ponticum* L. (*Ericaceae*) bulunmuştur. Bu tür aynı zamanda Asya kıtası için ilk rapordur çünkü Rusya uzak doğusunda daha önce *E. azaleae* olarak bilinen tür aslında *E. izuensis*'tir. Bu mantar conidium ve fruktifikasyon organı oluşturur. Bu nedenle *Erysiphe azaleae* Türkiye'de doğal yayılış gösteren orman gülleri için potansiyel tehlike arz eden bir külleme patojeni olarak düşünülmelidir.

Anahtar kelimeler: *Erysiphales*, yeni kayıt, *Rhododendron*

1. Introduction

Over recent decades, the number of powdery mildew species in Europe has increased due to the introduction of alien fungi of East Asian and North American origin. A newly introduced from America are, for example, species such as *Erysiphe azaleae* (U. Braun) U. Braun & S. Takam. (Ing, 2000; Heluta et al., 2004), *E. elevata* (Burrill) U. Braun & S. Takam. (Ale-Agha et al., 2004; Cook et al., 2004, Vajna et al., 2004; Heluta et al., 2009), *E. flexuosa* (Peck) U. Braun & S. Takam. (Ale-Agha et al., 2000; Zimmermannová-Pastirčáková et al., 2000; Piątek, 2002; Zimmermannová-Pastirčáková and Pastirčák, 2002; Heluta, Voytyuk, 2004), *E. symphoricarpi* (Kiss et al., 2002; Heluta et al., 2016), *Golovinomyces greeneanus* (U. Braun) V.P. Heluta (Heluta and Korytnyanska, 2011), *Podosphaera amelanchieris* Maurizio (Braun and Cook, 2012; Heluta and Hirylovich, 2016) etc. As previously reported (Heluta et al., 2013; Akata and Heluta, 2015), at least two of introduced species, North American *E. platani* and East Asian *E. syringae-japonicae*, migrated to Turkey via Europe. This process continues, as it is evidenced by the powdery mildew on *Rhododendron ponticum* L. (Fig. 1) found recently in Turkey. We should note that before our studies, any powdery mildew on

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* *Corresponding author* / Haberleşmeden sorumlu yazar: Tel.: +903122126720/1066; Fax.: +**903122126720**; E-mail: fungus@hotmail.com.tr
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rhododendrons was still not registered in this country (see Kabaktepe et al., 2015). So, we had found a new species for Turkey. Therefore, the goal of this study was to identify the fungus.

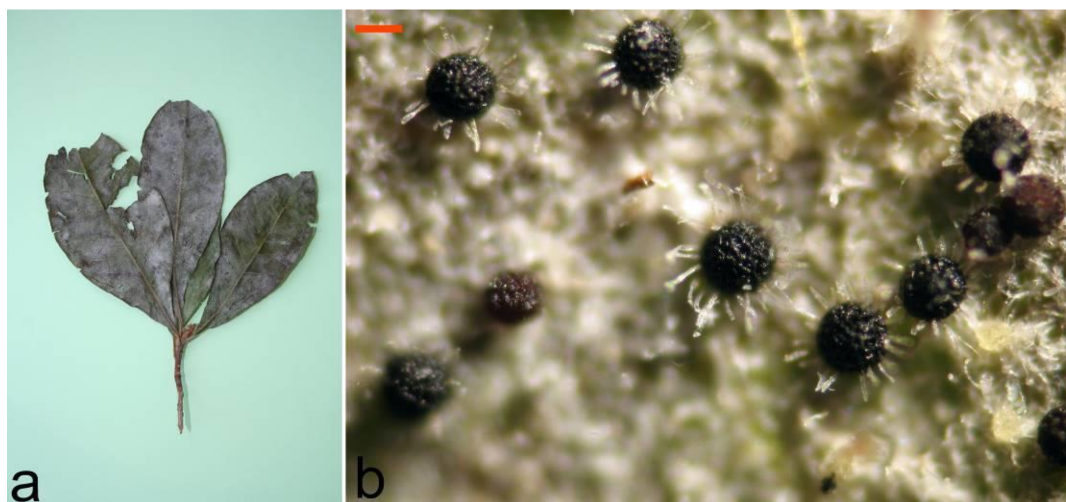

Figure 1. *Erysiphe azaleae* on *Rhododendron ponticum*: **a.** the host plant affected by the fungus; **b.**chasmothecia as viewed under the light microscope in reflected light (bar: b – 100 µm)

2. Materials and methods

The powdery mildew sample was collected on *Rhododendron ponticum* in Karagöl (Şavşat, Artvin, Turkey) in 2014. The herbarium specimen is deposited at the National Herbarium of the M.G. Kholodny Institute of Botany of the National Academy of Sciences of Ukraine (KW). Morphological features were examined and photographed using a light microscope «Primo Star» (Carl Zeiss, Germany) with the camera «Canon A 300» and the software «AxioVision 4.7». The mycelium, conidiophores and conidia were removed from the surface of infected leaves by a transparent adhesive tape. To restore shape and size, a piece of tape with these fungus structures was put in a droplet of 40 % lactic acid solution on a microscope slide (sticky side up), covered with a cover glass, gently heated to boiling point, then examined under the light microscope. For each morphological feature, 30 structures were measured and the data processed statistically. For scanning electron microscopy (SEM) dried pieces of infected *Rhodendron* leaves were fixed on stubs using double-sided adhesive tape, coated with gold, and examined using an EVO 40XVP (LEO Ltd., Cambridge, UK) scanning electron microscope at an accelerating voltage of 20 kV.

3. Results

As a result of microscopic examination of the collected specimen, it was revealed that fungus found on *Rhododendron ponticum* in Turkey has anamorph *Pseudoidium* and immature fruiting bodies with dichotomously branched appendages (Figs 1b, 2e, g, 3a). Thus, this species belongs to the genus *Erysiphe* sect. *Microsphaera* (former genus *Microsphaera*). By all features (appearance of affected leaves, chasmothecial size, morphology and location of the appendages), our specimen is consistent with samples of *E. azaleae* collected in Europe (Ukraine). So, in Turkey we registered a new powdery mildew species of North American origin. Obviously, the fungus migrated here via Europe.

Erysiphe azaleae **(U. Braun) U. Braun & S. Takam., Schlechtendalia 4: 5. 2000**

Syn.: *Oidium ericinum* Erikss., Meddn Kgl. Landbr.-Akad. Exper. **1**: 47. 1885. – *Microsphaera azaleae* U. Braun, Mycotaxon **14**(1): 370. 1982 (Figs 1–3).

Mycelium amphigenous, effuse, white, persistent, better developed on the upper surface of leaves, subtle on the lower surfaces of leaves. Hyphae thin, 3–4 µm wide. Conidiophores rather short, 75–105 µm, very rare longer, up to 180 µm long, consisting of a mainly cylindrical, usually flexuous-sinuosus or curved foot cell 50–70 µm long, followed by 1–2(–3) other cells. Conidia formed singly, mainly cylindrical, occasionally cylindrical-ellipsoid, (25–)27–44(–50) × 10.5–16 µm, with a length/breadth ratio of 2.1–4.4, germinated with a single terminal germ tube. Chasmothecia immature, scattered, hemispherical, depressed in the lower part, with a distinctly meshed peridial surface, (93–)98–130(–140) µm diam. Peridial cells rather distinct, polygonal, irregular in shape, (8–)11–25(–30) µm diam. Appendages semi-matured, numerous, over 30, equatorial, short, with dichotomously branched apices. Asci immature.

Specimen examined: Turkey, Karagöl, Şavşat (Artvin), *Rhododendron ponticum* L., 31.08.2014, Kabaktepe & Akata (KW 70694F).

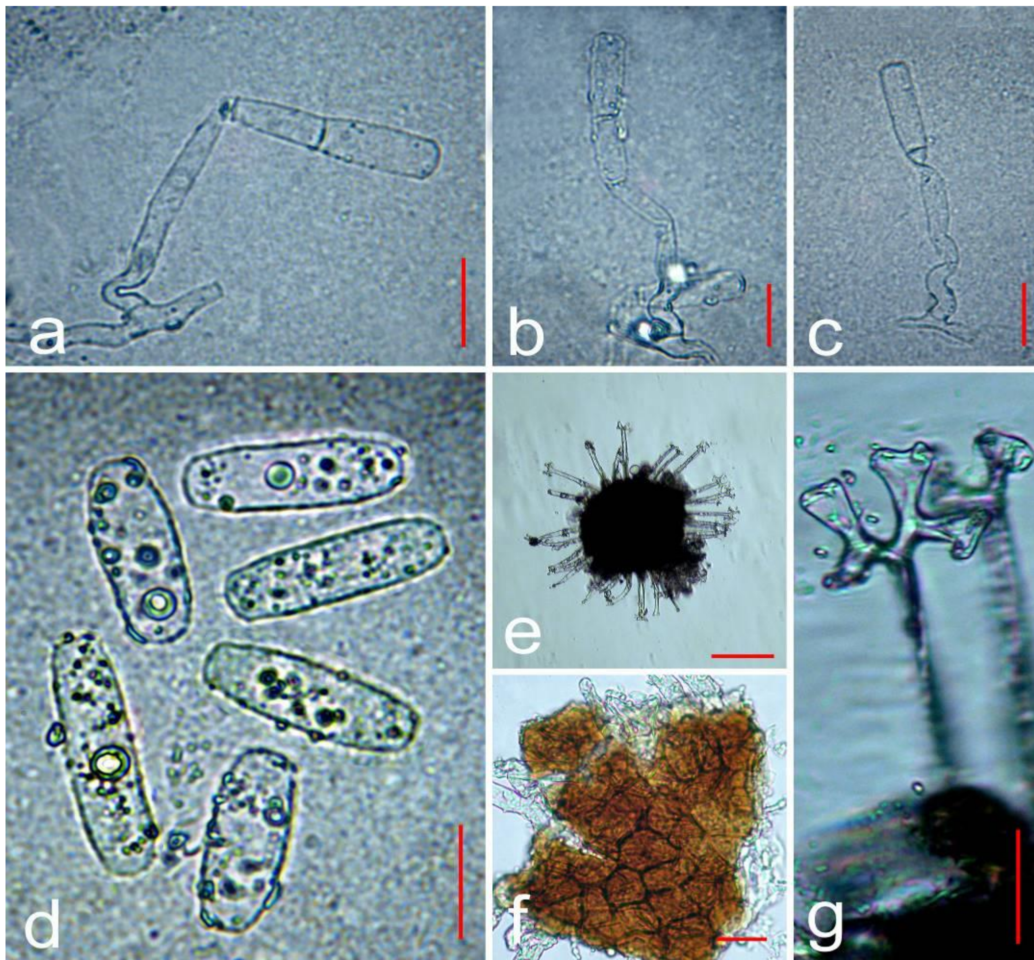

Figure 2. *Erysiphe azaleae* on *Rhododendron ponticum* as viewed by a light microscope: **a–c**. conidiophores, **d.** conidia, **e.** chasmothecium; **f.** peridial cells, **g.** appendage with dichotomously branched apex (bars: a–d, f–g – 20 µm, e – 100 µm)

4. Conclusions and discussion

Rhododendrons are popular ornamental shrubs and they are cultivated since 18th century. More than 850 *Rhododendron* species exist in the temperate zone of the northern hemisphere. Turkish native evergreen rhododendrons are distributed in Black Sea region and they can be observed from sea level up to altitudes of 3000 m (Avcı, 2004).

Several powdery mildew fungi are known on rhododendrons, namely *Erysiphe azaleae* (U. Braun) U. Braun & S. Takam., *E. digitata* (A.J. Inman & U. Braun) A.J. Inman & U. Braun, *E. izuensis* (Y. Nomura) U. Braun & S. Takam., *E. rhododendri* J.N. Kapoor, *E. vaccinii* Schwein., and *Phyllactinia enkianthi* Z.Y. Zhao (see Braun and Cook, 2012). *Erysiphe azaleae* and *E. vaccinii* were described from North America, *E. rhododendri* from India, *E. izuensis* and *Ph. enkianthi* from East Asia. *E. digitata* is a native European species, but it is known from the type collection only (Braun and Cook, 2012). None of these species has been found before in Turkey. However, one of them, namely *E. azaleae*, was introduced into Great Britain (Watling, 1985; Ing, 2000) and then spread throughout Europe. At present, this species also is recorded in Czech Republic (Bacigálová and Marková, 2006; Lebeda et al. 2007), Germany (Dietrich, 2006), Italy (Garibaldi et al., 2002), Latvia (Apine et al., 2013), Lithuania (Grigaliūnaitė and Pribušauskaitė, 2006), Norway (Talgø et al., 2011), Poland (Piątek, 2003, Shin and Mułenko, 2004; Werner and Karolewski, 2010), Slovakia (Bacigálová and Marková, 2006), Slovenia (Hauptman and Jurc, 2008), Switzerland (Bolay, 2001), and Ukraine (Heluta et al., 2004). In the latest of these countries, the fungus parasitizes both the exotic species and the native *Rhododendron luteum* Sweet. The development of the disease is epiphytotic and observed throughout the area of *Rh. luteum*. The spreading of *E. azaleae* has invasive symptoms. Infected plants largely lose their ornamental effect. So, this fungus is a very dangerous parasite of rhododendrons.

In 2014, *E. azaleae* was recorded by us in Turkey, in the natural plant community. Hitherto, this fungus is known here only from one locality. The fungus forms both conidia and fruiting bodies. Thus, *E. azaleae* should be considered as a potentially dangerous powdery mildew pathogen for introduced and native rhododendrons in Turkey.

It should be noted that *E. azaleae* was also reported from the Russian Far East (Bunkina, 1978, 1991; Braun and Cook, 2012). However, this information is based on only one sample, which many years ago was re-identified by

the first author of the article as *E. izuensis* (unpublished data). Thus, in this paper we present a new species not only for Turkey but also for the whole of Asia.

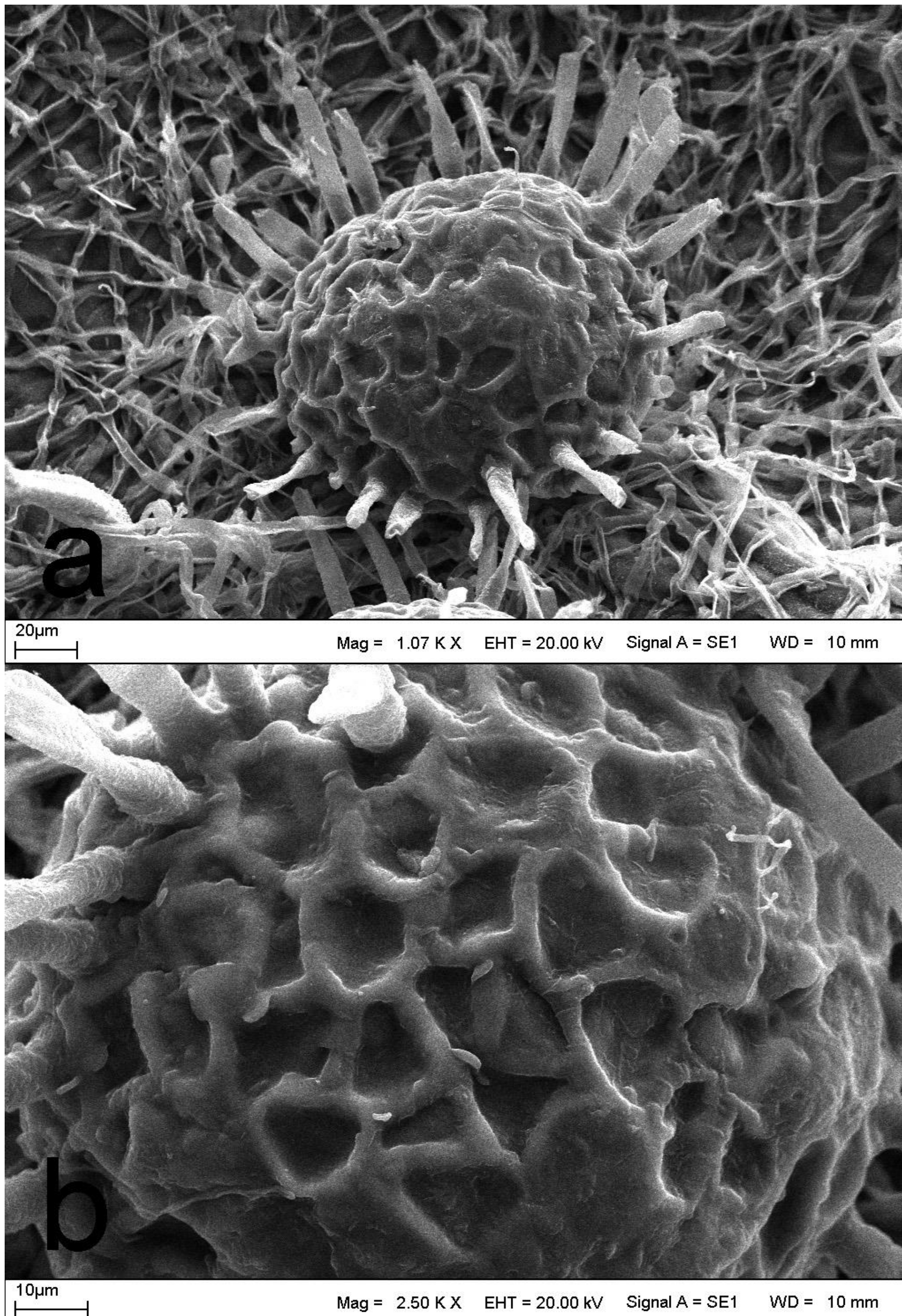

Figure 3. *Erysiphe azaleae* on *Rhododendron ponticum* as viewed by a scanning electron microscope: **a.** immature casmothecium, **b.** peridial surface of the chasmothecium

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Research article/Araştırma makalesi

Genotypic response on stability for yield and nitrogen use efficiency in triticale (X *Triticosecale* Wittmack) under different nitrogen regimes

Imren KUTLU [1], Nurdilek GÜLMEZOĞLU *[2]

[1] Eskişehir Osmangazi Univ., Agricultural Faculty, Department of Biosystem Engineering, Eskisehir, Turkey
[2] Eskişehir Osmangazi Univ., Agricultural Faculty, Department of Soil Science and Plant Nutrition, Eskisehir, Turkey

Abstract

Nitrogen (N) has major roles in plant physiology thus in crop yield and quality. Use of N efficiently in diverse environments has an importance for both economic and environmental friendly agricultural production. This study was carried out to evaluate the biomass and grain yield capacity of triticale (X *Triticosecale* Wittmack) genotypes and performance of nitrogen use efficiency (NUE) traits at different N rates and year, investigate the relative importance of genotypic variance components and heritability of yield and NUE traits, estimate the “genotype × environment” interactions and the stability of these interactions. Eleven triticale genotypes (six cultivars Karma, Melez, Mikham, Presto, Samursortu, Tatlicak, and five lines TVD-3, TVD-4, KTVD-9, TVD-17, TVD-25) were grown with three N rates (40, 80 and 160 kg N ha^{-1}) in two subsequent growing seasons. The experimental design was a split-plot design with four replicates. The calculated NUE indexes were agronomic efficiency, physiological efficiency, agro-physiological efficiency, apparent recovery efficiency and utilization efficiency for the triticale genotypes used in the experiment. It has been determined that eleven triticale genotypes differed for stability for biomass and yield, protein content and N efficiency traits examined under different environmental conditions. TVD-3, TVD-4 and TVD-17 genotypes were considered the best in terms of adaptation to all environments for NUE traits. Samursortu and TVD-25 were found to be the most stable for biomass and grain yield while any genotype was not found to be stable for protein content.

Key words: grain, genotype x environment, protein, biomass, N utilization

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Farklı azot rejimlerinde tritikalenin (X *Triticosecale* Wittmack) verim ve azot kullanım etkinliğinin stabilitesine genotipik yanıt

Özet

Azot (N), bitki fizyolojisi, ürün verimi ve kalitesinde önemli rol oynar. Azotun çeşitli çevrelerde etkin bir şekilde kullanılması, hem ekonomik hem de çevre dostu tarımsal üretim için önemlidir. Bu çalışma, tritikale (X *Triticosecale* Wittmack) genotiplerinin farklı N oranları ve yıllarda, biyokütle ve tane verimi kapasitesini aynı zamanda N kullanım etkinliği (NUE) özelliklerini değerlendirmek, verim ve NUE özelliklerinin genotipik varyans bileşenleri ve kalıtsallığının önemini araştırmak, bu özellikler için "genotip × çevre" etkileşimleri ve bu etkileşimlerin stabilitesini belirlemek amacıyla yürütülmüştür. Onbir triticale genotipi (altı çeşit; Karma, Melez, Mikham, Presto, Samursortu, Tatlıcık ve beş hat; TVD-3, TVD-4, KTVD-9, TVD-17, TVD-25), üç N dozu (40, 80 ve 160 kg N ha^{-1}) ve iki ardışık deneme sezonunda yetiştirilmiştir. Deneysel tasarım, dört tekrarlamalı bölünmüş parseller deneme desenine göre oluşturulmuştur. Tritikale genotiplerinin NUE indeksleri (agronomik etkinlik, fizyolojik etkinlik, agro-fizyolojik etkinlik N değerlendirme etkinliği ve N kullanım) hesaplanmıştır. Onbir triticale genotipinin farklı çevresel koşullar altında incelenen biyokütle, tane verimi, protein içeriği ve NUE özellikleri açısından istikrarı bakımından farklı olduğu belirlenmiştir. NUE özellikleri için tüm çevrelerde adaptasyon açısından TVD-3, TVD-4 ve TVD-17 genotipleri en iyi olarak kabul edilmiştir. Biyokütle ve tane verimi açısından Samur sortu ve TVD-25 genotiplerinin stabil oldukları belirlenirken, protein içeriği için hiçbir genotip stabil bulunmamıştır.

Anahtar kelimeler: tane, genotip x çevre, protein, biyokütle, azottan yararlanma

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* *Corresponding author* / Haberleşmeden sorumlu yazar: Tel.: +902223242991/4845; Fax.: +902223242990; E-mail: dgulmez@ogu.edu.tr
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1. Introduction

Environment consists of the factors such as climate conditions, region and agronomical applications including fertilizer applications and management which affect plant development. The environmental conditions can influence negative or positive crop genotypes growth and yield performance. Genotypes x environment interactions are highly significant in the growing and breeding of plant varieties because they can decrease genotypic stability values in different environments (Hebert et al., 1995). The stability was described as an adaptation of genotypes to unpredictable and temporary environmental conditions and a method has been developed to select stable genotypes which are unaffected or less affected by environmental changes (Allard and Bradshaw, 1964).

Yield stability always has been most attractive trait of all plant breeding programs because of the fluctuations in the mean annual yield, especially in the arid and semi-arid areas (Mohammadi et al., 2012). A new developed cultivar is released for production to farmers after testing genotypes in multi-environments because plant producers are most interested in a cultivar that gives consistent yields under different growing conditions (Naghavi et al., 2010; Sayar et al., 2013). Besides developing yield stability with different environmental conditions, improving nitrogen (N) use-efficient genotypes should be a significant aim for plant breeders. The reason behind this is applying the high rates of N fertilizers can cause the environmental pollution and economic loss (Al-Naggar et al., 2015) such as leaching of N and causing eutrophication of water (Vitousek et al., 1997) and increasing emissions of the greenhouse gas nitrous oxide (N_2O) from agricultural soils (Bouwman et al., 2002). If the cultivars which absorb N better are developed, both high crop yield can be gained with low fertilizer rates and pollution risks can be limited.

To determine the maximum grain and biomass production per unit of applied N, indexes of N use efficiency (NUE) are defined (Moll et al., 1982; Fageria and Baligar, 2001). NUE is usually dependent on interactions of genotype and environmental factors such as nutrient balance, water availability and applied fertilizer rate. Developing and identifying superior N-efficient genotypes is necessary to overcome economic and environmental concerns, and lack of adoption of more efficient N management strategies (Singh et al., 1998). Previous researchers remarked that N uptake and use efficiency were influenced by genotypic structure. They stated how crop yield was affected by different N rates (Gaju et al., 2011; Lemes da Silva et al., 2014; Hitz, 2015; Todeschini et al., 2016). Yi et al. (2011) reported that NUE and correlative indexes of 31 triticale genotypes were investigated at the tillering, jointing and heading stages under low and high N supplies. They found that triticale genotypes showed differences for NUE as high, low and middle NUE genotypes and the plant height and shoot biomass were significantly correlated with the NUE and suggested these as the indirect index to evaluate NUE of triticale. However, there is no stability study for triticale in different N rates and climate conditions and whether there is any consistency between yield stability and heritability of NUE traits of different triticale genotypes under diverse environmental conditions.

The purpose of this study were to (1) evaluate the biomass and seed yield capacity of triticale genotypes and performance of NUE traits at different N rates and year; (2) investigate the relative importance of genotypic variance components and heritability of yield and NUE traits; (3) estimate the genotype × environment interactions and the stability of these interactions using Eberhart and Russell's stability parameters under varying N environments.

2. Materials and methods

The field experiment was conducted at Faculty of Agriculture, Eskisehir Osmangazi University, Turkey, with N rates and triticale cultivars in the years of 2005/2006 and 2006/2007. The average annual precipitation and temperature (from October to July) for the area over the last 60 years and experimental years were presented in Figure 1. Soil samples (0-30 cm) were taken at sowing time and were air-dried, passed through a 2 mm sieve and analyzed for physical and chemical parameters using a standard procedure (Rowell 1996). Soil homogeneity was taken into consideration when the experiment design was established. The soil samples were analysed for texture (hydrometer method), organic matter (Walkley–Black method), pH (1:2.5 soil:water), lime (Scheibler calcimeter), total N (Kjeldahl method) and electrical conductivity (EC). Plant available Fe, Cu, Mn, and Zn concentrations were determined using method described by Lindsay and Norwell (1978) with an atomic absorption spectrometer (Analytik Jena novAA 350, Jena, Germany). Soil properties of research area across two years were similar with low organic matter (1.78%), alkaline (8.0), and moderate lime (6.1%). The available phosphorus and potassium of the soils were 8.1 and 198 mg kg^{-1}, respectively. The soils contained insufficient Zn (0.17 mg kg^{-1}), Mn (10.3 mg kg^{-1}) and sufficient Cu (1.27 mg kg^{-1}) but high Fe (4.55 mg kg^{-1}) concentrations. The soil texture was sandy clay loam and the soils had low in N (0.03%) supply to plants.

The four N rates (control, 40 (N_{40}), 80 (N_{80}) and 160 (N_{160}) kg N ha^{-1}) were applied to 11 hexaploid triticale genotypes (six cultivars Karma, Melez, Mikham, Presto, Samur sortu, Tatlicak, and five lines TVD-3, TVD-4, KTVD-9, TVD-17, TVD-25). The seeds were obtained from Bahri Dagdas International Agricultural Research Institute in Konya. The plots were arranged with 6 rows at 25 cm spaces. The 450 seeds per meter square were planted in October. The plants were fertilized with 60 kg P_2O_5 ha^{-1} as a triple superphosphate to all plots at sowing time. A split-plot with four replicates was designed. The N rates consisted of the main plots and triticale genotypes were sown as subplots. Nitrogen was not applied to control (N_0) plots. The half of the N rates was applied as an ammonium sulfate (26% N) at

planting while the rest of the N was treated as topdressing using ammonium nitrate (33% N) at tillering stage. The harvests were done in July.

Plants aboveground were removed at harvest for measuring of N concentration in grain and straw using by the Kjeldahl digestion method. The total N uptake was calculated by multiplying the dry weight by the N concentration in the grain and straw. The dry matter (biomass) and seed weight were weighted by drying the sampled plants.

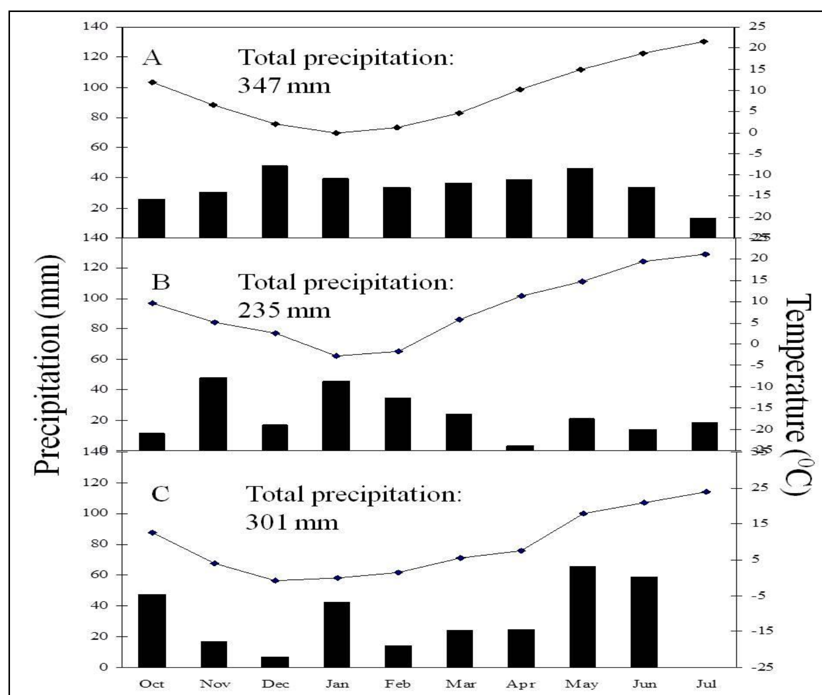

Figure 1. Total precipitation and temperature for 30-year average (A) and for 2005/06 (B), 2006/07 (C) at experiment field.

The NUE indexes (kg kg^{-1}) were calculated according to Fageria and Baligar (2001): (i) agronomic efficiency (the ratio of the increase in grain yield over N-control plots to the applied rate of N), (ii) physiological efficiency (the ratio of the increase in biomass yield over N-control plots to the increase in nutrient accumulation over N-control plots), (iii) agrophysiological efficiency (the ratio of the increase in grain yield over N-control plots to the increase in nutrient accumulation over N-control plots), (iv) apparent recovery efficiency (the ratio of the increase in nutrient accumulation over N-control plots to the applied rate of N x 100) (v) utilization efficiency (the multiplying of agro-physiological efficiency and apparent recovery efficiency).

The six environments (named E_1; 40 N kg ha^{-1}, E_2; 80 N kg ha^{-1}; E_3; 160 N kg ha^{-1} for 2005/2006 and the other three environments; E_4; 40 N kg ha^{-1}, E_5; 80 N kg ha^{-1}; E_6; 160 N kg ha^{-1} for 2006/2007) for statistical analysis were occurred from calculated NUE of triticale genotypes. A combined variance analysis was performed across the test environments. Broad sense heritability (H%) and variance components for examined traits were calculated as suggested by Demir and Turgut (1999). The linear regression model was used to describe genotypic stability (Eberhart and Russell 1966). All statistical analyses were performed using the SAS (Statistical Analyses Systems) program (SAS Institute 1999).

3. Results

A combined variance analysis of examined traits of the 11 triticale genotypes tested across six environments (Table 1) showed that 'environment', 'genotypes' and 'genotype x environment' interactions were highly significant ($p < 0.01$). While significant variations of 'genotype' and 'environment' indicated the existence of variability among the tested cultivars for all traits, highly significant 'genotype x environment' interactions suggested differential response of cultivars across testing environments and the need for stability analysis. This is an evidence that there were significant genetic background variations among triticale genotypes and the response of examined traits. Also, important variations were shown due to combination of N fertilization and climate changes over the years (environments). Many researchers (Anbessa et al., 2009; Sadras and Slafer, 2012; Lemes da Silva et al., 2014; Abd El Mohsen and Amein, 2016; Katar et al., 2016) agree with the finding that yield and agronomic traits are affected by genotypes, environmental factors and their interactions. Genotypic variance was smaller than environmental variance for all traits except grain yield (Table 1). This result indicated that these traits are influenced by environmental changes. High value of 'genotype x environment' interaction variance proved this opinion. The dominance of the environmental changes caused increased

phenotypic variance and decreased broad sense heritability degree for all traits (Table 1). The broad sense heritability (H%) was 69.0 for grain yield, although grain yield is a complex trait and many of them are affected by changes of environmental factors. For agronomic efficiency, physiological efficiency, agro-physiological efficiency, apparent recovery efficiency and utilization efficiency traits, small broad sense heritability pointed out that N fertilizer rates and climate changes must be considered besides selection according to genotype. To develop cultivars able to take up N from soils and fertilizers and to use it to produce more grain is a good way for improving NUE in cereals. However, one of the problems here is low degree of heritability. Another way improves N-efficiency in cereals is to have a better fertilizer management. The fertilizer efficiency can be improved by combining applications considering crop demand and climate changes (Barraclough et al., 2010). Climate has a major influence on N demand (crop growth and grain yield), and on the N supply (availability of N in soil and/or fertilizer). Presences of 'genotype x environment' interactions is a major obstacle to making proper fertilizer recommendations. Therefore, it is important to identify genotypes that remain the stable under changing climatic conditions and N applications.

Determining of stable triticale genotypes adapted to changeable environments is possible with the differential ranking of genotypes' performance across environments (Baker, 1988). The mean values of examined traits for 11 triticale genotypes tested across six environments are presented in Table 1. The means of all traits across the six environments showed substantial changes reflecting the presence of high 'genotype x environment' interactions. Mean agronomic efficiency, physiological efficiency, agro-physiological efficiency, apparent recovery efficiency and utilization efficiency varied from 3.04 (E_3)- 17.08 (E_1) kg kg^{-1}, 32.96 (E_6)- 78.34 (E_4) kg kg^{-1}, 7.50 (E_3)- 24.30 (E_1) kg kg^{-1}, 35.57 (E_6)- 75.11 (E_4) % and 12.55 (E_6)- 59.52 (E_4) kg kg^{-1} respectively. While grain yield and biological yield ranged from 2.90 (E_4)- 3.63 (E_2) and 16.62 (E_6)- 19.97 (E_2) ton ha^{-1}, the lowest and highest grain protein content values were gained from E_4 (2.90%) and E_2 (3.63%), respectively. Otherwise total N accumulation values were close to each other (Table 1).

Regression coefficients of genotypes versus the mean values of examined traits were illustrated (Figure 2). The mean agronomic efficiency of the 11 triticale genotypes ranged from 6.9 (Karma) kg kg^{-1} to 14.6 (Mikham) kg kg^{-1}. The highest physiological efficiency value was obtained from Samursortu (74.40 kg kg^{-1}). For agro-physiological efficiency, apparent recovery efficiency and utilization efficiency, the highest values were determined as 17.4 kg kg^{-1} (Presto), 74.4% (Mikham) and 48.6 kg kg^{-1} (Samursortu), respectively. The lowest utilization efficiency value was obtained from Karma as 26.1 kg kg^{-1} (Figure 2). Regression coefficients ranged between 0.65-1.21 for agronomic efficiency, 0.20-1.70 for physiological efficiency, 0.30-1.70 for agro-physiological efficiency, 0.60-1.60 for apparent recovery efficiency and 0.50-1.50 for utilization efficiency, respectively (Figure 2).

In addition, the mean values of total N accumulation were close to each other (0.14-0.17 ton ha^{-1}). Mean values of biomass and grain yield changed between 16.68 (Tatlicak)- 20.11 (TVD-25) ton ha^{-1} and 2.33 (KTVD-9)-3.88 (TVD-25), respectively. In addition, Tatlicak had the lowest grain protein content with 14.55% and TVD-25 had the highest grain protein content with 16.79 (Figure 2). Regression coefficients ranged between 0.49-1.65 for total N accumulation, 0.71-1.74 for biomass yield, -0.42-1.58 for grain yield and 0.15-1.57 for protein content, respectively (Figure 2). This broad variation in regression coefficients reflects the different responses of different genotypes to environmental changes. Akcura et al. (2005) and Sayar et al. (2013) found that regression coefficients (bi values) ranging from 0.46 to 1.56 for grain yield in wheat genotypes and 0.283 to 1.325 for dry-matter yield in vetch genotypes at different environmental conditions and they stated that the genotypes responses could be differed to environmental changes.

Eberhart and Russell (1966) expressed that regression coefficients (bi) approximating 1.0 combined with small value of S^2d (nearly zero) point out an average stability. When the regression coefficient approximating 1 is associated with the high mean values of traits, genotypes have general adaptability and when associated with low mean values of traits, genotypes are poorly adapted to all environments (Eberhart and Russell 1966). If regression values are above 1.0, it is considered that genotypes have below average stability with higher sensitivity to environmental change. So, such genotypes should be proposed for high yielding environments. Conversely, when regression coefficients decrease below 1.0, genotypes provide more tolerance to environmental change and increase adaptability to low yielding environments with above average stability (Eberhart and Russell, 1966; Akçura et al., 2006).

If Figure 2 is examined from this perspective, it is seen clearly that the cultivars Mikham and Samursortu had high mean performance and regression coefficient value for N utilization efficiency traits. So, these genotypes were regarded as sensitive to environmental changes and can be recommended for favorable conditions (high N rates). Genotypes KTVD-9 and Karma had less than unity of regression coefficient and had low mean values of N utilization efficiency traits. These genotypes are, therefore, insensitive to environmental changes and have adapted to the poor environments (low N rates). With its high mean values of N utilization efficiency traits and a regression coefficient that did not differ significantly from 1.0, TVD-3, TVD-4 and TVD-17 genotypes showed better adaptability to all environmental conditions. These genotypes can be described as the most stable genotypes for N utilization efficiency traits. This data is fundamental to understand the correlative role of genotypic variation on N utilization efficiency in different environments and the consequences of N fertilizer rates interactions on ecosystem processes. The results from Pregitzer's (2010) study show that across an environmental gradient *Populus angustifolia* genotypes can influence N mineralization through feedbacks between environmental variation, tree phenotype and soils. This result supported our opinion.

Table 1. Mean performance, variance analysis and variance components for examined traits of 11 triticale genotypes tested across 6 environments

	Agronomic efficiency	**Physiological efficiency**	**Agro-physiological efficiency**	**Apparent recovery efficiency**	**Utilization efficiency**	**Total N accumulation**	**Grain yield**	**Biological yield**	**Grain protein content**
E_1	17,08	62,62	24,3	71,91	46,57	0,14	3,46	18,21	13,47
E_2	10,71	63,25	15,12	72,58	45,25	0,16	3,63	19,97	14,76
E_3	3,04	48,7	7,5	39,96	19,52	0,17	3,26	19,47	15,94
E_4	8,65	78,34	11,43	75,11	59,52	0,15	2,9	17	15,5
E_5	9,24	53,44	12,04	74,44	41,49	0,18	3,3	17,93	16,64
E_6	3,68	32,96	9,49	35,57	12,55	0,18	3,15	16,62	17,49
Variance Analysis									
Environment(E)	1159,69**	10422,4**	1561,73**	15136,0**	13984,4**	0,015**	2,79**	76,88**	87,88**
Genotype (G)	162,38**	1994,7**	308,14**	821,8**	1133,0**	0,005**	4,03**	33,83**	14,36**
GxE	59,47**	1609,9**	139,77**	745,1**	1052,2**	0,001**	0,35**	4,87**	4,62**
Variance Components									
GV	4.29	16.02	7.01	3.18	3.36	0,0002	0,15	1,21	0,40
EV	25.01	200,28	32.32	327.06	293.91	0,0003	0,05	1,64	1,89
GxEV	14.86	402.40	34.94	186.20	263.25	0,0002	0,09	1,21	1,15
PV	31.77	283.38	45.16	361.29	341.12	0,001	0,22	3,04	2,49
H_{bs}	0.15	0.06	0.16	0.01	0.01	0,32	0,69	0,40	0.16

*p≤0.05, **p≤0.01, (GV: genotypic variance, EV: environmental variance, PV: phonotypic variance, H_{bs}: broad sense heritability)

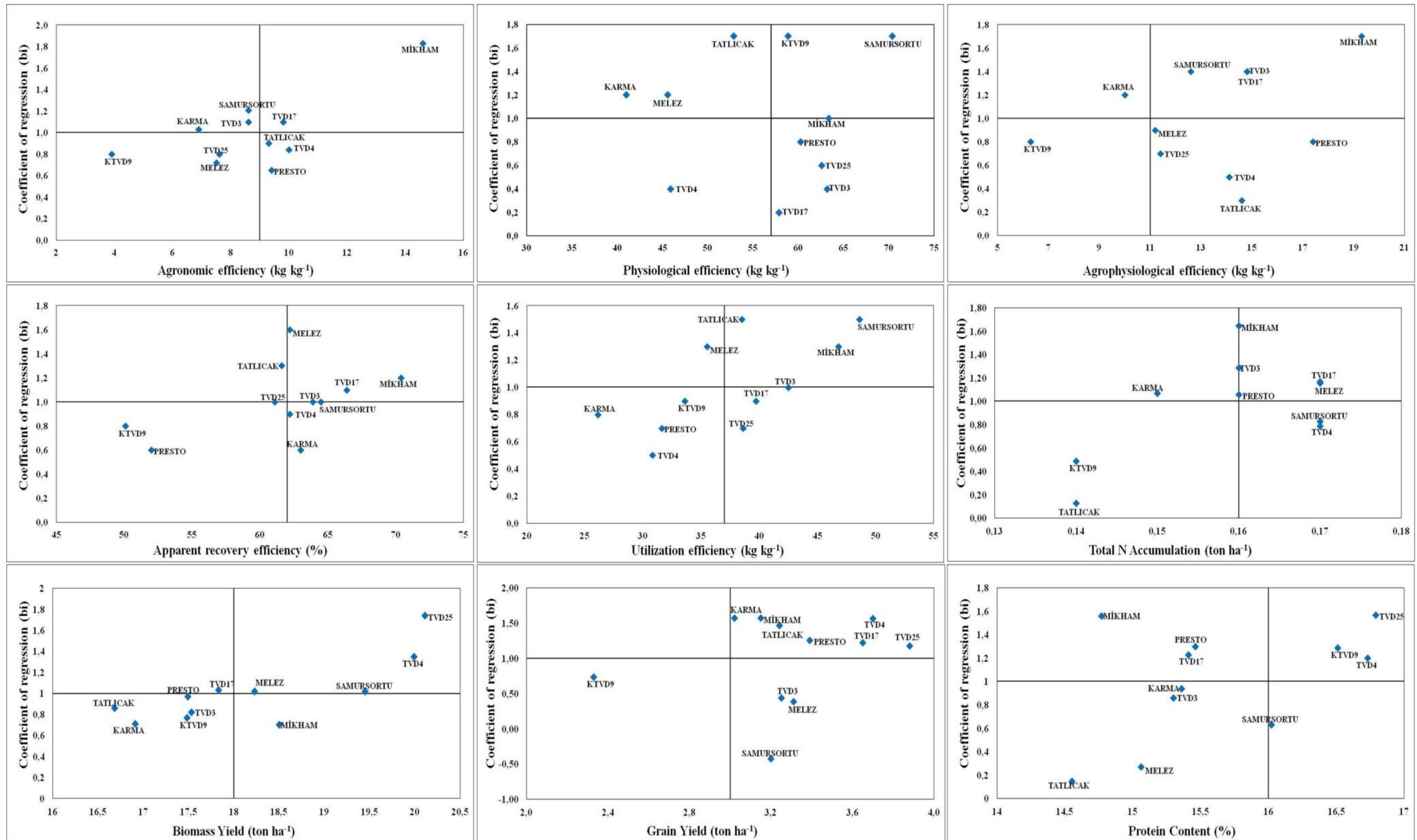

Figure 2. The relationship between the regression coefficients and mean value of examined traits for 11 triticale genotypes. The horizontal solid line represents the mean coefficient of regression and the vertical solid line denotes the mean value of examined traits.

Genotypes TVD-25, TVD-3 and Mikham had regression coefficients highly greater than unity for total Naccumulation over and equal mean values (Figure 2). For this reason, these genotypes are sensitive to environmental changes and can be suggested for growing under favorable conditions for high N accumulation. KTVD-9 and Tatlicak were determined as genotypes had poor adaptability to unfavorable environmental conditions with below unity bi value and low total N accumulation values. The other genotypes showed average stability for total N accumulation. Karma for biomass yield was insensitive to environmental changes and have adapted to the poor environments because it was less than unity (bi =1.0) regression coefficient and had low mean value. On the other hand, Samursortu could be considered as the most stable genotype for biomass yield (Figure 2). Although TVD-25 had regression coefficients remarkably greater than unity and over mean value for biomass yield, it had a high mean value, a regression coefficient equal to the unity (bi = 1) for grain yield. Therefore, this genotype was sensitive to environmental changes and could be recommended for cultivation under favorable conditions for biomass yield. In contrast, the same genotype could be considered as stable for grain yield. Samursortu, TVD-3, Melez were well adapted to unfavorable environmental conditions due to less than unity (bi =1.0) and had over mean grain yields. According to Figure 2, it could be concluded that Tatlicak's grain protein content would be affected from environmental changes. TVD-25, KTVD-9 and TVD-4 in favorable conditions give high grain protein content. Moreover, Samursortu's grain protein content is insensitive to environmental changes and have average adaptability to unfavorable environmental conditions

4. Conclusions and discussion

There were highly significant 'genotype x environment' interactions for all traits examined. Especially, the heritability of N use efficiency traits was very small and thus it was concluded that these traits are highly affected from environmental conditions. E1 and E4 (40 N rates) were determined as the best environment for N efficiency traits, however E2 (80 N rates) were the best environment for yield and protein content. TVD-3, TVD-4 and TVD-17 genotypes were considered the best in terms of adaptation to all environments for N utilization efficiency traits. In that case, these genotypes well respond even the low N fertilizer rates. Samursortu and TVD-25 were found to be the most stable for biomass and grain yield. Any genotype was not found to stable for protein content, however, Samursortu may be satisfactory with average adaptability and TVD-25 can be gained high grain protein content under favorable environments. For these genotypes, optimum N fertilizer rates (80 N) would be appropriate for high yield and protein content. Stable genotypes were different for N utilization efficiency traits and yield. This inconsistent values reflect the importance of understanding the type and magnitude of ‘genotype x environment’ interaction for N utilization efficiency traits in triticale production and breeding programs carried out under different environmental conditions in order to select a highly performance and genotypically stable genotype. Success in breeding studies would be improved by the selection of 'genotype x environment' according to N utilization efficiency traits besides yield stability. Such studies may be useful to develop flexible fertilizer management advice for farmers in diverse environments.

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Research article/Araştırma makalesi

Alkaliphilic bacterial diversity of Lake Van/Turkey

Nilgün POYRAZ [*1], Mehmet Burçin MUTLU [1]

[1] Anadolu University, Faculty of Science, Department of Biology, 26470, Eskisehir, Turkey

Abstract

Lake Van, situated in Turkey's eastern Anatolian region, is a saline soda lake and it covers the lowest part of a vast basin. The importance of Lake Van comes from being one of the largest soda lakes and third largest endorheic lake on Earth with a volume of 607 km^3. Although several biological studies have been done for Lake Van, research on microbial diversity of the lake is still missing. This paper contributes to scientific knowledge by research on alkaliphilic bacterial diversity of Lake Van. In this regard, culture-dependent and culture-independent methods were applied to the samples, which were taken from different locations close to the shores of surrounding cities. Isolation was performed on 10 different media. Bacterial diversity was analyzed by 16S rRNA gene PCR amplification, sequencing and blast analysis against NCBI database. Results showed that members of Proteobacteria (especially Gammaproteobacteria) and Actinobacteria phylums were represented dominantly. Culture-independent DGGE (Denaturing Gradient Gel Electrophoresis) and FISH (Fluorescence In-Situ Hybridization) methods were also used to detect the microbial populations in Lake Van. The results showed that the community was dominated by Bacteria.

Key words: Lake Van, alkaliphilic bacteria, ARDRA, DGGE, FISH

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Van Gölü'nün alkalifilik bakteriyel çeşitliliği

Özet

Van Gölü Türkiye Doğu Anadolu Bölgesi'nde yer alan, büyük bir havzanın alçak kısmını kaplayan tuzlu bir soda gölüdür. Van Gölü'nün önemi Dünya'nın en büyük soda göllerinden bir olmasından ve 607 km^3'lük hacmi ile Dünya'nın en büyük 3. kapalı havza gölü olmasından kaynaklanmaktadır. Van Gölü ile ilgili bazı biyolojik çalışmalar yapılmasına rağmen mikrobiyal çeşitliliği üzerine araştırmalar yeterli değildir. Bu yüzden bu çalışma Van Gölü'ndeki alkalifilik bakteri çeşitliliği üzerine yapılan araştırmalara bilimsel katkılar sağlamaktadır. Bu amaçla, gölün çevresindeki şehirlerin kıyılara yakın, farklı noktalardan alınan su örneklerine kültür bağımlı ve kültürden bağımsız yöntemler uygulanmıştır. Kültür çalışmaları için 10 farklı besi yeri kullanılmıştır. Bakteriyel çeşitlilik, 16 S rRNA geninin PCR ile çoğaltılması, dizileme ve NCBI veri tabanı kullanılarak incelenmiştir. Sonuçlar, Proteobacteria (özellikle Gammaproteobacteria) ve Actinobacteria filumlarının baskın bir şekilde bulunduğunu göstermiştir. Mikrobiyal populasyonları belirlemek için kültür bağımsız yöntemler olan DGGE (Denatüre Edici Gradiyent Jel Elektroforezi) ve FISH (Fluoresan In Situ Hibridizasyon) kullanılmıştır. Sonuçlar kommunitede bakterilerin yoğun olduğunu göstermiştir.

Anahtar kelimeler: Van Gölü, alkalifilik bakteri, ARDRA, DGGE, FISH

1. Introduction

Lake Van, or Van Gölü in Turkish, is situated at about 43ºE longitude and 38.5ºN latitude in the eastern part of Anatolian peninsula, Turkey. The lake has a surface area of 3574 km^2, a volume of 650 km^3, a maximum depth of 450 m, and a maximum length of 130 km (Cukur, 2014). It is located at an altitude of 1649 m and it covers the lowest part of a large basin surrounded by high mountains in the south, highlands and mountains in the east and volcanic cones in the

* *Corresponding author* / Haberleşmeden sorumlu yazar: Tel.: +902223350580/4711; Fax.: +902223204910; E-mail: nilgunpoyraz@anadolu.edu.tr
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west. The lake does not have any natural outlet and, hence, it gathers surface water from all around the lake (Kadioglu et al., 1997). Lake Van, besides being closed basin, is a saline soda lake and is distinguished from other lakes in the world with the importance it owns. Considering its properties and volume, it is one of the largest soda lake and third largest closed lake on Earth (Reimer et al., 2009) as well as being the largest lake of Turkey.

There is basic information about the ecosystem of the lake and it has not been studied in detail due to difficulties of access. Fruit and grain-growing agricultural areas surround Lake Van region. Lake Van basin also lies along the flyways of many migratory birds and this closed basin hosts 213 species of the bird fauna found in Turkey (Yılmaz and Aslan, 2004). The only fish, known to live in Lake Van is *Chalcalburnus tarichi* or the Pearl Mullet, which is endemic to the Lake Van basin (Danulat and Kempe, 1992) and is rated in "'Near Threatened" species (Freyhof, 2014). It feeds on phyto and zooplanktons (Sarı, 2008) and there are 103 phyto and 36 zooplanktons species recorded in Lake Van (Danulat and Kempe, 1992).

Rains and streams that feed the lake are among the water sources of Lake Van. At the north shore Delicay and Zilan, at the east shore Karasu, and at the south shore Engil and Gevas streams pour into the lake. Lake Van, as a saline lake, has different soda chemistry. This is due to the fact that alkali cations maintain the charge balance of bicarbonate and carbonate ions in addition to alkaline earth ions (Reimer et al., 2009). One of the reasons for Lake Van being salty is the increase of salt concentration as a result of evaporation, and the salinity ratio of the lake is 0.224% (Ciftci et al., 2008). Soda lakes are naturally occurring alkaline environments and they exhibit good examples of extreme conditions. These kinds of lakes are the representatives of the highly stable pH environments in the world (Jones et al., 1998). The chemistry of soda lakes is quite unique because of the presence of large amounts of carbonate/bicarbonate concentration in the form of Na_2CO_3 and $NaHCO_3$ (Jones et al., 1998). Soda lakes host a substantial number of microbial communities and studies on the microbial diversity of soda lakes help to improve the understanding of the biology of extreme environments. The studies also have the potential for the discovery of novel microorganisms and enzymes, which may be used, in the scope of biotechnological applications. Alkaliphilic microorganisms are quite common among the microbial communities and they show optimal growth at least 2 pH units above neutrality. However, some alkaliphilic microorganisms exhibit more than one pH optima (Horikoshi, 2008). As a result, stability at high pH makes these microorganisms important. Furthermore the enzymes of alkaliphilic microorganism show activity at a high pH and salinity values. Selected examples for the industrial applications of alkaliphilic microorganisms are enzymes such as proteases, lipases, and cellulases have been used for the production of biotechnologically advanced laundry detergents (Horikoshi, 2008). Alkaline protease is also used in the food industry, pharmaceuticals, and medical diagnosis (Kanekar et al., 2002). Apart from enzymes, alkaliphilic microorganisms can be used for the biodegradation of organic and inorganic pollutants and hydrocarbons (Sorokin et al., 2012).

In recent years, studies on alkaliphilic microorganisms of soda lakes have attracted increasing research interest and various methods were employed in order to investigate microbial diversity. Among these studies, researchers used culture-dependent methods, culture-independent methods or a combination of those (Foti et al., 2008; Deshmukh et al., 2011; Mwirichia et al., 2011). Diversity of specific alkaliphilic microorganisms was also investigated using 16S rRNA and functional genes (Sorokin and Kuenen, 2005; Antony et al., 2012; Tourova et al., 2014). Apart from these, high-throughput sequencing of the 16S rRNA amplicon techniques were utilized in order to examine the structure and diversity of various microbial communities (Simachew et al., 2005; Matyugina and Belkova, 2015). Although many of isolates have already been identified and studied from different soda lakes worldwide, alkaliphilic diversity of the Lake Van has not been studied in detail until now. Assorted studies have been conducted for Lake Van and Lake Van region such as observation of environmental impacts (Ciftci et al., 2008), influence of climatic change (Kadioglu et al., 1997), examination of lake sediments, determination of lake water quality and the levels of heavy metals (Bilgili et al., 1995; Öztürk et al., 2005), plant ecology and diversity (İlcim et al., 2013). On the other hand, the knowledge is quite limited on the microbial diversity of the lake. Among the small number of example microbial studies on Lake Van, investigation of bacterial diversity of microbiolite samples (López-García et al., 2005) and determination of enzymes and protein characteristics of alkaliphilic *Bacillus* species (Berber and Yenidünya, 2005) can be quoted.

The goal of this study is to evaluate the diversity and structure of alkaliphilic bacteria in the world's largest soda lake, Lake Van, in order to contribute to scientific knowledge and to form a baseline for prospective research. In the scope of this study, both culture-dependent and culture-independent techniques were used to provide an efficient investigation for alkaliphilic bacteria. In this regard, employed techniques are Polymerase Chain Reaction (PCR), Denaturing Gradient Gel Electrophoresis (DGGE) and Fluorescence In-Situ Hybridization (FISH). The results of this study reveal the presence of Proteobacteria species (especially Gammaproteobacteria) and Actinobacteria phylums in Lake Van.

2. Materials and methods

2.1. Sample collection and analysis

Lake Van consists of two sections; the main body of water is separated from its much shallower northern arm by a narrow passage. This fact raises various risks such as influence of environmental conditions on sample content

depending on time, temperature and water level decrease. Therefore, the sampling areas in this study, which was conducted under financial constraints, were only selected from the main body of water. Water samples were obtained from four different locations and these locations are the shores of Lake Van being close to the end points in four different directions as east, west, north and south. The names of these shores to be used as sampling locations are Van (approx. 38.6ºN, 43.3ºE), Tatvan (approx. 38.5ºN, 42.3ºE), Ahlat (approx. 38.8ºN, 42.6ºE) and Edremit (approx. 38.3ºN, 43.1ºE) (Figure 1). Water samples (5L) were taken approximately 1-2 m away from the shore and at 1 m depth. Samples were immediately transported with sterile bottles and stored at room temperature. Salinity (Eclipse Hand Refractometer), chemistry (Perkin Elmer Optical Emission Spectrophotometer Optima 4300 DV) and pH (Mettler Toledo) of the samples were determined.

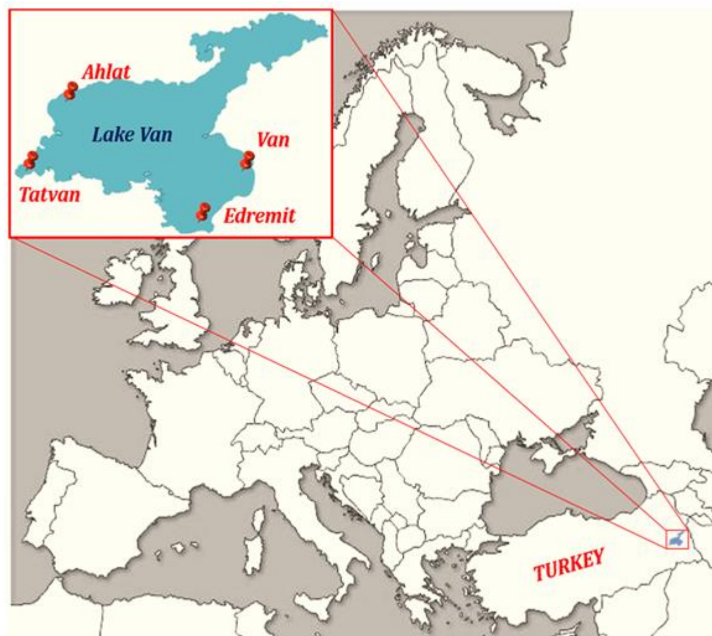

Figure 1. Lake Van sampling regions

2.2. Isolation and cultivation of alkaliphilic bacteria

In order to isolate alkaliphilic bacteria from water samples, enrichment culture technique was applied. For this purpose, 100 µl water sample from each location was inoculated to plates containing 10 different media. These media are Modified Growth Medium (MGM) in different NaCI concentrations such as 2-3-5-10-12% (Dyall-Smith, 2009), Horikoshi I and II medium (Horikoshi, 2008), Gauze Agar (Selyanin et al., 2005), Actinomycetes Isolation Agar (Sigma) and Glycerol Yeast Extract Agar. All plates were incubated at 37°C for 4-15 days. Different colony morphotypes were isolated by replication and the obtained pure cultures stocked in glycerol at -85ºC.

2.3. PCR amplification and ARDRA analysis

For PCR amplification of the 16S rRNA gene, boiling method was applied to isolates from different media. Briefly, a loop full of cells from pure culture was transferred to tube containing 200 µl of sterile distilled water and was suspended. After waiting for 5-10 minutes in the water bath set to 100°C, cells were lysed and then samples were centrifuged at 10000 rpm. Finally, the supernatant was transferred to sterile tube as DNA template for PCR reaction and was kept at -20°C for use. Each of isolates' DNA extracts and Bacteria specific universal primer set 27F (5'-AGAGTTTGATCATGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT3') (Lane et al., 1985) were combined for PCR reaction of 16 S rRNA genes. To control of the quality and signals of PCR products, 1% agarose gels were prepared and products were visualized under UV light after ethidium bromide staining. Then, Amplified ribosomal DNA restriction analysis (ARDRA) with the enzyme *Hinf I* (New England Biolabs) was applied to PCR products to group and characterize of isolates (Vaneechoutte et al., 1992). Reaction mixtures were incubated at 37°C overnight. Banding patterns were analyzed by using 2% agarose gel, which was run for 4 hours in 1X TBE (Tris-Boric acid-EDTA) buffer at 50 volts. Finally, restriction patterns were compared and grouped for sequencing.

2.4. Nucleic acid extraction

Total DNA was extracted from water samples combining previously described protocols (Nogales et al., 1999; Cifuentes et al., 2000; Mutlu et al., 2008). For collection of microorganisms by filtration, 200 ml water sample were passed through GV filter (Millipore, Isopore GTTP04700) with a pore diameter of 0.22 mm, using vacuum membrane

filter system. After cutting the one filter into small pieces, small pieces of membrane filters were transferred to RNase- and DNAse-free 2 ml tube, Extraction buffer (100 mM Tris-HCI pH: 8, 100 mM EDTA), lysozyme (3 mg/ml) were added and incubated at 37°C for 15 minutes in shaking incubator at 150 rpm. Then, proteinase K (150 mg/ml) and 10% sodium dodecyl sulfate (SDS) were added and incubated at 37°C for 40 minutes in shaking incubator. Following the incubation, 5 M NaCl and CTAB (10% CTAB - Cetil Trimethyl Ammonium Bromide, 0.7 M NaCl) was added and mixed by inverting the tube. Later, samples were immersed into liquid nitrogen for 1-2 minutes and were incubated at 65°C. This step was repeated for 3 times. After these steps phenol: chloroform: isoamyl alcohol (25:24:1) was added and centrifuged at 13000 rpm at 4˚C for 5 minutes for extraction and purification of nucleic acids. Then, sodium acetate (3 M, pH 4.8), isopropanol and $MgCI_2$ (1 M) were added and centrifuged at 13000 rpm for 30 minutes at 4˚C. 70 % alcohol was added on pellet and centrifuged again at 13000 rpm for 15 minutes at 4˚C. After ethanol precipitation, the pellet was dried at room temperature and suspended with sterile water. To control the quality of nucleic acids, 1% agarose gels were prepared and products were visualized under UV light after ethidium bromide staining (Lane et al., 1985). Finally, samples were stored at -85 °C.

2.5. DGGE analysis

Total nucleic acid extraction products from environmental water samples were used for amplification of the 16 S rRNA gene fragment for DGGE analysis. Towards this aim, 341F-GC (5'-GCclamp-CCTACGGGAGGCAGCAG-3') and 907R (5'-CCGTCAATTCCTTTRAGTTT-3') (Muyzer et al., 1993) bacteria primer combinations were used. DGGE was performed using a denaturing gradient of 30 - 60% denaturants in polyacrylamide gel with modifications of Muyzer et al.'s procedure in Ingeny System, (Leiden, NL) (Muyzer et al., 1993). Individual bands were excised and re-amplified with 341F and 907R primer combination. At last, PCR products were sequenced.

2.6. Sequence analysis and nucleotide sequence accession number

Sequence analysis was performed using 27F, 907R and 1492R primers on Beckman CEQ 8000 DNA analyzer. (Beckman Coulter, Fullerton, CA). Then 16S rRNA gene sequences were compared to reference sequences at NCBI (http://www.ncbi.nlm.nih.gov) by using BLAST programme (Altschul et al., 1990). Finally, representative sequences were determined and stored in GenBank with the accession numbers KM248755 to KM248759, KM362990 to KM363002 and KM388972 to KM388984. Phylogenetic analysis was performed with these sequences and sequences stored in GenBank were used to build tree by the "phylogeny.fr" tool. (Dereeper et al., 2008; Dereeper et al., 2010).

2.7. FISH analysis

Fixation of water samples was applied according to the previous protocol of Anton et al., (1999). Hybridization, DAPI staining, and microscopic examination were applied as decribed in protocol of Snaidr et al., (1997). 16S rRNA-targeted oligonucleotide probe EUB338, which is specific for members of the Bacteria domain, was used for hybridization (Amann et al., 1990). The filters were dried, DAPI staining was applied and microscopic examination was performed by fluorescence microscopy (Leica DM6000B).

3. Results

3.1. Chemical characterization of water samples

The salinities of water samples varied from 1.2% to 1.8% and pH varied from 9.5 to 10.2. The pH of the samples was alkaline. Na^+ and K^+ were the most abundant ions followed by the Mg^{2+} (**Hata! Başvuru kaynağı bulunamadı.**).

Table 1. Sampling regions, salinity and ionic features of water sample

Water Sample	Name	pH	Salinity (%)	Na^+ (ppm)	K^+ (ppm)	Ca^{2+} (ppm)	Mg^{2+} (ppm)	Cd (ppm)
M	Central	9.5	1.7	7003	430.4	<	86.25	169.5
T	Tatvan	9.8	1.8	8323	493.1	0.313	86.81	0.2354
A	Ahlat	9.6	1.8	11870	721.2	0.095	87.26	0.214
E	Edremit	10.2	2.0	9221	647.1	0.380	87.24	0.2564

3.2. Isolation and characterization of alkaliphilic bacteria

One hundred and twenty-four isolates were determined in different colors and morphology using low percentage of salt containing modified growth medium (MGM), alkaline nutrient agar, Horikoshi I and II medium, starch casein agar, actinomycetes isolation medium, Gauze agar and glycerol yeast extract agar medium. Approximately 1500 base pairs of bacterial 16S rRNA fragments were amplified with PCR analysis for 93 isolates from the shores of Van, Edremit, Ahlat and Tatvan. For these 93 isolates enzymatic restriction analysis were applied. When ARDRA results comprise for each gel individually; 6 different profiles from 18 isolates which was isolated from Van (Central), 7 different profiles from 17 isolates which was isolated from Ahlat, 12 different profiles from 34 isolates which was isolated from Edremit (**Hata! Başvuru kaynağı bulunamadı.**), 7 different profiles from 24 isolates which was isolated from Tatvan.

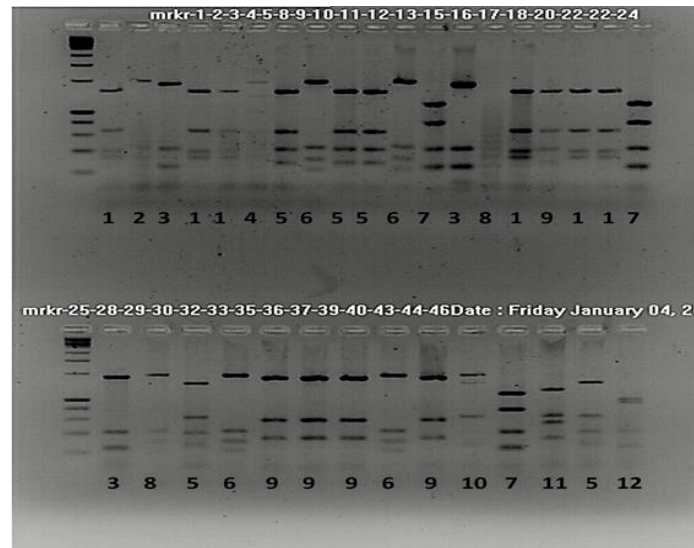

Figure 1. Gel image of ARDRA profiles from Edremit sampling region

The ARDRA profiles were found to be highly different when analyzing the samples. The nucleotide sequences of the purified PCR products were identified. 16S rRNA gene sequences were compared with reference sequences at NCBI using BLAST. BLAST results showed that, members of Proteobacteria and Actinobacteria were represented dominantly (Table 2). *Halomonas, Alkalimonas, Marinobacter, Rhodococcus* and *Vibrio* in Bacteria domain were detected as abundant species (**Hata! Başvuru kaynağı bulunamadı.**).

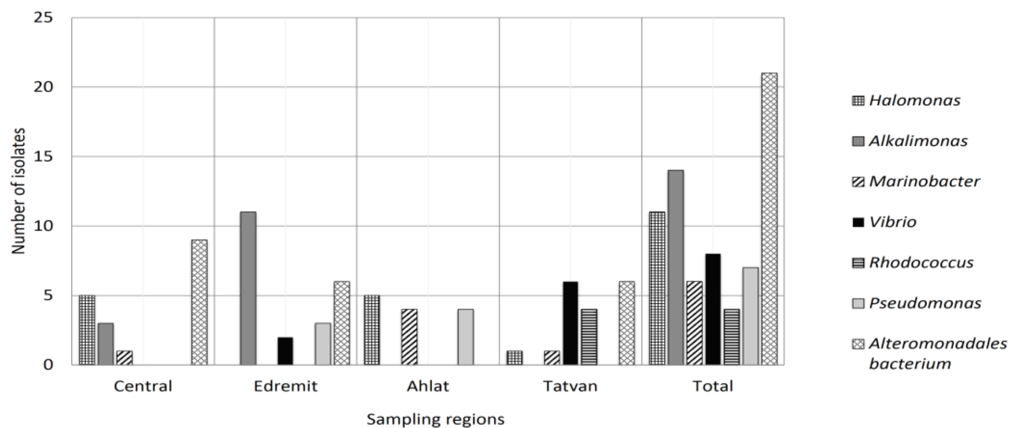

Figure 2. Bar chart of dominant isolates in Lake Van

In addition to these, *Rhodococcus sp.*, *Pseudomonas sp.*, *Rheinheimera sp.*, *Agrococcus sp.*, *Planococcus sp.*, *Belliella sp.*, and *Bacillus sp.* were obtained from results. Phylogenetic analysis was performed with sequences, and

partial sequences from analysis were used to build tree (Figure 4). Many of the isolates were classified under Proteobacteria phylum. (Table 2)

Table 2. Isolates and their closest matches in GenBank

Isolates	**Nearest neighbour**	**Similarity (%)**	**Accession**	**Source**	**Phylum**
M 7	*Halomonas sp.* 15-7 16S	99 (852/858)	HM598402.1	soda meadow saline soil	Proteobacteria(Gammaproteobacteria)
M 4	*Halomonas sp.* IB-559	99 (1416/1437)	AJ309560.1	high concentration of sodium nitrite	Proteobacteria(Gammaproteobacteria)
M 3	*Halomonas sp.* 10043	98 (1172/1190)	EU432575.1	mud volcano	Proteobacteria(Gammaproteobacteria)
M 5	*Marinobacter excellens* strain KMM	99 (1419/1432)	NR_025690.1	sediments of the sea	Proteobacteria(Gammaproteobacteria)
M 49	*Alkalimonas collagenimarina* strain AC40	98 (1424/1456)	NR_041515.1	deep-sea sediment	Proteobacteria(Gammaproteobacteria)
T 32	*Halomonas sp.* AMP-12	98 (1145/1178)	HM104378.1	activated sludge	Proteobacteria(Gammaproteobacteria)
T 35	Uncultured bacterium	99 (722/726)	FJ152919.1	alkaline saline soils	
T 52	*Marinobacter mobilis* strain B17	98 (724/744)	GQ214550.1	mangrove sediment	Proteobacteria(Gammaproteobacteria)
T 58	*Vibrio metschnikovii*	99 (764/772)	AB681962.1	culture collection	Proteobacteria(Gammaproteobacteria)
AT 28	*Rheinheimera sp. UDC526*	99 (723/725)	JQ895023.1	sea water	Proteobacteria(Gammaproteobacteria)
AT 33	*Rhodococcus sp.* BS-15	99 (690/698)	AB808578.1	sediment of the deep sea	Actinobacteria
AT 46	*Agrococcus jenensis* strain 1RN-3D2	99 (725/734)	EU379252.1	commercial airline cabin air	Actinobacteria
AT 49	*Rhodococcus sp.* A105-53B	98 (702/716)	KC422659.1	high arsenic groundwater sediment	Actinobacteria
AT 59	*Rhodococcus sp.* Dza15	99 (693/695)	JQ977264.1	rhizoplane	Actinobacteria
AT 63	*Rhodococcus sp.* D48	99 (684/689)	AY582940.1	deep-sea sediment	Actinobacteria
A 21	*Marinobacter sp.* LNM-5	99 (672/676)	AB758589.1	*Porphyra yezoensis*, red alga	Proteobacteria(Gammaproteobacteria)
A 23	*Gamma proteobacterium* E-116	97 (1154/1184)	FJ764790.1	haloalkaline lake	Proteobacteria(Gammaproteobacteria)
E 20	*Alkalimonas delamerensis* strain:1E1	99 (684/690)	NR_044879.1	soda lakes	Proteobacteria(Gammaproteobacteria)
E 36	*Gamma proteobacterium* O-010	99 (1029/1934)	DQ812540.1	seawater	Proteobacteria(Gammaproteobacteria)
E 40	*Nitrincola sp.* LAR05R9	99 (728/730)	JX945779.1	soda lakes	Proteobacteria(Gammaproteobacteria)
E 43	*Vibrio metschnikovii* strain SU1	98 (658/671)	HQ658055.1	textile effluent	Proteobacteria(Gammaproteobacteria)
2E 21	*Vibrio metschnikovii* strain SU1	99 (892/901)	HQ658055.1	textile effluent	Proteobacteria(Gammaproteobacteria)
2E 32	*Planococcus sp.* PP_B2A.1	99 (753/756)	KC250890.1	marine diatom	Firmicutes (Bacilli)
2T 30	*Bacillus okhensis* strain Kh10-101	98 (892/910)	NR_043484.1	saltpan	Firmicutes(Bacilli)
E 11	*Alkalimonas delamerensis* strain 1E1	98 (1019/1038)	NR_044879.1	soda lakes	Proteobacteria(Gammaproteobacteria)
AE 24	*Rheinheimera tuosuensis* strain TS-T4	97 (952/979)	NR_133840.1	saline lake water	Proteobacteria(Gammaproteobacteria)
AE 28	*Pseudomonas sp.* Ri83	98 (987/1005)	AM905940.1	groundwater from well	Proteobacteria(Gammaproteobacteria)
E 4	*Belliella sp.* LW3	99 (908/916)	FM956478.1	soda lake	Bacteroidetes/Chlorobi group (Bacteroidetes)
A 9	*Pseudomonas sp.* H-107	99 (954/959)	KF021789.1	marine bacteria	Proteobacteria(Gammaproteobacteria)

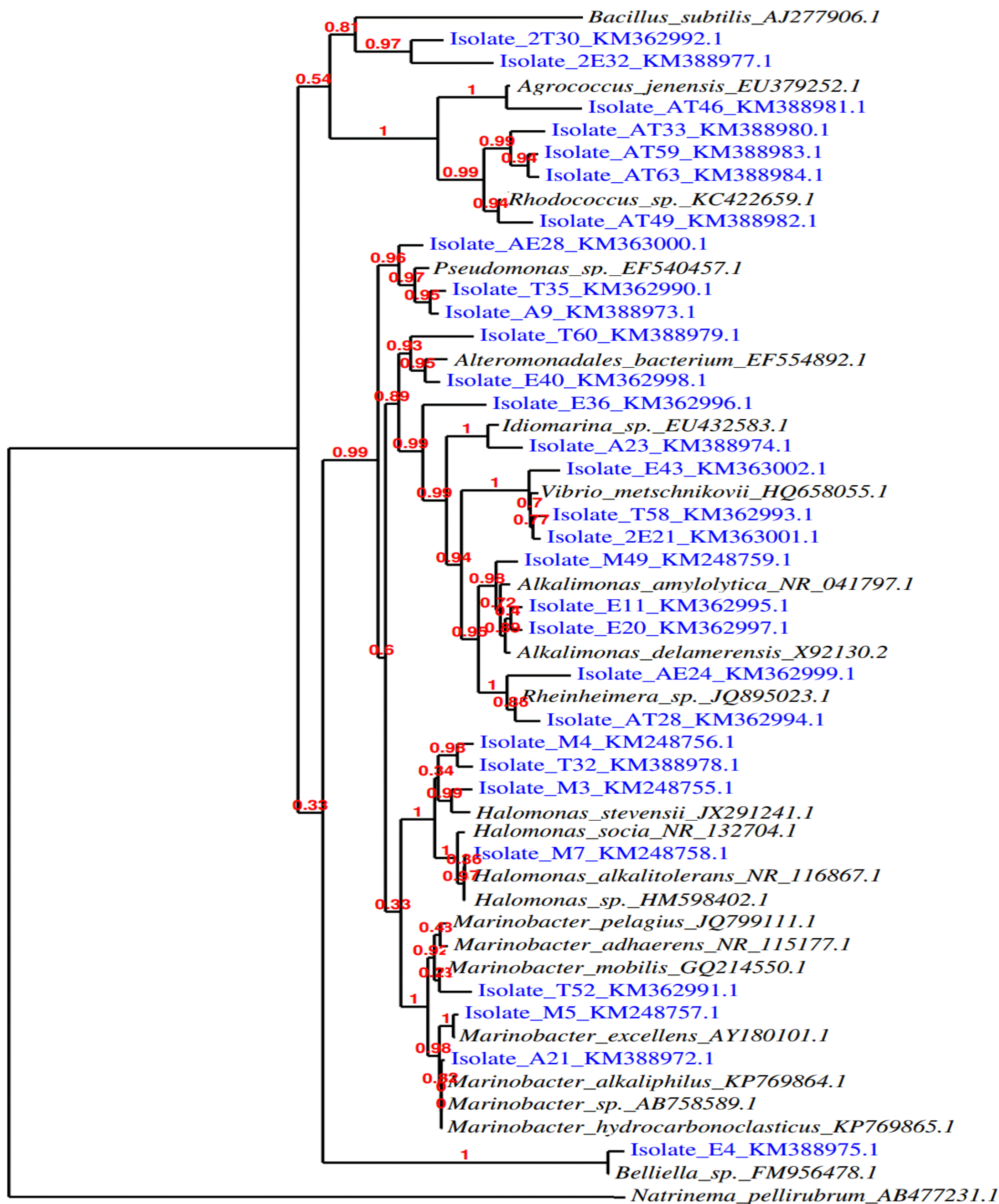

Figure 3. Phylogenetic tree based on 16S rRNA gene sequences from isolates belonging to Bacteria (indicated by blue colour). The sequence of *Natrinema pellirubrum* was selected as the outgroup. The horizontal scale bar shows the number of substitutions per site.

3.3. DGGE analysis

Amplicons obtained from environmental genomic DNA (water samples from Van Lake) were then analyzed by DGGE. Selected 18 bands were excised from the gel, re-amplified and 7 of these bands were sequenced. Denaturing gradient gel electrophoresis (DGGE) was performed to study population dynamics (**Hata! Başvuru kaynağı bulunamadı.**). As result of this gel in sequence analysis *Citrobacter sp.* and uncultured bacterium clones were identified (As shown in Table 3).When gel was evaluated some of bands were common in all samplings and some

patterns were represent dominantly, but all of bands were not able to sequence in detailed. For reason that was troubles in re-amplification of DGGE bands.

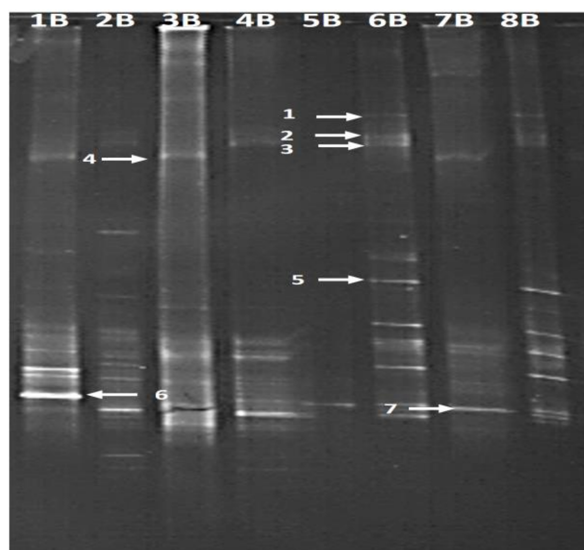

Figure 4. DGGE analysis of bacterial diversity in Lake Van samples (1B and 3B: Tatvan, 2B and 4B: Ahlat, 5B and 7B Van Central, 6B and 8B Edremit)

Table 3. DGGE bands and their closest similar sequences in GenBank

Bands(Bacteria)	Closest Relative in Gene Bank	Similarity %
1	*Citrobacter* sp. P074 16S ribosomal RNA gene, partial sequence (KC252814.1)	%95
2	*Citrobacter* sp. ChDC B352 16S ribosomal RNA gene, partial sequence (KF733678.1)	%99
3	*Kluyvera* sp. UIWRF0577 16S ribosomal RNA gene, partial sequence (KR190084.1)	%92
4	Uncultured bacterium clone SINP1135 16S ribosomal RNA gene, partial sequence (HM127404.1)	%94
	Loktanella vestfoldensis strain HME9315 16S ribosomal RNA gene, partial sequence (KF911340.1)	%93
5	Uncultured bacterium clone 5o46 16S ribosomal RNA gene, partial sequence (EU644798.1)	%95
	Flavobacteriaceae bacterium X15M-6 16S ribosomal RNA gene, partial sequence (KJ782428.2)	%93
6	Uncultured bacterium clone D11 16S ribosomal RNA gene, partial sequence (JF683447.1)	%74
	Polaribacter sp. NBRC 110213 16S ribosomal RNA gene, partial sequence (KM502317.1)	%73
7	Uncultured bacterium DGGE gel band C-2_Bac_a 16S ribosomal RNA gene, partial sequence (EF622424.1)	%95
	Bacteroidetes bacterium Omega 16S ribosomal RNA gene, partial sequence (KF830693.1)	%87

3.4. FISH analysis

After the fluorescence microscopy examination for FISH, DAPI stained cells and EUB338 bacteria probe hybridized cells were compared. EUB338 probe's positive signals obtained for Bacteria. Presence of the long and short rods, curved rods, spiral-shaped cells, coccus-shaped cells and spindle-shaped cells were detected in the samples (**Hata! Başvuru kaynağı bulunamadı.**- 11).

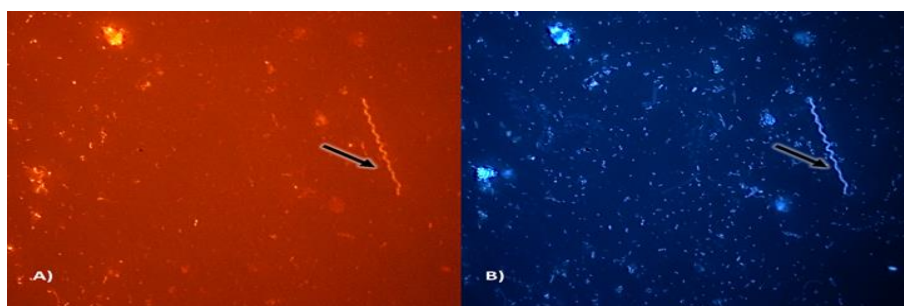

Figure 5.Visualization of microorganisms in Tatvan hybridizing with EUB338 (A) and DAPI (B)

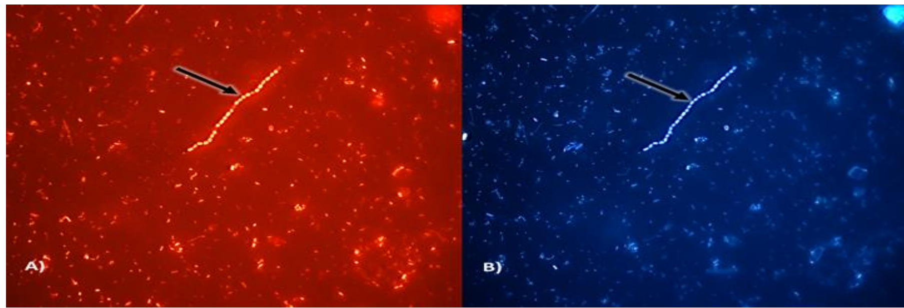

Figure 6.Visualization of microorganisms in Tatvan hybridizing with EUB338 (A) and DAPI (B)

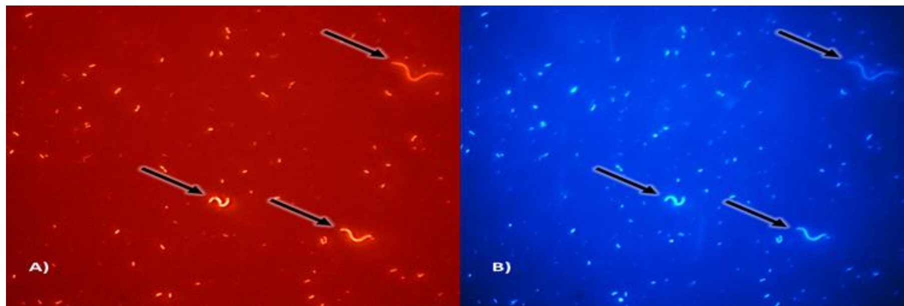

Figure 7. Visualization of microorganisms in Edremit hybridizing with EUB338 (A) and DAPI (B)

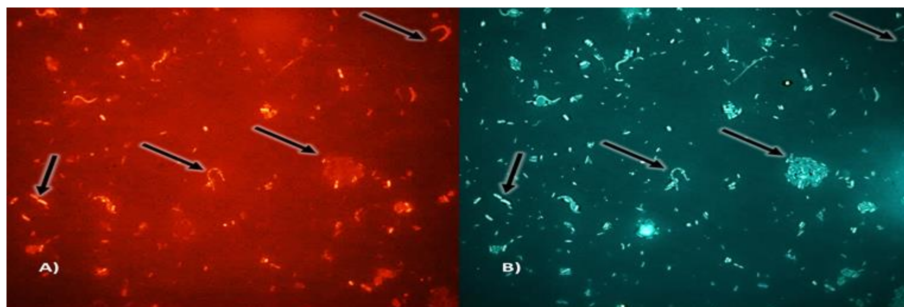

Figure 8. Visualization of microorganisms hybridizing in Edremit with EUB338 (A) and DAPI (B)

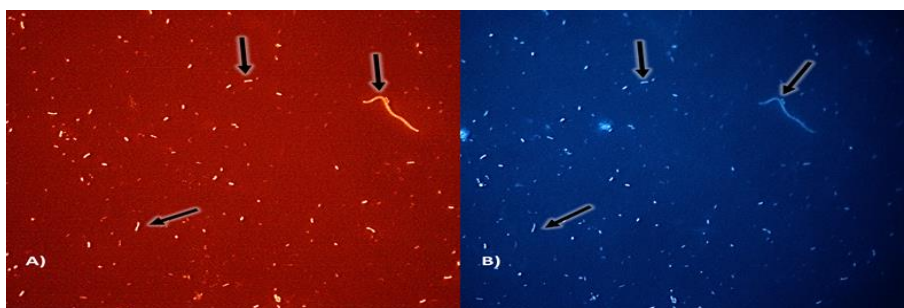

Figure 10. Visualization of microorganisms hybridizing in Ahlat with EUB338 (A) and DAPI (B)

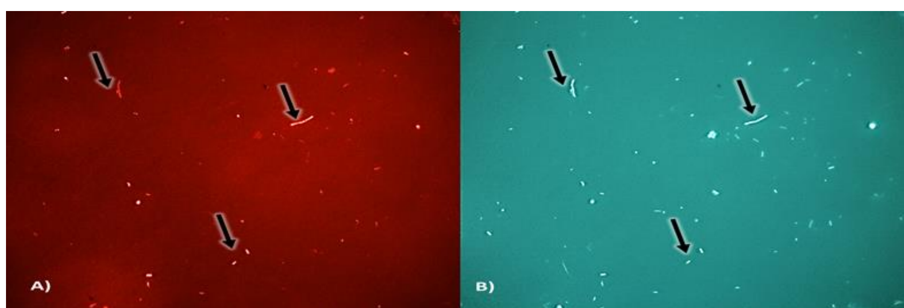

Figure 11. Visualization of microorganisms hybridizing in Van (Central) with EUB338 (A) and DAPI (B)

4. Conclusions and discussion

Soda lakes, particularly Eastern Rift Valley lakes (Kenya and Tanzania), Texcoco Lake (Mexico), Lonar Lake (India) and Kulunda Steppe soda lakes (Altai, Russia) have been the subjects of relatively detailed microbial diversity analysis (Kanekar et al., 2002; Jan-Roblero et al., 2004; Foti et al., 2008; Deshmukh et al., 2011; Mwirichia et al., 2011; Antony et al., 2012). However, until recently, the microbial diversity of the Lake Van has been limited. Early preliminary study from microbiolites of the Lake Van indicated some details for alkaliphilic bacterial diversity (López-García et al., 2005).

The results presented here indicated considerable microbial diversity of Lake Van. For these results, we used both culture dependent and culture independent studies. Culture-dependent techniques state of the sampling methods and culture conditions and these types of factors effect the range of species in the laboratory. 10 different media and incubation aerobically at 37°C were applied. The Gram-negative bacterial species were observed in Lake Van. The highest actively bacterial species were observed in Lake Van belonged to members of the Proteobacteria especially Gammaproteobacteria and the majority are related to *Halomonas* genus. Members of the genus *Halomonas* show growth different salt concentration and pH range so isolation from different environments can be easily. The other Gram-negative bacteria were *Alkalimonas, Marinobacter, Vibrio* and *Pseudomonas sp.* Previous studies have documented Proteobacteria isolates from other soda lakes (Jan-Roblero et al., 2004; López-García et al., 2005; Foti et al., 2008; Deshmukh et al., 2011; Mwirichia et al., 2011; Simachew et al., 2015; Matyugina and Belkova, 2015).

Gram-positive isolates were less diverse, a few Lake Van isolates related to the Actinobacteria, or high GC Gram positives. Most of them were related to environmental sequences and isolates from deep-sea sediment, high arsenic groundwater sediment, rainwater sample and sea water. Most abundant *Actinobacteria* genus was *Rhodococcus.* Many species can degrade most of environmental pollutants and transform or synthesize compounds for useful applications (Bell et al., 1998). *Rhodococcus* in soda lakes reported before in Lake Magadi, Kenya (Ronoh et al., 2013). Another species of the genus *Dietzia* isolated from Lake Van. *Dietzia* reported previously from soda lakes (Duckworth et al., 1996; Kanekar et al., 2002). The other Gram-positive bacteria were *Rheinheimera sp.*, *Agrococcus sp.*, *Planococcus sp.* and *Bacillus sp.*

These bacteria play important roles in biogeochemical cycles. The heterotrophic bacteria, can degrade organic matter which are produced by the autotrophic bacteria. *Bacillus sp.*, *Dietzia sp.*, and *Alkalimonas* species were isolated from Lake Van. These bacteria are aerobic hydrolytics. Haloalkaliphiles were the most abundant groups isolated from soda lakes (Sorokin et al., 2014). *Halomonas* and *Actinobacteria* were abundant in Lake Van. They utilize monomeric organic compounds. Another important biogeochemical cycle is denitrification. Mostly salt-tolerant alkaliphilic *Halomonas* play key role in denitrification process in soda lakes (Sorokin et al., 2014). Our results showed that *Halomonas* was the most abundant genus and play key role in Lake Van's biogeochemical cycles. In addition Actinobacteria with role in nutrient cycling in their environment has ecological significance. For example, its role in bacterial nitrate reduction is important for the nitrogen cycle in the lake ecosystem. Organotrophic *Actinobacteria* form an important step between nitrogen and carbon cycle by using nitrate (Foti et al., 2008).

We used culture-independent techniques such as DGGE and FISH. An advantage of DGGE method is that DNA can be recovered from the gels and allow sequencing and identification of the population (Rees et al., 2004). DGGE method could be carried out only for the domain Bacteria. Some of bands were selected and recovered from the gel, re-amplified, and sequenced. Bacterial bands belong to *Citrobacter sp*. and uncultured DGGE bands. DGGE bands showed similarity some sequences obtained from meromictic soda lake (Soap Lake) in Washington State, soda lakes of the Kulunda Steppe (Altai, Russia) and Tibetian Lake (Qinghai Lake). *Citrobacter* isolation sources' are raw water and activated sludge. *Citrobacter* species are thought to be environmental contaminants and these species may be live intestinal tracts of man and animals. These bacteria can be isolated from soil, sewage, water and food (Nayar et al., 2014). As a similar to our results, *Citrobacter freundii* has been reported before in a study about phylogenetic diversity of soda lake alkaliphiles (Duckworth et al., 1996). Although these results, DGGE method in our study still needs optimizations for detection of alkaliphilic population dynamics. Also, fluorescently labeled rRNA-targeted oligonucleotide probes have become useful materials for culture-independent identification of bacteria. FISH can be used without the need to cultivation and define the microorganisms in an environment (Wagner et al., 2003). FISH analysis of samples from Lake Van indicated that Bacteria domain was abundant. After signal examination with Bacteria specific probe, the curved bacillus cells, long rods, cocci, vibrio and spirillum cells were observed in our samples.

In conclusion, with this work, we investigated alkaliphilic bacteria in Lake Van, Turkey and this study indicated industrial potential of cultures that could be used as efficient pigment, antibiotic producers and hydrocarbon degraders. Alkaliphilic bacterial group were detected based on comparative 16S rDNA analyses, culture-independent techniques DGGE and FISH. Although there are many limitations, culture-dependent approaches are still a prefered method of studying microbial ecology in natural environments. A conclusion based on culture-dependent methods can lead to the result that Proteobacteria and Actinobacteria members in Van Lake are active and play an important role in

the cycling of nutrients. These are seems to be dominant in Lake Van. Hybridization with Bacteria specific 16S rRNA-targeted oligonucleotides and DGGE methods help to demonstrate population dynamics. These results show that the diversity of alkaliphilic microorganisms in Lake Van is quite high and diverse. Future studies should aim to isolate members of the uncultured bacteria. In addition such as detection of functional genes and metagenomics can be applied to obtain detailed phylogenetic and physiological diversity of Lake Van.

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Research article/Araştırma makalesi

Seasonal differences in the muscle fatty acid profiles of two freshwater fish species (*Scardinius erythrophthalmus, Squalius cephalus*)

Leyla KALYONCU *[1], Zerrin ABUOĞLU [1]

[1] University of Selcuk , Biology Department , Science Faculty, Konya, Turkey

Abstract

Fatty acid compositions of the muscle lipids and its seasonal variations in *Scardinius erythrophthalmus* (rudd) and *Squalius cephalus* (chub) in Sapanca and Terkos Lake, were examined using a gas chromatographic method. Palmitic acid is saturated fatty acid and the other dominant fatty acid is stearic acid in both species. SFA contents in rudd were between 26.79% and 41.54% in all seasons. Oleic acid C18:1 ω9 was identified as the main monounsaturated fatty acid (MUFA) in both fishes for four seasons. It was found that chub had a high amount of C18:1 (15.09% to 28.56 %) in all seasons compared to rudd. It was noticed from the present data that docosahexaenoic acid (DHA) (22:6ω3) was predominant PUFA in muscle lipids of rudd. According to these data, it can be concluded that both freshwater fishes are a good sources for eicosapentaenoic acid (EPA) and DHA.

Key words: muscle, fatty acid, seasonal changes, fish

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İki tatlısu balık türünün (*Scardinius erythrophthalmus, Squalius cephalus*) kas yağ asidi bileşiminde mevsimsel farklılıklar

Özet

Sapanca ve Terkos göllerinde bulunan *Scardinius erythrophthalmus* (kızılkanat) ve *Squalius cephalus* (kefal)'un kas lipidlerinin yağ asit kompozisyonu ve mevsimsel değişimleri gaz kromatoğrafik yöntemle incelenmiştir. Palmitik asit doymuş yağ asitidir ve diğer dominant yağ asidi her iki türde de stearik asitdir. Kızılkanattaki doymuş yağ asidi içeriği bütün mevsimlerde %26.79 ile % 41.54 arasındadır. Oleik asit C18:1 ω9 her iki balık türünde, bütün mevsimlerde primer doymamış yağ asidi olarak bulunmuştur. Kefalde, oleik asit C18:1(%15.09-%28.56) kızılkanata nazaran bütün mevsimlerde yüksek miktara sahiptir. Kızılkanat kas lipitlerinde dokosahexaenoik asit (DHA) (22:6ω3) dominant aşırı doymamış yağ asidi olarak tespit edilmiştir. Bu verilere göre her iki tatlı su balığının DHA ve eikosapentaenoik asit (EPA) yönünden iyi bir kaynak olduğu sonucuna varılmıştır.

Anahtar kelimeler: kas dokusu, yağ asidi, mevsimsel değişiklik, balık

1. Introduction

Fish use lipids rather than carbohydrates as energy source. They accumulate important amounts of lipids in liver, adipose tissues or their muscles "(Sheridan, 1988)". Fish lipids are quite rich in long-chain n-3 PUFAs, specially DHA and EPA "(Polak Juszczak and Komar Szymczk, 2009)".

Fish lipids have been recognized as a beneficial material for human health, during recent years. The omega 3 fatty acids are always present in fish flesh even in lean fish "(Ackman, 2002)". The omega 3 and omega 6 PUFAs are considered to be basic to the growth of children. These fatty acids are precursors for composite hormones known as eicosanoids, involved in a lot of metabolic processes of high importance for the human body, mainly related to cardiovascular activity "(Inhamuns and Franco, 2008)". n-3 PUFA cannot be synthesized by humans, these fatty acids absolutely must be taken with diet "Alasalvar et al., 2002". PUFA content, in particular, has been shown to be beneficial

* *Corresponding author* / Haberleşmeden sorumlu yazar: Tel.: +903322232773; Fax.: +903322412499; E-mail: lkalyoncu@selcuk.edu.tr
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in the reduction of coronary artery disease, respiratory distress in asthmatics and rheumatoid arthritis "Leaf and Weber, 1988; Broughton et al., 1997". DHA is essential for normal fetal brain and cognitive development as the formation of neuron synapses in the brain strongly depends on the integration of this fatty acid into growing neurons "(Jensen, 2006)". The fatty acid composition of fish lipids is affected by temperature, reproductive cycle, spawning, diet, geographical location and season "(Henderson and Tocher, 1987)". The fatty acid profiles is influenced by water temperature. There is a marked increase in unsaturated FA composition at low temperatures "(Henderson and Tocher, 1987)". At low temperatures in the water a higher level of unsaturation in cell membrane phospholipids is needed to maintain permeability and flexibility "(Lovell, 1991)". Marine fish have higher quantity of PUFAs specially EPA and DHA, compared with freshwater fish "(Özogul and Özogul, 2007)". Docosahexaenoic acid, eicosapentaenoic acid, and arachidonic acid (AA) are basic structural components of cell membranes "(Innis, 1991)". DHA and eicosapentaenoic acid, found only in fish and seafoods, have extremely useful properties for, in particular, the prevention of human coronary artery disease "(Leaf and Weber, 1988)".

Although there are a lot of studies that have published fatty acid composition of different fish species from various geographical regions, there is a small amount of information on the fatty acid composition of rudd and chub. The aim of this study was to determine the muscle fatty acid profile and ω3/ω6 fatty acids levels of rudd and chub.

2. Materials and methods

Scardinius erythrophthalmus (Linnaeus 1758) (rudd) samples investigated in this study have been taken from the Sapanca Lake which has a surface area of 46.9 km² located 12 km away from Sakarya city in the west of Sakarya. *Squalius cephalus* (Linnaeus 1758) (chub) have been taken from Terkos Lake which has 39 km^2 surface area and located about 40-50 km away from Istanbul in the north-west direction. In the present study, samples collected all four seasons were analysed. Fish species were caught in the middle of each month for each season during 2012-2013. They were obtained from local fisherman in four seasons. Gender differences were ignored. They were transported to the laboratories an ice in the frozen form, filleted and frozen. The fillets were homogenized in chloroform/methanol mixture (2:1, v/v).

Fish muscles were extracted by the method described by "Folch et al., 1957". BF_3 methanol was used for methylation "Moss et al., 1974".

HP Agilent 6890N model gas chromatography was used for fatty acids methyl esters. Detector and injector temperatures were 280^0C and 270^0C, respectively. Column temperature program was 190^0C for 35 min and then increased at a ratio of 30^0C/min up to 220^0C where it was maintained for 5 min. Helium was used as a carrier gas (2 ml/min). Identification of normal fatty acids was carried out by comparing sample FAME peak relative retention times with those obtained for Alltech standards. The data were represented as means±SD. The data were subjected to an analysis of variance at 0.05 significance level, using LSD.

3. Results

Fatty acid profiles of rudd and chub in 4 seasons is presented in Table 1 and Table 2. 29 fatty acids were found in muscle lipids of *S. erythrophthalmus* and 36 fatty acids were determined in muscles lipids of *S. cephalus*. The most abundant fatty acids were detected to be C16:0 (palmitic), C18:1ω9 (oleic), C16:1ω7 (palmitoleic), C18:2ω6 (linoleic), C20:5ω3(EPA), C20:4ω6 (AA), C22:6 ω3 in the fishes in four seasons. Oleic acid in fillet of rudd was determined to be 13.03% (spring), 16.87% (summer), 9.94% (autumn) and 8.56% (winter). In this study, the highest ratio of C 18:1 was determined in summer. Chub had a high amount of C18:1 (15.09% to 28.56 %) in all seasons compared to rudd. It was followed by C16:1 in two species.

Table 1. Total fatty acid profile of muscles of *Scardinius erythrophthalmus* from Sapanca Lake (%)

Fatty acids	**spring**	**summer**	**autumn**	**winter**
C14:0	1.32±0.01a	1.25± 0.03a	0.61±0.05b	1.01±0.07c
C15:0	0.31 ±0.02a	0.42±0.01a	0.28±0.01a	0.25±0.02a
C16:0	30.41±0.41a	20.41±0.03b	18.89±0.17b	24.21±0.99a
C17:0	0.59±0.01a	0.72±0.04b	0.52±0.01a	0.43±0.01c
C18:0	6.55±0.04a	6.27±0.09a	4.83±0.05b	4.98±0.13b
C20:0	0.14±0.03a	0.11±0.01a	0.24±0.01b	0.42±0.01c
C21:0	0.64±0.03a	0.44±0.02b	0.41±0.01b	0.45±0.02b
C22:0	0.82±0.54a	0.30±0.02b	0.77±0.04a	1.33±0.14c
C24:0	0.46±0.17a	0.27±0.25b	0.23±0.17b	0.72±0.19c
ΣSFA*	**41.54±0.65**	**30.30±0.14**	**26.79±0.20**	**33.79±1.22**
C14:1 ω5	0.11±0.01a	0.06±0.02b	0.17±0.00a	0.03±0.01b

Table 1. continued

C15:1 ω5	0.29±0.01a	1.23±0.03b	1.41±0.05b	0.49±0.03c
C16:1 ω7	5.28±0.07a	6.11±0.18a	4.81±0.11a	5.14±0.23a
C17:1 ω8	0.25±0.02a	0.73±0.03b	0.36±0.01a	0.27±0.01a
C18:1 ω9	13.03±0.03a	16.87±1.41a	9.94±0.02b	8.56±0.18b
C20:1 ω9	0.09±0.03a	0.83±0.72b	0.06±0.01a	0.13±0.10a
C22:1 ω9	0.45±0.77a	0.08±0.04b	__	0.07±0.06b
C24:1 ω9	__	0.23±0.34a	__	1.32±0.12b
ΣMUFA*	**19.50±0.69**	**26.50±2.21**	**16.75±0.17**	**16.00±0.02**
C18:2 ω6	8.04±0.75a	7.77±1.06a	9.74±0.02b	7.91±0.26a
C18:3 ω6	0.36±0.06a	0.20±0.01b	0.17±0.00b	0.01±0.01c
C18:3 ω3	3.24±0.17a	3.60±0.69a	5.75±0.05b	5.12±0.11b
C20:2 ω6	1.46±0.40a	0.58±0.01b	0.81±0.01b	1.46±0.04a
C20:3 ω6	0.82±0.54a	0.30±0.02b	0.77±0.04a	1.33±0.14c
C20:3 ω3	0.88±0.89a	2.61±0.42b	1.85±0.15c	1.56±0.23c
C20:4 ω6	7.16±0.12a	6.26±0.27b	7.78±0.02a	10.52±0.15c
C20:5 ω3	5.00±0.08a	5.86±0.57b	7.51±0.46c	6.25±0.09b
C22:2 ω6	0.16±0.28a	0.41±0.30a	__	0.45±0.74a
C22:5 ω3	__	0.72±1.24a	3.03±0.12b	2.93±0.41c
C22:5 ω6	0.71±1.24a	__	__	0.04±0.07b
C22:6 ω3	11.13±0.73a	14.88±0,79b	19.05±0.06c	12.63±0.46b
ΣPUFA*	**38.96±1,34**	**43.20±2.88**	**56.46±0,80**	**50.20±1.59**
Σω3	20.24±0.84a	27.68±3.35b	37.19±0.73c	28.49±1.02b
Σω6	18.72±0.80a	15.52±0.92b	19.27±0.08a	21.71±0.79c
Σ ω3/6	1.08±0.05a	1.79±0.28b	1.92±1.09b	1.31±0.05c

[a]average of analysed.
[b]means±S.D.
[c abc]within the lines, values in four seasons in a species of fish are significantly different at $p<0.05$.

Table 2. Total fatty acid profile of muscles of *Squalius cephalus* from Terkos (Durusu) Lake (%)

Fatty acids	**spring**	**summer**	**autumn**	**winter**
C10:0	__	__	__	0.01±0.00
C11:0	__	__	__	0.01±0.01
C12:0	__	0.02±0.02a	__	0.10±0.11b
C13:0	__	0.01±0.01a	__	0.13±0.09b
C14:0	0.61±0.22a	0.56±0.03a	0.92±0.15b	0.81±0.26b
C15:0	0.32±0.08a	0.62±0.11b	0.45±0.10c	0.47±0.10c
C16:0	14.88±2.61a	19.80±1.36b	19.07±2.16b	19.19±2.76b
C17:0	0.96±0.09a	0.60±0.05b	0.66±0.07b	0.80±0.10a
C18:0	3.77±0.30a	6.22±0.78b	4.10±0.53c	4.26±0.63c
C19:0	__	__	0.02±0.04a	0.06±0.12a
C20:0	0.77±0.63a	0.13±0.14b	0.06±0.07b	0.22±0.07c
C21:0	0.72±0.62a	0.32±0.15b	0.39±0.23b	0.73±0.01a
C22:0	0.53±0.44a	0.30±0.07b	0.29±0.03b	0.69±0.31c
C24:0	0.77±0.33a	0.55±0.37b	0.71±0.50a	0.71±0.20a
ΣSFA*	**23.33±1.28**	**29.12±1.66**	**26.66±2.56**	**28.18±3.96**
C14:1 ω5	0.20±0.07a	0,08±0,04b	0.10±0,66b	0.18±0.07a
C15:1 ω5	0.34±0.12a	0,22±0,13b	0,33±0.05a	0.47±0.21c
C16:1 ω7	5.19±1.24a	5.29±0.15a	9.86±2.37b	6.91±2.18c
C17:1 ω8	1.16±0.36a	0.20±0.02b	0.97±0.15c	0.31±0.12b
C18:1 ω9	28.56±7.44a	17.71±0.71b	19.80±4.09c	15.09±0.75b
C20:1 ω9	1.00±0.64a	0.94±0.10a	1.59±0.47b	1.37±0.11c

Table 2. continued

C22:1 ω9	__	0.10±0.10a	0.60±0.69b	0.02±0.02c
C24:1 ω9	__	0.68±0.10a	0.43±0.48b	0.82±0.95c
ΣMUFA*	**36.44±5.14**	**25.22±0.81**	**33.68**±6.67	**25.16±1.53**
C18:2 ω6	21.17±8.20a	5.66±0.81b	3.19±0.59c	4.48±1.26c
C18:3 ω6	0.77±0.93a	0.19±0.20b	0.10±0.03b	0.18±0.05b
C18:3 ω3	1.33±0.22a	0.95±0.23b	1.72±0.63c	1.67±0.49c
C20:2 ω6	0.21±0.13a	0.46±0.05b	0.44±0.04b	0.91±0.80c
C20:3 ω6	0.53±0.44a	0.40±0.41a	0.29±0.03b	0.07±0.03c
C20:3 ω3	1.76±0.35a	3.67±0.64b	3.14±0.52c	3.85±0.31b
C20:4 ω6	2.53±1.52a	8.93±0.49b	6.66±1.28c	7.84±1.61b
C20:5 ω3	4.93±4.98a	7.49±0.29b	5.83±0.83c	6.35±1.39b
C22:2 ω6	__	0.47±0.38a	0.12±0.08b	0.36±0.68a
C22:3 ω3	__	0.02±0.02a	__	0.07±0.03b
C22:4 ω6	__	0.23±0.16a	__	0.65±0.07b
C22:5 ω3	1.22±0.01a	1.93±0.12b	2.52±0.18c	2.90±0.53c
C22:5 ω6	__	0.51±0.75a	__	0.52±0.07a
C22:6 ω3	2.72±0.45a	14.78±1.18b	15.15±3.06b	14.61±4.26b
ΣPUFA*	**37.17±0.78**	**45.68±0.58**	**39.16±4.98**	**44.46±6.31**
Σ ω3	11.95±5.28a	28.83±0.57b	28.35±3.92b	29.45±6.62b
Σ ω6	25.20±6.06a	16.85±0.66b	10.79±1.13c	15.00±1.68b
Σ ω3/6	0.47±0.33a	1.71±0.09b	2.63±0.16c	1.96±0.52b

[a] average of analysed.
[b] means±S.D.
[c] [abc]within the lines, values in four seasons in a species of fish are significantly different at $p<0.05$.

4. Conclusions and discussion

Among saturated fatty acid and MUFA identified in present study the highest was palmitic acid and it was followed by oleic acid which is comparable with the findings of other studies. Similar results to this study were determined for MUFAs of total lipid of *S. cephalus* in Serban Dam Lake. Oleic acid (16.82%) and palmitoleic acid (13.60%) were the major MUFAs "(Bulut and Mert 2014)". Total monounsaturated fatty acid was found to be 31.27-34.56% in *Carassius gibelio* "(Bulut, 2010)", and 13.80%-21.36% in *Sander lucioperca* "Celik et al. 2005; Ozogul et al. 2007". Monounsaturated fatty acids was 22.40-23.87% in *S. lucioperca*, 36.10% and 37.15% in carp from Turkey "(Ozparlak, 2013)".

"Kalyoncu et al., 2010a,b" have reported seasonal differences in the fatty acid profile of carp and rainbow trout and identified oleic acid as major MUFA (25,01-29,28%) in carp, (23,65-34,06%) in rainbow trout. Similarly "Citil et al. 2014" have reported that fatty acid composition of *S. erythrophthalmus* from Isıklı Dam Lake and identified oleic acid as major MUFA (16.07%). Study has reported high levels of 18:1. According to "Steffens (1997)" C18:1 is a typical MUFA in fish tissues. The high amounts of C18:1, C16:1, and AA had been identified as a typical content of freshwater fish species oils "Andrade et al., 1995". In our study, the level of oleic acid, palmitoleic acid and arachidonic acids were determined higher than other fatty acids. C16:1 ω7 was the second high monounsaturated fatty acid (4.81-6.11%) in rudd. There were no differences between four seasons in terms of palmitoleic acid content ($p<0.05$). Although *S. erythrophthalmus* has the most constant fatty acid profile, there is quantitative differences. "Ackman (1989)" defined that highest values of palmitoleic acid is one of the typical of freshwater fish species. PUFA fractions were higher than the MUFAs and SFAs and in three seasons (winter, summer, autumn). A very high quantity of C16:0 (30.41%) increased the saturated fatty acid (SFA) content in spring, and a high quantity of, and C22:6, C20:5, C20:4 ω6 raised the PUFA amount in other seasons except spring. In this study, whereas total PUFA in chub was higher than total MUFA and SFA in four seasons, PUFA was higher in autumn, winter and summer in rudd. The levels of SFA, MUFA and PUFA were found to be 28.12%, 32.52%, 38.19% of lipid respectively in chub"(Bulut and Mert, 2014)".

Variations in fatty acids of freshwater and marine fishes should not only be considered with respires to species environment but also based on their diet specially whether a species is carnivorous, omnivorous, or herbivorous "Sargent et al., 1995; Ozogul and Ozogul, 2007". Rudd individuals of which it is stated that herbivores fed and

generally showed widespread in tropical regions. Chub is supplied with a variety of fish fry. In this study, differences in PUFA and MUFAs content in the chub and rudd may be attributed to this reason.

C 16:0 (palmitic acid) was main SFA in two species. Another heavy SFA was C18:0 (stearic acid). SFA ingredient ranges between 26.79% and 41.54% in rudd in four seasons. "Ozogul et al., 2007", "Donmez (2009)" and "Kalyoncu et al., 2010a" have also reported that prime saturated fatty acid was C 16:0 and the other was stearic acid in *C. carpio*. "Ackman et al., 1975" described that the levels of palmitic acid of fish was not affected by diet. However a lot of factors could influence fatty acid metabolism in fish, these: life mode and water of temperature. In general fishes have comparatively low in SFA (<30%) except for some species "(Ackman, 1989)". Results of in this study are in agreement with the other literature in data. Total saturated fatty acid was considerably higher in summer than in other seasons in chub. C 16:0 was the basic SFA, 18.89-30.41% for *S. erythrophthalmus* and 14.88-19.80% for chub in all seasons. Similar results to this study for different fish species have also been determined in other studies "Celik et al., 2005; Guler et al., 2008". Stearic acid was the second highest SFA (4.83-6.55%) in rudd and in chub (3.77-6.22%).

In this study, data demonstrated that ω3/ω6 rate was 1.08 (in spring), 1.79 (in summer), 1.92 (in autumn) and 1.31 (in winter) in rudd, 0.47 (in spring), 1.71 (in summer), 2.63 (in autumn) and 1.96 (in winter) in chub. According to "Valfre et al., (2003)" the ω3/ ω6 ratio varies between 1 and 4 in freshwater fish. ω3/ ω6 rate in muscle tissues in chub were determined to be 1.28 in winter (February 2010) "(Bulut and Mert, 2014)". ω6 fatty acids were lower than ω3 fatty acids in all seasons in rudd. ω3/ ω6 rate in rudd were found to be 2.15 "Citil et al., 2014". "Ozparlak (2013)" have determined this ratio in winter to be 3.19 (*S. lucioperca*), 2.36 (*C. gibelio*), 2.08 (*L. lepidus*) and 1.06 (*C. carpio)*. Hence, our results are in accordance with other former studies "Kalyoncu et al., 2009; Cakmak et al., 2012; Gorgun et al., 2013". Our study has revealed that *S. erythrophthalmus* is a well source due to its superior ω3/ω6 rate. In this study, PUFA levels have been found to be 38.96%-56.46% for rudd. The most abundant PUFA in rudd was DHA (17.94%) from Isıklı Dam Lake "Citil et al., 2014". AA level is fairly important. AA was found to be the third highest fatty acid in samples collected in winter (10.52%). Total opinion all of these percentage, rudd (38.96%, 43.20%, 56.46%, 50.20%) and chub (37.17%, 45.68%, 39.16%, 44.46% respectively) seems to be two fish species rich in point of the PUFAs. "Bowman and Rand (1980)" reported that AA is a vanguard for prostaglandin and tromboxan.

The present results shows that DHA (22:6ω3) is predominant PUFA in muscle lipids of rudd. The high level DHA (19.05%) increases the PUFA content in rudd in autumn. In spring, a high rate C18:2 ω6 (21.17%) raises in the PUFA amount in chub. All fish species were rich in C 18:2 n-6 which is fundamental in human nutrition as DHA, C 18:2 n-6 are not synthesised in the body but they are necessary for tissue development. The level of EPA and DHA were between 4.93-7.51% and 2.72-19.05% in both species and four seasons respectively. It is known that, in seawater fish, the EPA and DHA amounts are higher than in freshwater fish "Czesny et al., 1999". EPA and DHA, have extremely helpful properties for the avoid of human coronary artery disease "(Leaf and Weber 1988)". Thus among the n 3 series the chub and rudd are well sources of EPA and DHA and ω3/ω6 rate observed in the recommended values (1-4) in the species that we work with. DHA is basic for the development the eye retina and of the foetal brain "Birch et al., 2000" and it was identified that DHA decreases the concentration of LDL cholesterol in plasma "Childs et al., 1990". According to "Simopoulos (1991)", because n 3 fatty acids are fundamental in growth throughout the life cycle and development, omega 3 should be included in the diets of all humans. n3 and n6 fatty acids are not interconvertible in the human body and are significant components of practically their cell membranes. Whereas cellular proteins are genetically determined, the polyunsaturated fatty acid (PUFA) composition of cell membranes is to a great extent dependent on the dietary intake. *Scardinius erythrophthalmus* and *Squalius cephalus* were good nutrient of PUFA, particularly total ω-3 fatty acids.

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Research article/Araştırma makalesi

Antifeedant effects of *Ferulago longistylis* extracts from Erzincan on *Ephestia kuehniella*

Hilal BAKİ [*1], Ayşe Gül AKTAŞ [2], Çağrı BEKİRCAN [3], Ersan BEKTAŞ [1]

[1] Giresun University, Espiye Vocational School, Giresun, Turkey
[2] Giresun University, Faculty of Sciences, Giresun, Turkey
[3] Karadeniz Technical University, Faculty of Sciences, Trabzon, Turkey

Abstract

Ferulago longistylis Boiss. is an endemic species naturally grown in Eastern Anatolia Region especially Erzincan and Malatya provinces. In this study, antifeedant effects of chloroform, hexane and water extracts of *F. longistylis* obtained by using soxhlet extractor, against *Ephestia kuehniella* Zeller (Lepidoptera: Prelidae) was investigated. Both our country and all around the world, insect pests living in the stored products cause a serious decline in the quality of products. One of them is *Ephestia kuehniella* which is well-known and called as a flour moth. The use of plant extracts against insect pests is becoming increasingly popular. Accordingly, in this study, consumption of wheat flour eaten by *Ephestia kuehniella* larvae were determined by following. Larvae were cultivated in sterile petri dishes including 50, 100, 250 and 500 ppm for each hexane, chloroform and water extracts for 24 hours and the average of the difference between the final and initial weight of the petri dishes was determined as the amount of consumption. According to the results, the highest antifeedant effect was observed in the petri including 50 ppm water extract. Hexan extract at 50 ppm and doses of water extracts at 250 and 500 ppm showed low antifeedant effect when compared to the control. All of chloroform extracts showed similar effects to the control.

Key words: antifeedant, endemic, *Ephestia kuehniella*, Erzincan, *Ferulago longistylis*

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Erzincan ilinden toplanan *Ferulago longistylis* özütlerinin *Ephestia kuehniella* üzerindeki antifeedant etkisinin belirlenmesi

Özet

Ferulago longistylis BOISS. Türkiye'de Doğu Anadolu Bölgesinde özellikle Erzincan ve Malatya illerinde yayılış göstermektedir ve endemik bir bitkidir. Bu çalışmada *F. longistylis* bitkisinden soxhlet yöntemiyle elde edilen kloroform, hekzan ve su özütlerinin *Ephestia kuehniella* Zeller (Lepidoptera: Prelidae)'ya karşı beslenmeyi durdurucu (antifeedant) etkisi araştırılmıştır. Hem ülkemizde hem de dünyada depolanan ürünlerde görülen zararlı böcekler ürünlerin kalitesinde ciddi düşüşlere neden olmaktadır. Bu böceklerin arasında un güvesi olarak da bilinen *Ephestia kuehniella* önemli yer tutar. Zararlı böceklere karşı bitkisel ekstraktların kullanımı üzerine yapılan araştırmalar giderek artmaktadır. Bu amaçla, *Ephestia kuehniella* larvaları, besinleri olan buğday ununu ve 50, 100, 250 ve 500 ppm'lik derişimlerdeki hekzan, kloroform ve su ekstraktlarını içeren petrilerde 24 saat bekletildikten sonra, başlangıç ve son ağırlıkları arasındaki farkların ortalamaları alınarak tüketim miktarları belirlenmiştir. Elde edilen sonuçlara göre en yüksek antifeedant etki, su özütünün 50 ppm'lik derişiminde tespit edilmiştir. Hekzan özütünün 500 ppm, su özütünün 250 ve 500 ppm'lik derişimleri kontrole göre düşük antifeedant etki göstermiştir. Kloroform özütünün tüm derişimleri kontrole göre benzer etki göstermiştir.

Anahtar kelimeler: antifeedant, endemik, *Ephestia kuehniella*, Erzincan, *Ferulago longistylis*

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[*] *Corresponding author* / Haberleşmeden sorumlu yazar: Tel.: +904543101430; Fax.: +904543101431; E-mail: hilal.baki@giresun.edu.tr
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1. Introduction

The flour moth, *Ephestia kuehniella* Zeller (Lepidoptera: Prelidae) is harmful on flour both our country and all around the world. This pest causes a serious decline in the quality of flour and stored products (Dabbaoğlu, 2004). Nowadays, protection of agricultural products from production to consumption with minimal losses is also very important. Usually, losses in stored products caused by animal organisms is considered average 10% an annual and this rate is over the 10% in different regions (Çanakçıoğlu, 2010). Generally, insecticides are used to control of insects in storage in our country (Ferizli and Emekçi, 2010). Insecticides are chemicals and they have several advantages and disadvantages**.** Especially, their harmful impacts on the environment and non-target species cannot be ignored (Igbedioh, 1991). Repeated application of insecticides causes insecticide resistance on the pest and pesticide residues in storages and several health problems on practitioners. For these reasons, use of plant extracts against insect pests is becoming increasingly popular (Isman, 1995; Yankanchi and Gonugade, 2009; Rajopadhye et al., 2016). There are many studies about the use of plant extracts from various families against to insect pest (Liu et al., 2011; Özger et al., 2013; Akumefula et al., 2014; Metin and Bürün, 2015). Also, Apiaceae family is among these families, it is a cosmopolite family and it is the eighth largest family in Turkey (Yılmaz and Koyuncu, 2015). Nowadays, impacts of endemic species are being wondered. Although 37 of endemic species are endangered, endemism rate this family is 33% in Turkey (Özhatay et al., 2009). *F. longistylis* in Ferulago genus in Apiaceae family is endemic species for Turkey. Endemic plants in different geographies may have a stronger effect on pest control. Because of this, the aim of this study was evaluate the antifeedant effects of extracts of *F. longistylis* against stored product pest, *Ephestia kuehniella* larvae.

2. Materials and methods

Stored product pest species, *E. kuehniella* Zeller. (Lepidoptera: Prelidae) larvae were brought from Ankara University, Department of Plant Protection for the antifeedant test. Larvae of flour moth are 4th instars. An endemic plant, *F. longistylis* is collected from Erzincan during 2014 to 2015 in Turkey. Plant sample is dried in the shade in the laboratory with good air space. Dried aerial parts of the plant are used to extraction. *F. longistylis* extract, employing different solvent were obtained. Dried aerial parts of the plant were powdered. A portion (20 gm; 5 gm portions into 3 cartridges) of dried plant material was extracted with hexane in Soxhlet apparatus. Hexane phases were combined and evaporated down to dryness. Residual plant material was then extracted methanol for 4 h and solvent was evaporated. Methanol extract was further partitioned with chloroform and water mixture (1:1) to obtain more polar fractions. Two fractions were separated and chloroform was evaporated. Water phase was frozen at -80 °C and lyophilized (Christ Alpha 1-2 LD plus). The potential of the antifeedant effect of the extract against to *E. kuehniella* was determined by the antifeedant test. Wheat flour were used as test food. Hexane, chloroform and water extracts were prepared in 1000 ml and mixed with 1 gr of the wheat flour at a concentration of 50, 100, 250 and 500 ppm for the test. Each test group was set up with petri dishes (6 cm in diam.) each including 10 larvae (4th instar). The prepared three different solvent (chloroform, water and hexane) to test groups was set up with 3 replicates for each test dose and replicated over 3 days. Larvae were weighed in petri dishes and saved. Then 1 g of wheat flour with different extracts and dose are added to the center of the petri dishes and weighed and saved. In addition, one control group was also set up for each test solution. In total, three control groups were set up for each repeat. All test groups placed in climatic chamber (Aralab-FITOKLIMA D1200PLH) (Temperature 25 ± 1 C°, Humidity ± 65% and 14/10 Light/Dark photoperiod) After 24 h, it was weighed and recorded together with the petri dish. *E. keuhniella* larvae were removed from petri dishes and then weighted and recorded. This process was repeated for each of the three treatment days. The amount of food consumed was calculated depending on the initial fresh weight of each petri dishes with larvae. The antifeedant index was calculated according to following formula (Sadek, 2003).

$AFI=[(C-T)/(C+T)] \times 100$

C: as the consumption of flour in control groups and T: the consumption of flour in treated groups. The food consumed by the 10 larvae were given control groups were averaged, and the means were used as C for the calculations of the AFI for each observed.

The antifeedant indices at different treatments were compared using an analysis of ANOVA followed by Duncan test for multiple-comparison where significant differences were observed. A Pearson correlation coefficient test was carried to determine the AFI-Day and AFI-Dose relations between the treatments. All of these analysis performed with SPSS version 21.0 for Windows (Yang, 2014).

3. Results

Antifeedant effects of hexane, choloroform and water extracts of *F. longistylis* against *E. kuehniella* larvae were evaluated in this study. Results were compared with the control (Table 1). According to the results, 50 ppm hexane extract of *F. longistylis* had negative antifeedant effect to the control. The increasing concentration of hexane extracts of *F. longistylis* reduced the amount of consumption (Figure 1). Therefore, the highest antifeedant effect was

observed at concentration of 250 and 500 ppm (amount of consumption: 0.249 g) and this concentrations of hexane extract of *F. longistylis* showed similar effects when compared to control. Average consumption amount of flour from other hexane extract concentration of this species (50 and 100 ppm) were calculated 0.282 g, 0.260 g respectively (Table 1).

Antifeedant effect of increasing concentration of chloroform extracts of *F. longistylis* was not different. All concentration of chloroform extracts of *F. longistylis* showed similar antifeedant effect with the control. Average consumption amount of flour from all chloroform extract concentration of this species (50, 100 and 250 ppm) were calculated 0.231 gr (Table 1).

The highest antifeedant effect was determined in the *F. longistylis* at the concentration of 50 ppm amongst the tested water extract concentrations. Only the 50 ppm of water extracts of *F. longistylis* have positive antifeedant effect due to their lowest amount of consumption (0.217 gr). Consumption amount of flour from other hexane extract concentration of *F. longistylis* (100, 250 and 500 ppm) were calculated 0.233 gr, 0.240 gr and 0.242 gr respectively (Table 1). The amount of consumption also increased with increasing concentration and therefore antifeedant effect was decreased.

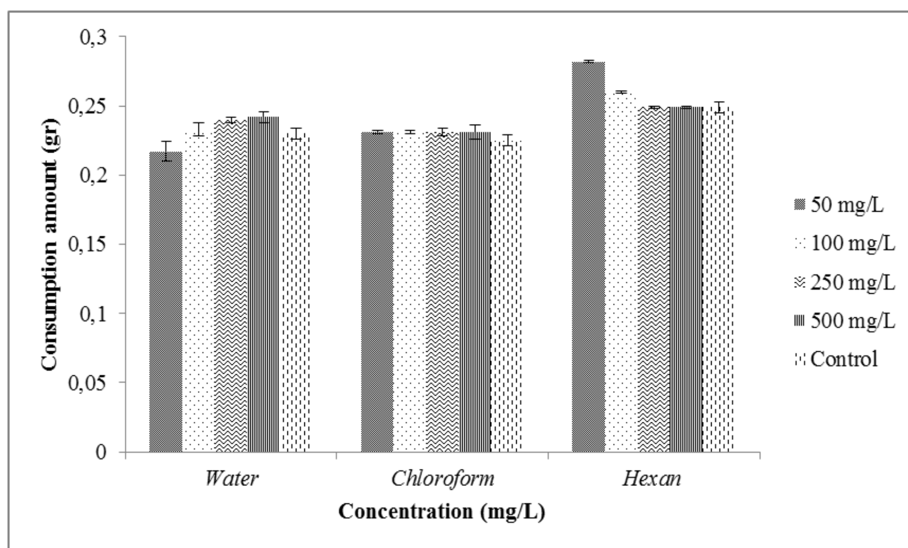

Figure 1. Consumption amounts of water, chloroform and hexan extracts (concentration of 50, 100, 250 and 500) of *F. longistylis.*

Finally, the highest antifeedant effect was observed at 50 ppm water extract. Doses of hexane extract at 50 ppm and water extracts at 250 and 500 ppm showed low antifeedant effects when compared to the control. All doses of chloroform extracts showed similar effects to the control. Increasing hexane extract concentrations of *F. longistylis* increases the antifeedant effect and increasing water extract concentrations declines the antifeedant effect.

Table 1. Consumption amounts of water, chloroform and hexane extracts of *F. longistylis*

	Concentration	Water	Chloroform	Hexane
F. longistylis	50 ppm	0,217±0,001a	0,231±0,003b	0,282±0,003c
	100 ppm	0,233±0,001b	0,231±0,004b	0,260±0,001b
	250 ppm	0,240±0,003c	0,231±0,002b	0,249±0,000a
	500 ppm	0,242±0,005c	0,231±0,001b	0,249±0,000a
	Control	0,230±0,004b	0,225±0,004a	0,249±0,007a

*Each values on concentrations are averages of three replicates. Similar letters in the same column are no different according to Duncan Multiple Comparison tests ($P < 0.005$).

4. Conclusions and discussion

Antifeedant effects of hexane, chloroform and water extracts of *F. longistylis* against *Ephestia kuehniella* larvae were studied. Results were statistically variable and different from the control group. In the literature, there are not a study about antifeedant effect of this plant (*F. longistylis*). Therefore, the results compared with the results of obtained from different plant extracts. The toxic effects of ethanol extracts of several plant extracts (*Tamarix smyrnensis* Bunge, *Scorzonera mollis* Bieb, *Scorzonera tomentosa* L., *Reseda alba* L., *Linum bienne* Miller, *Artemisia santonicum* L., *Prunus laurocerasus* L. and *Laurus nobilis* L.) on the large diamondback moth, *Plutella xylostella* L. was investigated by Ertürk et al., (2004). They reported that ethanol extract of A*rtemisia santonicum* had antifeedant and toxic effects on *P. xylostella* but alcohol extracts of *T. smyrnensis*, *S.mollis*, *S. tomentosa*, *R. alba*, *L. bienne*, *P. laurocerasus* and *L. nobilis* did no toxic effects. In our study, especially 50 ppm concentration of water extract of *F.*

longistylis showed antifeedant effect on *E. kuehniella* larvae and the increasing concentration of hexane extracts of *F. longistylis* reduced the amount of consumption of food. Antifeedant and toxicity effects of some plant extracts belongs to different families (*Origanum vulgare* L., Family (Labiatae), *Buxus sempervirens* L., Family (Buxaceae), *Sambucus nigra* L., Family (Caprifoliaceae), *Aesculus hippocastanum* L, Family (Hippocastanaceae), *Hypericum perforatum* L., Family (Compositae), *Viscum album* L., Family (Loranthaceae), *Diospyros kaki* L., Family (Ebenaceae), *Ocimum basilicum* L., Family (Labiatae), *Alnus glutinosa* Goertn, Family (Betulaceae) and *Achilea biebersteinii* Willd., Family (Compositae) on *Lymantria dispar* L. (Lep: Lymantridae) on the 2-3rd instar larvae of the *L. dispar has* reported by Ertürk et al., 2006. In that study showed that *O. vulgare* and *A. hippocastanum* extracts have high antifeedant activity on the larvae of*L. dispar*andthe highest consumption of diet was observed with alcohol extract from *V. album* and the minimum consumption was also with alcohol extract from *B. sempervirens.* The other tested extracts showed similar activity in the same study. In our study, 4th instar larvae of E. *kuehniella* were used and the highest consumption was observed with water extract (50 ppm concentration) and the minimum consumption was with hexane extract (50 ppm concentration) from *F. longistylis.* Çakır and Kıvan, (2012) conducted a study to determine the effects of sodium chloride (NaCl) on the sunn pest, *Eurygaster integriceps* Put. (Heteroptera, Scutelleridae) feeding and using as an insecticide enhancer in the laboratory. Their results showed that NaCl had an arresting effect over the sunn pest. Similarly, increasing concentrations of hexane extracts of *F. longistylis* has antifeedant effect on *E. kuehniella* larvae. Özger et al., (2013) reported that the compounds derived from the neem tree are a type of bioinsecticide that can be used as an alternative to synthetic insecticides. Liang et al., (2003) studied application of agroneemt, ecozint and neemixt insecticide that are originated from neem on feeding of *Plutella xylostella* larvae. They reported that these insecticides have high antifeedant effect on *P. xylostella larvae.* The larvae that fed on leaves treated with neem extract turned out to be smaller than the larvae that fed on leaves treated with water. Similarly, in our study, *E. kuehniella* larvae that fed 50 ppm of water extracts of *F. longistylis* are smaller than larvae fed all concentrations of hexane and chloroform extract. Yorulmaz Salman et al., (2015) studied the contact toxicities of the extracts with hexane, ethanol and methanol of *Ocimum basilicum* L.*, Thymus vulgaris* L.*, Mentha spicata* L.*, Melissa officinalis* L. and *Matricaria chamomilla* L. plants on adult, 3rd and 4th instar larvae of *Leptinotarsa decemlineata*. All of the plant extracts prepared with hexane, ethanol and methanol were found more effective in 3rd and 4th instar larvae of *L. decemlineata* than adult. The lowest effect was obtained from hexane extracts of *M. chamomilla* in their study, similarly the lowest effect was obtained from hexane extracts of *F. longistylis* in our study. Alkan and Gökçe, (2012) reported that hexane, ethyl acetate and methanol extracts of stem and flower of *Tanacetum abrotanifolium* caused reduction in feeding of both *Sitophilus oryzae* and *Sitophilus granarius* (Col.,Curculionidae), they known as granary weevil and rice weevil respectively. Karakoç ve Gökçe, (2013) indicated that antifeedant effects of 9 plant extracts on *Spodoptera littoralis* (Boisduval) (Lepidoptera: Noctuidae). They used aseton as a solvent and 4 of 9 plant extracts (*Delphinium consolida, Chrysanthemum segetum*, *Artemisia vulgaris, Tanacetum mucroniferium*) showed the strong antifeedant effect on *S. littoralis.* The highest antifeedant activity was seen on water extracts of 50 ppm concentration in our study. Antifeedant effect of *Salvia officinalis* and *Rosmarinus officinalis* extracts that they treated with imidakloprid, azadirachtin, *Bacillus thuringiensis* were tested on 4th instar larvae of *Leptinotarsa decemlineata* by Kara et al., (2014). The results indicate that bought plant extracts treated with azadirachtin have a potential in control of *L. decemlineata*.

Finally, antifeedant effects of concentration of 50, 100, 250 and 500 ppm of hexane, chloroform and water extracts of an endemic plant *F. longistylis* against *Ephestia kuehniella* larvae were different. Especially, concentration of 50 ppm water extracts of *F. longistylis* has strong antifeedant effect on *E. kuehniella* larvae. It can be used as an alternative control method. Otherwise, antifeedant effect of *F. longistylis* with the increase of the concentration of chloroform and hexane extracts increased. Therefore, in order to avoid loss of product during storage of agricultural products that consumed as food, plants which can be used for pest control and ıts concentrations should be determined instead of pesticides. Determination of the effective dose may occur expanding the use of herbal extracts at the same time scientifically important results and may reduce toxic effects.

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Research article/Araştırma makalesi

Contents of phenolic and flavonoid compounds in *Isatis demiriziana* Mısırdalı: an endemic to the Southeast Anatolia, Turkey

Özgür KARAKAŞ *[1], Mustafa Abdullah YILMAZ [2], Hilal SURMUŞ ASAN [3], Ahmet ONAY [3], Nazan ÇALAR [3]

[1] Şırnak University, Faculty of Agriculture, Department of Agricultural Biotechnology, 73300 Şırnak, Turkey
[2] Dicle University Science and Technology Research and Application Center (DÜBTAM), Dicle University, 21280 Diyarbakır, Turkey
[3] Department of Biology, Faculty of Science Dicle University, 21280 Diyarbakir, Turkey

Abstract

Isatis demiriziana Mısırdalı plant contains a number of compounds which has anticarcinogenic, antioxidant and other preventive effects. In this study, the flavonoid and phenolic contents in the plant samples harvested in the vegetative (leaf and root) and full flowering season (flower, leaf and root) were determined by LC-MS/MS. Among the 27 compounds studied, malic acid was found to be the most abundant compound in the methanolic extracts of samples and the amount of malic acid of vegetative root extracts were the highest (30124,37 µg g^{-1} dry-extract). Moreover, it was also determined considerable amount of salicylic acids and p-coumaric in the root extracts. This study is the first detailed study on the phenolic and flavonoid compounds of *I. demiriziana*. Based on the findings of this study, in further researches might be refered as an additional source in production of phenolic and flavonoid compounds.

Key words: Isatis, LC–MS/MS, phenolics, flavonoids, endemic

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Türkiye'de Güneydoğu Anadolu Bölgesi'ne endemik *Isatis demiriziana* Mısırdalı'ndaki fenolik ve flavonoid bileşiklerinin içerikleri

Özet

Isatis demiriziana Mısırdalı bitkisi antikanserojen, antioksidan ve başka koruyucu özelliklere sahip olan çok sayıda bileşik içermektedir. Bu çalışmada, *I. demiriziana*'nın vejetatif (yaprak ve kök) ve tam çiçeklenme (çiçek, yaprak ve kök) dönemlerinde hasat edilen bitki örneklerinin flavonoid ve fenolik içerikleri LC-MS/MS ile tespit edildi. Çalışılan 27 bileşik arasında, örneklerin metanolik ekstraktlarında malik asit miktarı en fazla düzeyde bulundu ve vejetatif köklerdeki malik asit miktarı (30124,37 µg g^{-1} kuru ekstrakt) en yüksek orana sahipti. Ayrıca kök ekstraktlarında büyük miktarda salisilik asit ve p-kumarik asit tespit edildi. Bu çalışma, *I. demiriziana*'nın fenolik ve flavonoid içerikleri üzerine yapılmış ilk detaylı çalışmadır. Bu çalışmanın sonuçlarına dayanılarak, ileriki çalışmalarda fenolik ve flavonoid bileşiklerin üretiminde bir ek kaynak olarak başvurulabilir.

Anahtar Kelimeler: *Isatis*, LC–MS/MS, fenolikler, flavonoidler, endemik

1. Introduction

Brassicaceae (Cruciferea) has about 350 genera and 3000 species, growing mostly in the North Temperate Zone and Mediterranean Region (Mabberley, 1987). *Isatis* genus belongs to Brassicaceae family and this genus is represented by 40 taxa which 24 of these are endemic to Turkey (Davis, 1988). Moreover, this genus has 31 species and 14 subspecies in Eastern and South-Eastern Anatolia (Mısırdalı, 1985). The chemical constitutients extracted from the roots and leaves of *Isatis* species have antiviral, anticancer, antibacterial, astringent, febrifuge and antirheumatic effects (Radwan et al., 2008; Vang, 1994; Kirtikau and Basu, 1983; Bown, 1995). They are also employed for different

* *Corresponding author* / Haberleşmeden sorumlu yazar: Tel.: +904865513032; Fax.: +904865513031; E-mail: okarakas80@gmail.com
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disorders such as encephalitis, meningitis, erysipelas, influenza, heat rash, mumps in the traditional medicine. It is reported that uses of *Isatis* species ethnobotanical in Eastern Anatolia (Polat et. al., 2012). Use of natural antioxidants including phenolic acids and flavonoids as preventive and therapeutic drug have been attracting considerable attention because of their antioxidant properties and anticarcinogenic potential. These compounds have several health promoting influences (Costa et al., 2012; Erdogan-Orhan et al., 2014; Prakash et al., 2007). Phenolic compounds act as antioxidants (Maillard et al., 1996). Phenolic compounds play a significant role in total antioxidant capacity of vegetables, fruits and grains (Jacobo-Velazquez and Cisneros-Zevallos, 2009). The benefits of flavonoids including reducing the risk of atherosclerosis developing have been clearly shown (Knekt et al., 1996). Flavonoids perform some important functions such as cell cycle inhibition, nitrogen fixation and filtration of UV rays (Kumar and Pandey, 2013). Several studies suggested that flavonoids have protective impacts against degenerative diseases such as cardiovascular diseases, cancers and other age-related diseases (Pandey, 2007; Kumar et al., 2013). Phenolic acids include two primary groups: Cinnamic and benzoic acid (Tarnawski et. al., 2006). The chlorogenic acid is the most significant cinnamic acid which is a combination of quinic and caffeic acids. Hydroxybenzoic acid derivatives are firstly present as vanillic acids but p-hydroxybenzoic and protocatechuic acids are more extensive (Kvasnicka et. al., 2008).

Study on several certain phenolic acids such as gallic acid, malic acid, p-coumaric acid, vanillic acid and syringic acid reveals that they have numerous advantage for human health. Malic acid plays a vital role in reversing muscle fatigue and mental clarity. Moreover, these actions can make it a beneficial treatment for sufferers of fibromyalgia (Russell et al., 1995). Vanillic acid and p-coumaric acid have hydroxide scavenging activity (Kang et al., 2006). Moreover, p-coumaric acid is a potent inhibitor of 5-S-cysteinyl dopamine induced neurotoxicity and this compound is used in treatment of Parkinson's disease (Vauzour et al., 2010). It was shown that the Gallic acid acted as an important agent in protection of renal damage causing death of tumor cells (Canbek et al., 2013). Salicylic acid plays a significant role in conservation against virus infection by inhibiting catalase resulting in the accumulation of H_2O_2 in plant cells (Chen et al., 1993).

Different parts of plants vary in terms of contents of phenolics and flavonoids, so each plant part needs to be analyzed to identify its potential advantage as a health product. It was reported that the chemical constituents in the leaves of some *Isatis* species possess antibacterial, antiviral, anticancer, febrifuge and astringent features (Karakoca et al., 2013) but there is no report on the phenolic and flavonoid contents of *I. demiriziana*. In this research, we aimed to determine the phenolic and flavonoid contents of different parts of *I. demiriziana* plants collected in the vegetative season (leaf and root) and full flowering season (flower, leaf and root). Liquid chromatography–tandem mass spectrometry (LC– MS/MS) technique was used to analyze the phenolic and flavonoid contents of *I. demiriziana*.

2. Materials and methods

2.1. Plant Material

Five different samples for *I. demiriziana* were collected from a height of 1300 meters from Ergani county of the Diyarbakır province and at vegetative (leaf and root) and full flowering season (flower, leaf and root). Voucher specimens were deposited at the Herbarium of Dicle University, Faculty of Science (voucher no. DUF-6050). Specimens were identified by Prof. Dr. Ömer SAYA, from the same institution. The samples were air-dried at root temperature and then the samples were pulverized with a laboratory mill and stored at 4^0C until the chemical tests were conducted. Quantitation and identification of phenolic and flavonoid compounds

2.2.1. Plant extract preparation for LC–MS/MS

The collected specimens were dried by air at room temperature, which were then powdered. The samples (100 g) were extracted three times with 300 mL of methanol for 24 h. Then, a rotary evaporator was used for removal of the solvent at 30°C. The remaining solid (Yield: 15.6%) was used to prepare a 1000 mg L^{-1} solution, which was then filtrated with a 0.2 µm microfiber filter to use for LC–MS/MS analysis.

2.2.2. Instruments and chromatographic conditions for LC–MS/MS

The phenolics were analyzed quantitatively by LC-MS/MS (Shimadzu LC/MS 8040 model). The liquid chromatograph has DGU-20A3R degasser, LC-30AD binary pumps, SIL-30AC autosampler and CTO-10ASvp column oven. The samples were separated chromatographically on a C18 reversed-phase Inertsil ODS-4 (150 mm × 4.6 mm, 3 µm) analytical column (40°C). The elution gradient comprised of mobile phase A:water, 0.1% formic acid and 5 mM ammonium formate and mobile phase B: methanol, 0.1% formic acid and 5 mM ammonium formate. The gradient program with the following ratios of solvent B was carried out t (min), B%: (0, 40), (20, 90), (23.99, 90), (24, 40), (29, 40). The solvent flow percentage was continued at 0.5 mL/min and injection volume was adjusted as 4 µL.

2.2.3. MS instrumentation

The samples were analyzed by MS employing a Shimadzu LC/MS 8040 model triple quadrupole mass spectrometer equipped (ESI source operating in both negative and positive ionization modes). Data obtained from LC–MS/MS were evaluated by Lab Solutions software (Shimadzu, Kyoto, Japan). The analyses were quantified by the multiple reaction monitoring (MRM) mode: the studied compounds were assayed following two or three transitions for each compound, the first one for quantitative uses and the second and/or the third one for checking of the finding. The optimum ESI conditions were determined as interface temperature; 350°C, DL temperature; 250°C, heat block temperature; 400°C, nebulizing gas flow (nitrogen); 3 L/min and drying gas flow (nitrogen); 15 L/min.

2.2.4. Analytical parameters for the validated LC–MS/MS method

Table 1 shows rectilinear regression equations and the linearity ranges of the studied standard chemicals. Correlation coefficients were higher than 0.99. Table 1 also displays the limit of detection (LOD) and limit of quantitation (LOQ) of the analytical method. LOD values of the compounds are between 0.05 and 25.8 g/L and LOQ values are between 0.17 and 85.9 g/L (the recoveries of the phenolics are between 96.9% and 106.2%).

Table 1. Analytical parameters of UHPLC-ESI-MS/MS method

No	**Analytes**	**RT[a]**	**Parent ion (m/z)[b]**	**Ionization Mode**	**R^{2c}**	**RSD%[d]**	**Linearity Range (mg/L)**	**LOD/LOQ (µg/L)[e]**	**Recovery (%)**	**U[f]**
1	Quinic acid	3.32	190.95	Neg	0.9927	0.0388	250-10000	22.3 / 74.5	103.3	4.8
2	Malic acid	3.54	133.05	Neg	0.9975	0.1214	250-10000	19.2 / 64.1	101.4	5.3
3	tr-Aconitic acid	4.13	172.85	Neg	0.9933	0.3908	250-10000	15.6 / 51.9	102.8	4.9
4	Gallic acid	4.29	169.05	Neg	0.9901	0.4734	25-1000	4.8 / 15.9	102.3	5.1
5	Chlorogenic acid	5.43	353	Neg	0.9932	0.1882	250-10000	7.3 / 24.3	99.7	4.9
6	Protocatechuic acid	5.63	152.95	Neg	0.9991	0.5958	100-4000	25.8 / 85.9	100.2	5.1
7	Tannic acid	6.46	182.95	Neg	0.9955	0.9075	100-4000	10.2 / 34.2	97.8	5.1
8	tr- caffeic acid	7.37	178.95	Neg	0.9942	1.0080	25-1000	4.4 / 14.7	98.6	5.2
9	Vanillin	8.77	151.05	Neg	0.9995	0.4094	250-10000	10.1 / 33.7	99.2	4.9
10	p-Coumaric acid	9.53	162.95	Neg	0.9909	1.1358	100-4000	15.2 / 50.8	98.4	5.1
11	Rosmarinic acid	9.57	358.9	Neg	0.9992	0.5220	250-10000	10.4 / 34.8	101.7	4.9
12	Rutin	10.18	609.1	Neg	0.9971	0.8146	250-10000	17.0 / 56.6	102.2	5.0
13	Hesperidin	9.69	611.1	Poz	0.9973	0.1363	250-10000	21.6 / 71.9	100.2	4.9
14	Hyperoside	10.43	463.1	Neg	0.9549	0.2135	100-4000	12.4 / 41.4	98.5	4.9
15	4-OH Benzoic acid	11.72	136.95	Neg	0.9925	1.4013	25-1000	3.0 / 10.0	106.2	5.2
16	Salicylic acid	11.72	136.95	Neg	0.9904	0.6619	25-1000	4 / 13.3	106.2	5.0
17	Myricetin	11.94	317	Neg	0.9991	2.8247	100-4000	9.9 / 32.9	106.0	5.9
18	Fisetin	12.61	284.95	Neg	0.9988	2.4262	100-4000	10.7 / 35.6	96.9	5.5
19	Coumarin	12.52	146.95	Poz	0.9924	0.4203	100-4000	9.1 / 30.4	104.4	4.9
20	Quercetin	14.48	300.9	Neg	0.9995	4.3149	25-1000	2.0 / 6.8	98.9	7.1
21	Naringenin	14.66	270.95	Neg	0.9956	2.0200	25-1000	2.6 / 8.8	97.0	5.5
22	Hesperetin	15.29	300.95	Neg	0.9961	1.0164	25-1000	3.3/ 11.0	102.4	5.3
23	Luteolin	15.43	284.95	Neg	0.9992	3.9487	25-1000	5.8 / 19.4	105.4	6.9
24	Kaempferol	15.43	284.95	Neg	0.9917	0.5885	25-1000	2.0 / 6.6	99.1	5.2
25	Apigenin	17.31	268.95	Neg	0.9954	0.6782	25-1000	0.1 / 0.3	98.9	5.3
26	Rhamnetin	18.94	314.95	Neg	0.9994	2.5678	25-1000	0.2 / 0.7	100.8	6.1
27	Chrysin	21.18	253	Neg	0.9965	1.5530	25-1000	0.05 / 0.17	102.2	5.3

RT: Retention time
[b]Parent ion (*m/z*)*:* Molecular ions of the standard compounds (mass to charge ratio)
[c]R^2: coefficient of determination
[d]RSD: relative standard deviation
[e]LOD/LOQ (µg/L): Limit of detection/Limit of quantification
[f] U (%): Percent relative uncertainty at 95% confidence level (k=2).
[g] Values in µg g^{-1} (w/w) of plant methanol extract
[h]N.D: not detected.

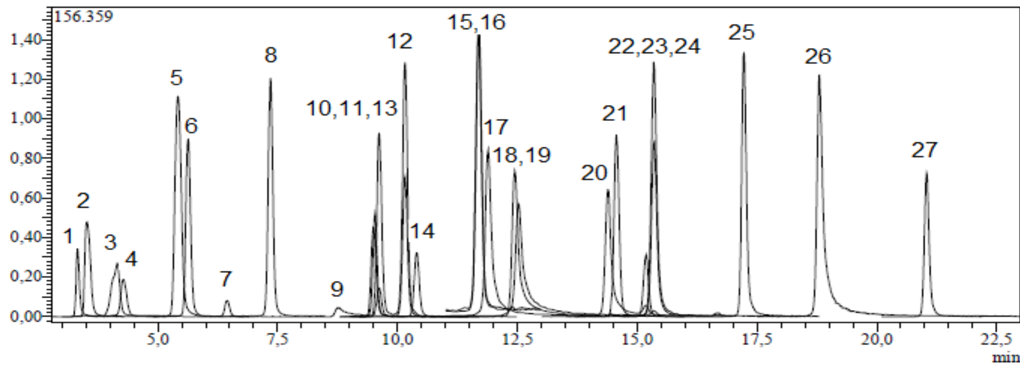

Figure 1. LC-MS/MS chromatograms of 250 ppb standard mix. quinic acid: 1, malic acid: 2, tr-aconitic acid:3, gallic acid:4, chlorogenic acid:5, protocatechuic acid:6, tannic acid:7, tr-caffeicacid:8, vanillin:9, *p*-coumaric acid:10, rosmarinic acid:11, rutin:12, hesperidin:13, hyperoside:14, 4-OH benzoic acid:15, salicylic acid:16, myricetin:17, fisetin:18, coumarin:19, quercetin:20, naringenin:21, hesperetin:22, luteolin:23, kaempferol:24, apigenin:25, rhamnetin:26, chrysin:27.

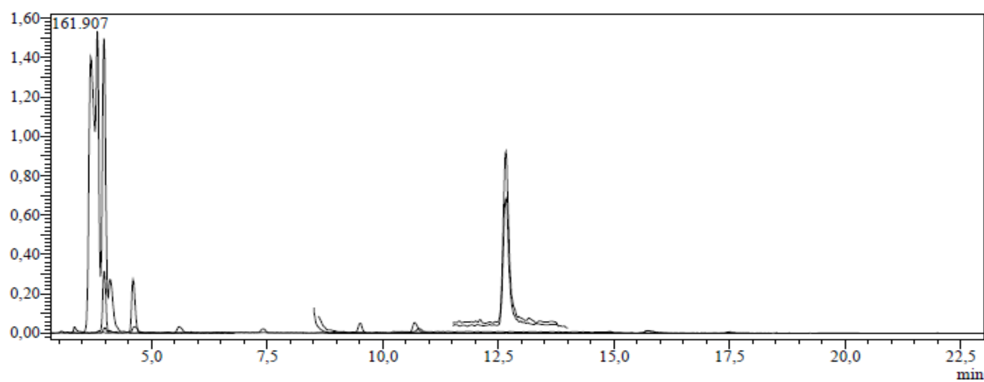

Figure 2. LC-MS/MS chromatogram of leaf in vegetative season.

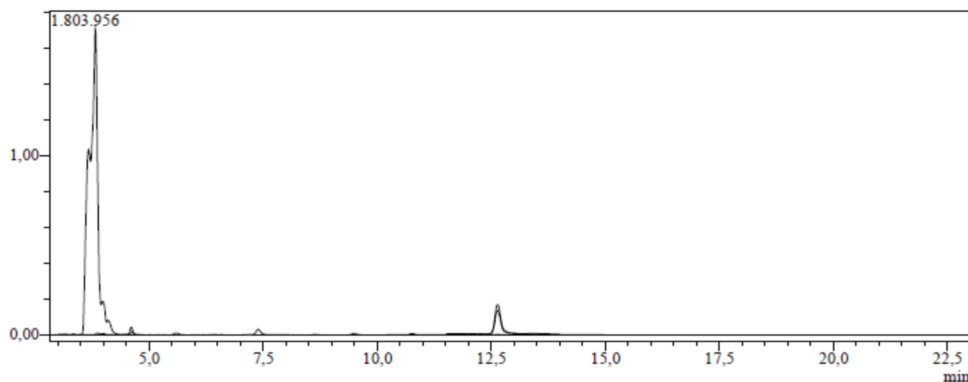

Figure 3. LC-MS/MS chromatogram of root in vegetative season

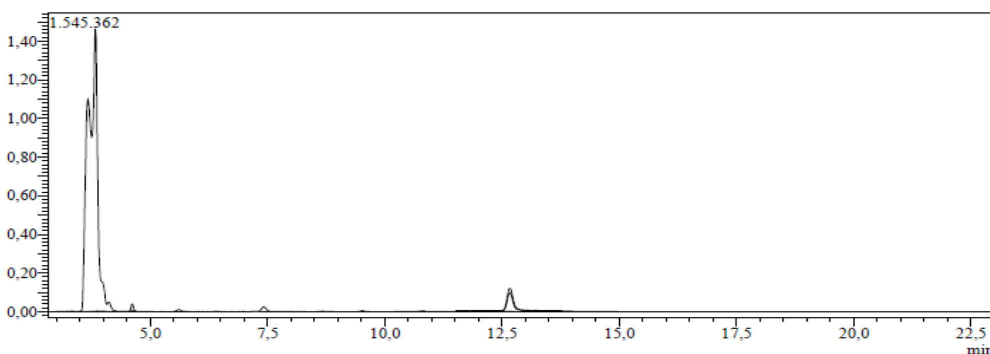

Figure 4. LC-MS/MS chromatogram of root in full flowering season

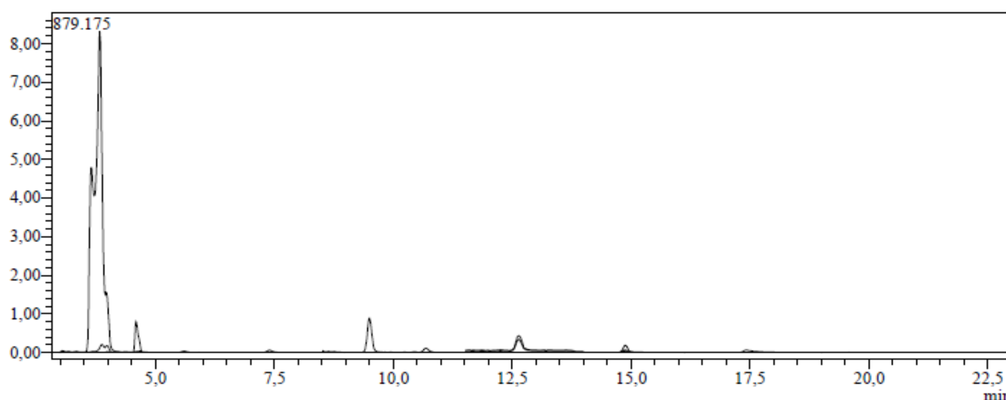

Figure 5. LC-MS/MS chromatogram of flower in full flowering season

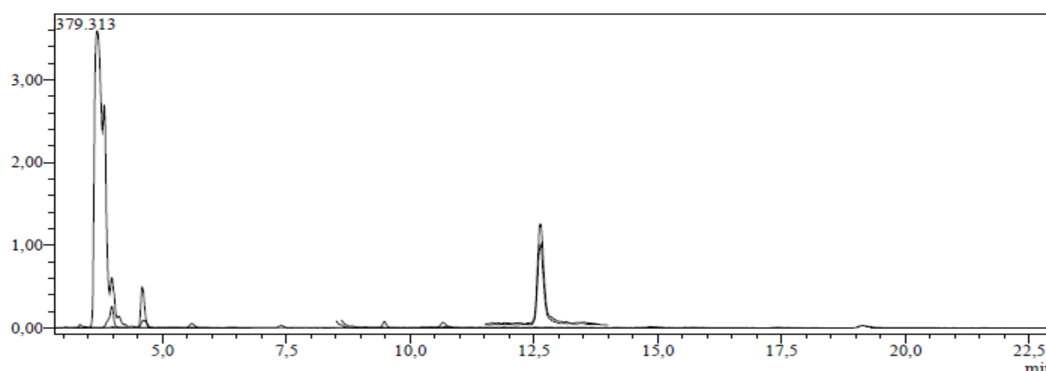

Figure 6. LC-MS/MS chromatogram of leaf in full flowering season

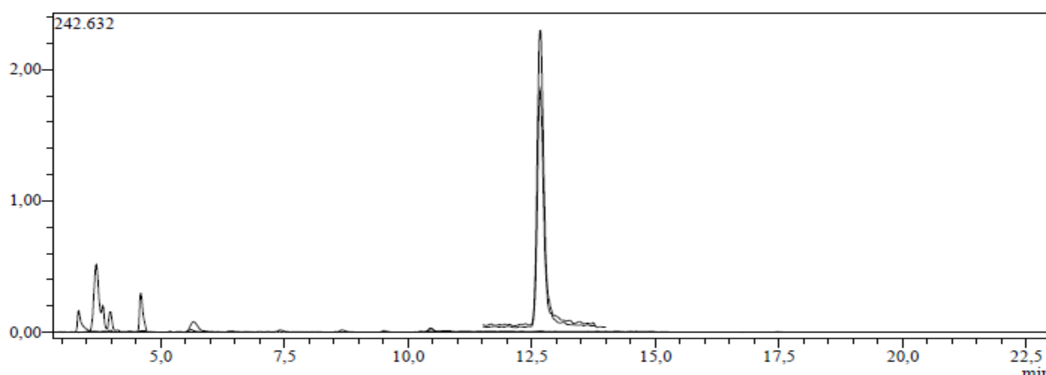

Figure 7. LC-MS/MS chromatogram of fruit

3. Results

3.1. Quantitative analysis of phenolics, flavonoids compounds by UHPLC-ESI-MS/MS

Results of phenolic and flavonoid content in the five samples of *I. demiriziana* have been presented in Table 2. Significant differences in the phenolic and flavonoid constituents of the different extracts of *I. demiriziana* were observed. LC–MS/MS analysis obviously display that the methanol extracts of *I. demiriziana* contain many phenolic and non-phenolic compounds Figure 2-7. Phenolic acids exist in most plants, and each plant can be adequately specific for the availability of various phenolic acids and their derivatives together with the other groups (Ziakova et. al., 2003).

It was determined that the main components of all plant samples were malic acid, quinic acid, tr-aconitic acid, vanillin, p-coumaric acid, 4-OH benzoic acid, salicylic acid, protocatechuic acid, tannic acid and tr-caffeic acid compounds. Out of non-phenolic compounds, *I. demiriziana* extracts include high amounts of quinic acid (221.18-1538.49 µg g^{-1}), tr-Aconitic acid (105.2 -298.62 µg g^{-1}), salicylic acid (35.94-216.27 µg g^{-1}), p-coumaric acid(19.13-1457.45 µg g^{-1}) and 4-OH Benzoic acid (27.99-176.57µg g^{-1}) and lower amounts of gallic acid (0.58-2.05 µg g^{-1}) (Table 2). Malic acid (MA) was single dominant compound among all samples studied. Among them, the vegetative root gave

the highest amount of malic acid with a 30124.37 μg g^{-1} extract (Table 2, Figure 3). This was followed by the flowering root, flower, the flowering leaf, the vegetative leaf and fruit stage with the amounts of 27733.72, 14.438.42, 6879.07, 3745.49 and 691.3 μg g^{-1} extract, respectively. Malic acid contents of the five samples of *I. demiriziana* were so different. This variation may be due to different organ or different growing stages of the samples studied and to gain more insight how growing stages and different organs influence phenolic content of this plant more studies are required. In prompting plant defense responses, present report declares that the metabolic levels of MA compounds play an important role (Huckelhoven, 2007). A corresponding induced defense response beginning intraplant signaling between roots and leaves was implicated in herbivory (Rasmann et al., 2005).

Quinic acid is a metabolite that responsible for metabolic response (inducible defense) to biotic stress (Murthy et al., 2009). It was the second highest phenolic acid determined as 994.56, 550.74, 1087.19, 221.18, 1538.49 and 487.62 μg g^{-1} extract in the vegetative leaf, vegetative root, flowering leaf, flowering root, flower and fruit samples, respectively. Besides, the amount of quinic acid that obtained from leaf extracts (vegetative leaf 994.56 μg g-1 and flowering leaf 1087.19 μg g-1) were greater amounts from root extracts (vegetative root 550.74μg g^{-1} and flowering root 221.18 μg g^{-1}). Similiarly, it is reported that the quinic acid and quercitol are present in high concentrations in wounded leaves of genus *Quercus* plants (Gargallo-Garriga et al., 2010). It is known that the *trans*-aconitic acid has antirheumatic and diuretic properties (Schnitzler, 2007) althought the distribition of this compound is rare (Nierhaus and Kinzel, 1971). The highest amount of *trans*-aconitic acid was obtained from flower stage with 298.62 μg g^{-1} (Figure 5). Salicylic acid (SA) is believed to be a plant signal molecule playing an important role in plant, development, growth and defense responses, and functioning in the commencement of systemic attained resistance (SAR) (Hahlbrock and Scheel, 1989; Ding et al., 2002). The vegetative and flowering root extracts contained significant amount of vanillin (124.09 and 311.94 μg g^{-1}), protocatechuic acid (59.4 - 66.91 μg g^{-1}) , tannic acid (40. 98- 25. 92 μg g^{-1}) and tr-caffeic acid (34. 09- 29. 41μg g^{-1}) respectively (Table 2, Figure 3,4). Vanillin is the main constituent of natural vanilla, a well-known food and cosmetic additive and has antioxidant and antimutagenic properties (Davidson and Naidu, 2000). Their collection is extremely sensitive to environmental situations such as water, light and nutrient availability, and pathogen infection (Harvell and Bosland, 1997). PCA is a natural phenolic acid and exist in several plants including mushrooms and microorganisms (Williams et al., 2012; Nguyen et al., 2013). It is known that the PCA has antiinflammatory and antioxidant (Liu et al., 2002; Syafni et al., 2012) and antibiotic activities (Nguyen et al., 2015). Tannic acid has antioxidant (Andrade et al., 2005), antimutagenic (Ferguson, 2001) and anticarcinogenic properties (Nepka et al., 1999). It is reported that the tannic acid induced by Rhizobia in rice, which is resistant to *Rhizoctonia (*Mishra et al., 2006*).*

Phenolic acid and flavonoids in plants have various functions such as protein synthesis, nutrient uptake, photosynthesis, allelopathy, enzyme activity, and structural components (Hung, 2016). Flavonoids are the largest group of phenolics having antimicrobial and antioxidant impacts (Lorenc et al., 2005). Along with their roles in plants, these compounds in human diet might introduce a number of benefits connected with reduced risk of chronic diseases including anti-inflammatory, anti-atherogenic, antiallergenic, antioxidant, anti-thrombotic, anti-microbial, vasodilatory and cardioprotective influences (Manach et al., 2004). In a study, Nahak et al. (2014) indicated that the phenolic constituent of a plant is usually a good sign of its antioxidant potential. It is found that the flower extracts of *I. demiriziana* have highest levels of flavonoids quercetin (7.98 μg g^{-1}), naringenin (22.96 μg g^{-1}), rhamnetin (72.74 μg g^{-1}), and hyperoside (49.19 μg g^{-1}) and non phenolics tr-aconitic acid (298.62 μg g^{-1}), and p-coumaric acid (1457.45 μg g^{-1}). Recently, Chang et al., (2016) stated that the acid hydrolysis extract of *I. indigotica* contained 61.02 mg/100g of p-coumaric acid, and 23.13 mg/100g of gallic acid. Similar propensity was also viewed for the flavonols, quercetin and hyperoside. The rutin mostly gathered at the fruiting and flowering phases (9.25- 29.67 μg g^{-1} respectively). Because they move as attractive to pollinators and/or to protect the reproductive structures against UV radiations and herbivores, probably, this accumulation pattern of quercetin, hyperoside and rutin may be associated with their biological roles (Gronquist et al., 2001; Kreft et al., 2003). Kaempferol (1.13- 0.13 μg g^{-1}), hesperetin (0-0.21 μg g^{-1}), luteolin (0-0.42 μg g^{-1}) and apigenin(0-0.35 μg g^{-1}) were the most abundant flavonoids in the exracts of *I. demiriziana* (Table 2).

According to our result, the highest amounts of hesperidin (27.61 μg g^{-1}), chlorogenic acid (110.4 μg g^{-1}), rutin (29.67 μg g^{-1}), 4-OH benzoic acid (176.57 μg g-1) and salicylic acid (216. 27 μg g^{-1}) were obtained from the fruit stage (Table 2, Figure 7). Hesperidin (Hsd) and hesperetin (Hst) have several biological activity such as antioxidant, anti-inflammatory and anti-cancer impacts. To struggle with different pathogens, these compounds play an important role in plant defense systems (Soares et al., 2015). Chlorogenic acid is widely employed in industries and medicine including food industries and the consumer chemicals (Kweon et al., 2001). It is a natural antioxidant and anticancer agent and has antiviral and antibacterial properties (Jiang et al., 2001). Karakoca et al., (2013) determined that the chlorogenic acid content was 1980.20 μg g^{-1} on the methanolic root extract of *I. floribunda.* Among twenty-seven references used; rosmarinic acid, myricetin, coumarin, fisetin and chrysin were not detected in *I. demiriziana* extracts employed in this study.

Table 2. Quantitative analysis of phenolic and flavonoid by LC-MS/MS in *I. demiriziana* (µg analyte/g extract)

Compounds	Vegetative-leaf	Vegetative-root	Flowering-leaf	Flowering-root	Flower	Fruit
Hesperidin	1.65	0.71	8.72	0.6	9.73	27.61
Coumarin	0	0	0	0	0	0
Quinic acid	994.56	550.74	1087.19	221.18	1538.49	487.62
Malic acid	3745.49	30124.37	6879.07	27733.72	14438.42	691.3
tr-Aconitic acid	105.15	129.5	187.85	114.53	298.62	105.2
Gallic acid	1.85	1.92	1.72	1.79	2.05	0.58
Chlorogenic acid	0.58	0.3	10.59	0.4	0.24	110.4
Protocatechuic acid	21	59.4	35.12	66.91	21.53	11.08
Tannic acid	4.53	40.98	25.68	25.92	4.53	4.63
tr-caffeic acid	2.35	34.09	2.83	29.41	7.08	1.86
Vanillin	26.31	124.09	24.64	311.94	6.39	133.67
Rosmarinic acid	0	0	0	0	0	0
p-Coumaric acid	77.77	129.35	98.44	85.92	1457.45	19.13
Rutin	0.46	0.69	5.41	0.20	9.25	29.67
Hyperoside	25.54	0.17	30.02	0.51	49.19	3.79
Myricetin	0	0	0	0	0	0
Fisetin	0	0	0	0	0	0
4-OH Benzoic acid	62.68	132.03	96.75	87.03	27.99	176.57
Salicylic acid	77.24	155.74	111.49	102.02	35.94	216.27
Quercetin	2.12	0.15	5.93	0	7.98	0.33
Kaempferol	1.13	0	0.13	0	0	0
Naringenin	1.26	0.04	1.5	0.04	22.96	0.16
Hesperetin	0.06	0.08	0.14	0	0.21	0.04
Luteolin	0.42	0	0.37	0.34	0	0.32
Apigenin	1.04	0.17	0.35	0.02	0	0.09
Rhamnetin	2.13	0	7.44	0	72.74	3.53
Chrysin	0	0	0	0	0	0

4. Conclusions and discussion

The present study can be deduced as the phenolic contents of different organs of *I. demiriziana* compared favorably with other plants. According to the results of this study, further investigations on *I. demiriziana* may be carried out to identify factors that will affect phenolic and flavonoids level in plant tissues, thus they might be grown and harvested under optimum circumstances to increase pleasing qualities chemical levels.

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Research article/Araştırma makalesi

Influence of surface kind on biofilm formation by *Salmonella* spp. and *Listeria monocytogenes* from food-contact surfaces

Bükay Yenice GÜRSU *[1], İlknur DAĞ [1], Şeref İSET [2], Gökhan DIKMEN [1]

[1] Eskisehir Osmangazi University; Central Research Laboratory Application and Research Center (ARUM) 26480, Odunpazari, Eskisehir,Turkey
[2] Eskisehir Food Control Laboratory Directorate, 26480, Eskisehir, Turkey

Abstract

Salmonella spp. and *Listeria monocytogenes* are important pathogenic bacteria, which are transmitted by food. It is known that both microorganisms may produce biofilm on biotic or abiotic surfaces. Bacteria in biofilms exhibit enhanced resistance to cleaning and sanitation. In this study, we investigated the biofilm producing ability of 8 *Salmonella* spp. and 6 *L. monocytogenes* isolates by microtiter plate and tube adherence method. All tested *Salmonella* spp. and *L. monocytogenes* strains produced biofilm but strains of *L. monocytogenes* exhibited a higher ability of biofilm formation. Concominantly with these two methods, adhesion and biofilm formation of selected strains to six different industrial surface was also assessed by scanning electron microscope (SEM). In addition, biofilm formation and development of selected two strains were also evaluated on granite surfaces and at five incubation periods (2th, 4th, 6th, 24th and 48th hours). Mature biofilm formation was determined after 24 and 48 hours. Granite, marble, wood and glass surfaces presented higher intensity of biofilm, compared to the steel and plastic surfaces. Especially granite and marble are the surfaces in which we found to be the most convenient for the biofilm formation.

Key words: Biofilm, *Salmonella, Listeria*, Surface Kind

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Besin temas yüzeylerinden elde edilen *Salmonella* spp. ve *Listeria monocytogenes* izolatlarının biyofilm oluşumu üzerine yüzey çeşidinin etkisi

Özet

Salmonella spp. ve *Listeria monocytogenes* besin yoluyla taşınan önemli patojenik bakterilerdendir. Her iki mikroorganizmanın da canlı ve cansız yüzeyler üzerinde biyofilm oluşturabildikleri bilinmektedir. Biyofilmde bulunan bakteriler temizleme ve sanitasyon işlemlerine artan bir direnç gösterirler. Biz bu çalışmada 8 *Salmonella* spp. ve 6 *Listeria monocytogenes* izolatının biyofilm oluşturma yeteneğini mikrotitre plaka ve tüp aderans metodlarıyla araştırdık. Test edilen tüm *Salmonella* spp. ve *L. monocytogenes* izolatları biyofilm oluşturmuş ancak *L. monocytogenes* daha yüksek derecede biyofilm oluşumu göstermiştir. Bu iki teste ilave olarak seçilen izolatların altı farklı endüstriyel yüzey üzerindeki adezyon ve biyofilm oluşumları da taramalı elektron mikroskopla tayin edilmiştir (SEM). Ayrıca seçilen iki izolatın biyofilm oluşum ve gelişimleri granit yüzeyler üzerinde ve beş farklı inkübasyon periyodunda değerlendirilmiştir (2., 4., 6., 24. ve 48. saatler). Olgun biyofilm oluşumu 24. ve 48. saatler sonrasında tespit edilmiştir. Granit, mermer, tahta ve cam yüzeyler, çelik ve plastik yüzeylere kıyasla daha yoğun biyofilm oluşturmuşlardır. Özellikle granit ve mermer biyofilm oluşumu için daha elverişli yüzeyler olarak bulunmuştur.

Anahtar kelimeler: biyofilm, *Salmonella*, *Listeria*, yüzey çeşidi

* *Corresponding author* / Haberleşmeden sorumlu yazar: Tel.: +902222393750/6415; Fax.: +902222393578; E-mail: bukayyenice@gmail.com
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1. Introduction

Biofilms are commonly defined as communities of microorganisms attached to a surface or interface and producing an extracellular matrix in which these microorganisms are embedded (Costerton et al., 1995). It is a dynamic and complex process which requires an extensive variation of gene expression and, consequently, major physiological changes (Davey et al., 2000). Briefly, biofilm formation has two key steps: (i) The reversible attachment of freely moving bacteria to a surface is followed by irreversible binding to the surface, growth of microcolonies and production of a polymer matrix. (ii) The maturation of the biofilm in a three dimentional structure often showing water filled channels and mushroom like structures (Botticella et al., 2013). Once they develop on any kind of surface, it is hard to eradicate them and they have the capability of spreading to other areas to form new colonies (Dag et al., 2014). In the food industry, pathogenic biofilms have been of considerable interest in the context of food safety. Because it allows microorganisms to persist in the environment and resist desiccation, UV light, and treatment with antimicrobial and sanitizing agent (Borucki et al., 2003). *Salmonella* spp. and *L. monocytogenes* are pathogenic bacteria capable of developing biofilms on food processing surfaces, a pathway leading to cross contamination of foods. Exposure of these pathogens to surfaces may take place either by direct contact with contaminated materials or indirectly through airborne microflora (Ciccio et al., 2015).

The major components of biofilm formation are the bacterial cells, the attachment surface and the surrounding medium. The surface properties are important for attachment and biofilm formation. The choice of surface material has significant influence in designing food contact and processing surfaces. Factors such as cleanability, roughness, wettability or disinfectability affect the adherence ability of cells and perform the hygienic property of material (Houdt and Michiels, 2010). The association of microorganisms to surfaces has been mainly analyzed in the laboratory. However, such studies still need to be standardized since they are difficult to carry out *in situ*, in food processing environments. The investigation of different experimental models allows the study of biofilms under defined and controlled conditions and are necessary fort he execution of reproducible experiments (Oliveria et al., 2010).

In order to control the *Salmonella* spp. and *L. monocytogenes* biofilm in the food industry, the greater understanding of the interactions between microorganisms and food processing equipment is required. Regarding these aspects, this study was carried out with the aim of evaluating the ability of *Salmonella* spp. and *L. monocytogenes* strains isolated from variety food samples, to form biofilms on six different surfaces (plastic, steel, marble, wood, granite and glass) at five different time periods (2. 4. 6. 24. and 48. hours) by Scanning Electron Microscope (SEM). The biofilm formations of strains were also suggested with microbiological methods.

2. Materials and methods

2.1 Organisms and growth medium

L. monocytogenes and *Salmonella* spp. isolates obtained from an accredited laboratory for this species (Eskisehir Food Control Laboratory). 490 several food sources samples screened for *Salmonella* spp. and 113 samples also tested for *L. monocytogenes*. Total of 14 strains, isolated from several food sources were used in this study; 8 *Salmonella* spp. and 6 *L. monocytogenes* strains. Obtained isolates were deposited in TSB (tyrpcase soy broth) with 12.5 % glyserol for stock. Organisms were cultivated in TSB at 35 °C for incubation and biofilm studies. *L. monocytogenes* ATCC7644 was used to control microorganisms as highly biofilm producer.

2.2. Biofilm formation

Biofilm producing ability of isolates were tested by microtiter plate and qualitative tube adherence method. In addition, adhesion and biofilm formation of selected strains to different surfaces was also assessed by scanning electron microscope (SEM).

2.2.1. Tube test

The qualitative method for biofilm formation was studied according to tube test described by Christensen et al (Christensen et al., 1982). A loopful of pure culture of strains were inoculated in test tube filled with 10 ml TSB containing 1% glucose. Tubes were incubated for 24 h at 35°C. After overnight incubation tubes were carefully drained and dried. The solution of crystal violet 0,1% was added to these tubes. After the staining, the solution was removed and washed and than placed upside to dry.

Positive results were determined as the presence of stained material adhered to the inner wall of the tube. The adherence was evaluated as no biyofilm (0), weak (+), moderate (++), or strong (+++). The observation of stained ring at the liquid–air interface was not considered to be positive. The tests were carried in triplicate for each isolate.

2.2.2. Microtiter –plate test

The method for assessment of biofilm formation on polystyrene microtiter plates was based on the techniques described by Stepanovic et al (Stepanovic et al., 2000). The wells of a sterile 96-well flat- bottomed polystyrene microplate, were added with 230 µl of the TSB medium. The negative controls contained only medium. Each strain was tested in triplicate. Wells were inoculated with 20 µl of overnight bacterial culture. After incubation for 24 h at 35°C the plate contents were discarded and rinsed three times with 300 µl of sterile distilled water. The content of the each well were fixed with 250 µl of methanol for 15 min , aspirated and air dried.The plates were stained for 5 min with 250 µl per well of Crystal violet (Gram-colour staining set for microscopy; Merck). The plates were washed with tap water to remove the excess of the stain. Later the plates were air dried, the dye bound to the adherent cells was resolubilized with 250 µl of 33% (v/v) glacial acetic acid each well. By using an automated ChroMate 4300 reader, the optical density (O.D.) of each well was measured at 450/630 nm. According to the values of optical density, isolates were categorized as no biofilm producers, weak, moderate or strong biofilm producers, as previously described (Stepanovic et al. 2004). Optical density cut-off (ODc) was determined. It is defined as average OD of negative control + 3× standard deviation (SD) of negative control. The strains were classified as follows: Absorbance/ COV (cut off value) < 1.125 non produced.

1.125 < Abs/ COV <2.00 weak
2.00< Abs/COV < 4.00 modarete
4.00 < Abs/COV < 6.00 high
All tests were carried out in triplicate and the results were averaged.

2.2.3. Scanning electron microscopy (SEM)

Granite, marble, wood, plastic, steel and glass surface samples were used in attachment and biofilm studies. They were cutted as surface area of each piece was 1cm^2 coupons and cleaned by 70% ethanol 10 min. They were washed with sterilized distilled water, dried for 2 hours at 60 ºC and autoclaved at 121 ºC for 15 minutes.

For scanning electron microscobic analysis, surface samples were placed in 2.5% glutaraldehyde (prepared in 0.1 M phosphate buffer, pH 7.4) for 24 hours at 4°C as a prefixation step. They were then rinsed twice with 0.1 M phosphate buffer (pH 7.4), postfixed using 1% osmium tetroxide for 1 hour at room temperature, and finally rinsed with distilled water. Next, the specimens were dehydrated using graduated concentrations of ethyl alcohol (30%, 50%, 70%, 90%, and 96%) for 15 minutes each followed by absolute alcohol for 30 minutes. All samples were air dried and coated with gold with a Polaron SC7620 Sputter Coater was used. Finally, each specimen was examined using a JEOL scanning electron microscope (JEOL JSM-5600LV).

3. Results

In our study, 8 *Salmonella* spp. and 6 *L. monocytogenes* strains were assayed for biofilm formation by using a microtiter plate assay and tube adherence assay. *L. monocytogenes* ATCC 7644 formed the strongest biofilm by two experiments and was used as quality control strains. Value of absorbance at 450/630 nm was 6.25 of control isolate. The results of the tube adherence test are presented in Table 1. In our study, all *Salmonella* spp. isolates showed the weak biofilm 8 (%100). For *L. monocytogenes* isolates, the number of strong biofilm producers were 4 (%67) and moderate were 2 (%33). Tube adherence method showed good correlation with the microtiter plate method for strongly biofilm forming *L. monocytogenes* isolates and total 3 (% 50) isolates were picked up as strong and 3(%50) were moderate biofilm producers. By microtiter plate method, 1 (12.5%) *Salmonella* spp. isolate were determined as moderate and 7 (87.5%) isolates were observed as weak biofilm producers. However, it was difficult to discriminate between moderate and weakly biofilm producing isolates by tube adherence method.

Table 1. Percent of isolates formed biofilm by tube adherence test

Isolates (n)	Adherence	Isolate number/percent values
Salmonella spp. (8)	strong	0 (%0)
	moderate	0 (%0)
	Weak/non biofilm producer	8 (%100)
Listeria monocytogenes (7)	strong	4 (%67)
	moderate	2 (%33)
	Weak/non biofilm producer	0 (%0)

In control assays, surface properties of samples were investigated by SEM. Based on the obtained results, each type of surface showed different degrees of roughness. The control micrographs of granite, steel, marble and wood surfaces showed some crevices. Plastic and glass surfaces were also more smooth (Fig. 1).

Then, isolates were allowed to form a biofilm on these surfaces. *Salmonella* spp. S8 and *L. monocytogenes* 13L isolates were also taken to study for SEM analysis. Figure 2 demonstrated that the biofilm formation of *Salmonella* spp. S8 isolate on different six surfaces at 24 hours. As also shown in figure; granite, marble, wood and glass surfaces presented higher intensity of biofilm, compared to the steel and plastic surfaces. This isolate clearly produced a dense, three-dimentional composite of cells on marble, wood, granite and glass surfaces (Fig. 2 c, d, e, f). The image in Figure 2e shows the EPS-embedded bacteria colonies on granite surface. However, it was observed that sparse aggregates of cells on steel and plastic surfaces (Fig 2a, b). Figure 3 demonstrated that the biofilm formation of *L. monocytogenes* 13L isolate on different surfaces at 24 hours. Similarly, three dimentional biofilm and EPS structures were determined on granite, marble, wood and glass surfaces. Colonization was less on plastic and steel surfaces. Figure 3F shows attachment and biofilm formation by *L. monocytogenes* cells.

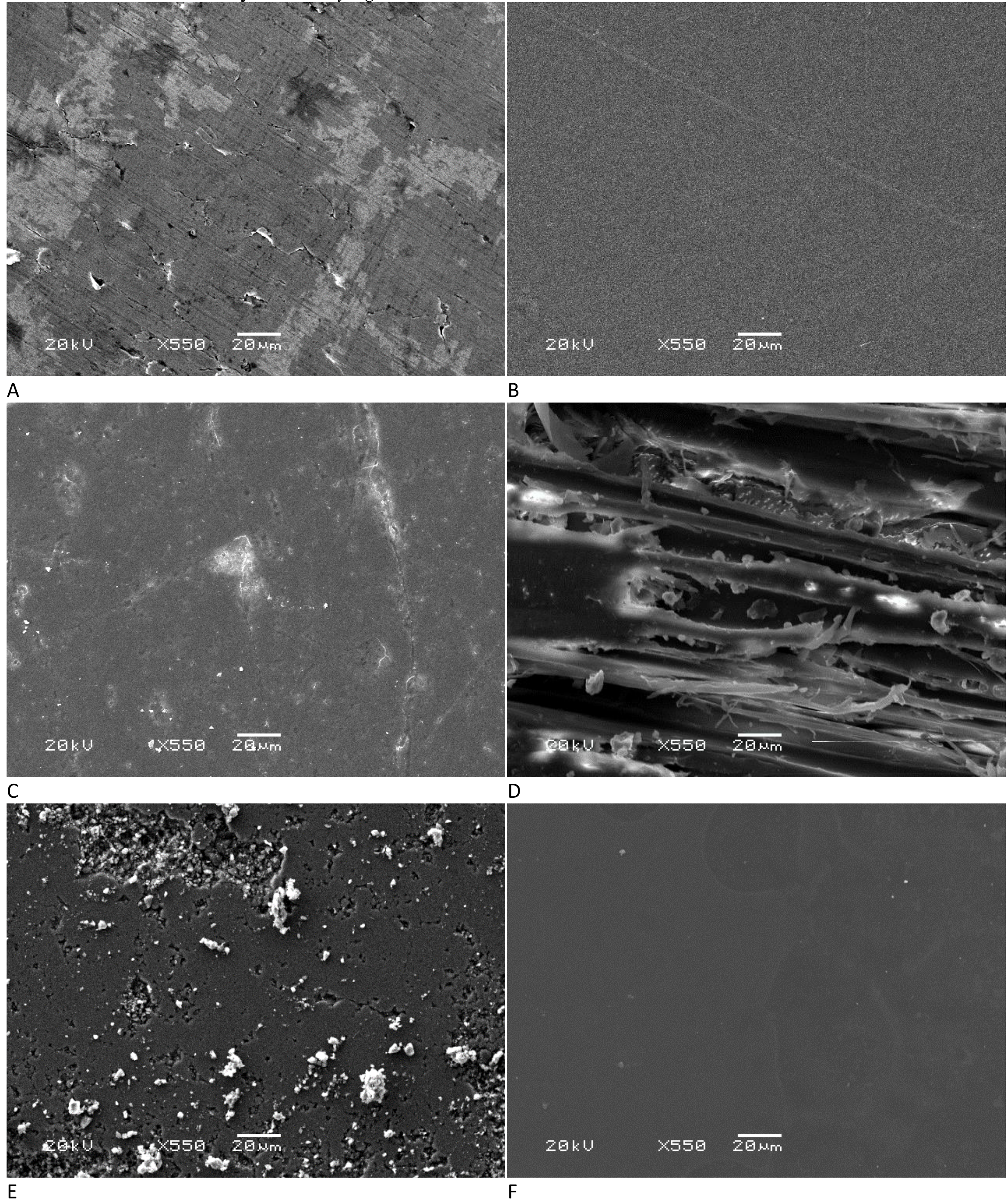

Figure 1. Control micrographs of all surfaces. steel (A), plastic (B), marble (C), wood (D), granite (E) and glass (F) surfaces. (Mag x550)

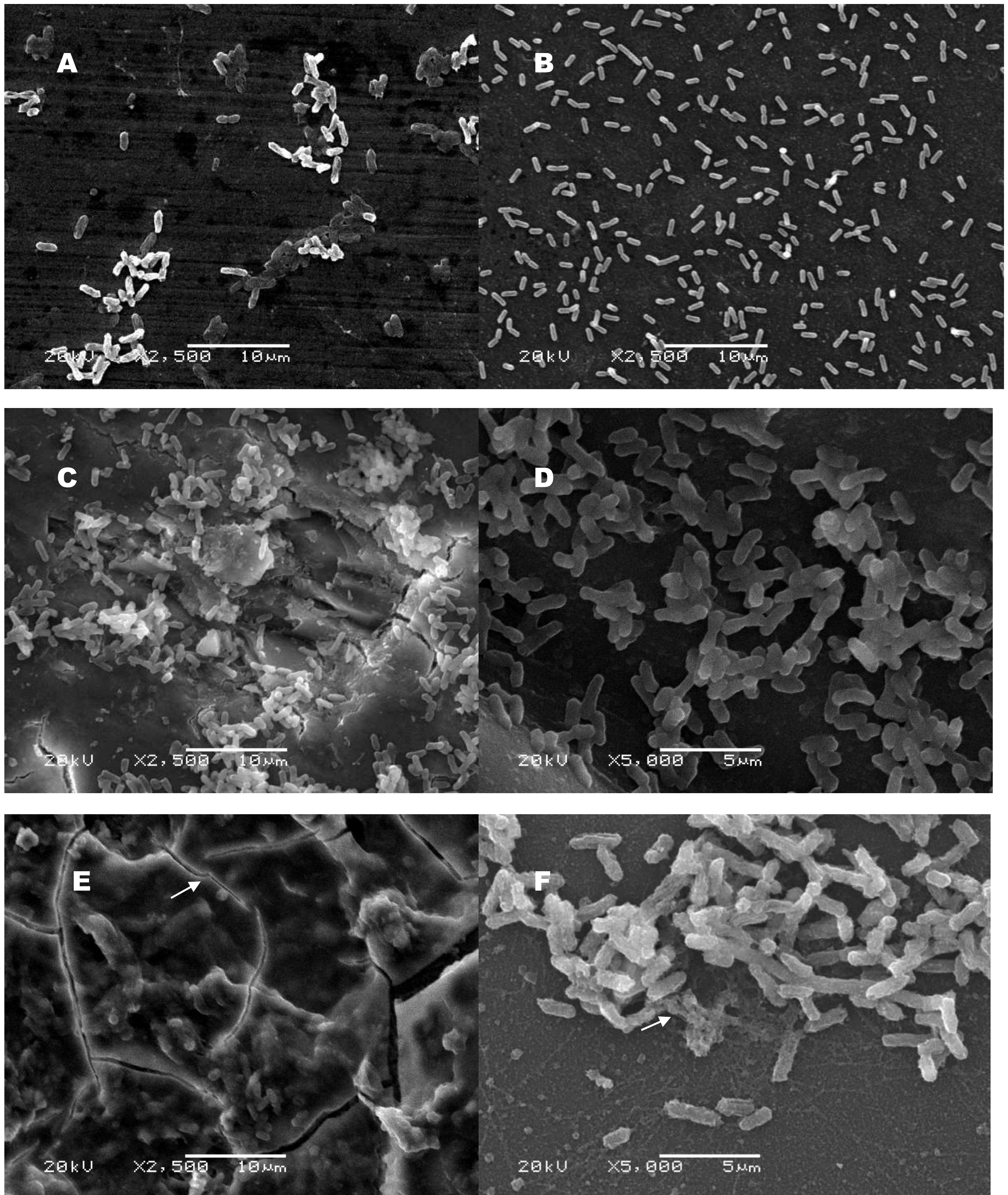

Figure 2. SEM images of strain *Salmonella* spp. S8 on steel (A), plastic (B), marble (C), wood (D), granite (E) and glass (F) surfaces. (A, B, C, E Mag x2500; D-F Mag x5000)

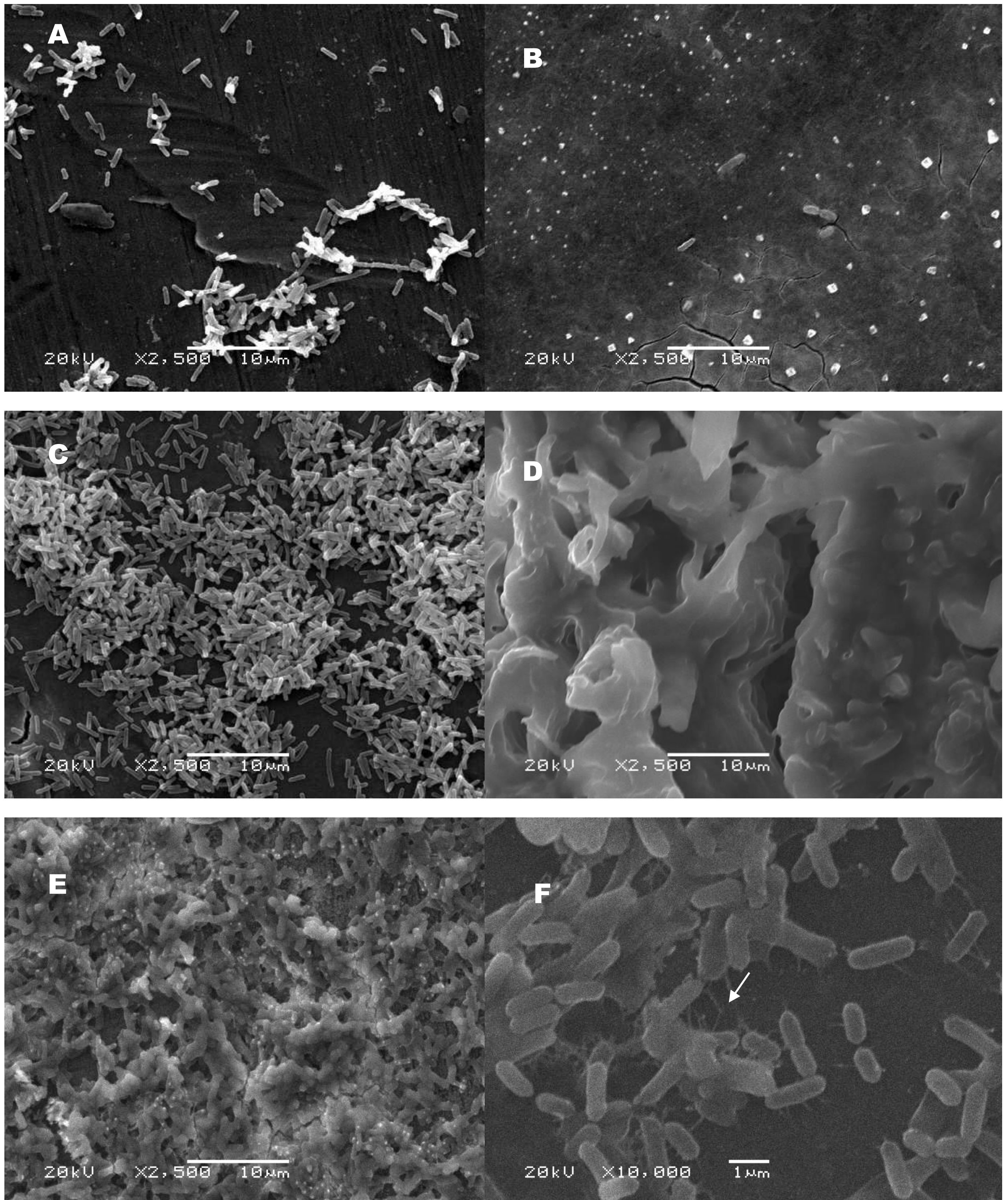

Figure 3. SEM images of strain *L. monocytogenes* 13L on steel (A), plastic (B), marble (C), wood (D), granite (E) and glass (F) surfaces. (A, B, C, D, E Mag x2500; F Mag x10000)

In the study, the adherence, biofilm formation and development of the test isolates on granite surfaces was assessed in five different incubation temperatures, 2., 4., 6., 24. and 48.h. Granite surface was chosen due to the their high biofilm performing capacity. As observed by SEM, *L. monocytogenes* 13L and *Salmonella* spp. S8 adhered to the

granite surface after 6 hours of contact (Figure 4, 5). Strain *Salmonella* spp. S8 showed distinctly more adherence than the *L. monocytogenes* 13L after 6 hours of contact, however, the distribution of the surface adhered cells occurred irregularly. Although in some areas several cells were adhered to surface, in some places, the bacterial adherence observed was not so evident. However mature biofilm formation was determined after 24 and 48 hours for both two isolates.

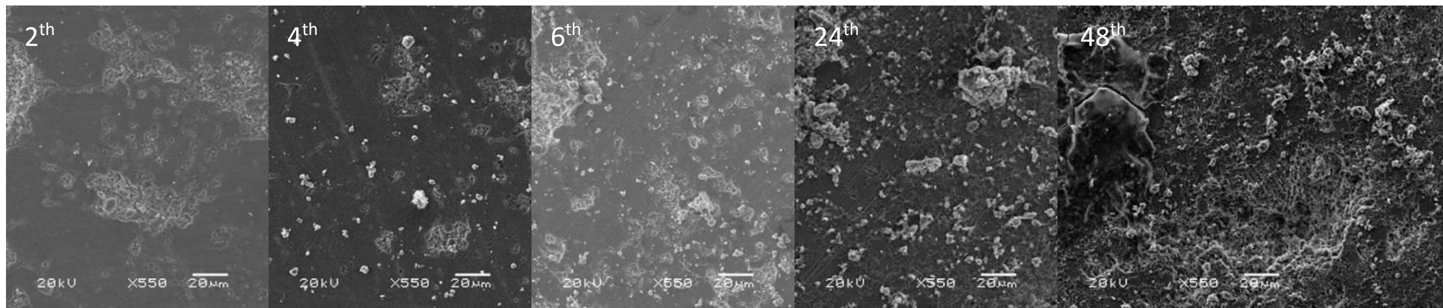

Figure 4. SEM images of the biofilm formation of *Salmonella* spp. S8 isolate on granite surfaces at five time point (Mag x550)

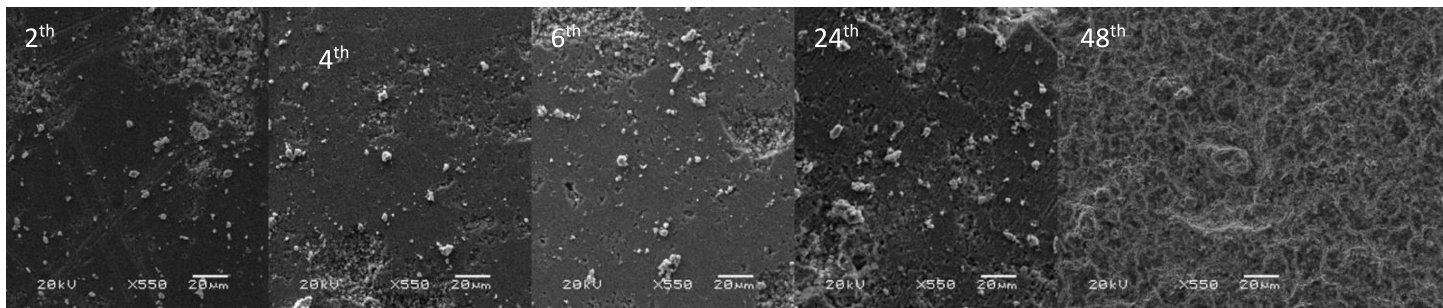

Figure 5. SEM images of the biofilm formation of *L. monocytogenes* 13 L isolate on granite surfaces at five time point (Mag x550)

4. Conclusions and discussion

The ability of many bacteria to adhere to surfaces and to form biofilms has major implications in the food industry, where biofilms create a persistent source of contamination. If the microorganisms from food contact surfaces that are not completely removed, they may lead to biofilm formation and also increase the biotransfer potential. The formation and development of biofilms is affected by many factors, including the spesific bacteria strain, material surface properties and variety environmental parameters. Biofilm cells are more resistant to antimicrobial agents than planctonic bacteria (Srey et al., 2013).

Owing to *Salmonella* spp and *L. monocytogenes* can be quickly transferred from biofilm to food, they have great concern for food safety (Botticella et al., 2013). Several studies reported that *L. monocytogenes* and *Salmonella* spp. may produce biofilms on variety food processing surfaces (Bonaventura et al., 2008). However differences in the degree of attachment and biofilm formation by these pathogens affected by various types of food-contact surfaces. Bacterial adhesion mostly depended on the nature of the inert surface and the bacterial surface property. However, in some situations, it has been difficult to explain the interactions between surface properties and the bacterial adhesion (Silva et al., 2008).

The present study represents our attempt to assess biofilm formation and development by *L. monocytogenes* and *Salmonella* spp. on six different industrial surfaces. In our study, the biofilm degrees of isolates were evaluated based on adherence ability and absorbance values of quality control isolate *L. monocytogenes* ATCC 7644. To detect biofilm forming ability on different surfaces, only selected isolates were examined by SEM. The results of the research indicated that all isolates used in the study were capable of forming biofilms. These findings were consistent with those of some of the earlier observations (Hood and Zottola, 1997; Wong, 1998; Chae and Schraft, 2000). Earlier studies have suggested that the topography of bacterial contact surface plays an important role in facilitating bacteria adhesion and biofilm formation (Simoes et al., 2010). Characklis et al. noted that the extent of microbial colonization appears to increase as the surface roughness increases (Characklis et al., 1990). Howell and Behrends noticed that the extent of microbial attachment is correlated with surface roughness (Howell and Behrends, 2006). This is because surface tension is diminished, and surface area is larger on rougher surfaces. Our study also shows, rough surfaces including granite,

marble and wood showed higher biofilm formation than the other surfaces. Actually in our study, each type of surface (wood > granite > marble > steel > plastic > glass) had different degrees of roughness by the control micrographs obtained from SEM. Especially wood showed to be the roughest surface, with many pores and deep crevices. Granite showed many small pores and crevices. Steel and marble had many oblique, straight line, and narrow crevices on the surface. Plastic and glass surfaces was remained without crevices. However in our study, each type of surface showed different degrees of biofilms (granite > marble > wood> glass> steel > plastic) surfaces. Although the wood had the roughest surface in our study, the most biofilm formation was observed in granite and marble surfaces. Silva et al demostrated that the adhesion ability of 10 isolates *L. monocytogenes* in eight materials commonly used in kitchens and evaluated the viability of the cells to adhere to different surfaces. Similarly, to the our findings, they showed all isolates adhered to surfaces. *L. monocytogenes* adhered most strongly to marble and granite, followed by stainless steel, glass, silestones and finally polypropylene surfaces (Silva et al., 2008).

On the other hand, the biofilm formation is related both the nature of the attachment and the properties of the environmental factors and bacterial cell (Houdt and Michiels, 2010). So, general predictions for the degree of biofilm formation on a particular material can not be made. For example, depending on the hydrophobicity of material, bacteria adhere differently to surfaces with different hydrophobicities. When Adetunji and Isola compared the biofilm formation by *L. monocytogenes* on glass, steel and wooden surfaces immersed in nutrient broth, biofilm formation was strongest on wooden surfaces, followed by steel and glass. It was theorized that *L. monocytogenes* might attach to wooden surfaces more readily because of its higher hydrophobicity (Adetunji and Isola, 2011). Because surfaces with high free surface energy, such as stainless steel and glass, are more hydrophilic. Araujo et al also investigated that the adhesion of *Bacillus cereus* strains isolated from dairy plants, to stainless steel, granite and glass surfaces. They showed the adhesion to the hydrophobic surfaces of granite and stainless steel was greater than adhesion to glass, which was classified as a hydrophilic surface. We also observed strong adhesion and biofilm formation in hydrophobic surfaces such as wood or steel. On the other hand, all plastics are naturally hydrophobic but plasticizer reagents are used to change the plastics' surface character and make them hydrophilic. In our study, we used the hydrophilic coated polypropilen (PP5) plastic and glass materials. Our data also suggested that the hydrophobicity of the surface material influence the initial adhesion and consequently the biofilm formation.

According to the our SEM results, there was an initial attachment of isolates after 6 hours. Fully mature biofilms, are produced after incubation for 24-48 hours. Leonhard et al showed that the initial microbial adhesion might be promoted by rough surfaces but it does not have an impact on later phases of biofilm formation. Also they reported that the surface character did not effect long term biofilm formation (Leonhard et al., 2014). In our research, we have investigated that the biofilm formation and developments until 48hours. But even at 48 hours, smooth surfaces such as plastic and glass showed the weaker biofilm formation compared to the rough surfaces.

Bacterial appendages such as flagella and fimbriae are responsible for bacterial motility, thus contributing to the growth and spread of biofilm development along surfaces. They also showed the hydrophobicity contributed the bacterial cell surfaces. As known, flagellar motility is critical for *L. monocytogenes* biofilm formation (Houdt and Michiels, 2010). In also our study, *L. monocytogenes* produced the stronger biofilm activity by tube adherence and microtiter plate assays than *Salmonella* spp. On the other hand, we have also observed the biofilm formation was less on glass and plastic surfaces than on other materials tested. Although there is some controversy about the effect of surface materials on biofilm formation, we think that bacterial appendages such as flagella and fimbriae may also provide an additional hydrophobicity to bacteria.

The adhesion and biofilm formation of *L. monocytogenes* and *Salmonella* spp. to abietic surfaces is a multifactorial phenomenon. Biofilm formation by these bacteria was affected from by factors such as type of surface, incubation time and hydrophobicity. Granite and marble, although widely used as in the food processing area or for domestic use, are highly convenient surfaces for biofilm formation. For this reason, they may not be preferable for areas where effective disinfection opportunities are not available. Whereas, usage of plastic and glass surfaces may promise a safer environment. Although usage of plastics has negative effect to environmental protection and global warming, our data showed less surface adhesion and biofilm formation in plastic surfaces. However, more studies are needs to better understanding of the microbiological and physicochemical factors in biofilm formation and development.

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Research article/Araştırma makalesi

Macrofungi biodiversity of Kütahya (Turkey) province

Hakan ALLI [1], Bekir ÇÖL, İsmail ŞEN *[1]

[1] Muğla Sıtkı Koçman University, Faculty of Science, Department of Biology, Menteşe, Muğla, Turkey

Abstract

In this study, determination of macrofungi biodiversity of Kütahya province is aimed and 332 species belonging to 57 families, 15 order, 5 classis and 2 divisio were identified from the study area as a consequence of routine field and laboratory studies between 2011 and 2014 years.

Key words: macrofungi, biodiversity, taxonomy, Kütahya, Turkey

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Kütahya yöresi makrofunguslarının biyoçeşitliliği

Özet

Bu çalışmada, Kütahya yöresinde yetişen makrofunguların belirlenmesi amaçlanmıştır ve, 2011 ve 2014 yılları arasında yapılan rutin arazi ve laboratuar çalışmaları sonucunda araştırma bölgesinden 57 familya, 15 takım, 5 sınıf ve 2 bölümde dağılım gösteren 332 tür belirlenmiştir.

Anahtar kelimeler: makrofunguslar, biyoçeşitlilik, taksonomi, Kütahya, Türkiye

1. Introduction

The studies on Turkish mycota have been carried out for more than one hundred years (Solak et al., 2015) and nearly 2400 macrofungi species have been documented in the checklists of Turkey (Solak et al., 2007; Sesli and Denchev 2008; Acar et al. 2015; Sesli et al., 2015; Solak et al., 2015; Akata et al. 2016; Doğan and Kurt 2016; Sesli et al. 2016). However, Turkish mycota have not yet been fully determined, and new macrofungi records and checklists of some limited areas have also been published by several researchers as a consequence of routine field and laboratory studies. Prior to this study, Kütahya province was the one of the areas in which macrofungi biodiversity was not determined.

Kütahya province (Figure 1) is located at the junction point of Euro-Siberian, Mediterranean and Irano-Turanian Region and this situation encourages biological diversity. Kütahya has rich vegetation and the forests including *Pinus nigra* J.F. Arnold., *P. brutia* Ten., *P. sylvestris* L., *Q. cerris* L., *Q. coccifera* L., *Cedrus libani* A. Rich., *Juniperus* sp. and *Castanea sativa* Mill. dominating the area. Also, *Pinus nigra* Arnold. subsp. *pallasiana* (Lamb.) Holmboe var. *pyramidata* (Acatay) Yaltirik (known as Ehrami Karaçamı), which is considered as endemic species of the region, is distributed in limited area of Kütahya (Ünaldı, 2004). Similarly, rainy climatic condition of the region throughout the year is suitable for macrofungi growth.

In the present study, therefore, we attempt to determine macrofungi biodiversity of Kütahya province in Turkey.

* *Corresponding author* / Haberleşmeden sorumlu yazar: Tel.: +902522111537; Fax.: +905547396639; E-mail: frapesle@gmail.com
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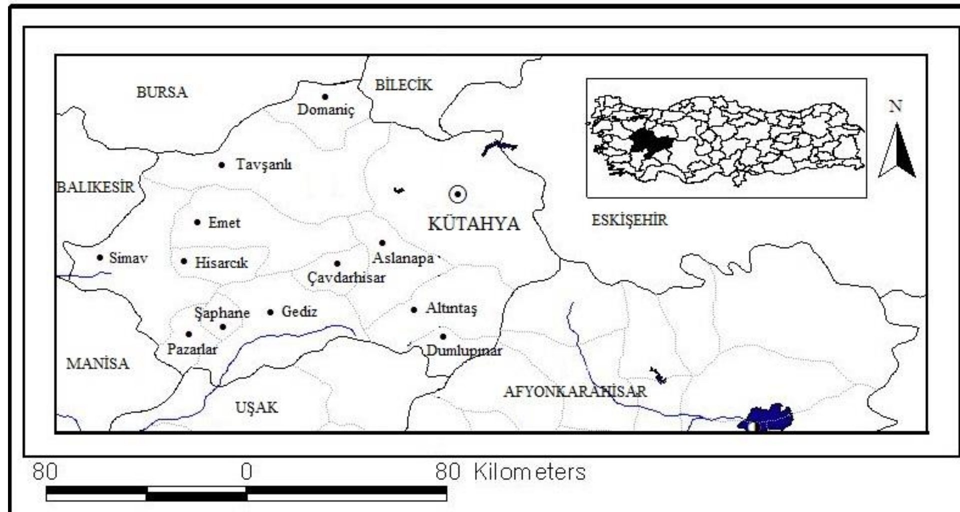

Figure 1. Kütahya province

2. Materials and methods

Specimens of macrofungi were collected from Kütahya province during routine field studies in 2011-2014. The field studies have been conducted to sixty-three different locations. The detailed features such as habitat, altitudes and coordinates of the locations are given below:

1. Çavdarhisar-Tavşanlı road 10th km. (Çavdarhisar), *Pinus nigra,* 1000 m. 39.307283N, 29.573267E
2. Domaniç, Çatalalıç area, *Fagus orientalis – P. nigr*a, 1430 m., 39.857958N, 29.632116E
3. Domaniç, Çubuk area, *P. nigra – Quercus* sp., 900 m., 39.782078N, 29.574031E
4. Domaniç, Domurköy area, *Platanus orinetalis – Populus* sp., 900 m., 39.802078N, 29.634001E
5. Domaniç, Durabey village, *P. nigra – F. orientalis – Quercus* sp., 1050 m., 39.834324N, 29.637105E
6. Domaniç Forest Man. Office, *P. nigra,* 900 m., 39.786894N, 29.582682E
7. Domaniç, Güney village, *P. nigra – C. libani – Populus* sp., 650 m., 39.758046N, 29.548644E
8. Domaniç, Kocayayla area, *F. orientalis – P. nigra,* 1500 m., 39.863081N, 29.658600E
9. Domaniç, Kaşalıç area, *P. nigra,* 1450 m., 39.872776N, 29.650184E
10. Domaniç-Tavşanlı road 20th km, *P. nigra,* 950 m., 39.708647N, 29.542144E
11. Emet, *Pinus brutia,* 750 m., 39.339904N, 29.280805E
12. Emet, Kurt Geçidi area, *P. brutia,* 800 m., 39.337229N, 29.297702E
13. Eski Gediz, *P. nigra,* 680 m., 39.041746N, 29.431453E
14. Gediz, Abide area, *P. brutia,* 660 m., 38.915167N, 29.3013E
15. Gediz, Alikahya village, *P. brutia – Quercus* sp., 630 m., 38.827682N, 29.271850E
16. Gediz-Çavdarhisar road 10th km., *P. nigra,* 650 m., 39.095132N, 29.473499E
17. Gediz, Dedeköy area, *P. nigra – Quercus* sp., 665 m., 38.976124N, 29.439453E
18. Gediz, Gölcük village, *P. brutia,* 636 m., 38.859332N, 29.281316E
19. Gediz, Gümele pond, *Platanus orientalis,* 640 m., 38.928933N, 29.470583E
20. Gediz, Ilıca area, *P. brutia – P. nigra,* 625 m., 38.939459N, 29.255291E
21. Gediz – Kütahya road 20th km. (Gediz), *P. nigra,* 635 m., 39.121085N, 29.528618E
22. Gediz, Murat Mountain, *P. nigra,* 1450 m., 38.965531N, 29.641926E
23. Gediz, Sazak village, *P. nigra – Quercus* sp., 750 m., 39.050711N, 29.364189E
24. Gediz – Simav road 20th km. (Gediz), *P. nigra – Quercus* sp., 630 m., 38.959721N, 29.235607E
25. Gümüş area (Center of Kütahya), *P. nigra,* 1081 m., 39.454137N, 29.747623E
26. Hisarcık, Beyköy village, *P. nigra – Quercus* sp., 780 m., 39.228595N, 29.206731E
27. Hisarcık, Gölcük Mountain, *P. nigra,* 1450 m., 39.160467N, 29.0856E
28. Hisarcık, Günlüce area, *P. nigra,* 900 m., 39.228323N, 29.192137E
29. Hisarcık, Saklar area, *P. nigra – Quercus* sp., 1200 m., 39.211389N, 29.178056E
30. Kütahya-Afyon road 10th km. (Center of Kütahya), *P. nigra,* 1110 m., 39.351390N, 30.056624E
31. Kütahya – Afyon road 25th km. (Center of Kütahya), *Quercus* sp., 1138 m., 39.317233N, 30.064333E
32. Kütahya, Pusan Hatıra Forest (Center of Kütahya), *P. nigra,* 1144 m., 39.196565N, 30.132875E
33. Seyitömer village (Center of Kütahya), *P. nigra,* 1040 m., 39.699533N, 29.878080E
34. Simav, *Castanea sativa – P. nigra,* 970 m., 39.085801N, 28.966575E
35. Simav, Akçakertik Geçidi, *P. nigra – Populus* sp. – *Quercus* sp., 1300 m., 39.115248N, 28.737826E
36. Simav, Aksaz area, *Quercus* sp., 810 m., 39.137097N, 28.754183E
37. Simav, Aksaz Forest Deposit, *P. nigra,* 805 m., 39.136010N, 28.739493E
38. Simav – Balıkesir road 10th km (Simav), *Quercus* sp., 795m., 39.127040N, 28.839079E
39. Simav, Beyce village Graveyard, *P. nigra – Quercus* sp., 819 m., 39.099415N, 28.937507E

40. Simav, Çağlaca area, *Quercus* sp., 980 m., 39.143957N, 28.721911E
41. Simav, Central Graveyard, *Quercus* sp., 972m., 39.092141N, 28.960965E
42. Simav, Demirci village, *Quercus* sp., 900 m., 39.098914N, 28.917085E
43. Simav, Donuzkıran area, *P. nigra,* 820 m., 39.123417N, 28.859983E
44. Simav – Gediz road turnout, *P. brutia,* 700 m., 38.935145N, 29.308367E
45. Simav – Gediz highway 20th km. (Simav), *P. nigra – Quercus* sp., 800 m., 39.061065N, 29.075162E
46. Simav, Gümüşsu area, *P. nigra,* 790 m., 39.140821N, 28.889741E
47. Simav, Kapıkaya village, *Cedrus libani,* 850 m., 39.135394N, 29.036687E
48. Simav, Martılı Plateu, *F. orientalis – P. nigra,* 1510 m., 39.249017N, 28.828133E
49. Simav, Samatköy area, *P. nigra,* 900 m., 39.025731N, 29.000490E
50. Simav – Selendi road 5th km. (Simav), *P. nigra – Abies* sp., 965 m., 39.071891N, 29.035335E
51. Simav, Yenikent, *P. brutia,* 700 m., 38.877658N, 29.289125E
52. Tatahmet village area (Center of Kütahya), Near stream area under *Salix* sp., 1021 m., 39.078767N, 30.212117E
53. Tavşanlı, Dağdemirli village, *P. nigra,* 900 m., 39.537274N, 29.316167E
54. Tavşanlı, Devekayası village, *P. nigra,* 852 m., 39.533529N, 29.605269E
55. Tavşanlı, Karacaören village, *P. nigra,* 865 m., 39.644529N, 29.303737E
56. Tavşanlı – Kütahya road 5th km., *P. nigra,* 1015 m., 39.538196N, 29.557228E
57. Tavşanlı – Kütahya road 10th km., *Quercus* sp., 1018m., 39.530519N, 29.602797E
58. Tavşanlı, Yeniköy area, *P. nigra,* 981 m., 39.620017N, 29.3369E,
59. Tunçbilek – Domaniç road 5th km. (Tunçbilek), *P. nigra – Quercus* sp., 941 m., 39.647316N, 29.492184E
60. Tunçbilek, Küçük village, *C. libani,* 970 m., 39.761424N, 29.551270E
61. Tunçbilek, Kozcağız area, *P. nigra – Quercus* sp., 981 m., 39.700326N, 29.515082E
62. Tunçbilek, Muhacir area, *Populus* sp. – *F. orientalis,* 945 m., 39.685235N, 29.504852E
63. Yoncalı (Center of Kütahya), *P. orientalis,* 1020 m., 39.500733N, 29.854567E

During the field studies, morphological (size, shape, color, smell, taste, color exchanging) and ecological features of specimens were recorded and photographed in their habitat. After the field studies, specimens were brought to the laboratory in suitable conditions. Microscopic characters of the specimens were determined by mounting the specimen in a %3 KOH solution and other solutions such as melzer reagents, cotton blue, acetocarmine and creysl blue analyzing the distinctive features by a microscope (Leica DM750).

The specimens were identified morphologically using the references of Dennis (1981), Breitenbach and Kränzlin (1984-2000), Capelli (1984), Candusso and Lanzoni (1990), Ellis and Ellis (1990), Hansen and Knudsen (1992), Noordeloos (1992), Pegler et al. (1995), Candusso (1997), Ladurner and Simonini (2003), Riva (2003), Neville and Poumarat (2004), Doveri (2007), Knudsen and Vesterholt (2008), Sanchez (2008), Knudsen and Vesterholt (2012) and Christensen and Heilmann-Clausen (2013).

The identified specimens were deposited at the Fungarium of Muğla Sıtkı Koçman University

3. Results

Systematics of the species were given in accordance with Cannon and Kirk (2007) and Index Fungorum (www.indexfungorum.org; accessed in 02 January 2017). The species are listed below.

ASCOMYCOTA Caval.- Sm.
Leotiomycetes O.E. Erikss. & Winka
Helotiales Nannf.
Lachnaceae Raitv.
1. *Lachnum virgineum* (Batsch) P. Karst., 14, 21, Inedible.

Rutstroemiaceae Holst-Jensen, L.M. Kohn & T. Schumach.
2. *Rutstroemia firma*(Pers.) P. Karst., 24, Inedible.

Pezizomycetes O.E. Erikss. & Winka
Pezizales J. Schröt.
Discinaceae Benedix
3. *Discina ancilis* (Pers.) Sacc., 27, 43, Inedible.
4. *Gyromitra esculenta* (Pers.) Fr., 58, Poisonous.
5. *Gyromitra gigas* (Krombh.) Cooke, 43, Poisonous.

Helvellaceae Fr.
6. *Helvella acetabulum* (L.) Quél., 18, 25, 32, 60, Edible.
7. *Helvella atra*J. König, 25, Inedible.
8. *Helvella costifera* Nannf., 32, Unknown.
9. *Helvella elastica* Bull., 21, Inedible.
10. *Helvella ephippium* Lév., 32, Inedible.
11. *Helvella lacunosa*Afzel., 7, 36, 43, 48, Inedible.
12. *Helvella latispora* Boud., 25, Inedible.
13. *Helvella palustris* Peck, 31, Unknown.
14. *Helvella leucomelaena* (Pers.) Nannf., 2, 11, 18, 62, Edible.
15. *Paxina queletii* (Bres.) Stangl, 3, 22, 34, Inedible.

Morchellaceae Rchb.
16. *Mitrophora semilibera* (DC.) Lév., 52, Edible.
17. *Morchella deliciosa* Fr., 18, Edible.
18. *Morchella elata* Fr., 10, Edible.
19. *Morchella esculenta* (L.) Pers., 18, 20, 25, Edible.

Pezizaceae Dumort.
20. *Sarcosphaera coronaria* (Jacq.) J. Schröt., 18, 30, 58, Poisonous.
21. *Peziza arvernensis* Roze & Boud., 2, Inedible.
22. *Peziza depressa* Pers., 11, Edible.
23. *Peziza domiciliana* Cooke, 34, Inedible.
24. *Peziza fimeti* (Fuckel) E.C. Hansen, 18, Unknown.

Pyronemataceae Corda
25. *Geopora sumneriana* (Cooke) M. Torre, 13, 60, Edible.
26. *Geopyxis carbonaria* (Alb. & Schwein.) Sacc., 18, Inedible.
27. *Scutellinia scutellata* (L.) Lambotte, 14, Inedible.

28. *Tarzetta catinus* (Holmsk.) Korf & J.K. Rogers, 5, Inedible.

Sordariomycetes O.E. Erikss. & Winka
Xylariales Nannf.
Diatrypaceae Nitschke

29. *Diatrypella favacea* (Fr.) Ces. & De Not., 5, Inedible.

Xylariaceae Tul. & C. Tul.

30. *Xylaria longipes* Nitschke, 5, Inedible.
31. *Xylaria hypoxylon* (L.) Grev., 5, Inedible.

BASIDIOMYCOTA Whittaker ex Moore
Agaricomycetes Doweld
Agaricales Underw.
Agaricaceae Chevall.

32. *Agaricus arvensis* Schaeff., 26, Edible.
33. *Agaricus comtulus* Fr., 41, Edible.
34. *Agaricus litoralis* (Wakef. & A. Pearson) Pilát, 46, Edible.
35. *Agaricus macrocarpus* (F.H. Møller) F.H. Møller, 53, Edible.
36. *Bovista limosa* Rostr., 27, Edible.
37. *Bovista nigrescens* Pers., 31, 43, Edible.
38. *Bovista plumbea* Pers., 2, 34, 47, Edible.
39. *Bovista pusilla* (Batsch) Pers., 43, Edible.
40. *Coprinus comatus* (O.F. Müll.) Pers., 18, 30, 63, Edible.
41. *Crucibulum leave* (Huds.) Kambly, 43, 58, Unknown.
42. *Cystoderma carcharias*(Pers.) Fayod, 14, 58, Inedible.
43. *Cystoderma fallax* A.H. Sm. & Singer, 43, Inedible.
44. *Cystodermella cinnabarina* (Alb. & Schwein.) Harmaja, 6, 14, 58, Inedible.
45. *Cystodermella granulosa*(Batsch) Harmaja, 24, 58, Inedible.
46. *Lepiota cristata* (Bolton) P. Kumm., 17, 58, Poisonous.
47. *Lepiota ermine* (Fr.) P. Kumm., 34, Poisonous.
48. *Lepiota ignivolvata* Bousset & Joss. ex Joss., 43, Inedible.
49. *Lepiota subgracilis* Wasser, 8, Poisonous.
50. *Leucoagaricus leucothites* (Vittad.) Wasser, 32, Inedible.
51. *Leucoagaricus littoralis* (Menier) Bon & Boiffard, 39, Edible.
52. *Leucocoprinus birnbaumii* (Corda) Singer, 21, Poisonous.
53. *Lycoperdon atropurpureum* Vittad., 32, Unknown.
54. *Lycoperdon lividum* Pers., 14, 24, Edible.
55. *Lycoperdon marginatum* Vittad., 31, Inedible.
56. *Lycoperdon molle* Pers., 43, 46, 58, Inedible.
57. *Lycoperdon nigrescens* Pers., 32, 43, Unknown.
58. *Lycoperdon perlatum* Pers., 43, 54, Edible.
59. *Lycoperdon pyriforme* Schaeff., 25, 34, Edible.
60. *Lycoperdon radicatum* Durieu & Mont., 13, 27, Edible.
61. *Lycoperdon umbrinum* Pers., 15, 43, 49, 59, Edible.
62. *Macrolepiota excoriata* (Schaeff.) Wasser, 2, 23, Edible.
63. *Macrolepiota konradii* (Huijsman ex P.D. Orton) M.M. Moser, 34, Edible.
64. *Macrolepiota mastoidea* (Fr.) Singer, 27, 38, 43, 56, Edible.
65. *Macrolepiota procera* (Scop.) Singer, 3, 24, 43, 58, 59, Edible.
66. *Tulostoma brumale* Pers., 25, Inedible.

Amanitaceae R. Heim ex Pouzar

67. *Amanita argentea* Huijsman, 2, Inedible.
68. *Amanita crocea* (Quél.) Singer, 31, Edible.
69. *Amanita eliae* Quél., 37, Poisonous.
70. *Amanita muscaria* (L.) Lam., 46, Poisonous.
71. *Amanita nivalis* Grev., 43, Poisonous.
72. *Amanita pantherina* (DC.) Krombh, 31, 37, 43, Poisonous.
73. *Amanita phalloides* (Vaill. ex Fr.) Link, 8, Poisonous.
74. *Amanita proxima* Dumée, 20, Poisonous.
75. *Amanita solitaria* (Bull.) Fr., 43, Poisonous.
76. *Amanita vaginata* (Bull.) Lam., 26, 43, Edible.
77. *Amanita verna* (Bull.) Lam., 37, Poisonous.

Bolbitiaceae Singer

78. *Bolbitius titubans* (Bull.) Fr., 39, 48, 57, Inedible.
79. *Conocybe aporos* Kits van Wav., 8, Inedible.
80. *Conocybe blattaria* (Fr.) Kühner, 34, Inedible.
81. *Conocybe filaris* (Fr.) Kühner, 58, Inedible.
82. *Conocybe rickenii* (Jul. Schäff.) Kühner, 18, Inedible.

Cortinariaceae R. Heim ex Pouzar

83. *Cortinarius rigens* (Pers.) Fr., 34, Inedible.
84. *Cortinarius vernus* H. Lindstr. & Melot, 43, Inedible.

Entolomataceae Kotl. & Pouzar

85. *Entoloma corvinum* (Kühner) Noordel., 18, Inedible.
86. *Entoloma hirtipes* (Schumach.) M.M. Moser, 2, Inedible.
87. *Entoloma incanum* (Fr.) Hesler, 31, Inedible.
88. *Entoloma juncinum* (Kühner & Romagn.) Noordel., 43, Inedible.
89. *Entoloma politum* (Pers.) Noordel., 27, Poisonous.
90. *Entoloma subradiatum* (Kühner & Romagn.) M.M. Moser, 36, Inedible.
91. *Entoloma vernum* S. Lundell, 8, 36, 43, Poisonous.

Fistulinaceae Lotsy

92. *Fistulina hepatica* (Schaeff.) With., 34, Edible.

Hydnangiaceae Gäum. & C.W. Dodge

93. *Laccaria bicolor* (Maire) P.D. Orton, 37, Edible.
94. *Laccaria proxima* (Boud.) Pat., 43, Edible.

Hygrophoraceae Lotsy

95. *Hygrocybe conica* (Schaeff.) P. Kumm., 2, Inedible.
96. *Hygrophorus agathosmus* (Fr.) Fr., 43, 58, Inedible.
97. *Hygrophorus hedrychii* (Velen.) K. Kult, 2, Inedible.
98. *Hygrophorus gliocyclus* Fr., 58, Edible.

Hymenogastraceae Vittad.

99. *Galerina badipes* (Pers.) Kühner, 43, 58, Inedible.
100. *Galerina marginata* (Batsch) Kühner, 2, 43, Poisonous.
101. *Hebeloma candidipes* Bruchet, 2, Inedible.
102. *Hebeloma crustuliniforme* (Bull.) Quél., 43, 58, Inedible.
103. *Hebeloma sinapizans* (Paulet) Gillet, 29, 58, Inedible.
104. *Psilocybe coronilla* (Bull.) Noordel., 46, 60, Inedible.

Inocybaceae Jülich

105. *Crepidotus cesatii* (Rabenh.) Sacc., 24, Inedible.
106. *Inocybe amethystina* Kuyper, 14, Poisonous.
107. *Inocybe bongardii* (Weinm.) Quél., 14, 18, Poisonous.
108. *Inocybe calida* Velen., 42, Poisonous.
109. *Inocybe catalaunica* Singer, 22, 43, Poisonous.
110. *Inocybe cervicolor* (Pers.) Quél., 14, 44, Poisonous.

111. *Inocybe dulcamara* (Pers.) P. Kumm., 44, Poisonous.
112. *Inocybe erubescens* A. Blytt, 53, Poisonous.
113. *Inocybe flocculosa* (Berk.) Sacc., 13, Poisonous.
114. *Inocybe geophylla* (Bull.) P. Kumm., 43, 58, Poisonous.
115. *Inocybe geraniodora* J. Favre, 18, Poisonous.
116. *Inocybe gymnocarpa* Kühner, 22, Poisonous.
117. *Inocybe hirtella* Bres., 43, Poisonous.
118. *Inocybe maculipes* J. Favre, 25, Poisonous.
119. *Inocybe mixtilis* (Britzelm.) Sacc., 3, 10, 31, Poisonous.
120. *Inocybe nitidiuscula* (Britzelm.) Lapl., 14, 15, Poisonous.
121. *Inocybe perbrevis* (Weinm.) Gillet, 43, Poisonous.
122. *Inocybe pseudohiulca* Kühner, 25, Poisonous.
123. *Inocybe queletii* Konrad, 3, Poisonous.
124. *Inocybe rimosa* (Bull.) P. Kumm., 30, 42, Poisonous.
125. *Inocybe taxocystis* (J. Favre & E. Horak) Senn-Irlet, 42, Poisonous.
126. *Inocybe tenebrosa* Quél., 5, Poisonous.
127. *Inocybe vaccina* Kühner, 2, 14, Poisonous.

Incertae sedis

128. *Leucocybe connata* (Schumach.) Vizzini, P. Alvarado, G. Moreno & Consiglio, 25, Inedible.
129. *Panaeolus acuminatus* (Schaeff.) Quél., 32, Poisonous.
130. *Panaeolus guttulatus* Bres., 14, Poisonous.
131. *Panaeolus olivaceus* F.H. Møller, 32, Poisonous.
132. *Panaeolus papilionaceus* (Bull.) Quél., 18, Poisonous.
133. *Panaeolus rickenii* Hora, 43, Inedible.
134. *Panaeolus semiovatus* (Sowerby) S. Lundell & Nannf., 18, Inedible.
135. *Rhizocybe vermicularis* (Fr.) Vizzini, G. Moreno, P. Alvarado & Consiglio, 43, 61, Inedible.

Lyophyllaceae Jülich

136. *Calocybe gambosa* (Fr.) Donk, 2, Edible.
137. *Calocybe gangraenosa* (Fr.) V. Hofst., Moncalvo, Redhead & Vilgalys, 58, Inedible.
138. *Rugosomyces carneus* (Bull.) Bon., 43, Inedible.

Marasmiaceae Roze ex Kühner

139. *Atheniella flavoalba* (Fr.) Redhead, Moncalvo, Vilgalys, Desjardin & B.A.Perry, 58, Inedible.
140. *Baeospora myosura* (Fr.) Singer, 14, 29, 34, 58, Inedible.
141. *Macrocystidia cucumis* (Pers.) Joss., 2, Inedible.
142. *Marasmius epiphyllus* (Pers.) Fr., 34, Inedible.
143. *Marasmius oreades* (Bolton) Fr., 39, 42, 43, 58, Edible.
144. *Marasmius wynnei* Berk. & Br., 2, 17, Inedible.
145. *Megacollybia platyphylla* (Pers.) Kotl. & Pouzar, 2, Edible.

Mycenaceae Overeem

146. *Hemimycena lactea* (Pers.) Singer, 29, Inedible.
147. *Hemimycena pithya* (Fr.) Dörfelt, 27, 34, Unknown.
148. *Mycena abramsii* (Murrill) Murrill, 2, Inedible.
149. *Mycena aetites* (Fr.) Quél., 43, 45, 51, 58, Inedible.
150. *Mycena amicta* (Fr.) Quél., 14, 29, Unknown.
151. *Mycena crocata* (Schrad.) P. Kumm., 2, 9, Edible.
152. *Mycena epipterygia* (Scop.) Gray, 27, 29, 43, 58, Edible.
153. *Mycena flavescens* Velen., 43, 58, Edible.
154. *Mycena galopus* (Pers.) P. Kumm., 14, Inedible.
155. *Mycena leptocephala* (Pers.) Gillet, 43, Inedible.
156. *Mycena metata* (Secr. ex Fr.) P. Kumm., 42, Unknown.
157. *Mycena polygramma* (Bull.) Gray, 58, Inedible.
158. *Mycena pura* (Pers.) P. Kumm., 9, 18, 34, Poisonous.
159. *Mycena rosella* (Fr.) P. Kumm., 58, Inedible.
160. *Mycena rubromarginata* (Fr.) P. Kumm., 14, Unknown.
161. *Mycena seynesii* Quél., 15, 18, 34, Inedible.
162. *Mycena silvae-nigrae* Maas Geest. & Schwöbel., 9, Inedible.
163. *Mycena viridimarginata* P. Karst., 27, Inedible.
164. *Mycena vitilis* (Fr.) Quél., 57, Unknown.
165. *Mycena xantholeuca* Kühner, 56, Inedible.
166. *Mycena laevigata* Gillet, 43, Inedible.
167. *Xeromphalina cauticinalis* (With.) Kühner & Maire, 6, Inedible.

Omphalotaceae Bresinsky

168. *Gymnopus androsaceus* (L.) J.L. Mata & R.H. Petersen, 24, 58, Unknown.
169. *Gymnopus dryophilus* (Bull.) Murrill, 14, 41, 57, 58, Edible.
170. *Gymnopus hariolorum* (Bull.) Antonín, Halling & Noordel., 24, Inedible.
171. *Gymnopus ocior* (Pers.) Antonín & Noordel., 34, 43, Poisonous.
172. *Marasmiellus ramealis* (Bull.) Singer, 25, Inedible.
173. *Rhodocollybia prolixa* (Hornem.) Antonín & Noordel., 43, Inedible.

Physalacriaceae Corner

174. *Hymenopellis radicata* (Relhan) R.H. Petersen, 2, Inedible.
175. *Mucidula mucida* (Schrad.) Pat., 8, Inedible.
176. *Rhizomarasmius epidryas* (Kühner ex A. Ronikier) A. Ronikier & Ronikier, 14, Inedible.
177. *Rhizomarasmius setosus* (Sowerby) Antonín & A. Urb., 34, Inedible.
178. *Strobilurus tenacellus* (Pers.) Singer, 1, 11, 14, 27, 58, Inedible.

Pluteaceae Kotl. & Pouzar

179. *Pluteus ephebeus* (Fr.) Gillet, 2, Inedible.
180. *Pluteus umbrosus* (Pers.) P. Kumm., 2, Inedible.

Pleurotaceae Kühner

181. *Hohenbuehelia tremula* (Schaeff.) Thorn & G.L. Barron, 43, Edible.
182. *Pleurotus eryngii* (DC.) Quél., 32, 43, Edible.
183. *Pleurotus ostreatus* (Jacq.) P. Kumm., 25, 34, 62, Edible.

Porotheleaceae Murrill

184. *Phloeomana speirea* (Fr.) Redhead, 24, 58, Inedible.

Psathyrellaceae Vilgalys, Moncalvo & Redhead

185. *Coprinellus disseminatus* (Pers.) J.E. Lange, 4, 17, 61, Inedible.
186. *Coprinellus domesticus* (Bolton) Vilgalys, Hopple & Jacq. Johnson, 52, Inedible.
187. *Coprinellus ephemerus* (Bull.) Redhead, Vilgalys & Moncalvo, 18, Inedible.
188. *Coprinellus micaceus* (Bull.) Vilgalys, Hopple & Jacq. Johnson, 32, 39, Inedible.
189. *Coprinellus xanthothrix* (Romagn.) Vilgalys, Hopple & Jacq. Johnson, 57, Inedible.
190. *Coprinopsis canoceps* (Kauffman) Örstadius & E. Larss., 58, Inedible.
191. *Coprinopsis lagopus* (Fr.) Redhead, Vilgalys & Moncalvo, 24, Inedible.

192. *Coprinopsis nivea* (Pers.) Redhead, Vilgalys & Moncalvo, 31, Inedible.
193. *Coprinopsis tigrinella* (Boud.) Redhead, Vilgalys & Moncalvo, 57, Inedible.
194. *Lacrymaria lacrymabunda* (Bull.) Pat., 17, Inedible.
195. *Parasola auricoma* (Pat.) Redhead, Vilgalys & Hopple, 41, Inedible.
196. *Parasola hemerobia* (Fr.) Redhead, Vilgalys & Hopple, 32, Inedible.
197. *Parasola plicatilis* (Curtis) Redhead, Vilgalys & Hopple, 38, Inedible.
198. *Psathyrella bifrons* (Berk.) A.H. Sm., 34, Inedible.
199. *Psathyrella candolleana* (Fr.) Maire, 14, 34, Inedible.
200. *Psathyrella corrugis* (Pers.) Konrad & Maubl., 34, 43, Inedible.
201. *Psathyrella obtusata* (Pers.) A.H. Sm., 8, Inedible.
202. *Psathyrella potteri* A.H. Sm., 43, Inedible.
203. *Psathyrella prona* (Fr.) Gillet, 34, Inedible.
204. *Psathyrella pseudogracilis* (Romagn.) M.M. Moser, 14, 59, Inedible.

Schizophyllaceae Quél.

205. *Schizophyllum commune* Fr., 3, 18, 62, Inedible.

Strophariaceae Singer & A.H. Sm.

206. *Agrocybe paludosa* (J.E. Lange) Kühner & Romagn. ex Bon., 14, 54, 62, Inedible.
207. *Agrocybe praecox* (Pers.) Fayod., 14, 27, 31, 43, Edible.
208. *Cyclocybe cylindracea* (DC.) Vizzini & Angelini, 14, 18, Edible.
209. *Hypholoma fasciculare* (Huds.) P. Kumm., 5, 9, Poisonous.
210. *Kuehneromyces mutabilis* (Schaeff.) Singer & A.H. Sm., 43, Edible.
211. *Leratiomyces squamosus* (Pers.) Bridge & Spooner, 8, 34, 41, Inedible.
212. *Pholiota lucifera* (Lasch) Quél., 34, Inedible.
213. *Protostropharia semiglobata* (Batsch) Redhead, Moncalvo & Vilgalys, 43, Inedible.
214. *Stropharia aeruginosa* (Curtis) Quél., 58, Inedible.
215. *Stropharia caerulea* Kreisel, 43, Inedible.

Tricholomataceae R. Heim ex Pouzar

216. *Bonomyces sinopicus* (Fr.) Vizzini, 54, 59, Edible.
217. *Clitocybe bresadolana* Singer, 39, 45, Edible.
218. *Clitocybe gibba* (Pers.) P. Kumm., 31, 36, 41, Edible.
219. *Clitocybe infundibuliformis* (Schaeff.) Quél., 9, Edible.
220. *Clitocybe odora* (Bull.) P. Kumm., 14, 43, 58, Edible.
221. *Clitocybe vibecina* (Fr.) Quél., 31, Inedible.
222. *Lepista nuda* (Bull.) Cooke, 21, 25, 31, 43, 60, Edible.
223. *Lepista personata* (Fr.) Cooke, 43, Edible.
224. *Leucopaxillus giganteus* (Quél.) Singer, 34, 37, Edible.
225. *Melanoleuca cognata* (Fr.) Konrad & Maubl., 24, 27, 43, Edible.
226. *Melanoleuca exscissa* (Fr.) Singer, 37, Edible.
227. *Melanoleuca graminicola* (Velen.) Kühner & Maire, 8, 43, 58, Edible.
228. *Melanoleuca grammopodia* (Bull.) Murrill, 59, Edible.
229. *Melanoleuca humilis* (Pers.) Pat., 58, Edible.
230. *Melanoleuca melaleuca* (Pers.) Murrill, 13, 26, 43, Edible.
231. *Melanoleuca paedida* (Fr.) Kühner & Maire, 43, Edible.
232. *Melanoleuca schumacheri* (Fr.) Singer, 39, Edible.
233. *Melanoleuca strictipes* (P. Karst.) Jul. Schäff., 34, Edible.
234. *Melanoleuca stridula* (Fr.) Singer, 21, 59, Edible.
235. *Melanoleuca subalpina* (Britzelm.) Bresinsky & Stangl, 43, Edible.
236. *Melanoleuca substrictipes* Kühner, 37, Edible.
237. *Myxomphalia maura* (Fr.) Hora, 58, Inedible.
238. *Resupinatus applicatus* (Batsch) Gray, 5, Unknown.
239. *Tricholoma acerbum* (Bull.) Vent., 14, Inedible.
240. *Tricholoma batschii* Gulden, 14, 58, 60, Inedible.
241. *Tricholoma caligatum* (Viv.) Ricken, 20, Inedible.
242. *Tricholoma focale* (Fr.) Ricken, 27, 43, Edible.
243. *Tricholoma gausapatum* (Fr.) Quél., 43, 58, Edible.
244. *Tricholoma scalpturatum* (Fr.) Quél., 14, Inedible.
245. *Tricholoma stans* (Fr.) Sacc., 8, Inedible.
246. *Tricholoma terreum* (Schaeff.) P. Kumm., 8, 16, 27, 30, 43, Edible.
247. *Tricholoma ustaloides* Romagn., 43, Inedible.
248. *Tricholoma virgatum* (Fr.) P. Kumm., 43, Inedible.
249. *Tricholomopsis decora* (Fr.) Singer, 43, Inedible.
250. *Tricholomopsis rutilans* (Schaeff.) Singer, 43, Inedible.

Tubariaceae Vizzini

251. *Tubaria conspersa* (Pers.) Fayod., 24, 25, 56, Inedible.
252. *Tubaria furfuracea* (Pers.) Gillet, 24, 29, 34, 58, Inedible.
253. *Tubaria romagnesiana* Arnolds, 8, 57, Inedible.

Auriculariales J. Schröt

Auriculariaceae Fr.

254. *Exidia glandulosa* (Bull.) Fr., 27, 43, Inedible.

Boletales E.-J. Gilbert

Boletaceae Chevall.

255. *Boletus erythropus* Pers., 2, Edible.
256. *Imleria badia* (Fr.) Vizzini, 2, Edible.
257. *Xerocomellus chrysenteron* (Bull.) Šutara, 36, Edible.
258. *Xerocomellus porosporus* (Imler ex G. Moreno & Bon) Šutara, 2, Inedible.

Diplocystidiaceae Kreisel

259. *Astraeus hygrometricus* (Pers.) Morgan, 11, 36, 40, 47, Inedible.

Gomphidiaceae Maire ex Jülich

260. *Chroogomphus helveticus* (Singer) M.M. Moser, 14, 25, 32, 34, 43, Edible.
261. *Chroogomphus rutilus* (Schaeff.) O.K. Mill., 27, 43, 58, Edible.
262. *Gomphidius glutinosus* (Schaeff.) Fr., 58, Edible.

Paxillaceae Lotsy

263. *Paxillus involutus* (Batsch) Fr., 23, 32, Poisonous.

Rhizopogonaceae Gäum. & C.W. Dodge

264. *Rhizopogon luteolus* Fr. & Nordholm, 9, 43, 58, Edible.
265. *Rhizopogon roseolus* (Corda) Th. Fr., 3, 14, 20, 25, 43, 58, Edible.

Sclerodermataceae Corda

266. *Pisolithus arhizus* (Scop.) Rauschert, 14, 43, 58, Inedible.

Suillaceae Besl & Bresinsky

267. *Suillus bellinii* (Inzenga) Kuntze, 14, 59, Edible.
268. *Suillus bovinus* (Pers.) Roussel, 5, 27, Edible.
269. *Suillus boudieri* (Quél.) Marchand, 15, Edible.

270. *Suillus collinitus* (Fr.) Kuntze, 8, 12, 14, 27, 28, 55, 58, Edible.
271. *Suillus granulatus* (L.) Roussel, 2, 8, 43, Edible.
272. *Suillus luteus* (L.) Roussel, 27, 43, 58, Edible.

Tapinellaceae C. Hahn

273. *Tapinella panuoides* (Batsch) E.-J. Gilbert, 43, Inedible.

Cantharellales Gäum.

Clavulinaceae Donk

274. *Clavulina rugosa* (Bull.) J. Schröt., 43, Edible.

Hydnaceae Chevall.

275. *Hydnum rufescens* Pers., 22, Edible.

Geastrales K. Hosaka & Castellano

Geastraceae Corda

276. *Geastrum quadrifidum* DC. ex Pers., 62, Inedible.
277. *Geastrum fimbriatum* Fr., 14, 15, 22, 25, Inedible.
278. *Geastrum pectinatum* Pers., 3, 29, 59, Inedible.
279. *Geastrum saccatum* Fr., 8, 21, 30, Inedible.
280. *Geastrum triplex* Jungh., 26, 29, Inedible.

Gloeophyllales Thorn

Gloeophyllaceae Jülich

281. *Gloeophyllum abietinum* (Bull.) P. Karst., 20, 43, Inedible.
282. *Gloeophyllum trabeum* (Pers.) Murrill, 30, Inedible.

Gomphales Jülich

Gomphaceae Donk

283. *Ramaria flava* (Schaeff.) Quél., 53, Edible.

Hymenochaetales Oberw.

Hymenochaetaceae Donk

284. *Fuscoporia torulosa* (Pers.) T. Wagner & M. Fisch., 15, Inedible.
285. *Hymenochaete rubiginosa* (Dicks.) Lév., 2, 35, Inedible.
286. *Phellinus hartigii* (Allesch. & Schnabl) Pat., 2, 39, Inedible.
287. *Phellinus igniarius* (L.) Quél., 43, 52, 63, Inedible.
288. *Porodaedalea pini* (Brot.) Murrill, 14, Inedible.

Incertae sedis

289. *Trichaptum abietinum* (Dicks.) Ryvarden, 43, Inedible.
290. *Trichaptum fusco-violaceum* (Ehrenb.) Ryvarden, 2, 56, 58, Inedible.

Schizoporaceae Jülich

291. *Schizopora paradoxa* (Schrad.) Donk, 36, Inedible.

Polyporales Gäum.

Fomitopsidaceae Jülich

292. *Rhodofomes roseus* (Alb. & Schwein.) Vlasák, 19, Inedible.
293. *Laetiporus sulphureus* (Bull.) Murrill, 16, 52, Edible.
294. *Phaeolus schweinitzii* (Fr.) Pat., 43, Inedible.

Ganodermataceae Donk

295. *Ganoderma adspersum* (Schulzer) Donk, 43, Inedible.
296. *Ganoderma lucidum* (Curtis) P. Karst., 34, Inedible.

Meruliaceae Rea

297. *Bjerkandera adusta* (Willd.) P. Karst., 51, Inedible.

Polyporaceae Fr. ex Corda

298. *Cerrena unicolor* (Bull.) Murrill, 17, Inedible.
299. *Cerioporus leptocephalus* (Jacq.) Zmitr., 8, Inedible.
300. *Cerioporus varius* (Pers.) Zmitr. & Kovalenko, 2, Inedible.
301. *Fomes fomentarius* (L.) J. Kickx f., 9, 19, 34, 63, Inedible.
302. *Lentinus arcularius* (Batsch) Zmitr., 43, 58, Inedible.
303. *Lentinus brumalis* (Pers.) Zmitr., 8, Inedible.
304. *Lentinus substrictus* (Bolton) Zmitr. & Kovalenko, 5, 13, 58, Inedible.
305. *Leptoporus mollis* (Pers.) Quél., 43, Inedible.
306. *Polyporus tuberaster* (Jacq. ex Pers.) Fr., 52, Inedible.
307. *Trametes versicolor* (L.) Lloyd, 22, 26, 34, Inedible.

Sparassidaceae Herter

308. *Sparassis crispa* (Wulfen) Fr., 8, Edible.

Russulales Kreisel ex P.M. Kirk, P.F. Cannon & J.C. David

Russulaceae Lotsy

309. *Lactarius decipiens* Quél., 43, Inedible.
310. *Lactarius deliciosus* (L.) Gray, 43, Edible.
311. *Lactarius salmonicolor* R. Heim & Leclair, 50, Edible.
312. *Lactarius sanguifluus* (Paulet) Fr., 28, 50, 58, Edible.
313. *Lactarius quieticolor* Romagn., 43, 50, Inedible.
314. *Russula aquosa* Leclair, 43, Inedible.
315. *Russula curtipes* F.H. Møller & Jul. Schäff., 58, Edible.
316. *Russula decipiens* (Singer) Bon, 42, Inedible.
317. *Russula delica* Fr., 43, 50, Edible.
318. *Russula integra* (L.) Fr., 43, Edible.
319. *Russula postiana* Romell, 42, Inedible.
320. *Russula rosea* Pers., 58, Edible.
321. *Russula risigallina* (Batsch) Sacc., 43, Edible.
322. *Russula sanguinea* Fr., 6, 43, Inedible.
323. *Russula solaris* Ferd. & Winge, 28, Poisonous.
324. *Russula vinosa* Lindblad, 43, Edible.
325. *Russula xerampelina* (Schaeff.) Fr., 14, Edible.

Stereaceae Pilát

326. *Stereum ochraceoflavum* (Schwein.) Sacc., 8, Inedible.
327. *Stereum hirsutum* (Willd.) Pers., 13, 34, 35, 43, 58, Inedible.

Thelephorales Corner ex Oberw.

Bankeraceae Donk

328. *Boletopsis leucomelaena* (Pers.) Fayod, 20, Edible.
329. *Hydnellum suaveolens* (Scop.) P. Karst., 26, Inedible.
330. *Sarcodon glaucopus* Maas Geest. & Nannf., 20, Inedible.

Thelephoraceae Chevall.

331. *Thelephora terrestris* Ehrh., 58, Inedible.

Dacrymycetes Doweld

Dacrymycetales Henn.

Dacrymycetaceae J. Schröt.

Dacrymyces variisporus McNabb, 8, 33, 43, Inedible.

4. Conclusions and discussion

In the current study, we aimed to determine the macrofungi biodiversity of Kütahya province. After the field and laboratory studies, 332 macrofungi species belonging to 2 divisio, 5 classis, 15 order and 57 families, have been identified. Also, 10 species are given as Incertae sedis (8 of them belong Agaricales order while 2 of them are

Hymenochaetales) because their taxonomic position are not clear and they have not been categorized in any families. Distributions of these species to families and classis are given in Figure 2.

Donuzkıran (Simav) and Yeniköy (Tavşanlı) are the richest area with respect to macrofungi biodiversity (Table 2). Some animal farms are located in Donuzkıran, and so the soil is fertilized by animal droppings. We think that these fertilizers cause higher the fungal diversity. Similarly, local people grow animals in the vicinity of Yeniköy area. Besides of this, it is showed that Kütahya province is in general quite rich in terms of macrofungi diversity.

Macrofungi species were evaluated by ecological niche in the field studies. According to these results, it was found that 90 species (27.10 %) are mycorrhizal, 168 species (50.60 %) are saprobe and 7 species (2.10 %) are parasitic only (Figure 3). Furthermore, 27 species were identified as both saprobe and parasitic, since these species can grow on dead and alive substrates enabling them to behave as saprobe or parasitic. However, ecological niches of 40 species (12.04 %) were not determined, because they were found on the ground among the plant debris and their substrates could not clearly be observed.

Macrofungi plays key roles in the ecosystems. Kütahya has a rich and strong forest ecosystem due possibly to the help of these macrofungi. Because, we found that the most of identified macrofungi are saprobe (51%) and mycorrhizal (27%) (Figure 3). Saprobe species play critical roles in the breakdown and recycling of plant debris especially cellulose and lignin (Deacon, 2006). Thus, macrofungi cause an increase in the level of soil nutrition, which results in encouraging the growth of plants. Also, mycorrhizal species support plant growth by supplying inorganic nutrition (Deacon, 2006). Both saprobe and mycorrhizal species support plant growth and healthy forests. Therefore, we can conclude that Kütahya may have a rich forest ecosystem due to rich macrofungal diversity.

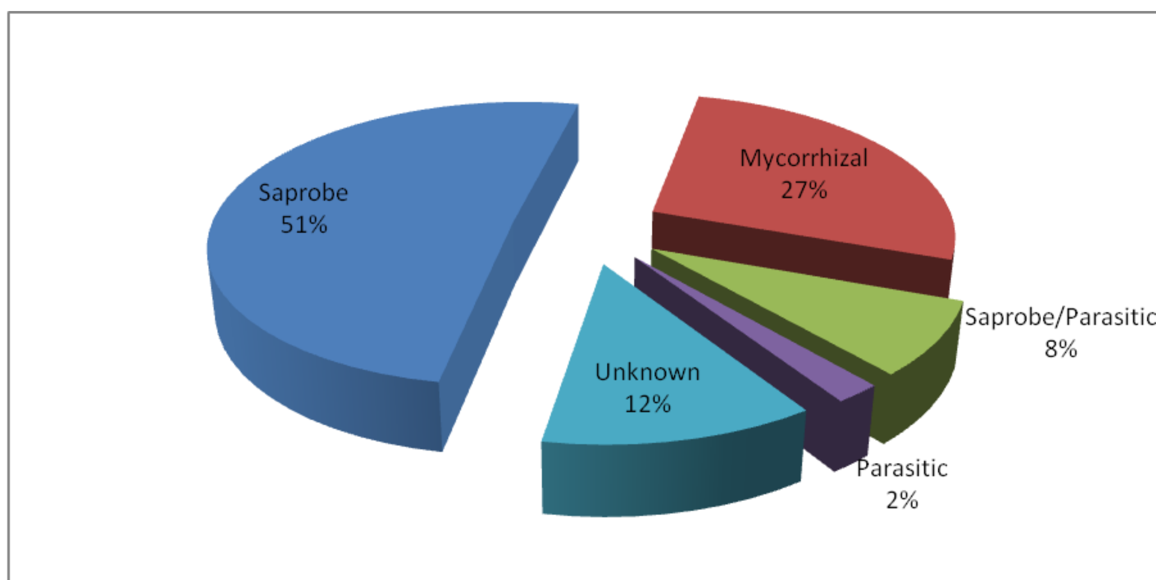

Figure 3. Percentage of the species by ecological niche

While 98 species are edible, 171 of them are inedible. 49 speciesspeciesare poisonous (Figure 4). Although 98 species are edible, only twelve species (*Agaricus litoralis, Choorogomphus rutilus, Hohenbuehelia tremula, Macrolepiota procera, Morchella deliciosa, M. elata, M. esculanta, Lactarius deliciosus, L. salmonicolor, L. sanguifluus, Lycoperdon perlatum, T. terreum*) are recognized and consumed by local people. The members of *Morchella* known as "dübecik, kuzu göbeği", *A. litoralis* as "kızılcamantar", *C. rutilus* as "ebe espiti", *H. tremula* as "kulak mantarı", *M. procera* as "dedebölük", *L. perlatum* as "paflak", *T. terreum* as "kara kafa" and the members of *Lactarius* as "espit". Similarly, *Morchella* sp. and *Lactarius* sp. are sold in the local bazaar. But a fatal mushroom poisoning has been observed after consuming *Amanita proxima* and the patient was reported dead. Also, some *Amanita* species were eaten by five local people. So, we also point out that mushroom poisoning could, at times, be an important health problem for the local people living in Kütahya.

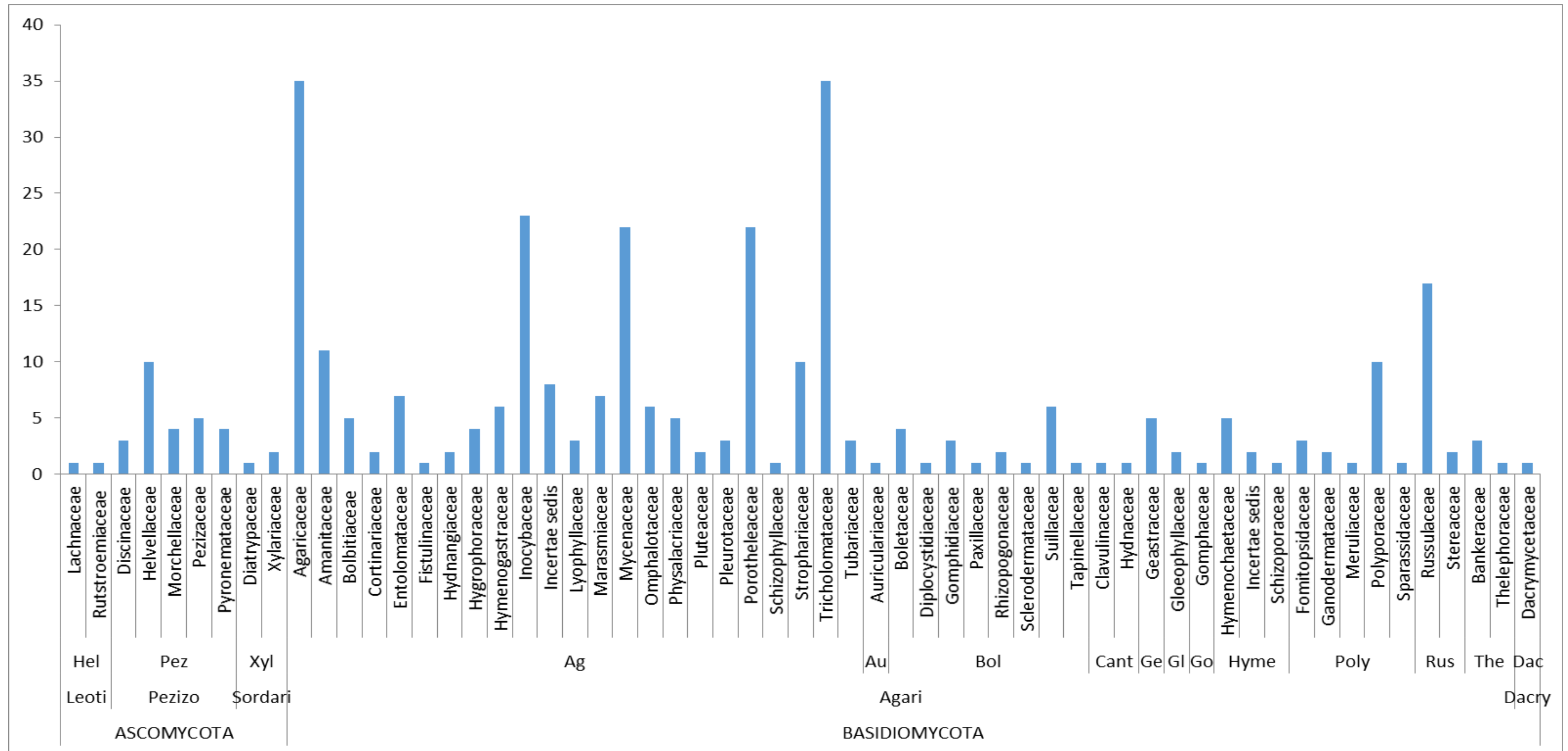

Figure 2. Distribution of species to families and classis (Hel: Helotiales, Pez: Pezizales, Xyl: Xylariales, Ag: Agaricales, Au: Auriculariales, Bol: Boletales, Cant: Cantharellaes, Ge: Geastrales, Gl: Gloeophyllales, Go: Gomphales, Hyme: Hymenochaetales, Poly: Polyporales, Rus: Russulales, The: Thelephorales, Dac: Dacrymycetales, Leoti: Leotimycetes, Pezizo: Pezizomycetes, Sordari: Sordariomycetes, Agari: Agaricomycetes, Dacry: Dacrymycetes).

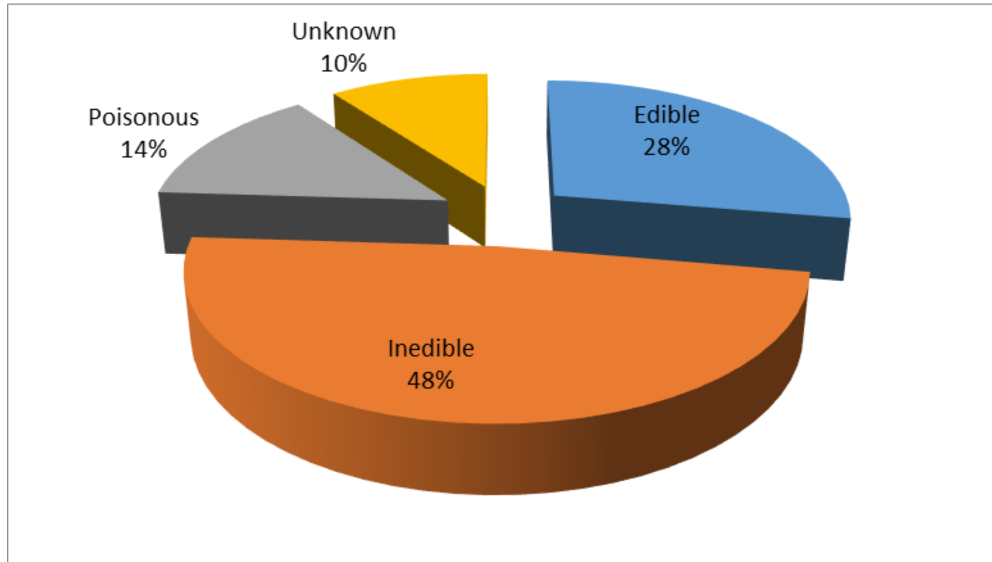

Figure 4. Edibility percentage of species

The species determined in this study reflect similarity to those of the other studies conducted in its environs and similarity percentages between this study and others are given in Table 1. Relatively high level of agreement between the studies may be due to similar climate and vegetation. In addition, it is shown that the similar species are cosmopolite, so they grow in different habitats.

Table1. Similarity percentages of study area and its environs

	Number of identical species	Total species	Similarity percentage (%)
Yamaç et al. (2007)	11	15	73
Oskay and Kalyoncu (2006)	23	34	67.64
Solak and Yılmaz (2002)	17	36	47.22
Yılmaz et al. (2003)	14	24	58.33
Köstekçi et al. (2005)	48	77	62.33
Türkoğlu and Yağız (2012)	45	100	45
Şen et al. (2014)	30	48	62.5

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Research article/Araştırma makalesi

The rediscovery of *Silene surculosa* Hub.-Mor. endemic for Turkey

Metin ARMAĞAN *[1], Ali Savaş BÜLBÜL [2]

[1] Adnan Menderes University, Buharkent Vocational School, Medicinal and Aromatic Plants Program, 09670, Buharkent, Aydın, Turkey
[2] Bartın University, Faculty of Science, Department of Molecular Biology and Genetics, Bartın, Turkey

Abstract

Silene surculosa Hub.-Mor. (Caryopyllaceae) was first collected in 1951 by Huber-Morath from Pülümür-Mutu (Tunceli). After that it was lastly collected from Pülümür by Davis and Hedge. Based on these two samples, it was introduced to the scientific world by Huber-Morath in 1967. It is an endemic species that is known from just two localities close together. Despite the investigations till today, there was found no trace of this species in the nature, and thus it has been evaluated as Extinct (EX) according to the World Conservation Union Red List Categories. By this study, firstly, the species was recollected in nature. Secondly, its description was rewritten comprehensively. Thirdly, its pollens and seeds were examined under the SEM (Scanning Electron Microscope) and LM (Light Microscope). Lastly, the new IUCN category for the species was suggested.

Key words: Tunceli, Caryophyllaceae, *Silene surculosa*, Turkey

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Türkiye için endemik olan *Silene surculosa* Hub.-Mor. türünün yeniden keşfi

Özet

Silene surculosa Hub.-Mor. (Caryopyllaceae) ilk olarak 1951 yılında Huber-Morath tarafından Pülümür- Mutu (Tunceli) arasından toplanmıştır. Son olarak Davis ve Hedge tarafından Pülümür'den toplanmıştır. Bu iki örneğe dayanılarak 1967 yılında Huber-Morath tarafından bilim dünyasına tanıtılmıştır. Sadece birbirine yakın iki lokaliteden bilinen endemik bir türdür. Günümüze değin yapılan araştırmalarda izine rastlanamayan bu türün IUCN kriterlerine göre doğada yok olduğu kabul edilmiştir. Bu çalışmayla, tür doğada yeniden toplanmış, deskripsiyonu yeniden yazılmış, polen ve tohumları SEM (Taramalı Elektron Mikroskobu) ve LM (Işık Mikroskobu)'de incelenmiş, ayrıca tür için yeni IUCN kategorisi önerilmiştir.

Anahtar kelimeler: Tunceli, Caryophyllaceae, *Silene surculosa*, Türkiye

1. Introduction

The Caryophyllaceae family has about 2,200 species of 86 genera all of the World (Bittrich, 1993) and it is represented by 494 species belong to 32 genera in Turkey (Özçelik and Muca, 2010). *Silene* L., the biggest genus of Caryophyllaceae, consists of 167 taxa in 31 sections and 72 of them are endemic to Turkey (Güner et al., 2012).

Silene surculosa Hub.-Mor. was firstly collected in 1951 from Pülümür by Huber-Morath. It is endemic and only known from two localities in the Flora of Turkey (Coode and Cullen, 1967). During my field works, we have ascertained six different populations along a line parallel to the Munzur Mountains.

This taxon was not found in studies carried on Tunceli and the other parts of Turkey until today (Yıldırımlı, 1995; Akgöz, 2013). Thus, this taxon was evaluated in category of EX that means no longer known to exist in the nature according to IUCN criteria (Ekim et al., 2000).

* *Corresponding author* / Haberleşmeden sorumlu yazar: Tel.: +903785011000; Fax.: +903785011018; E-mail: metinarmagan@gmail.com
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2. Materials and methods

All the samples belonging to *Silene surculosa*, were collected from Tunceli province during several excursions between 2014 and 2015. The specimen was determined using Flora of Turkey (Coode and Cullen, 1967) as a reference. Identification of the specimens was also confirmed by type photos in the herbaria of Geneva (G) and Kew (K). Descriptions of the species were re-written, based on its descriptions in Flora of Turkey (Coode and Cullen, 1967). Data about their ecology and newly proposed IUCN category are given (IUCN, 2014). All the specimens are kept in the herbarium of Adnan Menderes University (AYDN).

2.1. Light microscopy (LM)

By implementing Wodehouse (1935) method, pollen samples were taken from plants and then, slides were prepared by using basic fuchsine mixture. At least 30 pollen samples for the species were examined in LM and micrographs of these samples taken by Leica DM 750 digital imaging system. Different morphological characters of pollen parts, such as pollen diameter, pore diameter, distance between two pores and exine thickness, were measured by means of light microscopy. Seeds were examined in stereomicroscope and micrographs were taken with Olympus SZ2-LGB digital imaging system. After the examination of each species, measurements were done for seed shape, color and size. The seed terminology of Stearn (1992), Bojnanský and Fargašová (2007), Punt et al. (2007), Amini et al. (2011), and Mitra Arman (2013) was used.

2.2. Scanning Electron Microscopy (SEM)

For SEM, pollen grains and seeds were directly mounted on stubs and covered with gold and surface ornamentations of pollen grains were examined in detail with Jeol Tescan MAIA3 XMU model electron microscope in Bartın University Central Research Laboratory. Shape classification follows Erdtman (1969), based on P/E ratio in Table 1.

3. Results

Silene surculosa was re-discovered in the present study. In this paper, description of the species was re-identified after many samples from different populations were examined. Differences between female and hermaphrodite flowers were not mentioned in the Flora of Turkey (Coode and Cullen, 1967). It was found that anthophore is hairy. In addition, stem length, leaf lengths, flower lengths, etc. of the plant have been determined again with this study.

Silene surculosa Hub.-Mor. in Notes R.B.G. Edinb. 28: 2 (1967) (Figure 1-3).

Perennial, gynodioecious plants. Stems decumbent to ascending, up to 30 cm, some parts retrorse-puberulent, the stock bearing numerous sterile rosettes. Leaves all petiolate, fleshy, mucronate, spathulate, papillose-ciliate along the margins, the cauline leaves with sterile shoots in their axils (7-12 mm); basal leaves smaller than stem leaves (5-7 mm). Inflorescence a few-flowered panicle (3-15-flowered), sometimes shortly glandular-puberulent. Calyx 7-8 mm in functionally female flowers, 10-11 mm in hermaphrodite flowers, violet, densely glandular-pubescent. Petals white, the claw ciliate, c. 8 mm in functionally female flowers, c. 12 mm in hermaphrodite flowers. Anthophore hairy, 1.5-2.0 mm in functionally female flowers, 4.0-4.5 mm in hermaphrodite flowers. Capsule ovoid, 5 mm, included in the calyx.

Type: Turkey. B7Tunceli: Pülümür-Mutu, Serpentinschutt 8 km ob Mutu, 1760 m, 26 vi 1951, *Huber-Morath* 11170 (holo. G-00000093!).

Paratype: Turkey. B7Tunceli: above Pülümür, 1850 m, stony igneous mountainside, 08.06.1957, *Davis & Hedge 29289* (K-000728686!).

Examined specimens: *Silene surculosa* Hub.-Mor.: B7Tunceli: Pülümür, 11 km to northwest of Ardıçlı (Gersunut) village, Munzur mountains (near to border of Erzincan province), 2380 m, 18.06.2014, *Armağan 4900*; B7Tunceli: Ovacık, 6. km from Işıkvuran to Eskigedik village, 2250 m, 19.06.2014, *Armağan 4917*; B7Tunceli: Ovacık, 5. km from Ovacik-Tunceli road to Yakatarla village, 1620 m, 04.06.2014, *Armağan 4222*; B7Tunceli: Pülümür, 2.5 km from Kocatepe to Sarıgül village, 1970 m, 05.06.2014, *Armağan 4345*; B7Tunceli: Ovacık, 17 km from Ovacık to Hozat district, the east of Halitpınar village, 1895 m, 17.06.2014, *Armağan 4719*; B7Tunceli: Pülümür, 7 km from Pülümür to Tunceli, 1841 m, steppe, 29.05.2015, *Armağan 6496.*

Flowering and fruiting time: June

Distributions and Habits: *Silene surculosa* is an endemic species growing on serpentine rocks and alpine meadows at 1620-2380 m a.s.l. in Tunceli (Figure 4).

Conservation Status: *Silene surculosa* only occurs in Tunceli province (Turkey) where covers an area of about 1400 km^2. There is a distance about 65 km between two farthest localities of *S. surculosa*. The estimating distance between each small population is 10 km. We suggest that the *S. surculosa* should be placed under Endangered

[B1, B2 (a, b (iii))] category according to IUCN. The reason behind this suggestion is that Area of Occupancy (AOO) is less than 20 km^2 and the population is fragmented severally. (IUCN, 2014).

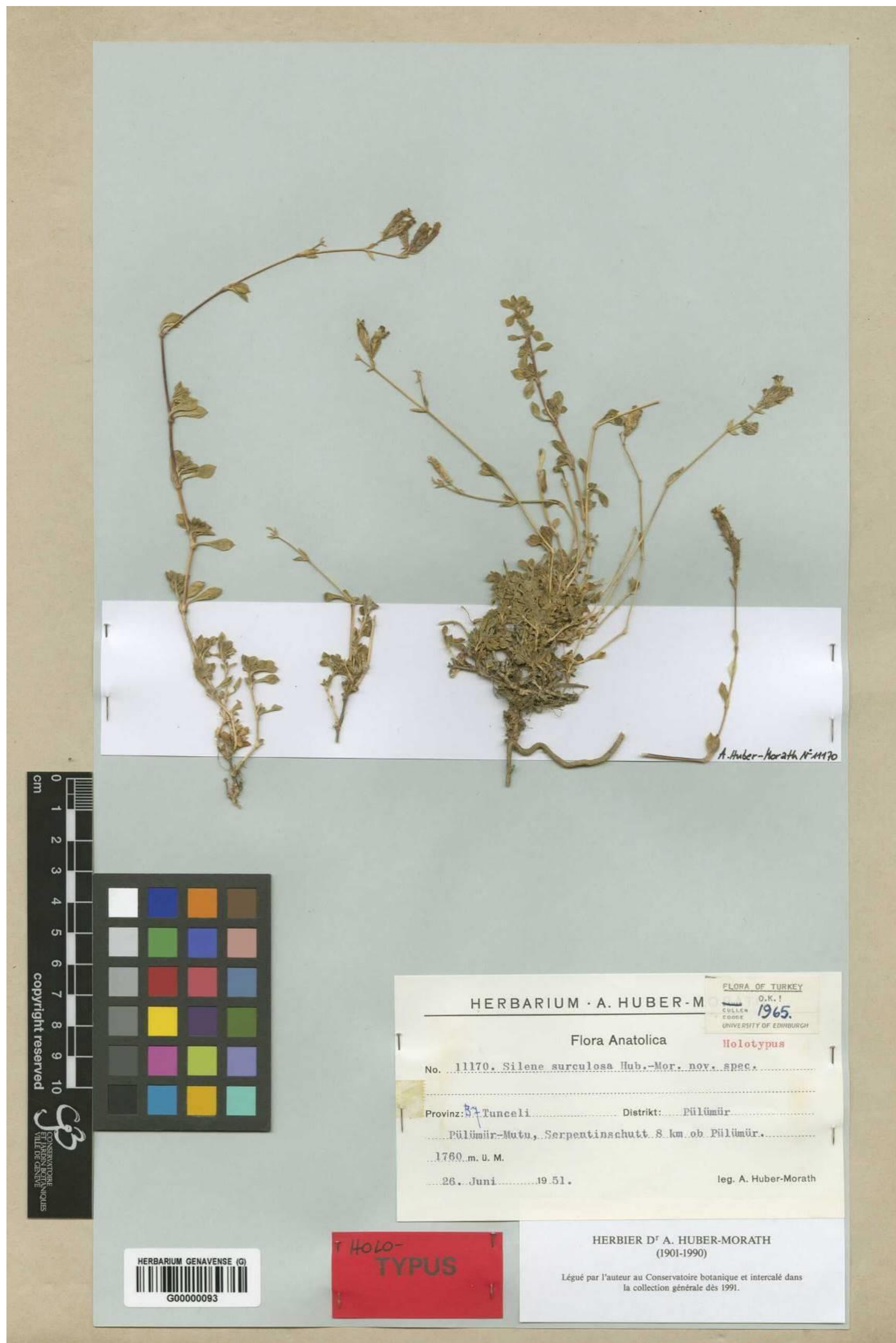

Figure 1. The holotype of *Silene surculosa* (G-00000093!)

Figure 2. The photo of *Silene surculosa* in nature

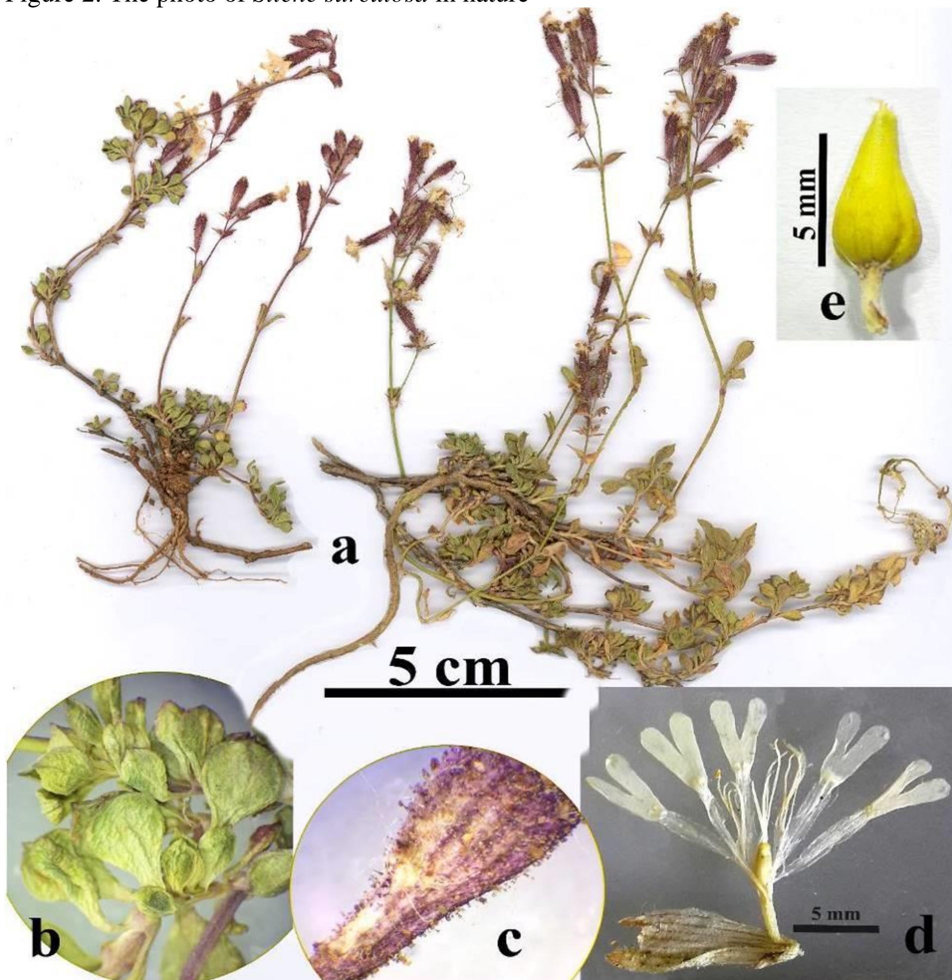

Figure 3. *Silene surculosa* (*Armağan 4345*): **a)** Habit, **b)** Basal leaves, **c)** Calyx, **d)** Hermaphrodite flower, **e)** Capsule

3.1. Pollen morphology

Pollen grains are radially symmetrical and isopolar. Polar axes is 30.57(23.70-41.09) µm, equatorial axes is 29.91 (22.75-40.68) µm. Pollen shape is P/E:1.02, subprolate. The pollen grains are operculate and pantoporate, generally 11-28 porate. Pores polar length of 4.58 (3.15-7.55) µm, equatorial width 4.15 (2.03-8.58) µm (plg/plt):1.1, prolate-spheroidal. There are 10-15 conical spinules on operculum. Distance between two pori is 4.88 (2.56-7.78) µm. The exine is tectate and 1.86 (1.09-2.63) µm in thickness. Intine thickness is 0.54 (0.43-0.6) µm. Pollen ornamentation was scabrate-punctate. There were 45-70 spinules in 100 µm^2 area. Supratectal spinules are 0.36 (µm) (see table 1, figure 5-6).

Table 1. Pollen morphology of species *Silene surculosa*

Polar axes (µm)	Mean	30.57 (23.70-41.09)
	Median	29.3
	Variation	19.84
	Standard error	0.99
	Standard deviation	4.45
Equatorial axes (µm)	Mean	29.91 (22.75-40.68)
	Median	28.63
	Variation	21.839
	Standard error	1.04
	Standard deviation	4.67
Exine thickness (µm)	Mean	1.86 (1.09-2.63)
	Median	1.81
	Variation	0.14
	Standard error	0.08
	Standard deviation	0.37
Intine thickness (µm)	Mean	0.54 (0.43-0.6)
	Median	0.55
	Variation	0.003
	Standard error	0.011
	Standard deviation	0.53
Pore length (µm)	Mean	4.58 (3.15-7.55)
	Median	4.04
	Variation	1.65
	Standard error	0.2873
	Standard deviation	1.28
Pore width (µm)	Mean	4.15 (2.03-8.58)
	Median	3.7
	Variation	2.682
	Standard error	0.3662
	Standard deviation	1.637
Pore distance (µm)	Mean	4.88 (2.56-7.78)
	Median	4.9095
	Variation	1.568
	Standard error	0.28
	Standard deviation	1.125
Pore number		20 (11-28)
Spinules on operculum		10-15
Spinules (100 µm^2)		45-70
Plg/plt		1.1
Operculum shape		Prolate-spheroidal
Pollen shape		Subprolate
Pollen dimension(P/E)		1.02
Pollen class		Pantoporate
Pollen aperture type		Tectate
Supratectal spinules(µm)		0.36
Pollen ornamentation		Scabrate-punctate

Figure 4. The distribution map of *Silene surculosa*

Figure 5. SEM of pollen morphology of *Silene surculosa*: **A)** General appearance, **B)** Pore, **C)** Ornamentation

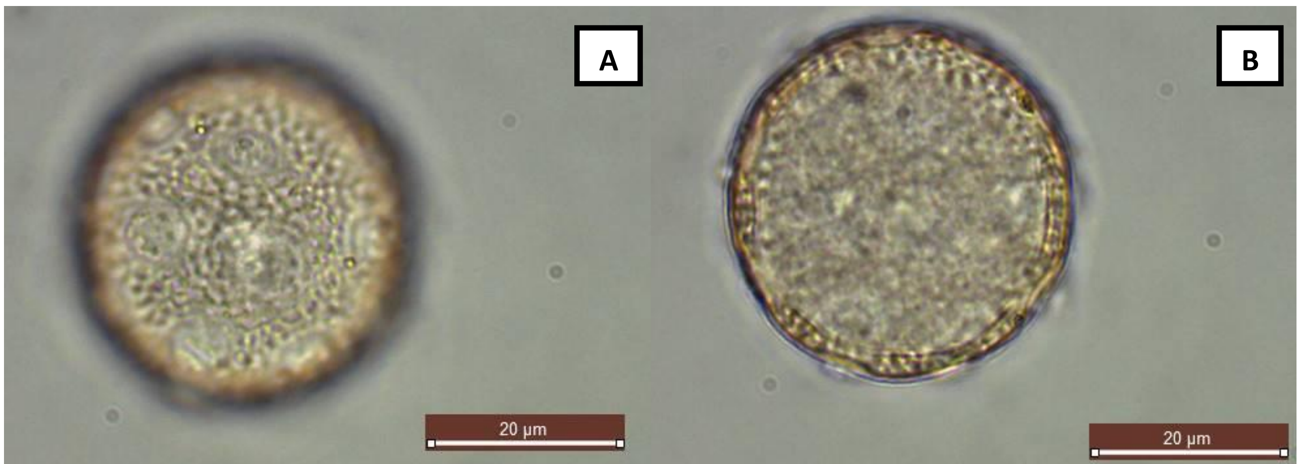

Figure 6. Light microscope micrographs of pollen grains of *Silene surculosa*: **A)** Polar view, **B)** Equatorial view

3.2. Seed morphology

The seed type of *Silene surculosa* is reniform-orbicular, seed length 1.227 (1.123-1.372) mm, seed width is 0.82 (0.6-1.035) mm. Seed color varies from brown or light brown. Seed surface type is slightly convex to straight and seed back is concave. Tubercles are mostly elongated. Seed surface granulation is medium, hylar zone type recessed, suture outline serrate (see table 2, figure 7-8).

Table 2. Seed morphology of species *Silene surculosa*

Seed type	Reniform-orbicular
Seed color	Brownish
Seed surface type	Slightly convex to straight
Seed back	Concave
Surface granulation	Medium
Shape of testa cell	Elongate
Hylar zone type	Recessed
Margin of testa cell	Serrate
Seed length (mm)	1.227 (1.123-1.372)
Seed width (mm)	0.82 (0.6-1.035)
Seed length/width ratio (mm)	1.49
Testa cell length (µm)	78-150
Testa cell width (µm)	34-55
Number of suture point per plate	20-38
Ridge	Conspicuous ridge

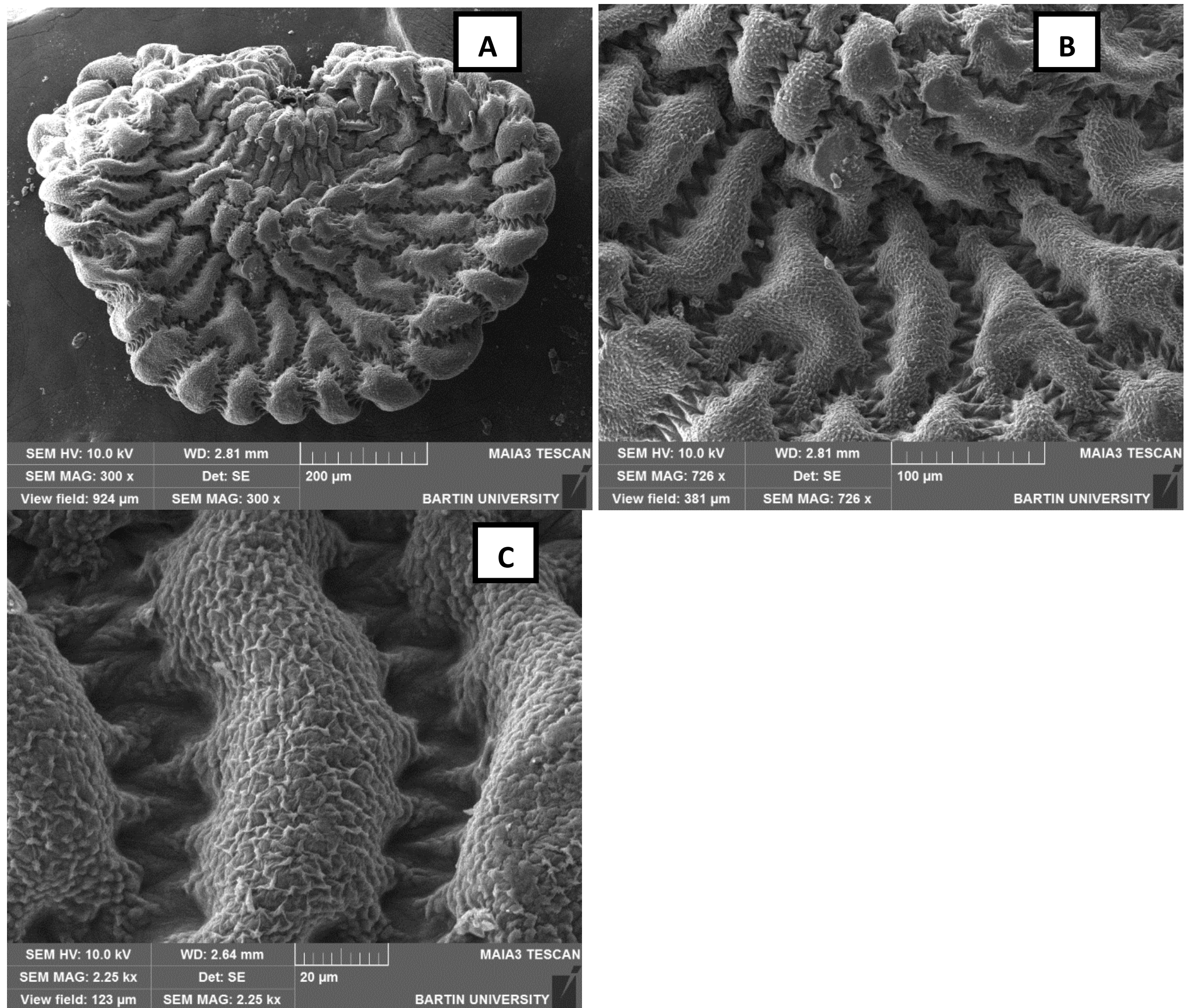

Figure 7. SEM of seed morphology of *Silene surculosa*: **A)** General appearance, **B)** Testa cells, **C)** Surface granulation and suture outline.

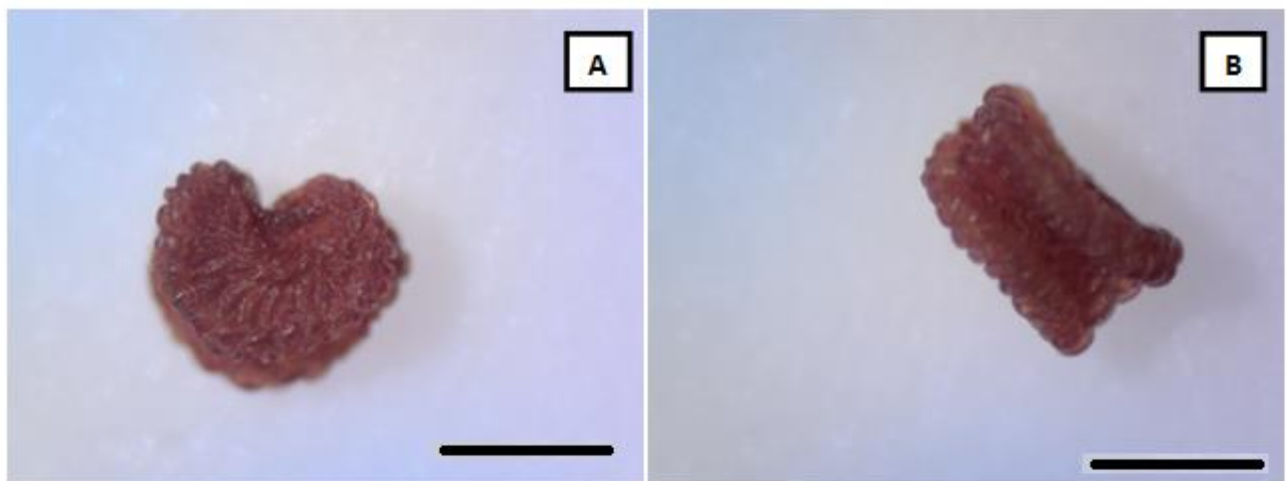

Figure 8. Stereo microscope micrographs of seed of *Silene surculosa*: **A)** Seed surface, **B)** Seed back, Scala bar=1 mm

4. Conclusions and discussion

Sect. *Spergulifoliae* Boiss. includes some perennials and usually gynodioecious taxon. Functionally female flowers' characteristics have shorter than hermaphrodite flowers of the same species. If collectors did not note the different sex forms in an area, as without enough material, the taxon of this section can easily be confused with each

other. Only carefully field studies can solve these taxonomic problems adequately. Because of this we prepared comprehensive description of *Silene surculosa* Hub.-Mor. by field observations and adequate samples.

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Research article/Araştırma makalesi

Toxicological effects of the entomopathogenic *Purpureocillium lilacinus* on the model organism, *Galleria mellonella*

Hülya ALTUNTAŞ [*1], Emine DUMAN [1]

[1] Department of Biology, Faculty of Science, Anadolu University, Eskişehir, 26470, Turkey

Abstract

The toxicological effects of entomopathogenic fungus *Purpureocillium lilacinum* (Thom) Luangsa-ard, Houbraken, Hywel-Jones and Samson on the *Galleria mellonella* L. (Lepidoptera: Pyralidae) larvae which have great importance in biological, physiological and biochemical studies as a model insect were investigated. For this purpose, larvae were injected with median lethal concentration (LC_{50}) 2.82 x 10^3 conidia/ml suspension. Hemolymph samples were collected after injection at 24, 48, 72 and 96 hours. Total protein analysis and specific enzyme activities of CAT and GST in the hemolymph were analyzed spectrophotometrically. Total protein amount in the hemolymph of the infected larvae decreased at all times when compared with control groups. Furthermore, the activity of GST in the hemolymph of the infected larvae increased at all times compared with untreated larvae, but the activity of CAT was similar with activity of untreated larvae. Consequently, increasing the activity of GST showed that entomopathogen organisms are effective on the antioxidant defense system of insects as a source of free radical. CAT activity in the absence of any change showed inactivation of CAT out of increased superoxide radicals based pathogenicity. Therefore, we suggest that the variance in the activity of CAT and GST in larvae may indicate a physiological adaptability to compensate for pathogen-induced stress.

Key words: *Galleria mellonella, Purpureocillium lilacinus*, entomopathogen fungus, catalase, glutathione S-transferase

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Entomopatojenik *Purpureocillium lilacinus'*un model organizma, *Galleria mellonella* üzerindeki toksikolojik etkileri

Özet

Biyolojik, fizyolojik ve biyokimyasal araştırmalarda model organizma olarak büyük öneme sahip olan *Galleria mellonella* L. (Lepidoptera: Pyralidae) larvaları üzerinde entomopatojenik fungus *Purpureocillium lilacinum* (Thom) Luangsa-ard, Houbraken, Hywel-Jones ve Samson'un toksikolojik etkileri araştırılmıştır. Bu amaçla ortalama letal konsantrasyon (LC_{50}) olan 2.82 x 10^3 spor/ml süspansiyonu kullanılarak son evre larvalara enjeksiyon yapıldı. Enjeksiyondan 24, 48, 72 ve 96 saat sonra larvalardan hemolenf alındı. Hemolenf örneklerinde toplam protein miktarı, katalaz (CAT) ve glutatyon S-transferaz (GST) aktiviteleri spektrofotometrik olarak analiz edildi. *P. lilacinus* ile enfekte olan larvaların toplam protein miktarı tüm saatlerde kontrol grupları ile karşılaştırıldığında azalış göstermektedir. Ayrıca fungal enfeksiyona bağlı olarak GST enzim aktivitesi zamanla artış gösterirken, CAT enzim aktivitesinde herhangi bir değişiklik olmadığı belirlenmiştir. Bu sonuçlar, larval GST aktivitesinde görülen artışın patojenite kaynaklı oksidatif stresin telafi edilebilmesi için fizyolojik bir uyum olabileceğine, CAT aktivitesinde hiçbir değişimin olmaması ise patojenite kaynaklı artan süperoksit radikallerinin enzimin inaktivasyonuna neden olabileceğine işaret etmektedir. Bu nedenle, CAT ve GST aktivitesinde görülen bu değişikliklerin nedeninin patojen kaynaklı strese karşı oluşan fizyolojik onarım mekanizmalarıyla ilişkili olduğunu düşünmekteyiz.

Anahtar kelimeler: *Galleria mellonella, Purpureocillium lilacinus;* entomopatojen fungus, katalaz, glutatyaon-S-tranferaz

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[*] *Corresponding author* / Haberleşmeden sorumlu yazar: Tel.: +902223350585; Fax.: +902223204910; E-mail: hyalcitas@anadolu.edu.tr
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1. Giriş

Biyolojik çeşitliğin en önemli unsurlarından birisi olan böceklerin ekolojik ortamlarında büyüme ve gelişimlerini etkileyen biyolojik ve biyolojik olmayan çeşitli etkenlerin olduğu bilinmektedir. Böceklerin gelişim sürecinde maruz kaldıkları önemli etkenlerden birisi de ksenobiyotiklerdir (Wu vd., 2004). Böceklerin ksenobiyotiklerin etkileri altında kalmaları, serbest oksijen radikallerinin oluşumuna ve dolayısıyla metabolik olayların bozulmasına neden olur. Serbest oksijen radikalleri, sahip oldukları paylaşılmamış elektronları nedeniyle yüksek aktiviteye sahip olan atom ve moleküllerdir. Bu radikaller hücre zarının doymamış yağ asitleri ile protein bileşimi üzerine zarar vermektedir (Halliwell ve Gutteridge, 2007). Serbest radikallerin zar ile etkileştiği durumda enzimler, hormonlar ve nörotransmitter maddeler de olumsuz yönde etkilenmektedir. Patofizyolojik durumlarda üretilen bu serbest oksijen radikalleri canlılarda antioksidan sistemler ile uzaklaştırılır (Felton ve Duffey, 1991; Felton ve Summers, 1995; Krishnan ve Sehnal, 2006). Omurgalılarda olduğu gibi böceklerde de enzimatik ve enzimatik olmayan savunma sistemleri vardır. Enzimatik sistemin başlıca elemanları süperoksit dismutaz (SOD), katalaz (CAT), glutatyon peroksidaz (GPx), glutatyon redüktaz (GR), glutatyon-S-transferaz (GST) enzimleridir (Krishnan ve Kodrik, 2006). Bu nedenle, böceklerin çevresel koşullara karşı başarılı adaptasyonları, etkili detoksifikasyon mekanizmaları ve bu maddelerin vücutlarından giderilmesi ile gerçekleşmektedir (Goryunova vd., 1991; Wu vd., 2004). Bu detoksifikasyon enzimlerinin rolü, böcekleri insektisitlerin, çeşitli bitkisel metabolitlerin veya entomopatojenik mikroorganizmaların olumsuz etkilerinden koruması ile sınırlandırılmamıştır. Örneğin, bazı hormonların, feromonların ve diğer biyolojik aktif maddelerin metabolizmasına da aracılık etmektedirler (Terriere, 1984; Feyereisen, 1999). Bu yüzden, böceklerde antioksidan enzim aktivitelerinde ortaya çıkabilecek farklılıklar sadece ksenobiyotiklere karşı gelişebilecek direnç mekanizmasını değil, aynı zamanda biyolojik uyum kapasitesini de göstermektedir (Terriere, 1984; Fuchs vd., 1993).

Böceklerde detoksifikasyon enzimlerinin inhibisyonu ya da aktivitesindeki değişikliklerin nedenleri arasında günümüzde ksenobiyotikler arasında da yer alan entomopatojenik funguslarla ilişkili enfeksiyonlar da olabilir (Kol’chevskaya ve Kol’chevkii, 1988; Xia vd., 2000; Serebrov vd., 2003). Entomopatojenik funginin zararlı böceklerde gösterdiği patojen etki konukçularının zayıflaması veya ölümleri ile ilişkilidir (Sanjaya vd., 2016). Entomopatojenik fungusların diğer mikroorganizmalar ile kıyaslandığında zararlı böcekler üzerinde daha fazla türde etkili olduğu bilinmektedir (Deacon, 1983). Ayrıca *Beauveria bassiana, Metarhizium anisopliae* ve *Lecanicillium lecanii* türlerinin tüm dünyada yaygın olarak bulunan entomopatojenik fungus türleri olduğu ve birçok zararlı böcek türünde insektisidal etki gösterdikleri daha önce yapılan çalışmalarda gösterilmiştir (Deacon, 1983; Sanjaya vd., 2016). Entomopatojenikler yaşayan, çoğalan ve infeksiyöz özellik taşıyan varlıklardır. Organizmaları enfekte etmeleri göz önüne alınarak spesifite çalışmaları özellikle de ekotoksitite analizleri titizlik ile yapılmalıdır. Ancak, entomopatojenik fungusların böcek popülasyonlarında geniş ölçüde yayılmış olmasına rağmen, literatürde enfeksiyona bağlı detoksifikasyon enzimlerinde meydana gelen değişiklikler ile ilgili çalışmalar oldukça sınırlı sayıdadır (Serebrov vd., 2006). Bu nedenle bu çalışmada doğal ortamını çok çeşitli toprakların oluşturduğu ve lila renkli koloniler üretmesi ile karakterize edilen bir entomopatojenik fungus türü olan *Purpureocillium lilacinus* (KUKENS WDCM101) [eski adı ile *Paecilomyces lilacinus* (Thom)] ve model böcek büyük bal mumu güvesi *G. mellonella* türü kullanılmıştır. Büyük bal mumu güvesi *G. mellonella*’nın özellikle de larval döneminin immünolojik, patojenik, genotoksik ve biyokimyasal çalışmalarda iyi bir fizyolojik model olduğu ve laboratuvar şartlarında kolayca kültüre edildiği bilinmektedir (Cook ve McArthur, 2013; Emre vd., 2013; Ergin vd., 2013).

Çalışmamızda model organizma olarak *G. mellonella* türüne ait son evre larvalar kullanılarak, böceklerin önemli detoksifikasyon enzimlerinden olan katalaz (CAT) ve glutatyon-S-transferaz (GST) aktivitelerinin fungal enfeksiyona bağlı değişimlerinin laboratuvar ortamında araştırılması amaçlanmıştır.

2. Materyal ve yöntem

2.1 Böcek kültürünün hazırlanması

G. mellonella kültürünün yetiştirilmesi 25$\pm$2 °C sıcaklık, % 60$\pm$5 bağıl nem ve 12:12 saat (aydınlık: karalık) fotoperiyot şartlarında sağlandı. Kültürün devamlılığı ve deneysel çalışmalarda kullanılacak son dönem larvaların seçimi için steril edilmiş 40 g yarısentetik besiyeri (Bronskill, 1961) içeren beş adet 1000 ml’lik her bir cam kavanoz içerisine *G. mellonella*’ya ait beş adet erkek, beş adet dişi ergin birey, çiftleşmek ve yumurta bırakmak üzere konularak beş gün bekletildi. Beşinci günün sonunda ergin bireyler kavanozlardan alındı. Yumurtaların açılmasından itibaren *G. mellonella* larvaları son evreye (0,17 ± 0.02 g) ulaşıncaya kadar takip edildi. Son evreye ulaşan larvaların bir kısmı deney grupları için kullanılırken diğer kısmı kültürün devamlılığının sağlanmasında kullanıldı.

2.2 Entomopatojenik fungus kültürünün hazırlanması

Çalışma kapsamında kullanılan entomopatojenik *P. lilacinus* Eskişehir ili çevresindeki tarım topraklarından elde edilmiş ve Pitt (1979)’a göre teşhis edilmiştir (Demirel vd., 2005). Kullanılan suş KUKENS kültür

kolleksiyonunda (WDCM101) muhafaza edilmektedir. Entomopatojenik *P. lilacinus*'un kültüre edilmesi için Malt Ekstrat Agar (MEA- Merck 1. 05398) besi ortamında 28 °C'de 14 gün süre ile inkübasyon gerçekleştirilmiştir.

2.3 Deney gruplarının hazırlanması

Entomopatojenik *P. lilacinus'*un *G. mellonella* üzerindeki toksik etkilerinin belirlenmesi amacıyla Şanal vd., (2011) tarafından belirlenen ortalama letal konsantrasyon (LC_{50}) olan 2.82 x 10^3 spor/ml spor konsantrasyonu kullanılmıştır. Spor süspansiyonunun hazırlanması için MEA ortamında 28 °C'de 14 gün süre ile geliştirilmiş olan *P. lilacinus* kültüründen steril koşullarda alınan biyokütle % 0.1'lik Tween 80 (Sigma) içerisine homojen olarak karıştırılmış ve Thoma lamı ile sayım işlemi gerçekleştirilmiştir (Halkman, 2005).

Entomopatojenik *P. lilacinus* ile enjeksiyon işlemi için son döneme ulaşan *G. mellonella* larvaları kullanılmıştır. 2.82 x 10^3 spor/ml olarak hazırlanan spor solüsyonu Helminton şırınga (10 µl) ile larvanın 1. arka ekstremitesinin kaidesinden vücut boşluğuna enjekte edildi. Kontrol grubunun oluşturulmasında ise % 0.1'lik Tween 80 kullanılarak, enjeksiyon işlemi aynı şekilde yapıldı. Enjeksiyon işleminin ardından steril edilmiş yarı sentetik *G. mellonella* besini (1 larva için 2 g besin) içerisine alınan larvalar stok kültür ile aynı fotoperiyot koşullarında inkübe edildi. Enjeksiyon işlemlerinden sonra inkübasyon saatleri olarak 24, 48, 72 ve 96. saatler seçildi. Belirlenen tüm saatler ve kontrol grupları için her bir tekrarda 10 larva olmak koşuluyla deneyler 3 kez tekrar edildi.

2.4 Larval hemolenf toplama ve ekstraksiyon işlemi

P. lilacinus'a ait spor solüsyonu ile enfekte olan ve olmayan son dönem larvalardan hemolenf toplama işleminden önce larvalar buz üzerinde bekletilerek hareketlerinin yavaşlatılması sağlandı. Bu işlemin ardından larvaların dış yüzeyi % 70'lik alkol içeren gazlı bez ile temizlendi ve larvaların ikinci ön ekstremite bölgesi streril diseksiyon iğnesi ile delinerek her bireyden 10 µl hemolenf mikrokapiller tüp ile toplandı. Elde edilen hemolenf örnekleri ise içerisinde 0.001 mg N-phenlythiourea (Sigma) (PTU) bulunan soğuk mini santrifüj tüplerine alındı.

Enfekte olan deney grupları ile enfekte olmayan kontrol gruplarına ait larval hemolenflerin ekstraksiyon işlemleri ise içerisinde soğuk homojenizasyon tamponu [0,15 M NaCl, 0,05 M fosfat tamponu (pH: 7,4)] bulunan tüplerde gerçekleştirildi. Larval hemolenflerin ekstraksiyonu için homojenizasyon tamponu ile dilüe (1: 3) edilen hemolenf örnekleri +4 °C'de, 10,000 g'de, 15 dakika santrifüj edildi. Santrifüj sonrası elde edilen süpernatant protein miktar tayini ile enzim aktivite deneylerinde kullanıldı.

2.5 Total protein miktar tayini ve enzim analizleri

P. lilacinus ile enfekte olan ve olmayan larval hemolenf örneklerinin ekstraksiyonu sonrası elde edilen homojenatlardan toplam protein miktar tayini Bradford (1976) yöntemine göre gerçekleştirildi. Bovine serum albümin (BSA) ile hazırlanan 1 mg/ml stok solüsyon ile 0,002 - 0,3 mg/ml aralığında standart protein çözeltileri hazırlandı. Hazırlanan standart protein çözeltileri ve homojenatlara ait toplam protein ölçümleri spektrofotometrede (Shimadzu, UV-2101PC) 595 nm'de ölçüldü.

G. mellonella larval hemolenfinden elde edilen homojenatlardan katalaz (EC 1.11.1.6) aktivitesinin belirlenmesi için Chance ve Maehly (1995) tarafından geliştirilen yöntem kullanıldı. Bu yöntemde, katalazın hidrojen peroksiti su ve moleküler oksijene yıkım hızının hesaplanması esas alınmıştır. Bu amaçla, homojenat (40 µg/ml), 30 mM hidrojen peroksit ve fosfat tamponundan (50 mM KH_2PO_4, 50 mM K_2HPO_4) (pH: 7,00) oluşan karışım kuvars küvetine alındıktan sonra spektrofotometrede (Shimadzu, UV-2101PC) 240 nm'de 3 dakika boyunca azalan hidrojen peroksit ölçümü yapıldı. Elde edilen azalış miktarlarından sabit sayı (ε240: 0,0394 mM/cm) kullanılarak spesifik katalaz enzim aktivitesi (U/mg) hesaplandı. Ölçümlerde kör olarak fosfat tamponu (pH: 7,0) kullanıldı.

Enfekte olan ve olmayan larvaların hemolenfinde glutatyon-S-transferaz (E.C.2.5.1.18) enzim aktivitesinin belirlenmesi Boyland ve Chasseaud (1969) tarafından geliştirilen yönteme göre gerçekleştirildi. Bu yöntem 1-chloro-2,4-dinitrobenzen (CDNB)'nin redükte glutatyon ile konjügasyonunu katalize eden toplam GST (mikrozomal ve sitozolik) aktivitesinin ölçülmesi esasına dayanmaktadır. Bu amaçla 200 µg/ml protein içeren homojenat ve kokteyl (100 mM CDNB, 100 mM GSH (glutatyon) ve fosfat tamponu pH: 6,5) karışımı hazırlanarak, aktivite ölçümü spektrofotometrede (Shimadzu, UV-2101PC) 340 nm'de 5 dakika süresince yapıldı. Ölçüm sonucunda CDNB'nin redükte glutatyon ile reaksiyona girmesine bağlı olarak tioether yapısının oluşumuyla ilgili yükselen absorbans değerleri elde edildi. Elde edilen absorbans değerleri ile ε340: 0,0096 μM^{-1} katsayısı kullanılarak spesifik enzim aktivitesi (U/mg) hesaplandı. Ölçümlerde kör olarak fosfat tamponu (pH: 6,5) kullanıldı.

2.6 İstatistik

P. lilacinus ile enfekte edilen ve kontrol grubuna ait enzim aktivite deneyleri her saat (24-48-72-96) için 10 adet son evre larva olacak şekilde 3 kez tekrar edilmiştir (n= 30). Tekrarlar sonucunda elde edilen her saatin enfekte grubu ve kontrolü Indepentent Sample T-test ile analiz edildi. Enzim aktivite farklılıklarında ise verilerin normal

dağılım gösterdiği istatistiksel olarak sınanmış ve tüm verilere One - Way Anova uygulanarak, % 95 güven aralığında Tukey gerçekten anlamlılık testi (Tukey's Honestly Significant) yapılmıştır (Windows versiyon 18.0, SPSS, Chicago, IL).

3. Bulgular

3.1 Enjeksiyon sonrası total protein miktarlarındaki değişimler

P. lilacinus ile enfekte edilen G. *mellonella* larval hemolenfindeki toplam protein miktarlarının tüm saatlerde kendi kontrol gruplarına göre azaldığı tespit edildi (p<0,05). Fakat kontrol ve enfekte grupların kendi aralarında protein miktarları karşılaştırıldığında istatistiksel olarak anlamlı bir farklılık olmadığı (p>0,05) belirlenmiştir (Tablo 1).

Tablo 1. *G. mellonella* larvalarında enjeksiyon sonrası (24, 48, 72, 96 saat) toplam protein miktar değişimi

Süre (Saat)	Ort. ± SH Protein Miktarı (mg/ml)	
	Kontrol	Enfekte
24	11,77±0,39x** a	10,25±0,15y a*
48	12,09±0,29x a	10,84±0,56y a
72	12,09±0,61x a	10,24±0,32y a
96	12,87±2,02x a	10,65±0,21y a

*Aynı sütunda (a) aynı harfle gösterilen değerler arasında istatistiksel olarak fark bulunmamaktadır (P>0.05, ANOVA Tukey HSD). Kontrol için F= 0,282, df= 3,8, P= 0,837; Enfekte için F= 1,393, df= 3,8, P= 0,314.
**Aynı satırda (x, y) aynı harfle ile gösterilen değerler arasındaki fark istatistiksel olarak önemsizdir (P>0.05, t testi).

3.2 Enjeksiyon sonrası antioksidan enzim aktivitelerindeki değişimler

P. lilacinus'a ait LC_{50} spor solüsyonu ile enfekte edilen *G. mellonella* larvalarının hemolenfinde, enfekte olmamış gruplarına göre tüm saatlerin sonunda GST enzim aktivitesinin arttığı belirlenmiştir (p<0,05). Ayrıca kontrol ve enfekte grupların kendi aralarında yapılan analizi sonucunda kontrole ait saatlerde istatistiksel olarak anlamlı bir fark yokken (p>0,05), enfekte gruplar arasındaki farkın istatistiksel olarak anlamlı olduğu (p<0,05) bulunmuştur (Tablo 2). Enfekte gruplarda GST aktivitesi 96. saate göre düzenli artış gösterirken, son saatte daha yüksek bir aktivite tespit edilmedi.

Tablo 2. *G. mellonella* larvalarında enfeksiyon sonrası (24-48-72 ve 96. saatte) GST enzim aktivitesi

Süre (Saat)	Ort. ± SH GST Enzim Aktivitesi	
	Kontrol	Enfekte
24	18,60±0,26x** a	22,66±0,01y a*
48	17,01±1,45x a	34,46±0,25y b
72	16,53±1,35x a	36,10±0,24y c
96	15,92±1,69x a	24,95±0,39y d

*Aynı sütunda (a-d) aynı harfle gösterilen değerler arasında istatistiksel olarak fark bulunmaktadır (P<0.05, ANOVA Tukey HSD). Kontrol için F= 0,785, df= 3,8, P= 0,535; Enfekte için F= 804,992, df= 3,8, P=0,000
**Aynı satırda (x-y) aynı harfle ile gösterilen değerler arasında istatistiksel olarak fark bulunmamaktadır? (P<0.05, t testi).

P. lilacinus'a ait LC_{50} solüsyonu ile enfekte edilen *G. mellonella* larval hemolenfinde 24, 48, 72 ve 96. saatte belirlenen CAT enzim aktivitelerinin kontrol gruplarına göre değişiklik göstermediği belirlenmiştir (p>0,05). Ayrıca kontrol ve enfekte gruplarının saatler arasında da CAT enzim aktivitesi yönünden anlamlı farklılık göstermediği görülmüştür (p>0,05) (Tablo 3).

Tablo 3. *G. mellonella* larvalarında enfeksiyon sonrası (24-48-72 ve 96. saatte) CAT enzim aktivitesi.

Süre (Saat)	Ort. ± SH CAT Enzim Aktivitesi	
	Kontrol	Enfekte
24	17,55±0,41x** a	18,47±1,10x a*
48	17,05±0,36x a	16,71±2,03x a
72	18,81±1,56x a	20,62±0,62x a
96	20,72±4,25x a	19,48±0,47x a

*Aynı sütunda (a) aynı harfle gösterilen değerler arasında istatistiksel olarak fark bulunmamaktadır (P>0.05, ANOVA Tukey HSD); Kontrol için F= 1,783, df= 3,8, P= 0,228; Enfekte için F= 2,707, df= 3,8, P= 0,116.
**Aynı satırda (x) aynı harfle ile gösterilen değerler arasındaki fark istatistiksel olarak önemsizdir (P>0.05, t testi).

4. Sonuçlar ve tartışma

Biyolojik sistemlerde endojen ve ekzojen kökenli stres faktörlerine bağlı olarak üretilen reaktif oksijen türlerine (ROS) karşı enzimatik ve moleküler antioksidan savunma sistemleri geliştirilmiştir (Urso ve Clarkson, 2003; Greathouse vd., 2005). Diğer canlılarda olduğu gibi böceklerde de ROS moleküllerine karşı özelleşmiş bileşikler ve enzimler bulunmaktadır (Grubor- Lajsic vd., 1997). Genel olarak birçok göreve sahip olan antioksidan enzimleri, patojenik ürünlerin detoksifikasyonunda tamir mekanizmasına ve biyolojik aktif ürünlerin metabolizmasına aracılık ettikleri için enfeksiyon durumlarında aktivitelerinde farklılıklar meydana gelebilir (Serebrov vd., 2006). Bununla birlikte böceklerin doğal populasyonunda yaygın olarak bulunan entomopatojenik mantar ve mikroorganizmalar da böceklerin detoksifikasyon sistemleri üzerinde etkili olabilmektedir (Pedro ve Candido, 1997; Hughes vd., 2004; Serebrov vd., 2006). Böcekler genellikle ksenobiyotiklere maruz kaldıkları için antioksidan enzimler ile ilgili yapılan çalışmaların çoğu bu yöndedir (İçen vd., 2005; Dere vd., 2015; Altuntas vd., 2016). Ancak, enfeksiyona bağlı detoksifikasyon mekanizmasıyla ilgili yapılan çalışmalar sınırlı sayıda olmakla birlikte, genellikle özel olmayan esterazlar ve fosfatazlarla ilgilidir (Kol'chevskaya ve Kol'chevkii, 1988; Xia vd., 2000; Serebrov vd., 2003). Fuchs vd. (2010) tarafından fungal patojenite çalışmalarında model organizma olarak kullanılan *G. mellonella*'nın *Caenorhabditis elegans* ve *Drosophila melanogaster*'e göre daha kullanışlı olduğu belirtilmiştir. Bu çalışmada ise entomopatojen özellikteki *P. lilacinus*'un *G. mellonella* son dönem larvalarında antioksidan enzimlerden katalaz (CAT) ve glutatyon-S-transferaz (GST) üzerindeki etkisinin belirlenmesi amaçlanmıştır. Böylece entomopatojenik fungus enfeksiyonu sonrası böceklerin antioksidan savunma sisteminde meydana gelen değişiklikler ile ilgili bilgiler literatüre kazandırılacaktır.

Parazitler, konak organizmada gelişimlerini ve çoğalmalarını sağlayabilmek için yüksek metabolik hıza ihtiyaçlar duyarlar. Bu durum ise konukçuda oksidatif strese ve parazitler tarafından üretilen büyük miktarda toksik madde ile yan ürünlerin ortaya çıkmasına neden olur (Becker vd., 2004). Multienzim yapısındaki GST enzimi, faz II detoksifikasyon enzimi olarak bilinmekte ve böceklerde stres koşulları altında hücresel detoksifikasyonun sağlanmasında önemli rol oynamaktadır. Özellikle oksidatif stres durumunda artış gösteren oksijen türlerinin zararlarına karşı hücrenin korunumunda, doku hasarının giderilmesinde ve ksenobiyotiklerin detoksifikasyonunda önemli rol oynamaktadır (Terriere, 1984; Krishnan ve Kodrik, 2006; Serebrov, 2006; Sanjaya vd., 2016) Daha önce yapılan bir çalışmada *Metarhizium anisoplia* mantarı ile enfekte edilen *G. mellonella* larval hemolenfinde GST enzim aktivitesinde artış tespit edilmiştir (Glupov vd., 2003). Lozinskaya vd. (2004)'nın gerçekleştirdikleri bir çalışmada ise *Vairimorpha ephestiae* ile enfekte edilen *G. mellonella* larvalarında GST enzim aktivitelerindeki değişimler araştırılarak, küfle enfekte edilmiş larval hemolenfte enzim aktivitesinin kontrol grubuna göre daha yüksek olduğu belirlenmiştir (Lozinskaya vd., 2004). Bizim çalışmamızda da benzer olarak *P.lilacinus* enfeksiyonu sonrası *G. mellonella* larval hemolenfindeki GST aktivitesinde artış meydana gelmiştir. Böylece elde ettiğimiz sonuçlar hem daha önce yapılan çalışmaları desteklenmekte hem de GST enziminin toksik fungal metabolitlerin eliminasyonu için gerekli olduğunu göstermektedir. Bu nedenle entomopatojenik funguslarla ilişkili ekotoksitite çalışmalarında GST enzim aktivitesindeki değişimlerin bir biyolojik belirteç olarak kullanılabileceğini önermekteyiz.

Fizyopatolojik şartlar altında reaktif oksijen türleri yüksek oranlarda üretilerek süperoksit radikallerinin birikmesine neden olmaktadır (Sies, 1997). Katalaz enzim aktivitesi ise hidrojen peroksit radikalinin yüksek miktarda oluştuğu durumlarda artarak, hücrede hidrojen peroksit artışına neden olan reaktif moleküllerin detoksifikasyonunu sağlamaktadır (Samashekaraiah vd., 1992). Ancak, CAT aktivitesinin, hücresel ortamda tekli oksijen, süperoksit ve peroksit radikali gibi serbest radikallerin yüksek miktarlarda birikmesine bağlı olarak inhibe edildiği bilinmektedir (Kono ve Fridovich, 1982). Kono ve Fridovich (1982) enzim aktivitesinde görülen inhibisyonu, reaktif oksijen radikallerinin hücresel membranlardaki doymamış yağ asitlerini ve proteinleri okside etmesi sonucu MDA üretiminin artmasına bağlı olarak proteinlerde denatürasyon meydana gelmesi ile ilişkilendirmiştir. Daha önce böceklerle ilgili yapılan bazı çalışmalarda da çeşitli kimyasalların ve fenolik karakterdeki bitkisel metabolitlerin larval dokularda bulunan CAT enziminin inhibisyonuna neden olduğu belirlenmiştir (Felton ve Duffey, 1991; Emre vd., 2013). Bizim çalışmamızda da benzer olarak *P. lilacinus* ile enfekte olan *G. mellonella* larvalarının CAT enzim aktivitesinde herhangi

bir değişiklik tespit edilmedi. Bu durumun fungal enfeksiyona bağlı olarak ortaya çıkan yüksek oksidatif hasarın larval hemolenfteki CAT aktivitesini engelleme yönünde etkili olduğunu düşünmekteyiz.

Sonuç olarak, bu çalışma entomopatojenik fungusların ekosistemin önemli elemanları olan böceklerde antioksidan enzim aktiviteleri üzerinde değişimlere neden olabileceğini ve bu durumun organizma ile çevresi arasındaki fizyolojik uyum kapasitelerini de etkileyeceğini göstermektedir. Ayrıca entomopatojenik fungus türlerinin zararlı böcek mücadelesinde kullanılabilirliğine ilişkin araştırmalarda çalışma sonuçlarımızdan faydalanılabileceğini önermekteyiz..

Teşekkür

Çalışma kapsamında kullanılan entomopatojenik *Purpureocillium lilacinus* (KUKENS WDCM101) Doç. Dr. Rasime Demirel tarafından sağlanmıştır. Kendisine teşekkür ediyoruz.

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Research article/Araştırma makalesi

Water quality assessment of Felent Stream (Kütahya/Turkey)

Arzu ÇIÇEK[1], Esengül KÖSE [2], Cem TOKATLI [*3] Özgür EMİROĞLU[4]

[1] Anadolu University, Applied Environmental Research Centre, Eskişehir, Turkey
[2] Eskişehir Osmangazi University, Department of Environmental Protection and Control, Eskişehir, Turkey
[3] Trakya University, Department of Laboratory Technology, Ipsala Vocational School, Edirne, Turkey
[4] Eskişehir Osmangazi University, Faculty of Science, Department of Biology, Eskişehir, Turkey

Abstract

Felent Stream is one of the most important branches of Porsuk Stream and it is affected by wastes of industrial, agricultural and thermal springs of Kütahya Province. In the present study, water quality of Felent Stream including upstream, downstream and reservoir parts of the system were investigated. For this purpose, temperature, pH, dissolved oxygen, ammonium nitrogen, nitrite nitrogen, nitrate nitrogen, sulphate, phosphate, chemical oxygen demand (COD), total phosphorus, calcium, magnesium and sodium parameters were determined on water samples collected seasonally (2010 – 2011) from seven stations selected on the basin considering the pollution sources. Also One –Way ANOVA test was applied to detected data in order to evaluate the significant water quality differences among the investigated stations. According to data observed, Felent Stream was found to be as under effect of a significant organic – thermal pollution caused form especially domestic wastes of Kütahya Province and thermal springs of Yoncalı Village.

Key words: water quality, Felent stream, one – way ANOVA

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Felent Çayı (Kütahya/Türkiye) su kalitesinin değerlendirilmesi

Özet

Felent Çayı, Porsuk Çayı'nın en önemli kollarından biridir ve Kütahya'nın sanayi, tarım ve kaplıca kaynaklı atıklarından etkilenmektedir. Bu çalışmada, sistemin yukarı, aşağı ve rezervuar kısımlarını içeren Felent Çayı'nın su kalitesi araştırılmıştır. Bu amaçla, havzada kirlilik kaynaklarını değerlendirilerek seçilen yedi istasyondan mevsimsel olarak alınan su örnekleri tıplanmış (2010 - 2011) ve bu numunelerde sıcaklık, pH, çözünmüş oksijen, amonyum azotu, nitrit azotu, nitrat azotu, sülfat, fosfat, kimyasal oksijen ihtiyacı (KOİ), toplam fosfor, kalsiyum, magnezyum ve sodyum parametreleri belirlenmiştir. Ayrıca, araştırılan istasyonlar arasındaki önemli su kalitesi farklılıklarını değerlendirmek için tespit edilen verilere Tek Yönlü ANOVA testi uygulanmıştır. Tespit edilen verilere göre, Felent Çayı'nın, özellikle Kütahya İli evsel atıklarından ve Yoncalı Köyü kaplıcalarından kaynaklanan önemli bir organik - termal kirliliğin etkisi altında olduğu tespit edilmiştir.

Anahtar kelimeler: su kalitesi, Felent çayı, tek yönlü ANOVA

1. Introduction

Lotic ecosystems play a significant role as a receiving environment of domestic – industrial – agricultural wastewater. Seasonal variations of rains, surface runoff, and groundwater flow have a strong effect on river discharges and concentration of pollutants in the river water. Water quality monitoring of lotic ecosystems helps to evaluate the pollution sources of the systems, provide an effective management of water resources and also protect the aquatic life (Shresta and Kazama, 2007; Strobl and Robillard, 2008; Tokatlı, 2013; Tokatlı et al., 2014; Köse et al., 2015; Tokatlı, 2015).

* *Corresponding author* / Haberleşmeden sorumlu yazar: Tel.: +902846161348; Fax.: +902846163534; E-mail: tokatlicem@gmail.com
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Felent Stream is one of the most important branches of Porsuk Stream that is the most important element of Sakarya River Basin. It has a Dam Lake (Enne Dam Lake) on the watershed boundaries and exposed to a significant pollution. Agricultural applications, thermal hotels located on the upstream of the basin and solid waste storage area located on the downstream of the basin are known as the main pollution factors for the system (Çiçek et al., 2013; Tokatlı et al., 2013).

The aim of this study was to evaluate the water quality of Felent Stream Basin by determining some limnologic parameters including temperature, pH, dissolved oxygen, ammonium nitrogen, nitrite nitrogen, nitrate nitrogen, sulphate, phosphate, chemical oxygen demand (COD), total phosphorus, calcium, magnesium, potassium, which are especially thought to best reflect the agricultural and organic pollution on the system.

2. Materials and methods

2.1. Study area

Kütahya Province is one of the most important locations in Turkey due to being the vanishing point of different phyto – zoo geographical regions (Irano – Turanian, Mediterranean, and European – Siberian). Kütahya, which is located at the junction of the Sakarya River Basin, in the Inner Anatolian part of the Aegean Region, extends between the souths – western edge of an alluvial plain watered by Felent Stream (Solak et al., 2012).

Felent Stream has a length of 35 km and the average flow rate of the stream is 0.56 m3/s. It is the most important branch of Porsuk Stream and exposed to a significant pollution (Anonymous, 2006; Tokatlı et al., 2012). The study area and selected stations on the Felent Stream Basin are given in Figure 1. Water samples were collected seasonally between the dates of February 2011 – January 2012.

F1 station was located quite close to the source of Felent Stream and away from pollution and any waste discharge. F2 station was located on the Köprüören Village and agricultural activities are intensively conducted around this station. F3 station was located on the Yoncalı Village, where known as the termal turistic place of Kütahya Province. F4 station was located on the Enne Dam Lake and water samples were collected from a close area to the output of the reservoir. F5 station was located on the Felent Stream after from the output of the Enne dam Lake. F6 station was located quite close to the solid waste disposal site of Kütahya Province. F7 station was located in the Kütahya Province and it was the last station before falling Porsuk Stream.

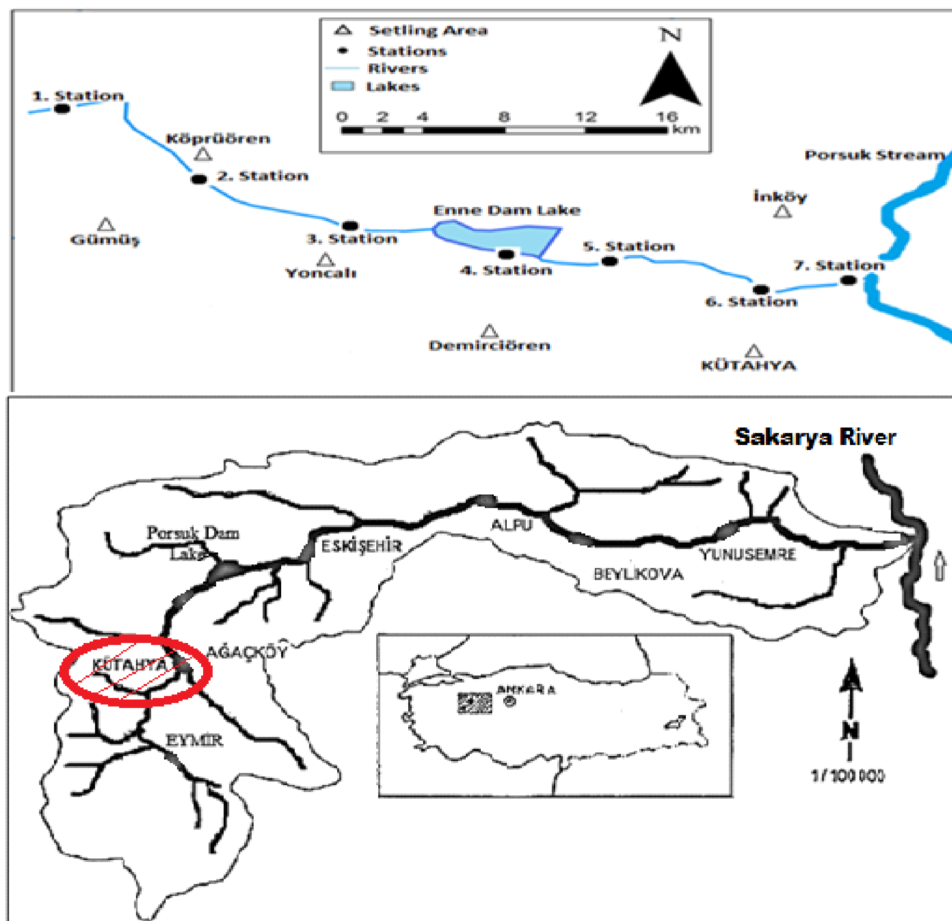


Figure 1. Porsuk Stream Basin, Felent Stream and selected stations

2.2. Physicochemical analysis

Measurements of temperature (T), pH, dissolved oxygen (DO) and electrical conductivity (EC) in water of Felent Sreek were performed in situ by using Hach branded (HQ40d) Portable Multi – Parameter Measurement Device during the field studies.

Ammonium nitrogen (NH_4–N), nitrite nitrogen (NO_2–N), nitrate nitrogen (NO_3–N) and sulphate (SO_4), phosphate (PO_4), chemical oxygen demand (COD) were measured by using Hach branded (DR 2800) Spectrophotometer Device.

Water samples of one liter that were taken at each sampling point were adjusted to pH 2 by adding 2 ml of nitric acid into each for determination of Ca, Mg, Na and K. Afterwards, the samples were filtered (cellulose nitrate, 0.45 µm) in such a way as to make their volumes to 100 ml.

For determination of total phosphorus in water, 100 ml from samples were transferred to a 250-ml beaker and 2 ml (1+1) of nitric acid and 1 ml (1+1) of hydrochloric acid were added. And then put on hot plate for evaporation to nearly dryness, making certain that the samples do not boil at 85^0C. Sample volume was come down to approximately 20 ml. Afterwards, the samples were filtered (cellulose nitrate, 0.45 µm) in such a way as to make their volumes to 50 ml with ultra-pure water. Total phosphorus, calcium, magnesium, potassium and sodium elements were measured by using ICP – OES device (U.S. EPA, 2001).

2.3. Statistical analysis

One – Way ANOVA test was applied to detected data by using SPSS 17 statistical packed program in order to evaluate the significant differences among the stations in terms of investigated water quality parameters at the 0.05 – 0.01 levels. Scatter – dot diagrams were applied to detected data by using SPSS 17 statistical packed program in order to provide a visual summary of investigated parameters to make them easy to compare with water quality standards.

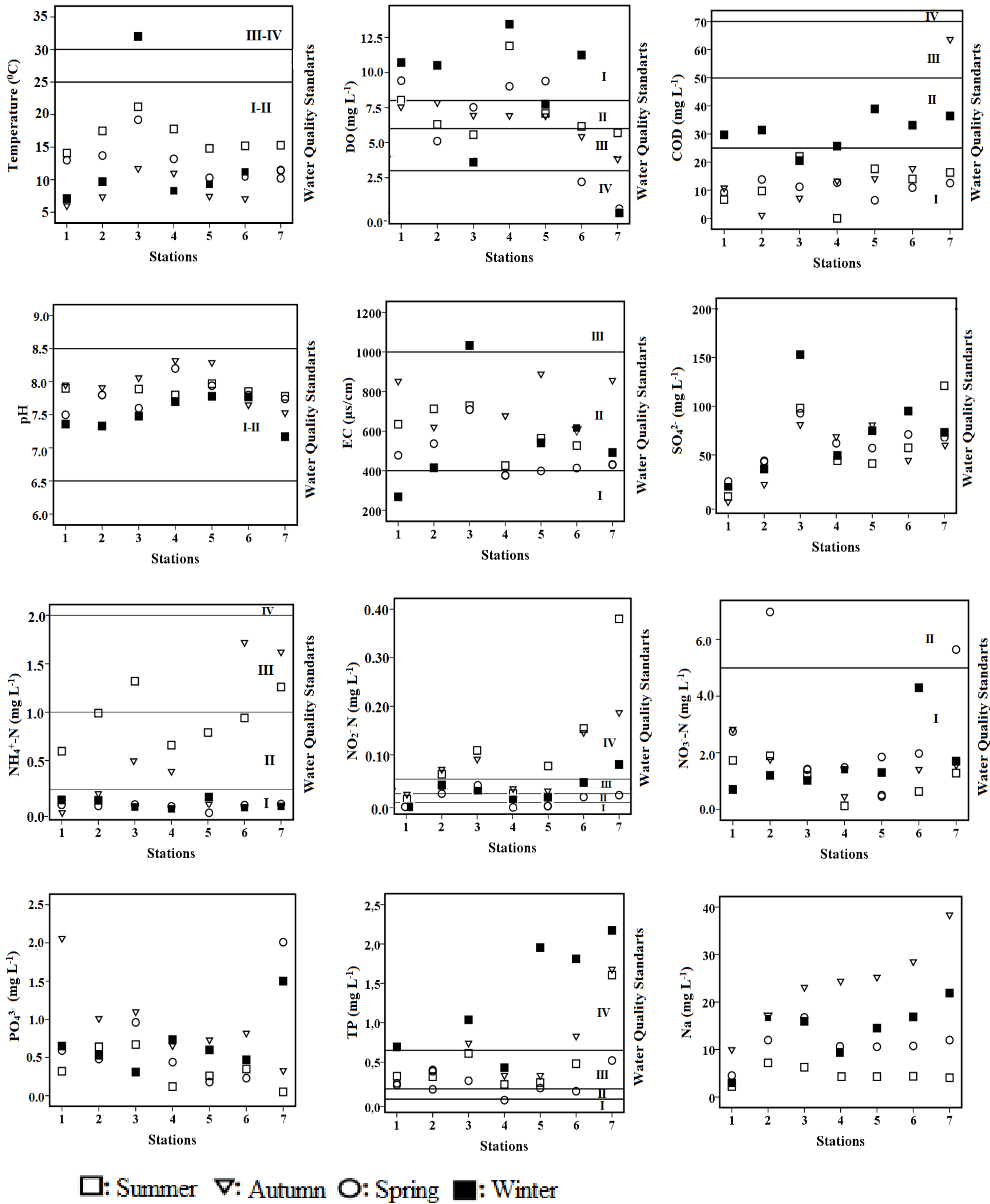
3. Results

Seasonally water quality values recorded in water of Felent Stream are given in Figure 2 and also annual water quality values with the results of One – Way ANOVA test are given in Table 1. Water quality regulations in Turkey separate the inland waters into four classes; Class I refers to high – quality water that can be used for high potential for drinking water and recreational purposes, Class II refers to less contaminated water that can be used as potential for drinking water and recreational purposes, Class III includes polluted water that can only be used as industrial water after treatment, and Class IV refers to heavily polluted water that should not be used at all (Turkish Regulations, 2012).

Ca, Mg, K and Na values in water of Felent Stream were stable in general and they were ranged between 20.99 – 201 mg L^{-1}, 6.87 – 74.61 mg L^{-1}, 1.31 – 60.5 mg L^{-1} and 2.23 – 38.35 mg L^{-1} respectively. According to the results of One – Way ANOVA test, no statistically significant differences was found among the stations in terms of Ca, Mg, K and Na levels. The temperature values detected in the 3rd station were significantly higher than the other stations ($p<0.05$) and the highest temperature value was measured in 3rd station as 32 ^{0}C in winter season. This station was located in the Yoncalı Village of Kütahya and there are many thermal hotels around there. Thermal waste waters of Yoncalı Village were being discharged into the Felent Stream on this point and this sudden increase of temperature value in water has a significant negative impact on the aquatic flora and fauna in this location. In a study performed in the same stream by Tokatlı et al (2012), water quality of this location was found to be in a very low level in terms of especially temperature values.

Changes in pH values in surface water ecosystems can be indicative of an industrial pollutant, photosynthesis or the respiration of algae that is feeding on a contaminant. Most ecosystems are sensitive to changes in pH and the monitoring of pH has been incorporated into the environmental laws of most industrialized countries (Ugwu and Wakawa, 2012). In a macroscopic point of view, Felent Stream has an alkaline water characteristics and pH data in water of Felent Stream were recorded between the values of 7.17 (in station 7th, winter season) and 8.29 (in station 5th, spring season). According to the results of One – Way ANOVA test, no statistical differences were determined among the stations in terms of pH values ($p<0.05$).

Electrical Conductivity (EC) in natural waters is the normalized measure of the water's ability to conduct electric current. This is mostly influenced by dissolved salts such as sodium chloride and potassium chloride (Ugwu and Wakawa, 2012). The highest EC values in Felent Stream were determined in 3rd station as 1033 µs/cm in winter season and according to the Turkish Regulations, Felent Stream has II. Class water quality in general in terms of EC values (Turkish Regulations, 2012). In a study performed by Solak et al. (2012), water quality of Felent Stream were investigated. According to the results of this study, Felent Stream had II. – III. Class water quality in general in terms of EC and temperature parameters as similar to the present study. They also reported that Felent Stream was significantly affected by domestic sewage and thermal tourism and this situation may cause these extreme temperature and EC values detected in especially Yoncalı Village. The amount of dissolved oxygen in the water at any time is depending on temperature of water, partial pressure of gas in the atmosphere in contact with the water surface, salinity of water, biological processes in water (Tanyolaç, 2009).



□: Summer ▽: Autumn ○: Spring ■: Winter

I-II-III-IV. Classis: Water Quality Standards for Turkish Regulations

Figure 2. Seasonally values of some water quality parameters.

The highest DO value was recorded in 4th station as 13.45 mg L^{-1} in winter season and the lowest DO values were recorded in 7th station in general in all seasons. The DO values detected in the 7th station were also significantly lower than the other stations ($p<0.05$). This station was located on the output of Kütahya Province and affected by domestic, industrial and agricultural wastes. Also it was the last sampling point before falling the Porsuk Stream. In a study performed in Porsuk Stream by Köse et al. (2016), DO values detected in the output of Kütahya Province were in a very low level (annual means of DO values was 2.74 mg L^{-1}).

The highest COD value was determined in 7th station as 63.6 mg L^{-1} in autumn season. But according to the results of One – Way ANOVA test, no statistically significant difference was determined among the stations in terms of COD levels ($p<0.05$). According to the Turkish Regulations, Felent Stream has I. – II. Class water quality in general in terms of COD levels (Turkish Regulations, 2012).

The highest NO_2–N was found as 0.38 mg L^{-1} in 7th station. According to Turkish Regulations (2012), 3th, 6th and 7th stations of Felent Stream has III. Class water quality in terms of nitrite levels and also NO_2–N values recorded in water of station 7th was significantly higher than other stations ($p<0.05$). In a study performed in Kütahya Province, trophic status of Upper Porsuk Stream Basin was investigated by using some diatom indices. According to the results of this study, water quality levels were found as eutrophic and organically polluted in general as similar to the present study (Solak, 2011). The highest ammonia nitrogen was determined in 6th station as 1.72 mg L^{-1} in autumn season and the lowest ammonia nitrogen was determined in 5th station, where was located on the output of the dam lake, as 0.028 mg L^{-1} in spring season. Nitrate values determined in the basin were in low levels in general and according to Turkish Regulations, Felent Stream has I. Class water quality in terms of nitrate values (Turkish Regulations, 2012).

The highest total phosphorus levels were determined in 7th station and also total phosphorus levels recorded in this station were significantly higher than the other stations ($p<0.05$). According to Turkish Regulations, 3rd, 5th, 6th and 7th stations of Felent Stream has IV. Class water quality in terms of this parameter (Turkish Regulations, 2012). These results may reflect that agricultural, domestic, and livestock activities conducted around the Felent Stream Basin adversely affect the water quality. As similar to the present study, Varol et al. (2012) reported that total phosphorus and nitrate levels detected in winter and spring seasons were higher than detected in summer and autumn seasons in Tigris River because of high surface runoff during the rainy seasons.

4. Conclusions and discussion

In this study, water quality of Felent Stream Basin was evaluated by investigating some water quality parameters and by using One – Way ANOVA test. As a result of the present study, it can be concluded that Felent Stream Basin is under effect of a significant agricultural, domestic and thermal pollution and this adverse situation also cause to reduce the water quality of Porsuk Stream. It can also concluded that the reservoir located on the watershed has a significant cleaning capacity and water quality of Felent Stream was increasing after output of the dam lake.

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Contents / İçindekiler

Yayın No./Sayfa No.

01. **1 The Herpetofauna of Karçal Mountains (Artvin/Turkey)**
02. **6 Flora of alpine grasslands of the Eğribel pass in the Giresun mountains (Turkey)**
03. **18 LC-MS/MS and GC-MS analyses of three endemic *Astragalus* species from Anatolia towards their total phenolic-flavonoid contents and biological activities**
04. **31 The bryophyte flora of Baskil district (Elazığ/Turkey)**
05. **39 Morpho-anatomical investigations on *Ajuga postii* Briq and *Ajuga relicta* P.H.Davis**
06. **49 Some geophyte plants determined in Bartın/Turkey**
07.**55 Mycorrhizal diversity in some species of *Dactylorhiza* genus (Orchidaceae)**
08. **65 Antimycobacterial activity of some lichen extracts**
09. **69 Nineteen new records for Turkish freshwater algal flora from Lake Taşkısığı and Lake Little Akgöl**
10. **79 *Erysiphe azaleae*, a new powdery mildew fungus introduced into Turkey**
11. **84 Genotypic response on stability for yield and nitrogen use efficiency in triticale (X *Triticosecale* Wittmack) under different nitrogen regimes**
12. **92 Alkaliphilic bacterial diversity of Lake Van/Turkey**
13. **104 Seasonal differences in the muscle fatty acid profiles of two freshwater fish species (*Scardinius erythrophthalmus, Squalius cephalus*)**
14. **110 Antifeedant effects of *Ferulago longistylis* extracts from Erzincan on *Ephestia kuehniella***
15. **115 Contents of phenolic and flavonoid compounds in *Isatis demiriziana* Mısırdalı: an endemic to the Southeast Anatolia, Turkey**
16. **124 Influence of surface kind on biofilm formation by *Salmonella* spp. and *Listeria monocytogenes* from food-contact surfaces**
17. **133 Macrofungi biodiversity of Kütahya (Turkey) province**
18. **144 The rediscovery of *Silene surculosa* Hub.-Mor. endemic for Turkey**
19. **153 Toxicological effects of the entomopathogenic *Purpureocillium lilacinus* on the model organism, *Galleria mellonella***
20. **160 Water quality assessment of Felent Stream (Kütahya/Turkey)**

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