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Antimicrobial activities of chitosan-based edible films produced by adding different macrofungi extracts and plants essential oils

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Farklı makromantar ekstraktları ve bitki uçucu yağları eklenerek üretilen kitosan bazlı yenebilir filmlerin antimikrobiyal aktiviteleri

Abstract: This research aims to reveal the antimicrobial activity of chitosan-based edible films containing different mushroom extracts and plant essential oils. In this study, edible films were produced using the essential oils of *Satureja cuneifolia* Ten., *Mentha longifolia* (L.) Hudson subsp. t*yphoides* (Brig.) Harley var. *typhoides* (L.) Hudson and extracts of *Amanita caesarea* (Scop.) Pers. and *Boletus reticulatus* Schaeff. collected from different localities of Osmaniye province. The antimicrobial activities of these films were investigated on *Escherichia coli* by using the Kirby-Bauer disk diffusion test. At the end of the research, it was determined that the edible film obtained by adding *S. cuneifolia* Ten. essential oil (3%) had the highest antimicrobial activity. And also, it could be said that all the edible films produced had antimicrobial activity.

Key words: Antibacterial activity, edible film, volatile, mushroom

Özet: Bu araştırma, farklı mantar ekstraktı ve bitki uçucu yağı içeren kitosan temelli yenilebilir filmlerin antimikrobiyal aktivitesini açığa çıkarmayı amaçlamaktadır. Bu çalışmada, Osmaniye'nin farklı lokalitelerinden toplanan *Satureja cuneifolia* Ten., *Mentha longifolia* (L.) Hudson subsp. *typhoides* (Brig.) Harley var. *typhoides* (L.) Hudson'un uçucu yağları ve *Amanita caesarea* (Scop.) Pers. ve *Boletus reticulatus* Schaeff. ekstraktları kullanılarak yenebilir filmler üretilmiştir. Üretilen bu filmlerin *Escherichia coli* üzerindeki antimikrobiyal aktiviteleri Kirby-Bauer disk difüzyon testi kullanılarak araştırılmıştır. Araştırma sonunda, *S. cuneifolia* Ten.'in uçucu yağının (%3) ilave edilmesiyle üretilen yenilebilir filmin en yüksek antimikrobiyal aktiviteye sahip olduğu belirlenmiştir. Bununla birlikte, üretilen tüm yenebilir filmlerin antimikrobiyal aktiviteye sahip olduğu söylenebilir.

Anahtar Kelimeler: Antibakteriyel aktivite, yenebilir film, aroma, mantar

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1. Introduction

The deterioration of synthetic plastic materials has been a major cause of the growing interest in edible films in recent years (Kumar et al., 2022). Plastics are among the waste elements that harm the environment when interacting with nature since they take a long time to break down in the natural world (Lakhiar et al., 2024). However, they swiftly deteriorate after being thrown away, edible coatings from consumable goods can improve food quality by extending its shelf life (Xu et al., 2005). Chitosan is one of the polymer materials that is commonly utilized in the creation of edible films (Ebrahimi et al., 2024; Singh et al., 2024). It is created by deacetylating chitin, which is found in large quantities in shrimp and crab shells (Priyadarshi et al., 2018; Campalani et al., 2024). Chitin becomes more reactive and soluble in diluted acetic and citric acids, as a result of the deacetylation process (Ma et al., 2021). A valuable and promising biomaterial, chitosan is a derivative of chitin and a helpful bioactive polymer that has gained attention recently (Safarzadeh et al., 2024). Important characteristics of the edible polymer chitosan include its adsorption, non-toxicity, biodegradability, and biocompatibility (Amin et al., 2024). Chitosan finds application in the food business because of its unique chemical and physical characteristics (Gutiérrez, 2017). Chitosan film is a viable substitute for commercial polymers used to prolong food shelf life because it is a rigid, flexible, semi-permeable substance that rips readily (Butler vd., 1996; Kittur vd., 1998). In recent years, studies on chitosan-based edible films have started to examine their antibacterial and antioxidant properties by adding essential oils derived from aromatic and medicinal plants (Altiok et al., 2010). The biological effects of essential oils differ since they are composed of a diverse range of chemicals (Wińska et al., 2019; Parveen et al., 2024).

Numerous experts have found that non-toxic biocompatible chitosan has antibacterial and antioxidant properties in earlier investigations (Begin and Calsteren, 1999; Xie et al., 2001; Rabea et al., 2003; Yen et al., 2008; Goy et al., 2009; Raafat and Sahl, 2009; Kong et al., 2010; Siripatrawan and

Harte, 2010; Leceta et al., 2013; Ke et al., 2021). Several investigations have been carried out to examine the antibacterial and antioxidant activities of mushroom extracts (Mau et al., 2002; Yang et al., 2002; Cheung et al., 2003; Gao et al., 2005; Gezer vd., 2006; Puttaraju et al., 2006; Barros et al., 2007; Jayakumar et al., 2009; Smolskaite et al., 2015). A few studies were conducted previously on edible films produced by adding essential oil and mushroom extract (Kumar et al., 2021; John, 2022; Kaya et al., 2022). However, there is no sufficient study in the literature on chitosan-based films containing the extracts of *Boletus reticulatus* Schaeff. and *Amanita caeserea* (Scop.) Pers. and the essential oils of *Satureja cunefolia* Ten., and *Mentha longifolia* (L.) Hudson *subsp. typhoides* (Brig.) Harley *var. typhoides* (L.) Hudson. This study aims to investigate the antimicrobial activities of chitosan-based edible films obtained by adding the extracts of *A. caesarea* (Scop.) Pers., and *B. reticulatus* Schaeff. macrofungi and the essential oils of *S. cunefolia* Ten., and *M. longifolia* (L.) Hudson *subsp. typhoides (Brig.) Harley var. typhoides* (L.) Hudson. plants collected from Osmaniye province.

2. Materials and Method

2.1. Sampling, extraction, production and characterization of edible films

The localities of plant and fungal species are given in Table 1. Edible film production steps and physical and chemical analyses of the produced films were mentioned in the studies conducted by Bülbül et al., (2023) and Gökyermez et al., (2023). Plant and fungal species were collected and identified by the last author.

2.2. Antimicrobial activity

In the study, *Escherichia coli* was used to determine the antimicrobial activities of the edible films. The Kirby-Baurer disc diffusion assay was used to determine the antibacterial activities of the produced edible films. According to the assay, the commercial Müller-Hinton Agar (MHA, Merck) medium was autoclaved at 121°C. After the processing, the obtained hot liquid (70 °C) medium was poured into sterile petri plates of 9 cm diameter. After the cooling of medium, The bacterial strain was spread on the surface of the rigid MHA plate with a swab and pre-incubated for 10 minutes. The inoculated samples were incubated at 25 °C for 15 minutes and then at 37ºC for 24 hours. The resulting inhibition zones were measured with a ruler in mm (Sedefoğlu et al., 2023). Assays were performed in three replicates.

2.3. Statistical analysis

The results were evaluated using the one-way analysis of variance Duncan test (95% confidence interval) with the help of the Statistical Package for the Social Sciences (SPSS) program (IBM Statistics, USA) (Version 18.0). In addition, the effects of the variables on the outputs were determined by two-way analysis of variance.

3. Results and Discussion

The research by Bülbül et al. (2023) and Gökyermez et al. (2023) displayed the edible films' physical (thickness, moisture content, water solubility, opacity, UV-Vis, FTIR, and SEM) and chemical analysis (total phenolic compound content, DPPH activity) results. However, *E. coli* was used to examine the films' antibacterial properties in the present study. As seen in Figure 1, it was determined that antimicrobial activity increased with the increase in the amount of mushroom extracts and essential oils added to the edible films. It was determined that the edible film obtained by adding *S. cuneifolia* Ten. essential oil (3%) had the highest antimicrobial activity. And also, it could be said that all the edible films produced had antimicrobial activity.

In several previous studies, chitosan-based edible films were produced using different samples, and their various activities (antioxidant and antimicrobial activities, etc.) were investigated (Yuan et al., 2016; Hromiš et al., 2017; Kaya et al., 2018; Xu et al., 2021; Sarfraz et al., 2024). It was determined that these produced films had antioxidant and antimicrobial activities.

Gómez-Estaca et al. (2009) produced a chitosan-based film utilizing clove oil and used this film to preserve raw sliced salmon and investigated its antibacterial properties against the bacterial strains (*L. acidophilus, P. fluorescens, L. innocua* and *E. coli*.) At the end of the study, a decrease in total bacterial growth was observed after 11 days of storage, and therefore it was stated that, the produced films are suitable for use as an active packaging substance applied to fish products. In the study conducted by Sánchez-González et al. (2009), chitosan-based edible films including different concentrations of *Melaleuca alternifolia* (Maiden & Betche) Cheel essential oil, were obtained and the water vapor permeability, mechanical, and optical features of dried films were investigated to evaluate the effect of incorporating essential oil into these films. In addition, composite films were photographed by utilizing a scanning electron microscope (SEM) and their antibacterial features were tested against two bacterial strains (*Listeria monocytogenes* and *Penicillium italicum*). It was found that, chitosan-based films have a significant antimicrobial activity. Handayasari et al (2019) produced chitosan-based films containing garlic oil and nitrite-added gelatin-chitosan. They also investigated the mechanical properties and antibacterial effect of the films. At the end of the study, the tensile strength of the film increased gradually with the increasing amount of chitosan, but the elongation at break decreased. Furthermore, with the addition of the oil, the antibacterial activity of the films increased significantly. In

Table 1. Localities of plant and fungal species

Samples	Localities	GPS coordinates	Collection Date
Mentha longifolia subsp. *typhoides* var. *typhoides*	Bahçe, Yukarı Kardere village, Mandal deresi place	37º 13'K, 36º 37'D, 878 m,	10.07.2021
Satureja cuneifolia	Düziçi, Baskonuş high plateau, Odunluk place	37º 21'K, 36º30'D, 1681 m,	07.09.2021
Amanita caesarea	Osmaniye, Zorkun high plateau, Karıncalı place	36º57'K, 36º20'D, 1324 m	08.10.2021
Boletus reticulatus	Osmaniye, Zorkun high plateau, Karıncalı place	36º57'K, 36º19'D, 1240 m	08.10.2021

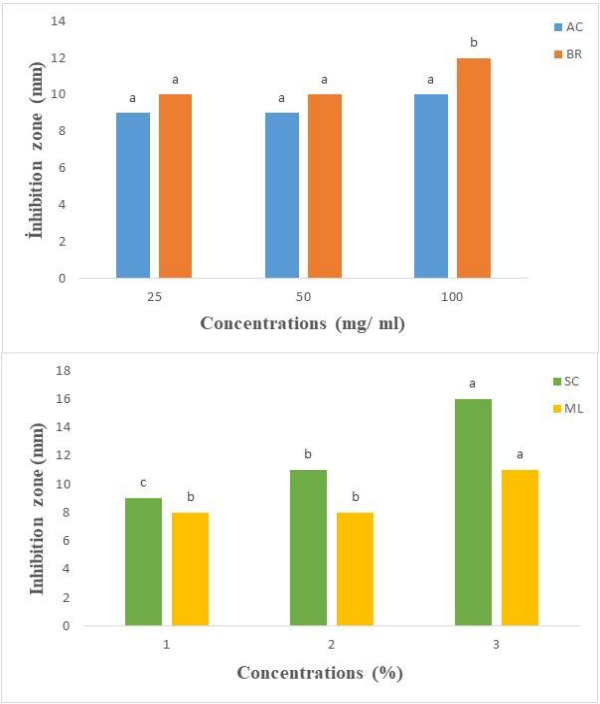

Figure 1. Antimicrobial activities of chitosan-based edible films (AC: *Amanita caesarea*, BR: *Boletus reticulatus*, SC: *Satureja cunefolia*, ML: *Mentha longifolia* subsp. *typhoides* var. *typhoides*). Mean with different letter in columns is significant at $p<0.05$.

the study by Şimşek et al. (2020), essential oils of *Eucalyptus globulus* Labill., *Schinus molle* L., and *Santolina chamaecyparissus* L. gathered in Osmaniye (province of Türkiye) were extracted by using a Clevenger apparatus. Carboxy-methyl cellulose films were obtained by utilizing different concentrations of the oils to investigate the physical, chemical, and antibacterial effects of these films. The conclusion of the study revealed that films with added essential oil exhibited significant changes in their physicochemical properties and antimicrobial effects compared to the control groups. In another study, the chitosan-based films with the included water-based extract of *Tricholoma terreum* (Schaeff.) P. Kumm. gathered from the İnönü plateau in Sakarya province of Türkiye, were produced by Koç et al (2020). The phenolic content, optical transparency, thermal analysis, thermo-gravimetric analysis, functional groups by FTIR, water contact angles, and solubility in water, along with antioxidant, antimicrobial, and anti-quorum sensing activities were tested to determine the potential of the films. As a result of this study, the phenolic content of 0.1 g of fungi extract included 2659.82 μg g^{-1} of p-coumaric acid and 2126.69 μg g^{-1} of gallic acid. Moreover, the researchers found that, the chitosan-based films including fungi extract have a thickness of 190 μm and a light brown color. Furthermore, the films have optical transparency ranging from 17% to 53% and water contact angles of 76.06 degrees. Lastly, the elongation of break lengths of the films was determined to increase by 111.1%, when compared to the control group. In terms of antioxidant activity, the films had more than double the effect of the control group. Similarly, the study showed that the films had remarkable antimicrobial activity compared to the control group as well. In the research conducted by Savin et al. (2020), chitosan-based films with added extract of *Ganoderma lucidum* (Curtis) P. Karst., which was obtained from Romania medica SRL laboratories exhibited substantial effects of antioxidant, cytotoxic, and antimicrobial. It was revealed that, the films had more effective antimicrobial activity against Gram-positive bacterial strains than against Gram-negative bacterial strains and that in terms of antioxidant tests such as ABTS and DPPH, the films showed a moderate level of activity.

Conflict of Interest

The authors have declared no conflict of interest.

Authors' Contributions

The authors contributed equally.

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References

Altiok D, Altiok E, Tihminlioglu F (2010). Physical, antibacterial and antioxidant properties of chitosan films incorporated with thyme oil for potential wound healing applications. Journal of Materials Science: Materials in Medicine 21: 2227-2236.

Amin T, Naik HR, Hussain SZ, Wani SM (2024). Polysaccharide Based Films for Food Packaging: Fundamentals, Properties and Applications. Singapore: Springer Verlag.

Barros L, Calhelha RC, Vaz JA, Ferreira IC, Baptista P, Estevinho LM (2007). Antimicrobial activity and bioactive compounds of Portuguese wild edible mushrooms methanolic extracts. European Food Research and Technology 225: 151-156.

Bégin A, Van Calsteren MR (1999). Antimicrobial films produced from chitosan. International Journal of Biological Macromolecules 26(1): 63-67.

Butler BL, Vergano PJ, Testin RF, Bunn JM Wiles JL (1996). Mechanical and barrier properties of edible chitosan films as affected by composition and storage. Journal of Food Science 61(5): 953-956.

Bülbül M, Süfer Ö, Bozok F (2023). Bazı Aromatik Bitkilerin Uçucu Yağlarını İçeren Yenebilir Film Üretimi. In Talaz O (edt.) Fen Bilimleri ve Matematik Alanında Gelişmeler. Ankara: Platanus Publishing. pp: 99-128.

Campalani C, Bertuol I, Bersani C, Calmanti R, Filonenko S, Rodríguez-Padrón D, Perosa A (2024). Green extraction of chitin from hard spider crab shells. Carbohydrate Polymers 345: 122565.

Cheung LM, Cheung PC, Ooi VE (2003). Antioxidant activity and total phenolics of edible mushroom extracts. Food Chemistry 81(2): 249-255.

Ebrahimi SS, Hamzeh Y, Ashori A, Roohani M, Marlin N, Spigno G (2024). Ozone-activated lignocellulose films blended with chitosan for edible film production. International Journal of Biological Macromolecules 270: 132285.

Gao Y, Tang W, Gao HE, Chan E, Lan J, Li X, Zhou S (2005). Antimicrobial activity of the medicinal mushroom Ganoderma. Food Reviews International 21(2): 211-229.

Gezer K, Duru ME, Kivrak I, Turkoglu A, Mercan N, Turkoglu H, Gulcan S (2006). Free-radical scavenging capacity and antimicrobial activity of wild edible mushroom from Turkey. African Journal of Biotechnology 5(20): 1924-1928.

Gómez-Estaca J, López-Lacey A, Gómez-Guillén MC, López-Caballero ME, Montero P (2009). Antimicrobial activity of composite edible films based on fish gelatin and chitosan incorporated with clove essential oil. Journal of Aquatic Food Product Technology 18(1-2): 46-52.

Goy RC, Britto DD, Assis OB (2009). A review of the antimicrobial activity of chitosan. Polímeros 19: 241-247.

Gökyermez M, Süfer Ö, Bozok F (2023). Edible film production containing *Amanita caesarea* and *Boletus reticulatus* extracts. In: Akpınar A (edt.), Research on Mathematics and Science- III. Özgür Publications. pp. 1-26.

Gutiérrez, TJ, Tomy J (2017). Chitosan applications for the food industry. In: Ahmed S, Ikram S (eds), Chitosan: Derivatives, Composites and Applications. pp. 183-232.

Handayasari F, Suyatma NE, Nurjanah S (2019). Physiochemical and antibacterial analysis of gelatin–chitosan edible film with the addition of nitrite and garlic essential oil by response surface methodology. Journal of Food Processing and Preservation 43(12): e14265.

Hromiš NM, Lazić VL, Popović SZ, Šuput DZ, Bulut SN (2017). Antioxidative activity of chitosan and chitosan-based biopolymer film. Food and Feed Research 44(2): 91-100.

Jayakumar T, Thomas PA, Geraldine P (2009). *In-vitro* antioxidant activities of an ethanolic extract of the oyster mushroom, *Pleurotus ostreatus*. Innovative Food Science & Emerging Technologies 10(2): 228-234.

John S (2022). Fungal Biopolymers as an alternative construction material. In: John S (edt.) Fungal Biopolymers and Biocomposites, Prospects and Avenues Singapore: Springer Nature. pp. 169-188.

Kaya E, Kahyaoglu LN, Sumnu G (2022). Development of curcumin incorporated composite films based on chitin and glucan complexes extracted from *Agaricus bisporus* for active packaging of chicken breast meat. International Journal of Biological Macromolecules 221: 536-546.

Kaya M, Ravikumar P, Ilk S, Mujtaba M, Akyuz L, Labidi J, Erkul SK (2018). Production and characterization of chitosan based edible films from *Berberis crataegina*'s fruit extract and seed oil. Innovative Food Science & Emerging Technologies 45: 287-297.

Ke CL, Deng FS, Chuang CY, Lin CH (2021). Antimicrobial actions and applications of chitosan. Polymers 13(6): 904.

Kittur FS, Kumar KR, Tharanathan RN (1998). Functional packaging properties of chitosan films. Zeitschrift für Lebensmitteluntersuchung und-Forschung A 206: 44-47.

Koc B, Akyuz L, Cakmak YS, Sargin I, Salaberria AM, Labidi J, Kaya M (2020). Production and characterization of chitosan-fungal extract films. Food Bioscience 35: 10054.

Kong M, Chen XG, Xing K, Park HJ (2010). Antimicrobial properties of chitosan and mode of action: a state of the art review. International Journal of Food Microbiology 144(1): 51-63.

Kumar A, Hasan M, Mangaraj S, Pravitha M, Verma DK, Srivastav PP (2022). Trends in edible packaging films and its prospective future in food: a review. Applied Food Research 2(1): 100118.

Kumar H, Bhardwaj K, Sharma R, Nepovimova E, Cruz-Martins N, Dhanjal DS, Kuča K (2021). Potential usage of edible mushrooms and their residues to retrieve valuable supplies for industrial applications. Journal of Fungi 7(6): 427.

Lakhiar IA, Yan H, Zhang J, Wang G, Deng S, Bao R, Wang X. (2024). Plastic pollution in agriculture as a threat to food security, the ecosystem, and the environment: An overview. Agronomy 14(3): 548.

Leceta I, Guerrero P, Cabezudo S, de la Caba K (2013). Environmental assessment of chitosan-based films. Journal of Cleaner Production 41: 312-318.

Ma Q, Gao X, Bi X, Xia M, Han Q, Peng M, Wang M (2021). Combination of steam explosion and ionic liquid pretreatments for efficient utilization of fungal chitin from citric acid fermentation residue. Biomass and Bioenergy 145: 105967.

Mau JL, Lin HC, Chen CC (2002). Antioxidant properties of several medicinal mushrooms. Journal of Agricultural and Food Chemistry 50(21): 6072-6077.

Parveen A, Abbas MG, Keefover-Ring K, Binyameen M, Mozūraitis R, Azeem M (2024). Chemical Composition of Essential Oils from Natural Populations of *Artemisia scoparia* collected at different altitudes: Antibacterial, mosquito repellent, and larvicidal effects. Molecules 29(6): 1359.

Priyadarshi, R., Kumar, B., Negi, YS. (2018). Chitosan film incorporated with citric acid and glycerol as an active packaging material for extension of green chilli shelf life. Carbohydrate polymers 195: 329-338.

Puttaraju NG, Venkateshaiah SU, Dharmesh SM, Urs SMN, Somasundaram R (2006). Antioxidant activity of indigenous edible mushrooms. Journal of Agricultural and Food Chemistry 54(26): 9764-9772.

Raafat D, Sahl HG (2009). Chitosan and its antimicrobial potential–a critical literature survey. Microbial Biotechnology 2(2): 186-201.

Rabea EI, Badawy MET, Stevens CV, Smagghe G, Steurbaut W (2003). Chitosan as antimicrobial agent: applications and mode of action. Biomacromolecules 4(6): 1457-1465.

Safarzadeh S, Mozafari MR, Naghib SM (2024). Chitosan-incorporated bioceramic-based nanomaterials for localized release of therapeutics and bone regeneration: An overview of recent advances and progresses. Current Organic Chemistry 28(15): 1190-1214.

Sánchez-González L, Vargas M, González-Martínez C, Chiralt A, Cháfer M (2019). Characterization of edible films based on hydroxypropylmethylcellulose and tea tree essential oil. Food Hydrocolloids 23(8): 2102-2109.

Sarfraz MH, Hayat S, Siddique MH, Aslam B, Ashraf, A, Saqalein M, Muzammil S (2024). Chitosan based coatings and films: A perspective on antimicrobial, antioxidant, and intelligent food packaging. Progress in Organic Coatings 188: 108235.

Savin S, Craciunescu O, Oancea A, Ilie D, Ciucan T, Antohi LS, Oancea F (2020). Antioxidant, cytotoxic and antimicrobial activity of chitosan preparations extracted from *Ganoderma lucidum* mushroom. Chemistry & Biodiversity 17(7): e2000175.

Sedefoglu N, Er S, Veryer K, Zalaoglu Y, Bozok F (2023). Green synthesized CuO nanoparticles using macrofungi extracts: Characterization, nanofertilizer and antibacterial effects. Materials Chemistry and Physics 309: 128393.

Simsek M, Eke B, Demir H (2020). Characterization of carboxymethyl cellulose-based antimicrobial films incorporated with plant essential oils. International Journal of Biological Macromolecules 163: 2172-2179.

Singh M, Saroj R, Kaur D (2024). Optimized chitosan edible coating for guava and its characterization. Measurement. Food 14: 100145.

Siripatrawan U, Harte BR (2010). Physical properties and antioxidant activity of an active film from chitosan incorporated with green tea extract. Food Hydrocolloids 24(8): 770-775.

Smolskaitė L, Venskutonis PR, Talou T (2015). Comprehensive evaluation of antioxidant and antimicrobial properties of different mushroom species. LWT-Food Science and Technology 60(1): 462-471.

Wińska K, Mączka W, Łyczko J, Grabarczyk M, Czubaszek A, Szumny A (2019). Essential oils as antimicrobial agents-myth or real alternative?. Molecules 24(11): 2130.

Xie Y, Xie Z, Zhou N (2001). The study on anti-*Helicobacter pylori* mechanisms of chitosans *in vitro*. Chinese Journal of Digestion 24(11): 655-658.

Xu D, Chen T, Liu Y (2021). The physical properties, antioxidant and antimicrobial activity of chitosan–gelatin edible films incorporated with the extract from hop plant. Polymer Bulletin 78: 3607-3624.

Xu YX, Kim KM, Hanna MA, Nag D (2005). Chitosan–starch composite film: preparation and characterization. Industrial crops and Products 21(2): 185-192.

Yang JH, Lin, HC, Mau JL (2002). Antioxidant properties of several commercial mushrooms. Food Chemistry 77(2): 229-235.

Yen MT, Yang JH, Mau JL (2008). Antioxidant properties of chitosan from crab shells. Carbohydrate Polymers 74(4): 840-844.

Yuan G, Chen X, Li D (2016). Chitosan films and coatings containing essential oils: The antioxidant and antimicrobial activity, and application in food systems. Food Research International 89: 117-128.

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Determination of efficient DNA recovery method in *Pyracantha coccinea* for use in forensic botany

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Pyracantha coccinea'da etkili DNA geri kazanım yönteminin belirlenmesi ve adli botanikte kullanımı

Abstract: Forensic Botany aims to uncover the connection between crime scenes, perpetrators, or victims using plant materials. Since the presence of metabolites in plants varies among different species, DNA isolation methods should be adjusted specifically for each plant species and even each plant tissue. In this study, the objective was to determine the most suitable DNA isolation method for the fruits of firethorn (*Pyracantha coccinea* M.Roem.) growing in Türkiye without using liquid nitrogen. In the study, the fruits of Firethorn were divided into two groups as fresh (12 pieces) and dried (12 pieces), which each fruit was subjected to different homogenization methods using a single sample for each analysis. After completing the DNA isolation of the samples, the DNA quantities were measured to determine which method yielded optimal results. The highest DNA quantity of 4.80 ng/µl was obtained from dried fruits homogenized using a mortar and tissue lyser with beads, while the lowest DNA quantity of 0.13 ng/µl was obtained from fresh fruits homogenized only with a kitchen blender. The findings of the study indicate that for both fresh and dried fruits, homogenization with a mortar and tissue lyser with beads would result in a high recovery of DNA from plant materials. This study provides an optimization for DNA isolation in forensic cases where the Firethorn plant may be involved.

Key words: Forensic sciences, forensic botany, plant DNA extraction, homogenization methods, *Pyracantha coccinea* (Firethorn)

Özet: Adli Botanik disiplini bitki materyallerini kullanarak olay yeri, fail veya kurban arasındaki bağlantıyı ortaya çıkarmayı hedeflemektedir. Bitkilerde bulunan ikincil metabolitlerin varlığı farklı bitki türlerinde değişiklik gösterdiğinden DNA izolasyon yöntemleri her bitki türüne hatta her bitki dokusuna özel olarak ayarlanmalıdır. Çalışmamızda, Türkiye'de yetişen Ateş Dikeni (*Pyracantha coccinea* M.Roem.) bitkisinin meyveleri için sıvı nitrojen kullanılmadan en uygun DNA izolasyon metodunun belirlenmesi amaçlanmıştır. Çalışmamızda, ateş dikeni bitkisinin meyveleri taze (12 adet) ve kurutulmuş (12 adet) olarak iki gruba ayrılmış ve her analizde tek meyve kullanılarak farklı homojenizasyon yöntemlerine tabi tutulmuştur. DNA izolasyonu tamamlanan örneklerin DNA miktarları ölçülerek hangi metodun optimum sonuç verdiği belirlenmiştir. En yüksek DNA miktarı 4,80 ng/µl olarak havan ve boncuklu doku öğütücü ile homojenize edilen kurutulmuş meyvelerden elde edilirken, en düşük DNA miktarı ise 0,13 ng/µl olarak yalnızca mutfak robotu ile homojenize edilen taze meyvelerden elde edilmiştir. Çalışmamızın bulguları hem taze hem de kurutulmuş meyveler için, homojenizasyonun havan ve boncuklu doku öğütücü ile gerçekleştirilmesi halinde bitki materyallerinden yüksek miktarda DNA geri kazanımı olacağı yönündedir. Yaptığımız çalışma, Ateş Dikeni bitkisinin dahil olabileceği adli vakalarda DNA izolasyonu için optimizasyon sunmaktadır.

Anahtar Kelimeler: Adli bilimler, adli botanik, bitki DNA izolasyonu, homojenizasyon metotları, *Pyracantha coccinea* (ateş dikeni)

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1. Introduction

Forensic botany is one of the sub-disciplines of forensic sciences that examines various aspects of plants, such as their morphological, anatomical, chemical, and genetic characteristics. DNA can be obtained from the leaves, stems, roots, seeds, flowers, and fruits of plants. Just as identification can be performed for human DNA, it can also be done for animal and plant DNA. The identification of plant materials found at a crime scene can directly link a suspect to the crime scene or the victim. After identifying plant species by examining the morphological and histological features of plant materials, DNA analysis is conducted to perform identification.

In addition to identifying plant specimens, determining which region they belong to, and whether a crime scene is a primary or secondary location, are also within the scope of forensic botany (Aquila et al., 2014). In this context, forensic botany has long held its place within forensic sciences (Craft et al., 2007; Wickneswari and Rajanaidu, 2011).

The first step in examining plant samples is to identify the species. However, since the morphology and anatomy of plant samples obtained from a crime scene are often fragmented or deteriorated, species identification is not always possible. In such cases, the examination should be supported by molecular biology analysis methods (Aquila et al., 2014). In the analysis of plant samples, it is common to encounter the issue of the samples being present in limited quantities as well as the tissue integrity being

compromised. Therefore, the analysis must be conducted with great care. Additionally, since there is no specific identification procedure for each plant species, researchers apply the general protocols provided in the isolation kits or the methods they use in their laboratories. Consequently, using protocols specifically developed for plant species will ensure optimal results in DNA analyses. In the study, which aims to address this deficiency, it was focused on the initial steps of identification (DNA isolation and quantification) using a selected plant with specific parameters.

In this study, the fruits of the Firethorn (*Pyracantha coccinea*) shrub, an evergreen plant with red-orange berries and thorny stems, commonly used for ornamental and security purposes in Atatürk Arboretum Garden borders were used. The plant's perennial and shrubby-thorny structure, which makes it easy to transfer and spread widely, has been an important factor that increases the probability of its presence at the crime scene, and for this reason it was chosen as material in this study (Akguc et al., 2010). The fruit samples of each plant were divided into two groups, fresh and dried, and different methods were used in DNA isolation to investigate which method provided the highest DNA yield. The quantities of the isolated DNA samples were measured, and the results were compared to gain perspective for method optimization. This way, the Firethorn shrub, which can easily grow, will illuminate the path for cases involving theft, murder, sexual assault, etc., with its thorny stem and small fruits.

After analysing the plant species, homogenization, which involves physically breaking down the cell walls found in plants, is required for DNA isolation. Among the commonly used methods in the homogenization step is grinding with liquid nitrogen. However, in this study, an alternative method using a bead tissue grinder and a kitchen blender without liquid nitrogen was used, and the results were examined.

2. Materials and Method

In this study, fruits of the Firethorn (*Pyracantha coccinea*) plant, an evergreen plant with thorny stems and striking orange-red berries that can easily grow in forested areas, found in various regions of Türkiye, were used.

In this context, Firethorn fruits were collected from 12 different shrubs in the province of İstanbul. Of the 12 plants that formed the sample of this study, five were collected from the Atatürk Arboretum in Sarıyer, İstanbul, which provides a reliable resource for educational and scientific research, and the remaining seven were collected from the surroundings of Üsküdar, İstanbul, where the species were identified according to morphological parameters. The fruit samples, carefully collected to prevent contamination, were placed in different paper envelopes, sealed, and transported to the laboratory with ice packs. The fruit samples collected from each shrub were divided into two groups for analysis as fresh and dried in room conditions (24 ºC, %60 humidity) and subjected to different storage conditions. The samples to be studied fresh were stored at +4ºC and analysed within 48 hours of collection, while the dried samples were analysed 12 days after collection, allowing for air circulation and protection from sunlight, with minimal contact.

For each sample to be isolated, the starting material was adjusted to a single fruit (~100 mg). The first stage of the isolation step, homogenization, was performed using a mortar, kitchen blender/hand blender (Sinbo, İstanbul-Türkiye), and bead tissue grinder (TissueLyser LT Qiagen, Hilden-Germany) without liquid nitrogen. Half of the fresh fruits were ground in a mortar and pulverized in a bead tissue grinder according to the "Purification of DNA from Plant Tissues" protocol found in the device manual, while the other half was homogenized using only the kitchen blender (QIAGEN® Technologies, 2009). For dried fruits, the samples that were ground in the blender were also ground in a mortar. The DNA of the samples homogenized with different combinations was isolated using the "Fruit" protocol contained in the i-genomic Plant DNA Extraction Mini Kit (iNtRON Biotechnology, 2012). In order to avoid contamination, the blender and mortar were sterilized by keeping them under bleach and UV light after each sample process.

The DNA concentration of the isolated samples was determined using the Qubit 4 Fluorometer (Invitrogen, Massachusetts-United States) device according to the protocol included in the Qubit™ dsDNA HS Assay Kit (Thermo Fisher Scientific Inc, Massachusetts-United States) (Thermo Fisher Scientific Inc., 2022) (Figure 1).

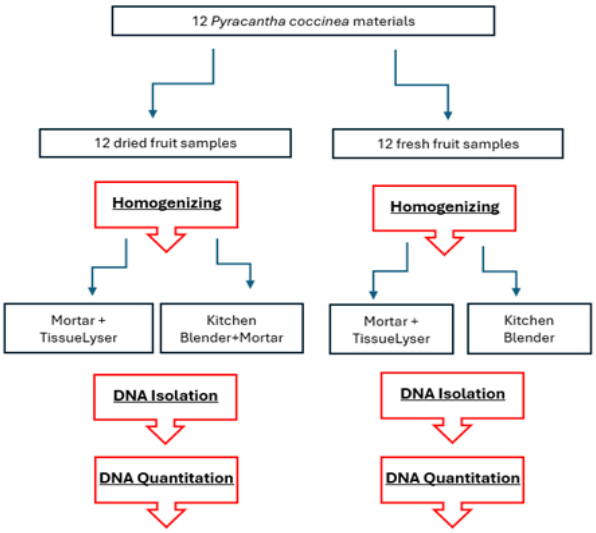

Figure 1. Flow chart of the study.

For statistical analysis Wilcoxon Signed-Rank Test in SPSS Statistics for Windows, version 20.0 (SPSS Inc., Chicago, Ill., USA) program was used to compare sample results.

3. Results

To find the most suitable DNA isolation method in this study, fruits taken from each plant were divided into two groups: fresh and dried (12 days) and subjected to different homogenization steps (mortar grinding, bead tissue grinder grinding, and blending). A single fruit was used for each analysis, and DNA isolation was performed on a total of 24 fruits. After the DNA isolation of the fruits was completed, DNA quantification was performed using the Qubit 4 Fluorometer (Invitrogen) device according to the Qubit™ dsDNA HS Assay Kit (Invitrogen) manual, and the results were compared.

Table 1 presents the DNA quantification results of the fruit samples, for which DNA isolation was performed on fresh material.

The DNA quantification results of the fruit samples, which were dried and had DNA isolation performed 12 days after collection, are shown in Table 2.

Table 1. Summary of DNA Quantities Obtained from Fresh Firethorn Fruits.

		Sample	DNA amount (ng/µl)	Mean value	Standart deviation
Fresh fruit samples	Mortar + Tissue lyser	1	0.28[b]	0.63	0.40
		2	0.45		
		3	0.70		
		4	0.58		
		5	0.36		
		6	1.39[a]		
	Kitchen blender	7	0.26	0.35	0.20
		8	0.64[a]		
		9	0.19		
		10	0.50		
		11	0.13[b]		
		12	0.38		

[a]= maximum value, [b]= minimum value

Table 2. DNA Recovery from dried firethorn fruits.

		Sample	DNA amount (ng/µl)	Mean value
Dried fruit samples	Mortar + Tissue lyser	1	2.32	3.23
		2	4.80[a]	
		3	4.43	
		4	1.55[b]	
		5	3.49	
		6	2.77	
	Kitchen blender + Mortar	7	1.59	1.25
		8	1.35	
		9	1.07	
		10	2.08[a]	
		11	1.02	
		12	0.363[b]	

[a]= maximum value, [b]= minimum value

The DNA amounts obtained as a result of DNA isolation using different methods on fresh and dried fruits of firethorn plant are shown comparatively in Figure 2.

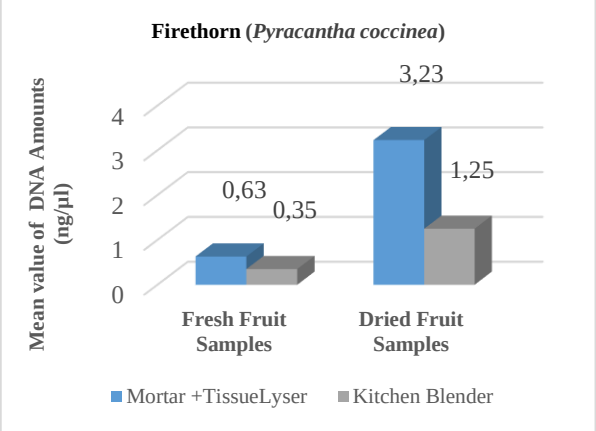

Figure 2. Comparison chart of DNA quantities from fruit samples.

The methods used in the preparation of dried and fresh fruit samples were evaluated among themselves using the Wilcoxon Signed-Rank Test in SPSS Statistics for Windows, version 20.0 (SPSS Inc., Chicago, Ill., USA). As a result of the test, insignificant results were obtained with a value of 0.1116 (>0.05) in fresh samples and significant results were obtained with a value of 0.046.

Table 3. Wilcoxon Signed-Rank Test results

Test statistics	Method comparison between fresh samples	Method comparison between dried samples
Z	-1,572a	-1,992a
Asymp. Sig. (2-tailed)	0,116	0,046

4. Discussions

Forensic botany, one of the subfields of forensic sciences, is not yet the primary discipline used in solving forensic cases, but new developments and approaches in this field are emerging daily.

Plant materials (e.g., leaves, fruits, seeds) frequently play a role in criminal cases such as murder, theft, and sexual assault due to their unnoticed presence. This places a responsibility on investigators to first detect and identify the plant species, and then conduct genetic analysis to obtain definitive results. Like human DNA, plant DNA can be analyzed, but the process begins with DNA isolation. Isolating pure, high-quality DNA from plants is critical, though often difficult due to contamination from secondary metabolites. Therefore, DNA isolation methods must be tailored to each plant species and tissue (Sahu et al., 2012).

Unlike humans, plant cells have a cell wall composed of cellulose. Therefore, the cell wall must be mechanically broken down to isolate DNA from plant samples. This step, known as homogenization, is typically performed using liquid nitrogen according to protocols in DNA isolation kits. However, liquid nitrogen can be problematic due to its difficulty of use, cost, and it's not being available in every laboratory. Additionally, the protocol in the DNA isolation kit manual suggests using 100 mg of fruit samples that have been lyophilized with liquid nitrogen (iNtRON Biotechnology, 2012). However, fruits collected from crime scenes or victims may not be fresh or available in the desired quantity. Therefore, alternatives to address potential issues should be developed, and method optimization for DNA isolation from the easily cultivated Firethorn (*Pyracantha coccinea*) plant was aimed in this study. This study is one of the pioneering works in DNA recovery for *Pyracantha coccinea* species.

The main factors affecting DNA quantities in this study are the homogenization methods used and the amount of starting material.

For fresh fruits, the highest DNA concentration obtained from samples homogenized with a mortar and bead mill was 1.39 ng/µl, while the highest concentration from samples homogenized with a household blender was 0.643 ng/µl. This difference is attributed to incomplete homogenization of samples processed with the blender and the bead mill's superior homogenization compared to the blender. For dried fruits, samples processed with the blender were additionally homogenized with a mortar, closing the gap in this stage. In order to avoid

contamination, the blender and mortar were sterilized by keeping them under bleach and UV light after each sample process.

For dried fruits, the highest DNA concentration obtained from samples homogenized with a mortar and bead mill was 4.8 ng/µl, while the highest concentration from samples using a household blender and mortar was 2.08 ng/µl. Using the mortar in both homogenization methods provided an opportunity to compare the bead mill and blender. The contribution of grinding larger pieces from blender-processed samples in the mortar supported homogenization, though it also resulted in minimal tissue loss, which likely had a slight effect on DNA quantities. It was observed that the DNA amounts obtained were suitable for the next step, PCR, for identification (QIAGEN®, 2010).

As an alternative method, there are studies showing that homogenization with the help of a blender can be performed for a wide range of plants and that these methods lead to good performance (Guillemaut and Maréchal-Drouard, 1992). However, as observed, this is not the case for Firethorn berries. It was not found to be an efficient method for plant samples with softer tissues, such as firethorn berries. Supporting our conclusion, another study reported that leaf tissues can be mechanically disintegrated, typically by grinding or beading for homogenization. However, it has been reported that too much mechanical force is applied in these methods, cutting the DNA and reducing its yield and quality. It has also been reported that mechanical force applied to plant samples causes DNA molecules to break into smaller fragments (Couch and Fritz, 1990).

For each DNA isolation, a single fruit of the Firethorn plant was used without measuring the quantities. The manual of the isolation kit suggests that after lyophilizing and grinding the fruit samples in a mortar, a starting amount of 100 mg dry weight is used. However, in this study, although the weight of a single fresh fruit was approximately 100 mg, it contained a high amount of water, resulting in a lower dry weight of the starting material. Additionally, although dried samples were worked with smaller starting material amounts, the DNA quantities yielded better results. These findings suggest that dried samples have considerable potential for DNA isolation.

4.1. Sufficiency of obtained DNA quantities for amplification

The minimum DNA amount required for plant amplification varies depending on the specific application and the quality of the DNA. A balance must be established between the amount of template DNA and the required degree of amplification. A very low amount of template DNA will require more amplification cycles, increasing the chance of errors. On the other hand, a high amount of template DNA can result in low yield and "dirty" PCR with many non-specific amplifications.

Uchiyama et al. (2013) used 5 ng DNA in their study to develope 32 EST-SSR markes for *Abies firma.*

Another study involving maize, tomato, and soybean used GenomePlex® Whole Genome Amplification (WGA) Kit with 1 ng/µl DNA concentrations for whole genome amplification, resulting in PCR products with an average length of 400 bp (Sigma-Aldrich, 2024).

According to QIAGEN® Multiplex PCR Handbook; the kit optimized at DNA concentrations between 1 ug and 1 ng (QIAGEN®, 2010) .

Based on the information provided and the referenced studies, there are no established standards for DNA amplification in plant samples. It is suggested that an amplification method should be used depending on each plant species, quantity, quality of the DNA obtained, and the intended analysis. With the DNA concentrations obtained from this study, it was concluded that a partial profile, which contains only a subset of genetic markers, could be obtained from some of the fresh samples and a full profile, which refers to the complete set of genetic markers used to uniquely identify an individual plant, from all dried samples using the GenomePlex® Whole Genome Amplification (WGA) Kit.

The increasing research in forensic botany and the significant role of plant materials in solving forensic cases are well-recognized. Techniques and methods are continuously evolving to enhance the usability of this field. Examples of these developments include genetic maps for plant origins, protocols specifically developed for plant species, and C-value databases created for plant DNA (Leitch et al., 2019).

Additionally, the chloroplast genome of *Pyracantha fortuneana* was analyzed using high-throughput sequencing technology and high genome similarity was found between the red and white flower phenotypes. Four mutational hotspots were identified and proposed as potential molecular markers for *Pyracantha* species. This information is important for the molecular taxonomy of *Pyracantha* species and for forensic botanical applications (Ding et al., 2022).

In addition, in the study by Huang et al., *Hamaecyparis taiwanensis* individuals were identified with 99.99% confidence level using 30 SSR markers to detect illegal logging. This success of genetic marker systems emphasizes the importance of DNA profiling in forensic botany. (Huang et al., 2020).

In forensic botany, it is crucial not only to define the plant species morphologically and anatomically but also to utilize molecular biology and genetic analyses when presenting evidence to courts and prosecutors. Genetic analyses can be used to prove that plants are endangered or endemic species, determine the origin of plant materials, or confirm if plant samples match. These analyses are carried out by targeting specific regions of plant DNA, DNA sequences, genetic polymorphisms, or profile matches.

This study aimed to optimize the homogenization methods used in DNA isolation from Firethorn (*Pyracantha coccinea*) fruits.

When working with firethorn fruits, drying and then homogenizing in a mortar followed by bead mill homogenization and using silica-based spin column-based plant DNA isolation kits yields the highest concentrations of DNA. However, in cases with time constraints, working with fresh fruit samples is also feasible. By homogenizing fresh fruit samples in a mortar and then in a bead mill and using spin column-based plant DNA isolation kits, it is possible to obtain high concentrations of DNA.

This study provides an important perspective on method

optimization for DNA isolation of Fire Thorn species. In future studies, it is important to increase the number of samples and to examine the discrimination power between individuals by identification. In future studies, the study will be improved by increasing the number of samples and performing other identification steps.

Conflict of Interest

Authors have declared no conflict of interest.

Authors' Contribution

TUS designed the experiment, supervised and edited. EA conducted the experiment, prepared the article. NS revised and edited the article.

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References

Akguc N, Ozyigit İİ, Yasar U, Leblebici Z, Yarcı C (2010). Use of *Pyracantha coccinea* Roem. as a possible biomonitor for the selected heavy metals. International Journal of Environmental Science and Technology 7(3): 427-434.

Aquila I, Ausania F, Serra A, Di Nunzio C, Boca S, Capelli A, Magni P, Ricci P (2014). The role of forensic botany in crime scene investigation: Case report and review of literature. Journal of Forensic Sciences 59: 820-824. https://doi.org/10.1111/1556-4029.12401.

Couch J, Fritz P (1990). Isolation of DNA from plants high in polyphenolics. Plant Molecular Biology Reporter 8: 8-12.

Craft KJ, Owens JD, Ashley MV (2007). Application of plant DNA markers in forensic botany: Genetic comparison of *Quercus* evidence leaves to crime scene trees using microsatellites. Forensic Science International 165(1): 64-70. https://doi.org/10.1016/j.forsciint.2006.02.035

Ding SX, Li JC, Hu K, Huang ZJ, Lu, RS (2022). Morphological characteristics and comparative chloroplast genome analyses between red and white flower phenotypes of *Pyracantha fortuneana* (Maxim.) Li (*Rosaceae*), with implications for taxonomy and phylogeny. Genes 13(12): 2404.

Guillemaut P, Maréchal-Drouard, L (1992). Isolation of plant DNA: A fast, inexpensive, and reliable method. Plant Molecular Biology Reporter 10: 60-65. https://doi.org/10.1007/BF02669265

Huang CJ, Chu FH, Huang YS, Hung YM, Tseng YH, Pu CE, Chao CH, Chou YS, Liu SC, You YT, Hsu SY, Hsieh H C, Hsu CT, Chen MY, Lin TA, Shyu HY, Tu YC, Chen CT (2020). Development and technical application of SSR-based individual identification system for *Chamaecyparis taiwanensis* against illegal logging convictions. Scientific Reports 10(1): 22095.

Intron Biotechnology (2012). i-genomic Plant DNA Extraction Mini Kit Protocol. Intronbio.com Website: https://intronbio.com:6001/inc/downfile.php?filename=F1523839442.pdf&orgfilename=IDR-RP0014_i-genomic%20Plant.pdf&filedir=product_protocol [accessed 20 August 2024].

Leitch I, Johnston E, Pellicer J, Hidalgo O, Bennett M. (2019). Plant DNA C-Values Database. cvalues. Science.kew.org Website: https://cvalues.science.kew.org/ [accessed 20 August 2024].

QIAGEN Sample & Assay Technologies (2009). TissueLyser LT Handbook. Qiagen.com/us Website: https://www.qiagen.com/us/resources/download.aspx?id=eb067bcc-0638-4f75-bf00-b54094d858fe&lang=en [accessed 20 August 2024].

QIAGEN Sample & Assay Technologies (2019). Multiplex PCR Handbook. Qiagen.com/us Website: https://www.qiagen.com/at/resources/download.aspx?id=a541a49c-cd06-40ca-b1d2-563d0324ad6c&lang=en&srsltid=AfmBOoqTpC4_TFwlF5UBTu7wnA6Xqf0RjHuDXmfl2zrh_x3_u5YfaCbZ [accessed 20 August 2024].

Sahu SK, Thangaraj M, Kathiresan K (2012). DNA extraction protocol for plants with high levels of secondary metabolites and polysaccharides without using liquid nitrogen and phenol. ISRN Molecular Biology 2012: 1-6. https://doi.org/10.5402/2012/205049.

Sigma Aldrich. (2023). Plant DNA Extraction & WGA Amplification Protocol. Sigmaaldrich.com/TR Website: https://www.sigmaaldrich.com/TR/en/technical-documents/protocol/genomics/dna-and-rna-purification/extraction-protocol-plant-tissues# [accessed 20 August 2024].

Thermo Fisher Scientific Inc. (2022). Qubit™ dsDNA HS Assay Kit (Invitrogen) User Guide. Thermofisher.com Website: https://www.thermofisher.com/document-connect/document-connect.html?url=https://assets.thermofisher.com/TFS-Assets%2FLSG%2Fmanuals%2FQubit_dsDNA_HS_Assay_UG.pdf [accessed 20 August 2024].

Uchiyama K, Fujii S, Ishizuka W, Goto S, Tsumura Y. (2013). Development of 32 EST-SSR markers for Abies firma (Pinaceae) and their transferability to related species. Applications in Plant Sciences, 1: 1200464. https://doi.org/10.3732/apps.1200464.

Wickneswari R, Rajanaidu N (2011). Genetic identification of oil palm: A tool for the sustainable management of palm oil. Journal of Plantation Research 25(3): 305-312.

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Evaluation of antifungal activities of the ethanolic extract of the macrofungus *Suillus collinitus* against dermatophytes

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Suillus collinitus makrofungusunun etanolik ekstraktının dermatofitlere karşı antifungal aktivitelerinin değerlendirilmesi

Abstract: In this study, the ethanolic crude extracts obtained from the macrofungus *Suillus collinitus* (Fr.) O. Kuntze (*Suillaceae, Bolatales*) were investigated for their antifungal activities against dermatophytes, including the isolates *Microsporum gypseum* (E. Bodin) Guiart & Grigoraki, *Trichophyton rubrum* (Castell.) Sabour. and *Epidermophyton floccosum* (Harz) Langeron & Miloch., using both the agar well diffusion method and tube dilution methods. The ethanol extracts demonstrated strong antifungal activity, with mean inhibition zones observed at different concentrations. The widest zone of inhibition was recorded at 1500 mg/mL, measuring 20.8 mm for *M. gypseum*, 18.2 mm for *T. rubrum*, and 16.8 mm for *E. floccosum*. The MIC values observed were 50 µg/mL for *M. gypseum*, 60 µg/mL for *T. rubrum*, and 70 µg/mL for *E. floccosum*, respectively. Terbinafine as the comparison antifungal agent gave MIC values of 10 µg/mL for both *E. floccosum* and *T. rubrum*, and 20 µg/mL for *M. gypseum*. These findings against dermatophytes support our observations regarding the use of this macrofungus among the public. Besides, the findings of this screening study are a preliminary step to further pharmaceutical researches on the relevant macrofungus in the future.

Key words: *Epidermophyton floccosum*, *Microsporum gypseum*, *Trichophyton rubrum*, agar well diffusion, MIC.

Özet: Bu çalışmada, makrofungus *Suillus collinitus* (Fr.) O. Kuntze (*Suillaceae, Boletales*) türünden elde edilen etanollü ham ekstraktların, *Microsporum gypseum* (E. Bodin) Guiart & Grigoraki, *Trichophyton rubrum* (Castell.) Sabour. ve *Epidermophyton floccosum* (Harz) Langeron & Miloch. izolatları gibi dermatofitlere karşı antifungal aktiviteleri hem agar kuyucuk difüzyon yöntemi hem de tüp dilüsyon yöntemleri kullanılarak incelenmiştir. Etanol ekstraktları, farklı konsantrasyonlarda belirgin bir antifungal aktivite sergilemiştir. En geniş inhibisyon zonu, 1500 mg/mL'de gözlemlenmiş olup, *M. gypseum* için 20.8 mm, *T. rubrum* için 18.2 mm ve *E. floccosum* için 16.8 mm olarak kaydedilmiştir. Gözlemlenen MIC değerleri sırasıyla *M. gypseum* için 50 µg/mL, *T. rubrum* için 60 µg/mL ve *E. floccosum* için 70 µg/mL olmuştur. Karşılaştırma amaçlı kullanılan antifungal ajan terbinafin, *E. floccosum* ve *T. rubrum* için 10 µg/mL, *M. gypseum* için ise 20 µg/mL MIC değerleri vermiştir. Dermatofitlere karşı elde edilen bu bulgular, bu makromantarın halk arasında kullanımını desteklemektedir. Ayrıca, bu tarama çalışmasının bulguları, gelecekte ilgili makromantar üzerinde yapılacak daha ileri farmasötik araştırmalar için ön adım niteliğindedir.

Anahtar Kelimeler: *Epidermophyton floccosum*, *Microsporum gypseum*, *Trichophyton rubrum*, agar kuyu difüzyon, MIC

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1. Introduction

Fungal pathogens called dermatophytes are responsible for a group of disorders collectively referred to as dermatophytosis. Three genera of fungi–*Epidermophyton* Sabour., *Microsporum* Gruby, and *Trichophyton* Malmsten –cause the majority of dermatophytic infections. Human keratin is broken down by microorganisms including *M. gypseum* (E. Bodin) Guiart & Grigoraki, *T. rubrum* (Castell.) Sabour., eventually leading to tissue digestion. Additionally, these organisms can lead to superficial infections affecting the outer layers of the skin, hair, and nails. It is crucial to acknowledge that treating fungal infections can be quite difficult, as the fungi tend to become resistant to the antifungal drugs that are currently supplied (Shikwambana and Mamokone, 2020; Gupta et al., 2023).

Terbinafine is the primary treatment for superficial mycoses; however, the increasing incidence of terbinafine resistance throughout Europe and the occurrence of side effects such as epigastralgia, nausea and tachycardia necessitate the development of different antifungal agents against dermatophytes (Keller, 2012; Moreno-Sabater et al., 2022). Macrofungi contain active compounds that can treat fungal infections, making their screening for fungicidal agents crucial due to the rise in fungal pathogen resistance (Kumar et al., 2021). In Türkiye, many macrofungi are used to treat skin diseases, including those caused by fungal pathogens. However, information on these macrofungi is primarily limited to local communities within specific regions.

Suillus collinitus (Fr.) O. Kuntze (*Suillaceae, Boletales*) is found throughout Europe. It is an ectomycorrhizal fungus, forming mutually beneficial symbiotic relationships with several species of *Pinus*. Although not widely regarded as highly valuable, *S. collinitus* is reported to be edible when thoroughly cooked. To reduce the risk of an adverse

reaction to these mushrooms, some people have found it advisable to discard the cap skin of all species from the *Suillus* Gray genus (Watling and Hills, 2005).

During routine field excursions, it was established that *S. collinitus* is employed for the treatment of injuries. It was also noticed that this fungus's extracts, prepared as a pomade, were used to counteract foot peeling and nail thickening.

Consequently, the purpose of this work was to examine the antifungal properties of the ethanolic extract of *S. collinitus* against dermatophytes, which has traditionally been used by locals to treat fungal diseases.

2. Materials and Method

2.1. Microorganisms

The dermatophytes used in this study came from the culture at the Mycology Research Laboratory in the Biology Department, Düzce University, Düzce, Türkiye. *M. gypseum, T. rubrum* and *E. floccosum* (Harz) Langeron & Miloch. as dermatophytes were used as test fungal pathogens. All fungi were stored on Sabouraud Dextrose Agar (SDA Agar) (Oxoid, Hampshire, England) slants in the refrigerator at 4 °C, before use.

2.2. Macrofungal materials

The macrofungus *S. collinitus* was collected from Bolu-Abant Road, Bolu, Türkiye (40°39’31’’ N, 31°24’17’’ E, Alt. 920 m) in July, 2023. Voucher specimens (BD-505-1) of the macrofungus was deposited in the Department of Biology of Düzce University in the author’s collection.

2.3. Preparation of crude extracts

The macrofungal samples were pulverized after being dried at 40 °C in an oven. Using Soxhlet apparatus, each batch of dry powdered macrofungal material (50 g) was extracted for 24 hours using 150 mL of 95% ethanol from Merck, Darmstadt, Germany. First, Whatman filter paper No. 1 was used to filter the extracted mixture. A 32.8% ethanol yield was obtained by vacuum-evaporating the filtrate solvent in a rotary evaporator set at 55 °C. The dehydrated extract was kept at -20 °C in sterile screw-capped vials with labels. Dimethyl sulfoxide (DMSO) was used to dissolve the ethanol, which was found as sticky black solids, to a final concentration of 1 g/mL for first screening.

2.4. Preparation of inoculum

The fungal test microorganisms are revitalized at 27 °C on PDA (Potato Dextrose Agar) (Oxoid, Hampshire, England) for 14 days. Spores of revitalized fungus were collected from cultures on agar plates after 7 days of incubation as described by Broekaert et al. (1990). The sporangial suspension concentration was estimated with regards to the conidium and spores forming fungi, and micro-dilution standardized by the Clinical and Laboratory Standards Institute (CLSI, 2018) which involves an inoculum of spores adjusted spectrophotometrically to 2.5×10^5 cfu/mL at wavelength of 530 nm of 0.11 O.D. (Castilho et al., 2015). The fungal spore suspension was stored in 20% glycerol at -4 °C to avoid contamination and growth.

2.5. Determination and antifungal activity

The antifungal activity of the macrofungus *S.collinitus* extract was determined by the agar well diffusion method (Holder and Boyce, 1994; Jamuna et al., 2013). The PDA medium was prepared according to the manufacturer’s instructions (39 g/L). The fungal culture was evenly spread over the medium by sterile cotton swabs. A sterile cork borer made wells (6 mm) in the medium. A volume of 200 µL of extracts was transferred into each well, incubated at 30 °C for 48-72 h, and the plates were then observed for the formation of clear zones around the wells indicating the presence of antifungal activity (Ambikapathy et al., 2011). The zone of inhibition was measured using caliber (all inhibition minus 6 mm of the well) and recorded. Ethanol (96%) and Terbinafine obtained from pharmacy 200 µL each were used as the negative and positive control, respectively.

2.6. Minimum ınhibitory concentration (MIC)

MIC values of *S. collinitus* extract were determined using the tube dilution method (Koneman et al., 1997). The fungal spore inoculum of 100 µL of 2.5×10^5 dilution was inoculated into test tubes with (1800 µL) Malt Extract Broth (Oxoid, Hampshire, England) in eight different test tubes and the macrofungal extract was serially diluted, ranging from 100 mg/mL to 800 mg/mL. A volume of 100 µL of each extract dilution was mixed in each incubated test tube, incubated at 30 °C for 48-72 h, and then examined for visual turbidity. The results of the extracts were compared with a standard positive control (Terbinafine 20 µg/mL).

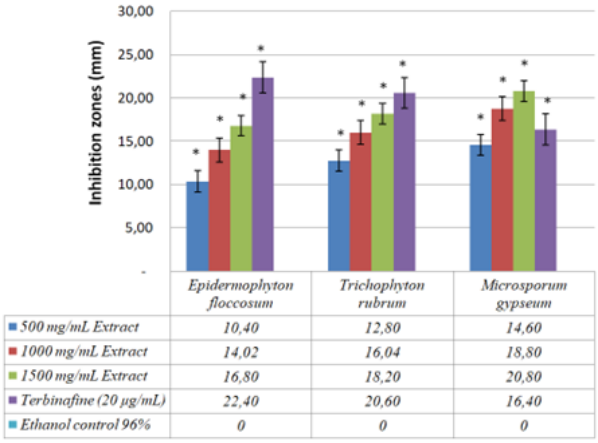
2.7. Data analysis

Statistical analyses were conducted using IBM SPSS Statistics Version 27. Experimental results obtained from the agar well diffusion assay were expressed as the mean of triplicate measurements (n=3). One-way analysis of variance (ANOVA) was used to evaluate significant differences among the groups. For data sets exhibiting normal distribution as evaluated by normality tests, Tukey's post hoc test was applied. Additionally, a Kruskal-Wallis test was conducted to compare the MIC values among *E. floccosum*, *T. rubrum*, and *M. gypseum*. Statistical significance was defined as $p<0.05$ with a 95% confidence interval.

3. Results

According to the data in Figure 1, the antifungal activity of the tested macrofungal extract on three dermatophyte fungi species increased with the applied concentration. The effects on *E. floccosum*, *T. rubrum* and *M. gypseum* tended to increase at 500, 1000 and 1500 mg/mL extract concentrations, respectively. However, this activity remained at lower levels compared to terbinafine used as positive control. For example, for *E. floccosum*, the effect of the macrofungal extract at 500 mg/mL concentration was 10.4 mm, while this effect reached 16.8 mm at 1500 mg/mL. In contrast, the effect of terbinafine at 20 µg/mL was higher at 22.4 mm. Similarly, the activity of *T. rubrum* extract increased with increasing concentration, but terbinafine showed higher antifungal activity. Notably, *M. gypseum* extract showed a stronger antifungal effect (20.8 mm) at the highest concentration than the standard Terbinafine (16.4 mm). The ethanol control group did not show any antifungal activity, confirming that the test conditions were appropriate. Inhibition zones (mm) were analyzed using one-way ANOVA followed by Tukey's post hoc test to determine the differences between the extract concentrations (500, 1000, and 1500 mg/mL) and the

standard antifungal agent Terbinafine (20 µg/mL) for each dermatophyte species. The results revealed statistically significant differences (*p<0.05) between the extract concentrations and Terbinafine, as well as among the extract concentrations themselves.



	Epidermophyton floccosum	*Trichophyton rubrum*	*Microsporum gypseum*
500 mg/mL Extract	10,40	12,80	14,60
1000 mg/mL Extract	14,02	16,04	18,80
1500 mg/mL Extract	16,80	18,20	20,80
Terbinafine (20 µg/mL)	22,40	20,60	16,40
Ethanol control 96%	0	0	0

Figure 1. Inhibition zones (mm) observed in agar well diffusion assay using different concentrations of test extracts and standard antifungal agent Terbinafine against selected dermatophytes. Statistical analysis using One-way ANOVA followed by Tukey's post hoc test revealed significant differences in the zones of inhibition between the extract concentrations and Terbinafine (* p<0.05). Error bars represent standard deviation (n=3).

In conclusion, the antifungal effect of the macrofungal extract on dermatophytes was observed, but it should be noted that it was more limited than terbinafine.

Regarding the MIC results (Table 1), the extract exhibited high to moderate (80-100% inhibition) antifungal activity against all three dermatophyte species, ranging from 50 µg/mL for *M. gypseum*, 60 µg/mL for *T. rubrum*, and 70 µg/mL for *E. floccosum*. Furthermore, Terbinafine, the comparative antifungal agent, resulted in MIC values between 10 µg/mL for *E. floccosum* and *T. rubrum*, and 20 µg/mL for *M. gypseum*. What's noteworthy is that the extract's effectiveness against all dermatophytes was not as strong as that of the standard Terbinafine. A Kruskal-Wallis test was conducted to compare the MIC values of the test extract in three dermatophyte species, *E. floccosum*, *T. rubrum* and *M. gypseum*. The analysis did not reveal any statistically significant difference between the groups (p>0.05).

Table 1. MIC values of the ethanolic extract of *S. collinitus* and standard Terbinafine*

Test Microorganisms	**MIC (µg/mL)**	**Standard (Terbinafine 20 µg/mL)**
Epidermophyton floccosum	70	10
Trichophyton rubrum	60	10
Microsporum gypseum	50	20

4. Discussions

The objective of this study was to evaluate the antifungal properties of an ethanolic extract of *S. collinitus* against dermatophytes. The findings indicate that the antifungal activity of the extract increased in a concentration-dependent manner in the three species tested (E. *floccosum*, *T. rubrum*, and *M. gypseum*). However, the activity generally remained lower than that of terbinafine. Interestingly, the zone of inhibition of M. gypseum at the highest concentration (20.8 mm) exceeded that of terbinafine (16.4 mm), indicating possible species-specific interactions. Such findings are critical for determining targeted applications for *S. collinitus* extracts in antifungal therapy. The increased zones of inhibition at higher extract concentrations support the hypothesis that the potency of the extract is concentration-related. However, the limited efficacy compared to terbinafine highlights the need for further improvements. MIC results further confirm this pattern, with the extract showing MIC values of 50 µg/mL for *M. gypseum*, 60 µg/mL for *T. rubrum*, and 70 µg/mL for *E. floccosum*. In contrast, terbinafine exhibited significantly lower MIC values, confirming its superior antifungal potential.

The results of this study are partially consistent with previous reports on the antimicrobial and antifungal properties of *S. collinitus*. Recent investigations have emphasized the antimicrobial capabilities of mushrooms, and various antibacterial compounds have been identified, primarily found in higher Basidiomycetes and certain Ascomycetes. These active compounds are secondary metabolites, including terpenoids, quinone or lactone derivatives, alkaloids, and other similar compounds. Additionally, high molecular weight molecules like polysaccharides or proteins contribute to mushrooms' anti-infectious properties. The bioactive chemicals also include certain derivatives that are sulfurated and chlorinated, which are frequently found in macromycetes (Hamers et al., 2020).

Remarkably, the literature review revealed that just one study had assessed the antibacterial activity of *S. collinitus*. According to Yamac and Bilgili (2006), disk diffusion and microdilution methods were used to investigate the antimicrobial activities of dichloromethane, ethyl acetate, and ethanol extract obtained from *S. collinitus* against test microorganisms such as *Salmonella typhimurium* NRRL-B-4440, *Escherichia coli* ATCC 25922, *Enterobacter aerogenes* NRRL-B-3567, *Pseudomonas aeruginosa* ATCC 27853, *Staphylococcus aureus* ATCC 25923, *Staphylococcus epidermidis* NRLL-B-4337, *Bacillus subtilis* NRRL-B-558 as bacterial cultures, *Candida albicans* ATCC 10259, and *Saccharomyces cerevisiae* NRRL-Y-2034 as the yeast cultures. The *S. collinitus* dichloromethane extract was found to be the most effective against bacteria and yeast. The dichloromethane extract exhibited the greatest antifungal activity (>20 mm) against *S. cerevisiae* NRRL-Y-2034. For *S. aureus* ATCC 25923, the inhibitory zone varied between 15-20 mm. The inhibitory zone, which measured 10-15 mm, had a moderate antifungal efficacy against *Candida albicans* ATCC 10259. The MIC values of these extracts ranged from 31.25 to 250 µg/mL.

Compared to our study, this difference, which was partially seen in the same species, was due to genotype, chemotype, and geographical origin, it is thought that parameters cause environmental and soil conditions. One of the reasons for this difference can be explained by the difference in the strains and protocols of the test microorganisms used in the studies. The fungal compounds responsible for the resulting antifungal effect are thought to originate from terpenoids,

quinone or lactone derivatives, and alkaloids, which are macrofungal secondary compounds.

The limited number of studies conducted on *S. collinitus*, have begun to reveal the medicinal potential of this mushroom. According to one study, the methanolic extract of *S. collinitus* induced apoptosis in a human breast tumor cell line and had a p53-mediated influence on the normal cell cycle distribution (Vaz et al., 2012).

In another study, the reducing and radical-scavenging capacities of *S. collinitus* were assessed, along with its ability to prevent lipid peroxidation in liposome solutions. *S. collinitus* was also shown to have a high tocopherol content (Heleno et al., 2010). Furthermore, Akata et al. (2012) reported that *S. collinitus* has a 71.94% free radical scavenging rate of 1,1-diphenyl-2-picrylhydrazyl (DPPH). Moreover, Froufe et al. (2011) found the median effective concentration (EC50) values (14.05, 2.97, and 1.20 mg/mL) for radical scavenging, lipid peroxidation activity suppression, and *S. collinitus* reducing power, indicating the plant's strong antioxidant capacity. In another study, Emsen et al. (2019) used chromosomal aberration (CA), micronucleus (MN), nuclear division index (NBI), and mitotic index (MI) analyses to investigate the effects of acetone and water extracts of *S. collinitus* on genotoxicity and proliferation of human cells.

In conclusion, although *S. collinitus* has been shown to have strong antioxidant, antimicrobial and antifungal properties, it appears that this fungus exhibits a more limited effect compared to effective agents like terbinafine, particularly in treating dermatophyte fungal infections. However, these findings provide important insignts into the potential medicinal use of *S. collinitus* and highlight the need for further research to more comprehensively evaluate its biological activities.

Conflict of Interest

The authors declared no conflicts of interest.

Authors' Contribution

All authors participated in the conceptualization, design, analysis, and writing of the study. They have all reviewed and approved the final manuscript.

References

Akata I, Ergönül B, Kalyoncu F (2012). Chemical compositions and antioxidant activities of 16 wild edible mushroom species grown in Anatolia. International Journal of Pharmacology 8(2): 134-138.

Ambikapathy V, Gomathi S, Panneerselvam A (2011). Effect of antifungal activity of some medicinal plants against *Pythium debaryanum* (Hesse). Asian Journal of Plant Science and Research 1(3): 131-134.

Broekaert WF, Terras FRG, Cammue BPA, Vanderleyden J (1990). An automated quantitive assay for fungal growth inhibition. FEMS Micro Letters 69(1-2): 55-60.

Castilho AL, Caleffi-Ferracioli KR, Canezin PH, Siqueira VLD, de Lima Scodro RB, Cadoso RF (2015). Detection of drug susceptibility in rapidly growing mycobacteria by Resazurin broth microdilution assay. Journal of Microbiological Methods 111: 119-121.

CLSI (2018). Methods for antifungal disk diffusion susceptibility testing of yeasts. 3rd ed.CLSI guideline M44. Wayne, PA: Clinical and Laboratory Standards Institute.

Emsen B, Türel A, Uzun Y (2019). Effects of *Suillus collinitus* (Fr.) Kuntze extracts on genotoxicity and proliferation of human lymphocytes. Anatolian Journal of Botany 3(2): 59-63.

Froufe HJC, Abreu RMV, Ferreira ICFR (2011). QCAR models to predict wild mushrooms radical scavenging activity, reducing power and lipid peroxidation inhibition. Chemometrics and Intelligent Laboratory Systems 109(2): 192-196.

Gupta C, Das S, Gaurav V, Singh PK, Rai G, Datt S, Tigga RA, Pandhi D, Bhattacharya SN, Ansari MA, Dar SA (2023). Review on host-pathogen interaction in dermatophyte infections. Journal of Medical Mycology 33: 101331.

Hamers V, Huguet C, Bourjot M, Urbain A (2020). Antibacterial compounds from mushroom: a lead to fight ESKAPEE Pathogenic Bacteria? Planta Medica 87(5): 351-367.

Heleno SA, Barros L, Sousa MJ, Martins A, Ferreira ICFR (2010). Tocopherols composition of Portuguese wild mushrooms with antioxidant capacity. Food Chemistry 119(4): 1443-1450.

Holder IA, Boyce ST (1994). Agar well diffusion assay testing of bactericidal susceptibility to various antimicrobials in concentrations non-toxic for human cells in culture. Burns 20(5): 426-429.

Jamuna S, Subramaniam P, Karthika K (2013). In vitro antifungal activity of leaf and root extracts of the medicinal plant, *Hypochaeris radicata*. International Journal of Pharmacy and Pharmaceutical Sciences 5(3): 758-761.

Keller KA (2012). Therapeutic review: terbinafine. Journal of Exotic Pet Medicine 21(2): 181-185.

Koneman EW, Allen SD, Janda WN, Schreckenber PC, Win WC (1997). Antimicrobial susceptibility testing. In Koneman EW, Allen SD, Janda WM, Schreckenberger PC, Winn, Jr WC (eds.) Color Atlas and Textbook of Diagnostic Microbiology (5th ed) Philadelphia: Lippincott. Pp. 785-856.

Kumar K, Mehra R, Guiné RPF, Lima MJ, Kumar N, Kaushik R, Ahmed N, Yadav AN, Kumar H (2021). Edible mushrooms: A comprehensive review on bioactive compounds with health benefits and processing aspects. Foods 10(12): 2996.

Moreno-Sabater A, Normand AC, Bidaud AL., Cremer G, Foulet F, Brun S, Bonnal C, Ait-Ammar N, Jabet A, Ayachi A, Piarroux R, Botterel F, Houzè S, Desoubeaux G, Hennequin C, Dannaoui E (2022). Terbinafine resistance in dermatophytes: a French multicenter prospective study. Journal of Fungi 8(3): 220.

Shikwambana N, Mamokone M (2020). A survey of antifungal activity of selected south African plant species used for the treatment of skin infections. Natural Product Communications 15(5): 1-10.

Watling R, Hills AE (2005). British Fungus Flora. Agarics and Boleti 1. Boletes and their allies. Edinburgh: Royal Botanic Garden.

Vaz JA, Ferreira ICFR, Tavares C, Almeid, GM, Martins A, Helena Vasconcelos M (2012). *Suillus collinitus* methanolic extract increases p53 expression and causes cell cycle arrest and apoptosis in a breast cancer cell line. Food Chemistry 135(2): 596-602.

Yamac M, Bilgili F (2006). Antimicrobial activities of fruit bodies and/or mycelial cultures of some mushroom isolates. Pharmaceutical Biology 44(9): 660-667.

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Karyology of *Prunella grandiflora* and *Prunella vulgaris* from Türkiye

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Türkiye'den *Prunella grandiflora* ve *Prunella vulgaris*'in karyolojisi

Abstract: *Prunella* L. species are distributed in very different habitats since they can adapt to variable ecological conditions. These conditions cause variations in morphological characters. It is very important to support morphological and taxonomic characters with genetic data. So far, various diploid numbers have been reported in the chromosomal records of Turkish *Prunella* species. The situation is particularly complex in *P. vulgaris* L. and *P. grandiflora* (L.) Turra species. The aim of this study is to eliminate the complexity by presenting karyological data of these species. The chromosome number and chromosome lengths of *P. grandiflora* and *P. vulgaris* were reported for the first time from Turkey. The diploid and basic numbers were detected as $2n = 28$ and $x = 7$ by ploidy levels of $4x$ in *P. grandiflora* and *P. vulgaris*. As a result, the karyology of the genus *Prunella* was evaluated by comparing previous and present results. The listed data provided important contributions to the cytotaxonomy of the genus *Prunella*: (i) the basic number was x = 7, (ii) the most common diploid number was $2n = 28$, and (iii) high polyploidy rates. The polyploidy probably played an important role in the speciation of the genus.

Key words: basic number, karyotype, polyploidy, *Prunella*

Özet: *Prunella* L. türleri değişken ekolojik koşullara uyum sağlayabildikleri için çok farklı habitatlarda dağılım göstermektedir. Bu koşullar morfolojik karakterlerde çeşitliliğe neden olmaktadır. Morfolojik ve taksonomik karakterlerin genetik verilerle desteklenmesi çok önemlidir. Şimdiye kadar Türkiye *Prunella* türlerinin kromozom raporlarında çeşitli diploid sayılar bildirilmiştir. Özellikle *P. vulgaris* L. ve *P. grandiflora* (L.) Turra türlerinde durum karmaşıktır. Bu çalışmanın amacı, bu türlerin karyolojik verilerini sunarak karmaşıklığı ortadan kaldırmaktır. *P. grandiflora* ve *P. vulgaris*'in kromozom sayısı ve kromozom uzunlukları Türkiye'den ilk kez rapor edilmiştir. Diploid ve temel sayılar *P. grandiflora* ve *P. vulgaris*'te $2n = 28$ ve $4x$ ploidi seviyesi ile $x = 7$ olarak tespit edilmiştir. Sonuç olarak, *Prunella* cinsinin karyolojisi önceki ve mevcut sonuçları karşılaştırarak değerlendirildi. Listelenen veriler *Prunella* cinsinin sitotaksonomisine önemli katkılar sağladı: (i) temel sayı $x = 7$'dir, (ii) en yaygın diploid sayı $2n = 28$'dir ve (iii) yüksek poliploidi oranları. Poliploidi muhtemelen cinsin türleşmesinde önemli bir rol oynamıştır.

Anahtar Kelimeler: temel sayı, karyotip, poliploidi, *Prunella*

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1. Introduction

The genus *Prunella* L. (Lamiaceae) contains eight species (12 taxa) and five hybrids distributed in Europe, Asia, North America, and Northwest Africa (Harley et al., 2004; Govaerts, 2023). In Türkiye, there are 4 species growing, and there are no endemic species (Dirmenci, 2012; Çelenk et al., 2024). Since the species can adapt to variable ecological conditions, they are distributed in very different habitats (from low altitudes such as sea levels to high altitudes such as alpine zones). Variable ecological conditions cause variable morphological features in structures such as inflorescence, leaves, and indumentum. Herbaceous, square stem, not aromatic, rhizomatous; leaves simple to pinnatifid; flowers blue, purple, cream, or white; inflorescences ovoid to an oblong spike; 2-lipped corolla and calyx; stamens 4, lower pair longer are important morphological and taxonomic characters (Edmonson, 1982; Harley et al., 2004).

It is very important to support morphological and taxonomic characters with genetic data. The cytotaxonomy contributes to the solution of taxonomic problems by using cytogenetic data such as the number of chromosomes (basic and diploid), the sizes of chromosomes or their parts (long arm, short arm, total chromosome, relative and total haploid lengths), karyotype formula and karyotype asymmetry (intrachromosomal and interchromosomal) (Peruzzi and Eroğlu, 2013; Eroğlu, 2015; Martin et al., 2022). In order to reveal systematic and evolutionary relationships more clearly, the morphological characteristics of the genus *Prunella* should be supported by cytotaxonomic data.

In the genus *Prunella*, the most frequent chromosome number is $2n = 4x = 28$. The diploid numbers of the taxa belonging to the genus *Prunella* vary from 14 to 32, such as $2n = 14$, 21, 24, 28, 30, and 32. The species are diploid ($2x = 14$), triploid ($3x = 21$), and poliploid ($4x = 24$, 28, and 32) (Hruby, 1932; Böcher, 1949; Fernandes and Leitão, 1984; Malik et al., 2017; Javadi and Safikhani, 2023; Mirzadeh Vaghefi and Jalili, 2023).

So far, various diploid numbers have been reported in the chromosomal reports of Turkish *Prunella* species. The

situation is particularly complex in *P. vulgaris* L. and *P. grandiflora* (L.) Turra species. The aim of the present study was to present the karyological data of these species to eliminate the complexity.

2. Materials and Method

2.1. Plant samples

The *Prunella* species were collected from natural habitats in Türkiye. The distribution map was generated by Google Maps (Fig. 1). The habitat and flower photos of the species are given in Figure 2. The plant samples were deposited in Balıkesir University, Necatibey Faculty of Education, Department of Biology Education. The collection information is given below.

Prunella grandiflora: Türkiye, Balıkesir, Edremit, Kazdağı, Kartalçimeni hill, 1750 m, 28.07.2007, Dirmenci 3473. ***Prunella vulgaris***: Türkiye, Adana: 10 km from Saimbeyli to Himmetli, 800 m, 20.07.2007, Dirmenci 3569 & Akçiçek.

2.2. Cytogenetic procedure

Prunella seeds were germinated between moist filter papers at room temperature (germination). Germinated root tips were treated with saturated water solution of α - bromonaphtalene overnight at 4ºC (pretreatment), fixed in ethanol–glacial acetic acid solution (3:1) (fixation), hydrolyzed with 1N HCl at room temperature (hydrolysis), squashed in 45% acetic acid (preparation), and stained with 2% aceto-orcein (staining).

Permanent slides were made with the standard liquid nitrogen method. Slides were dried for 24 h at room temperature and mounted in Depex (Martin et al., 2023).

At least 10 metaphase plates were analyzed to detect diploid chromosome numbers by Software KaryoType.

3. Results

Figure 3 represents the small metaphase chromosomes of *Prunella* species. The chromosome numbers ($2n = 28$) of *Prunella grandiflora* and *P. vulgaris* are confirmed. The chromosome numbers and total chromosome lengths are reported for the first time from Türkiye. Due to the small size of chromosomes and the indistinct centromere, only total chromosome lengths were measured without long and short arm lengths.

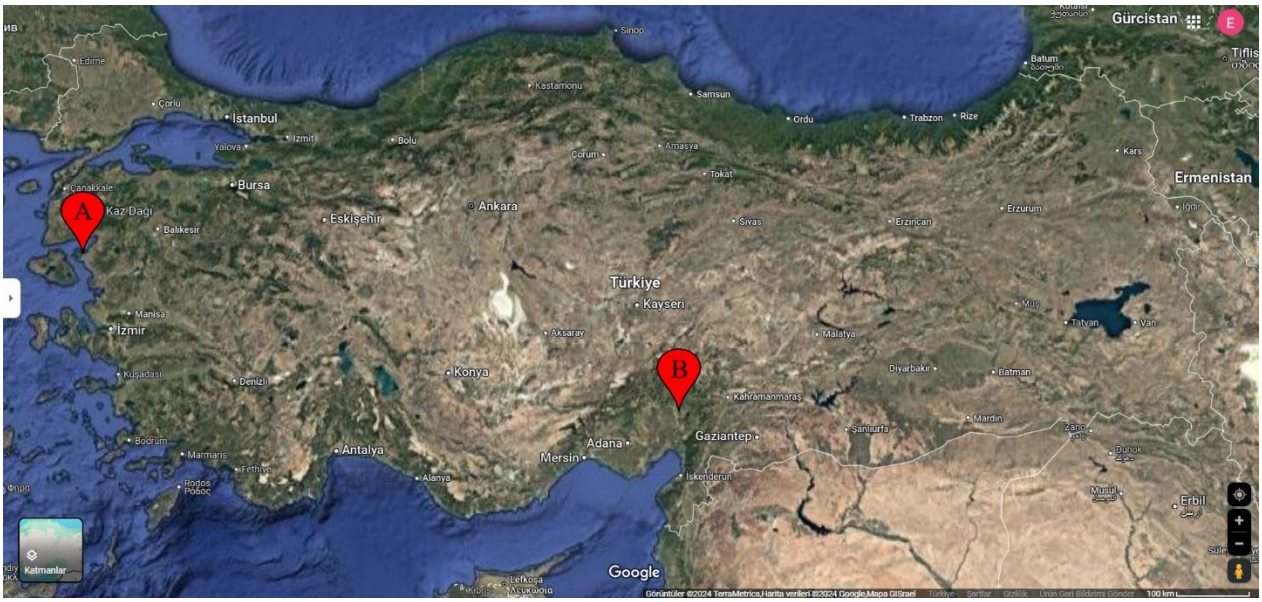

Figure 1. Distribution map of the studied species in Turkey. (A) *Prunella grandiflora* and (B) *Prunella vulgaris*

Figure 2. Habitat and flowers of *Prunella* species. (A) *Prunella grandiflora* and (B) *Prunella vulgaris*

3. Results

Figure 3 represents the small metaphase chromosomes of *Prunella* species. The chromosome numbers (2*n* = 28) of *Prunella grandiflora* and *P. vulgaris* are confirmed. The chromosome numbers and total chromosome lengths are reported for the first time from Türkiye. Due to the small size of chromosomes and the indistinct centromere, only total chromosome lengths were measured without long and short arm lengths.

In *Prunella grandiflora*, the total haploid length (THL) and mean haploid length (MHL) are 12.59 and 0.90 µm, respectively (Table 1). The chromosomes are quite small, and chromosome lengths range from 0.38 to 1.41 µm. (Table 2). In *P. vulgaris*, the total haploid length (THL) and mean haploid length (MHL) are 12.98 and 0.93 µm, respectively (Table 1). The chromosomes are quite small, and chromosome lengths range from 0.42 to 1.33 µm (Table 2).

4. Discussions

The chromosome number and chromosome lengths of *Prunella grandiflora* and *P. vulgaris* were reported for the first time from Türkiye. The different chromosome numbers for these species (especially *P. vulgaris*) were presented in the literature, which were 2*n* = 14, 21, 24, 28, 30, and 32 (Hruby, 1932; Böcher, 1949; Fernandes and Leitão, 1984; Malik et al., 2017; Javadi and Safikhani, 2023; Mirzadeh Vaghefi and Jalili, 2023). The most common somatic number in *Prunella* species was 2*n* = 28 (Probatova et al., 1991; Vitek et al., 1992; Melnikov, 2019), which was also presented in this study. It was reported that the number 2*n* = 32 proposed by Hruby (1932) was incorrect because the chromosome examination methods at that time did not allow a correct distinction between short and long chromosomes, and long chromosomes could be counted excessively (Böcher, 1949). However, presented all diploid numbers cannot be ignored.

In the genus *Prunella*, the basic chromosome number was *x* = 7 (Böcher, 1949; Magulaev, 1979; Melnikov, 2019). However, *x* = 6 and 8 cannot be ignored. The basic number was detected as *x* =7 by ploidy levels of 4*x* in *P. grandiflora* and *P. vulgaris*. Magulaev (1979) reported the diploid (2*n*= 2*x* = 14), triploid (2*n* = 3*x* = 21), and tetraploid (2*n* = 4*x* = 28) plants in the Caucasian population and suggested that the polyploidy as the main factor in the widespread occurrence of *P. vulgaris*. There is a positive correlation between polyploidy and environmental adaptation, and the polyploidy is one of the main mechanisms effective in the evolution process of plants. The polyploid organisms generally exhibit higher vitality and superior characteristics than their diploid relatives (Eroğlu, 2022).

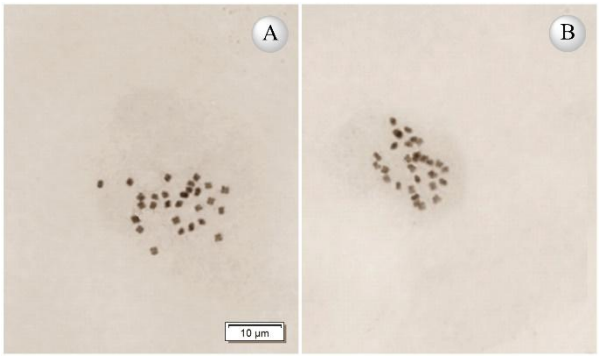

Figure 3. Metaphase chromosomes of *Prunella grandiflora* (A) and *Prunella vulgaris* (B). Scale bar 10 µm

Table 1. The karyological parameters of *Prunella* species

Parameters	*P. grandiflora*	*P. vulgaris*
Basic number (*x*)	7	7
Diploid number (2*n*)	28	28
Ploidy level	4*x*	4*x*
Total haploid length (µm)	12.59	12.98
Mean haploid length (µm)	0.90	0.93

Table 2. Total chromosome lengths of *Prunella* species

P. grandiflora		*P. vulgaris*	
Chromosome	**Length (µm)**	**Chromosome**	**Length (µm)**
1	1.41	1	1.33
2	1.32	2	1.28
3	1.24	3	1.25
4	1.19	4	1.16
5	1.13	5	1.13
6	1.02	6	1.07
7	0.95	7	0.94
8	0.88	8	0.92
9	0.79	9	0.83
10	0.71	10	0.78
11	0.63	11	0.72
12	0.51	12	0.65
13	0.43	13	0.50
14	0.38	14	0.42

The karyotype asymmetry is one of the most effective parameters in understanding karyotype evolution (Eroğlu et al., 2019). While the early stages of karyotype evolution are periods in which symmetrical karyotypes are dominant, karyotypes become asymmetrical in the later process (Martin et al., 2023). Intrachromosomal (M_{CA}) and interchromosomal asymmetry (CV_{CL}) values could not be calculated because arm lengths (short and long) could not be measured. There are no records of karyotype asymmetry in the genus *Prunella*, and this remains a general problem or deficiency.

In this study, the karyology of the genus *Prunella* was evaluated by comparing previous and present results. The listed data will provide important contributions to the cytotaxonomy of the genus *Prunella*: (i) the basic number was *x* = 7, (ii) the most common diploid number was 2*n* = 28, and (iii) high polyploidy rates. The polyploidy probably played an important role in the speciation of the genus.

Conflict of Interest

The authors have declared no conflict of interest.

Authors' Contribution

All authors contributed to the study's conception and design. All authors read and approved the final manuscript.

References

Böcher TW (1949). Racial divergences in *Prunella vulgaris* in relation to habitat and climate. New Phytologist 48: 289-313.

Çelenk S, Dirmenci T, Özcan T (2024). A micromorphological study of some *Prunella* L. (*Lamiaceae*) taxa and its taxonomic implications. Micron 183: 103647.

Dirmenci T (2012). *Prunella* L. In: Güner A, Aslan S, Ekim T, Vural M, Babaç MT (eds.). Türkiye bitkileri listesi (Damarlı bitkiler). İstanbul: Nezahat Gökyiğit Botanik Bahçesi ve Flora Araştırmaları Derneği, pp. 574.

Edmonson JR (1982). *Prunella* L. In: Davis PH (ed.). Flora of Turkey and East Aegean Islands, Vol. 7. Edinburgh: Edinburgh University Press, pp. 295-297.

Eroğlu HE (2015). Which chromosomes are subtelocentric or acrocentric? A new karyotype symmetry/asymmetry index. Caryologia 68(3): 239-245.

Eroğlu HE, Sefalı A, Ünal M, Kelkitoğlu A, Martin E (2019). Cytogenetical analyses of some species of the genus *Sisymbrium* (*Brassicaceae*) in Turkey. Anatolian Journal of Botany 3(1): 21-24.

Eroğlu HE (2022). The polyploidy mechanism as response to global climate change. Doğan H, Fidan H (eds.). Global climate change: agriculture and food science perspective. Ankara: İksad Publishing House.

Fernandes A, Leitão MT (1984). Contribution à l'étude cytotaxinomique des Spermatophyta du Portugal XVIII-*Lamiaceae*. Memorias da Sociedade Broteriana 27: 27-75.

Govaerts R (2023). The world checklist of vascular plants (WCVP). Royal Botanic Gardens, Kew. Website: https://www.gbif.org/dataset/f382f0ce-323a-4091-bb9f-add557f3a9a2 [accessed 15 November 2024].

Harley RM, Atkins S, Budantsev A, Cantino PD, Conn BJ, Grayer R, Harley MM, De Kok R, Krestovskaja T, Morales R, Paton AJ, Ryding O, Upson T (2004). *Labiatae*. In: Kadereit JW (ed.). The families and genera of vascular plants, Vol. 7. Berlin: Springer, pp. 167-275.

Hrubý K (1932). Cytologie a anatomie československých Brunell. Preslia 11: 40-44.

Javadi H, Safikhani K (2023). Chromosome numbers in 31 taxa of Asteraceae, Brassicaceae, Poaceae, Hypericaceae, Lamiaceae, Fabaceae and Plantaginaceae from wetlands of Hamadan region in Iran. Botanica Pacifica 12(1): 183-188.

Malik RA, Gupta RC, Singh V, Bala S, Kumari S (2017). New chromosome reports in *Lamiaceae* of Kashmir (Northwest Himalaya), India. Protoplasma 254(2): 971-985.

Martin E, Eroğlu HE, Hamzaoğlu E, Koç M, Kelkitoğlu A, Öztürk E, Tanhaş E, Maşa M, Bozkurt H, Yavaş FN (2022). New chromosomal data of the genus *Dianthus* section Dentati (*Caryophyllaceae, Sileneae*). Anatolian Journal of Botany 6(1): 34-38.

Martin E, Hamzaoğlu E, Koç M, Eroğlu HE (2023). Karyotype analysis of *Dianthus muglensis* (*Caryophyllaceae, Sileneae*). Bozok Journal of Science 1(1): 11-15.

Magulaev AY (1979). Chromosomal numbers of flowering plants of the North Caucasus. Msg. 3. In: Galushko AI (ed.). Flora of the North Caucasus and questions of its history. T. 3. Stavropol: Stavropol State Ped. Inst., pp. 101-106.

Melnikov DG (2019) The genus *Prunella* (*Lamiaceae*) in the Caucasus. Botanicheskii Zhurnal 104: 1269-1282.

Mirzadeh Vaghefi SS, Jalili A (2023). The chromosome counts of some species of wetland plants from Northwest Iran: chromosome number. Caryologia 75(4): 67-76.

Peruzzi L, Eroğlu HE (2013). Karyotype asymmetry: again, how to measure and what to measure? Comparative Cytogenetics 7: 1-9.

Probatova NS, Sokolovskaja AP, Rudyka EG (1991). Chromosome numbers in some species of vascular plants from the Soviet Far East and other regions of the USSR. Botanicheskii Zhurnal 76: 1174-1178.

Vitek E, Kiehn M, Pascher K, Starlinger F, Greimler J, Stocker U, Lehner S, Beinhofer P, Blaha A (1992). Beiträge zur Flora von Österreich-weitere Chromosomenzählungen. Verhandlungen der Kaiserlich-Königlichen Zoologisch-Botanischen Gesellschaft in Wien 129: 215-226.

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Lipoic acid confers osmotic stress tolerance to maize seedlings by upregulating the enzymes of antioxidant defense and glyoxalase systems

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Lipoik asit, antioksidan savunma ve glioksalaz sistemlerinin enzimlerini artırarak mısır fidelerine ozmotik stres toleransı kazandırır

Abstract: Exogenous application of lipoic acid (LA), which is a special antioxidant substance, alleviates abiotic stress damage in plants. However, the mechanism of action of LA in stress tolerance is still not fully understood. Here, the effect of exogenous LA on the coordination of antioxidant defense and glyoxalase systems to alleviate osmotic stress damage was investigated. LA (12µM) was applied to the roots of 21-day-old seedlings in Hoagland nutrient solution for 8 hours and then the seedlings were exposed to 10% polyethylene glycol (PEG_{6000}) for 3 days. Seedlings grown in Hoagland nutrient solution for 28 days were used as the control group. Exogenous LA under osmotic stress was found to increase the fresh and dry weights of the leaves, leaf relative water content, and non-enzymatic compounds such as ascorbate and glutathione (GSH) while significantly decreasing the contents of hydrogen peroxide (H_2O_2) and methylglyoxal (MG). LA application also increased some antioxidant enzyme activities such as ascorbate peroxidase (1.2-fold), glutathione peroxidase (1.3-fold), glutathione reductase (1.4-fold), and monodehydroascorbate reductase (1.8-fold). LA significantly induced the relative expression levels of the genes coding the antioxidant enzymes. Furthermore, LA stimulated the enzyme activities of the glyoxalase system (glyoxalase I (Gly I) (1.3-fold) and glyoxalase II (Gly II) (1.1-fold). Additionally, the relative expression levels of the *Gly I* and *Gly II* genes were consistent with the findings of the Gly I and Gly II activities. Moreover, exogenous LA induced the expression level of *Gly II* (1.4-fold) more than that of *Gly I* (1.3-fold). As a result, LA mitigates osmotic stress damage in maize by enhancing the activity of antioxidant and glyoxalase systems, enabling the rapid removal of reactive oxygen species and the toxic compound MG, thereby providing a protective mechanism. Further investigation of the effects of LA on crops exposed to abiotic stresses will contribute to improve the stress tolerance and increase agricultural yields.

Key words: Lipoic acid, antioxidant system, glyoxalase system, osmotic stress, maize, gene expression

Özet: Özel bir antioksidan madde olan lipoik asidin (LA) ekzojen uygulaması, bitkilerdeki abiyotik stres hasarını hafifletir. Bununla beraber, LA'nın stres toleransındaki etki mekanizması henüz tam olarak anlaşılamamıştır. Bu çalışmada, osmotik stres hasarını hafifletmek için antioksidan savunma ve glioksalaz sistemlerinin koordinasyonuna exojen LA uygulamasının etkisi incelenmiştir. 21 günlük fidelerin köklerine Hoagland besin solüsyonunda 8 saat süreyle LA (12 µM) uygulandı ve ardından fideler 3 gün süreyle %10'luk polietilen glikole (PEG_{6000}) maruz bırakıldı. Kontrol grubu olarak 28 gün boyunca Hoagland besin solüsyonunda yetiştirilen fideler kullanıldı. Osmotik stres altında eksojen LA'nın, yaprakların taze ve kuru ağırlıklarını, yaprakların bağıl su içeriğini ve askorbat ve glutatyon (GSH) gibi enzimatik olmayan bileşenleri artırdığı, ayrıca hidrojen peroksit (H_2O_2) ve metilglioksal (MG) içeriklerini azalttığı bulunmuştur. LA uygulaması, askorbat peroksidaz (1.2 kat), glutatyon peroksidaz (1.3 kat), glutatyon redüktaz (1.4 kat) ve monodehidroaskorbat redüktaz (1.8 kat) gibi bazı antioksidan enzim aktivitelerini de artırmıştır. LA, antioksidan enzimlerin kodlayan genlerin bağıl ekspresyon seviyelerini de önemli derecede indüklemiştir. Ayrıca, LA, glioksalaz sistemi enzim aktivitelerini (glioksalaz I (Gly I) (1.3 kat) ve glioksalaz II (Gly II) (1.1 kat)) artırmıştır. Ayrıca, *Gly I* ve *Gly II* genlerinin bağıl ekspresyon seviyeleri, Gly I ve Gly II aktiviteleri ile tutarlıdır. Dahası, eksojen LA, *Gly I*'ye (1.3 kat) kıyasla *Gly II*'nin (1.4 kat) ekspresyon seviyesini daha fazla indüklemiştir. Sonuç olarak, LA, antioksidan ve glioksalaz sistemlerinin aktivitesini artırarak mısırdaki osmotik stres hasarını hafifletir, reaktif oksijen türlerinin ve toksik bileşik MG'nin hızla uzaklaştırılmasını sağlayarak koruyucu bir mekanizma sunar. LA'nın abiyotik streslere maruz kalan ürünler üzerindeki etkilerinin daha fazla araştırılması, stres toleransının iyileştirilmesine ve tarımsal verimin artırılmasına katkı sağlayacaktır.

Anahtar Kelimeler: Lipoik asit, antioksidan sistem, glioksalaz sistem, osmotik stres, mısır, gen ekspresyonu

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1. Introduction

Drought is one of the most unpredictable and uncontrolled environmental stresses and causes many devastating effects on plants (Anjum et al., 2017). Among all other abiotic stresses, drought stress is considered one of the serious threats limiting plant production, growth, and productivity (Junaid et al., 2023). Damage caused by stress factors varies depending on the type of plant, its tolerance and its adaptation ability (Kadıoğlu et al., 2011). In response to stress factors, plants have developed complex physiological and chemical strategies to adapt to sudden environmental changes. Some physiological, biochemical and molecular changes also occur during drought stress (Krasensky and Jonak, 2012).

It is well-established that one of the primary mechanisms plants use to tolerate stress is their antioxidant system, which protects them from the damaging effects of reactive oxygen species (ROS). This system contains a variety of

enzymatic and non-enzymatic antioxidants that scavenge ROS. The enzymatic antioxidant defense system includes superoxide dismutase (SOD), glutathione peroxidase (GPX), catalase (CAT), ascorbate peroxidase (APX), and glutathione reductase (GR), all of which are part of the ascorbate-glutathione cycle. It comprises enzymes like MDHAR and DHAR, as well as non-enzymatic substances such AsA, GSH, tocopherol, carotenoids, and phenolics (Sharma et al., 2012). Many studies have revealed that tolerance to osmotic stress in some agricultural plants is connected with enhanced antioxidant capability (Sezgin et al., 2019; Altansambar et al., 2024; Sezgin Muslu, 2024).

Like ROS, methylglyoxal (MG), a reactive oxidizing compound, increases under abiotic stress, which can damage the ultrastructural components of the cell, cause mutation, and finally cause programmed cell death (Hasanuzzaman et al., 2017). MG-detoxifying enzymes are divided into two groups: GSH-dependent and GSH-independent. In the GSH-dependent glyoxalase pathway, MG is detoxified to the nontoxic compound by two sequential reactions catalyzed by glyoxalase I (Gly I) and glyoxalase II (Gly II). In the first step, MG is converted to S-D-lactoylglutathione (SLG) using GSH, and in the final step, Gly II converts the GSH into the system and hydrolyzes SLG to form D-lactate. Thus, MG detoxification of plants ensures the maintenance of GSH homeostasis. In the GSH-independent pathway, MG is irreversibly converted to D-lactate by glyoxalase III (Gly III) in a single step without using GSH (Singh and Dhaka, 2016). The antioxidant system in cooperation with the glyoxalase system is known to increase abiotic stress tolerance in plants (Nahar et al., 2015a). Studies showing the regulatory role of exogenous applications on antioxidant and glyoxalase systems to induce oxidative stress tolerance are still insufficient. Some studies were showing that applications of some antioxidant compounds increased tolerance to some stress factors by stimulating antioxidant and glyoxalase systems (Hossain et al., 2010; Nahar et al., 2015b, c). The activities of these two systems could change depending on the type of plants under different stress factors (Hasanuzzaman et al., 2019; Zaid et al., 2019; Sezgin Muslu and Kadıoğlu, 2021).

Maize, one of the plants with C4 metabolism, is a grain product grown all over the World (Farhad et al., 2009). It grows in the Mediterranean and Black Sea Regions and encounters abiotic stresses during the growth period (Tanyolaç et al., 2007). Researchers reported that water deficiency negatively affected germination and seedling growth in maize (Mohammadkhani and Heidari, 2008). To maintain the economic value of maize, it is necessary to develop genotypes that can tolerate stress or to apply some chemical substances with protective properties externally. Ensuring stress tolerance by applying chemicals to plants has a low cost (Hamdia and Shaddad, 2010), and antioxidants as chemicals are particularly important (Ashraf and Foolad, 2007). One of the antioxidant compounds that play a role in regulating the redox state of plants is lipoic acid (LA).

Unlike other antioxidant substances, LA, a unique short-chain fatty acid with two sulfur atoms, has strong antioxidant properties in both reduced (dihydrolipoic acid, DHLA) and oxidized forms (Sudesh et al., 2002). DHLA is a more effective antioxidant than oxidized lipoic acid and plays a role in directly scavenging ROS such as superoxide, hydroperoxyl and hydroxyl radicals (Navari-Izzo et al., 2002). It has been determined that LA, which plays a role in respiration and indirectly in carbon fixation and nitrogen assimilation in plants, increases glutathione, the most important water-soluble antioxidant (Taylor et al., 2004). It has recently been revealed that different plants can resist some stress factors (drought, salt, alkaline, and cadmium) with LA application (Elkelish et al., 2021; Youssef et al., 2021; Ramadan et al., 2022; Yadav et al., 2005; Daler and Kaya 2024). On the other hand, studies on maize under osmotic stress have shown that exogenous LA stimulates photosystem II activity and enhances water-deficit tolerance by modulating osmoprotectant metabolism (Sezgin et al., 2019; Saruhan Güler et al., 2021). In addition, it was reported that LA stimulated some antioxidant enzyme activities and their expression levels in the maize seedlings under osmotic stress (Terzi et al., 2018; Gümrükçü Şimşek et al., 2024). As far as we know, no reports exist on how LA stimulates the glyoxalase system in plants exposed to stress factors. We aimed to determine which of these systems (antioxidant and glyoxalase) LA has a greater effect. Therefore, in the current study, it was hypothesized that LA regulates the activity of the enzymes of the two systems and thus provides coordination of the antioxidant and glyoxalase systems. Therefore, the present study was designed to evaluate the role of LA in the coordination of antioxidant defense and glyoxalase systems. This is the first study to demonstrate that LA alleviates osmotic stress-induced oxidative damage in maize seedlings by stimulating the antioxidant and glyoxalase defense systems.

2. Materials and Method

2.1. Growing of plants, LA and stress applications

Maize (*Zea mays* L.) seeds were used in our study. For surface sterilization, the seeds were treated with 0.1% $HgCl_2$ for 3 min and then washed three or four times with sterilized distilled water**.** The seeds were grown in a hydroponic medium containing Hoagland nutrient solution (Hoagland and Arnon, 1950) in a growth chamber with light intensity (400-430 µmol $m^{-2}s^{-1}$), temperature (18-20 ^{o}C) and humidity (50-70%). LA (12µM) (Sezgin et al., 2019) was applied to the roots of 21-day-old seedlings in Hoagland nutrient solution for 8 hours. Then, the plants were exposed to osmotic stress and provided with 10% polyethyleneglycol (PEG_{6000}) for 72 hours. The osmotic stress applied to the plants was gradually increased. They were exposed to 3% PEG medium for 1 day, 6% PEG medium for 3 days and 10% PEG medium for 3 days, respectively (Gümrükçü Şimşek et al., 2024). In this current study, experimental groups were designed in four different ways: seedlings grown in Hoagland nutrient solution for 28 days (Control); 21-day-old seedlings exposed to osmotic stress ((3% PEG (1 day), 6% PEG (3 days) 10% PEG (3 days)) (PEG); after LA pretreatment for 8 hours, the seedlings were kept in Hoagland nutrient solution for 7 days (LA); after LA pretreatment for 8 hours, the seedlings exposed to the osmotic stress mentioned above (LA+PEG). After the applications, the leaves were harvested. Leaf fresh and dry weights were measured, and leaf relative water content analyses were completed immediately after sampling**.**

2.2. Determination of fresh and dry leaf weights

Leaf fresh weights (FW) of maize plants were weighed and recorded in 5 replicates from each experimental group. The leaves, whose fresh weights were calculated, were dried in an oven at 75°C for 48 hours, and the dry weights (DW) of the leaves were calculated.

2.3. Leaf relative water content (LRWC)

Determination of LRWC was made according to Castillo (1996). After measuring the fresh weight of the leaves of the plants, their turgid weights were taken by soaking them in water overnight. Then, LRWC was determined by keeping them in an oven set at 80 ºC, recording their dry weights and substituting them in the formula below.

Leaf RWC (%) = (Fresh weight-Dry weight/Turgid weight-Dry weight) x100

2.4. Detection of the presence of H_2O_2 by 3,3′ - Diaminobenzidine (DAB) staining method

To quantify H_2O_2, the DAB staining procedure was modified by Daudi and O'brien (2012). The leaves of the samples were treated with DAB made with Tween 20 and sodium phosphate buffer (pH 7.0). The leaves were then inserted in test tubes and incubated on a laboratory shaker at 90 rpm. After incubation, the leaves were boiled in ethanol, acetic acid, and glycerol in a water bath at 95 °C for 15 min. The leaves were placed in a fresh bleach solution for 30 min before being inspected and photographed for colour changes.

2.5. Determination of MG content

Leaf samples (0.1 g) were homogenized in perchloric acid and then centrifuged at 16,000 xg for 15 min. Before using this supernatant for the analysis, it was neutralized by immersing it in a saturated potassium carbonate solution at ambient temperature for 15 min before centrifuging again at 16,000 xg for 15 min. The supernatant was treated with 1 mL of 1,2-diaminobenzene and perchloric acid. The derivatized MG was measured at 335 nm after 25 min (Yadav et al., 2005).

2.6. Determination of changes in antioxidant capacity

Fresh leaves (0.1 g) were extracted in 1.8 mL extraction buffer (50 mM K_2HPO_4 (pH 7.0), 1 mM EDTA, 1% PVPP, and ascorbate for APX). The extract was centrifuged, and the supernatant was used to assess enzyme activity and protein content. Protein determination was carried out spectrophotometrically by Bradford (1976). GPX activity by measuring a 100 mM potassium phosphate buffer (pH 7.0), 0.1mM EDTA, 5 mM guaiacol, 20 mM H_2O_2, and enzyme extract (50 µL) in a 1 mL total volume at 470 nm for 1 min (Urbanek et al., 1991). The APX activity was assessed using Nakano and Asada's (1981) method, which measured the decrease at 290 nm of a 1 mL reaction mixture containing 50 mM potassium phosphate buffer (pH 7.0), 250 µM AsA, 5 mM H_2O_2, and enzyme extract (20 µL). GR activity was evaluated using Foyer and Halliwell's (1976) method, which involved adding enzyme extract (50 µL) to a combination of 50 mM Tris-HCl (pH 7.8), 1 mM GSSG, and 200 µL 0.25 mM NADPH in 200 µL 0.5 mM EDTA. It was assessed by a decrease in oxidation at 340 nm over 5 min. MDHAR was computed by measuring at 340 nm the 1 mL reaction mixture created by adding 50 mM potassium phosphate buffer (pH 7.8), 150 µM NADH, and 500 µM AsA to the sample extract (100 µL). The measured values were extrapolated from data collected without ascorbate oxidase (Hossain et al., 1984).

To assess DHAR activity, 0.1 g of fresh leaves were homogenized in an extraction buffer (1.8 mL) comprising 50 mM Tris-HCl (pH 7.4), 100 mM NaCl, 2 mM EDTA, and 1 mM $MgCl_2$). The extract was centrifuged at 15,000 xg for 10 min. The enzyme activity was measured by measuring 1 mL of a mixture containing 50 mM K_2HPO_4/KH_2PO_4 (pH 6.5), 0.5 mM DHA, 1 mM GSH, and enzyme extract (100 µL), as described by Hossain and Asada (1984). DHAR activity was evaluated by measuring the rise in absorbance at 265 nm.

Fresh leaf samples (0.1 g) were homogenized with m-phosphoric acid. The extract was centrifuged at 15,000 xg for 10 min and then added to a reaction medium containing citrate-phosphate buffer (pH 6.2). AsA content was determined using the protocol of Liso et al. (1984).

To determine GSH concentration, plant leaf samples (0.1 g) were extracted in metaphosphoric acid with EDTA. The extract was centrifuged at 15,000 xg for 15 min, and the supernatant was utilized to measure GSH content. It was determined using the "glutathione assay kit" (Cayman Chemical) protocol described by Tietze (1969).

2.7. Determination of the changes in activities of glyoxalase system enzymes

To extract enzymes, leaf samples (0.1 g) were homogenized in a potassium phosphate buffer (pH 7.0) containing KCl, AsA, β-mercaptoethanol, and glycerol were centrifuged at 13,000 xg for 10 min, and the supernatants were utilized to assess the activity. Gly I activity was assessed by Hasanuzzaman et al. (2011). The reaction began with the addition of MG, and the rise at 240 nm in 1 min was recorded. Gly II activity was determined by measuring GSH production at 412 nm for 1 min, as described by Principato et al. (1987).

2.8. Real-Time PCR Analyzes

Fresh leaf samples were thoroughly shredded with a tissue disintegrator. Then, the RNA isolation kit protocol was applied to obtain total RNA. The amount and purity of the RNA samples were determined using a nanodrop. Using the cDNA transcription kit (Applied Biosystems), cDNA was obtained from the total RNA samples that had been isolated. To determine the gene expressions of the samples, analyses were completed on the CFX Connect Real-Time PCR System device, using cDNA results and iTaq Universal SYBR Green Supermix (Bio-Rad) solution according to the manufacturer's instructions. With a final volume of 20 µL, the reactions are follows: (10 µL Supermix (2X), 1 µL forward and reverse primers (10 µM), 1 µL cDNA, and 7 µL nuclease-free water). The Real-Time PCR process steps were modified according to iTaq Universal SYBR Green Supermix' instructions as follows: an initial step of 1 min at 95°C, followed by 44 cycles of 15 sec at 95°C, 30 sec at 60°C, and 30 sec at 72°C. A melt curve was generated with 0.5°C increments from 65°C to 95°C. A melting curve analysis was performed at the end of each PCR reaction to confirm the presence of a single peak, ensuring the absence of primer dimers and non-specific product formation. Primers belonging to the genes shown in Table 1 were used to determine the expression

levels of genes belonging to enzymes responsible for antioxidant defense and glyoxalase system (Zhao et al., 2017; Talaat et al., 2022). The data obtained from the analysis were normalized to the *β-Actin* reference gene and presented as relative gene expression using the $2^{-\Delta\Delta CT}$ method, following the protocol outlined by Bookout and Mangelsdorf (2003). Each biological replication was analyzed as 3 technical replicates and the mean technical error was accepted as 0.5 (±1) Cq values. The Cq values of these genes were examined using the Bio-Rad CFX Manager Software (version 3.1).

2.9. Statistical Analysis

The analyses were done in triplicate. The results of the experiments are presented as mean ± standard deviations. One-way ANOVA was used for statistical studies in SPSS (version IBM 23). P< 0.05 was considered statistically significant. Relative gene expression during qRT-PCR analysis was examined using Bio-Rad CFX Manager 3.1.

3. Results

3.1. Leaf dry and fresh weight, and LRWC

LA application increased leaf fresh weight under both stressful and non-stressful conditions. Moreover, it was determined that LA application did not statistically change leaf dry weight and LRWC under non-stressful conditions. However, under stressful conditions, LA significantly increased leaf dry weight and LRWC (Fig. 1a-c).

3.2. H_2O_2 and MG contents

As illustrated in Figure 2a, the brown speck indicated the existence of H_2O_2. Brown spots on PEG-treated leaves significantly increased as compared to controls. However, under a non-stressful situation, LA-treated seedlings had fewer brown patches than the control treatment. Moreover, LA-treated leaves showed fewer brown spots under non-stress conditions than PEG-treated leaves.

Table 1 Primer sequences of genes whose expression levels were determined

Target gene	Sequences 5'-3'
Ascorbate peroxidase (APX)	F: GCCTTCTTCAGCTCCCAAGT R: TGCAAAAGACCACATGCAGC
Glutathione peroxidase (GPX)	F: CGCTATGCTCCAACCACTTC R: GCTCTCAGAGCAATGTTCATACAG
Glutathione reductase (GR)	F: ATG GTG GGA CTT GCG TGA TA R: GCA TCA ACT AGA CTG CCT GC
Monodehydroascorbate reductase, (MDHAR)	F: CTG TAA AGG CGA TCA AGG GC R: ACC TTG CCG TCC TTA ATC CA
Dehydroascorbate reductase (DHAR)	F: GCT GAT CTC TCT CTG GGT CC R: GCG CCA TCC AGC AAT TAC AT
Glyoxalase I (Gly I)	F: TGAGGCAGTTGATCTGGCG R: CCCGAGTCTTCACTGTAGTTCC
Glyoxalase II (Gly II)	F: CACATGGATGTTTGCTGGTC R: CGTGCATCATCAAAATGGTC
β-Actin	F: ACCAGTTGTTCGCCCACTAG R:GAAGATCACCCTGTGCTGCT

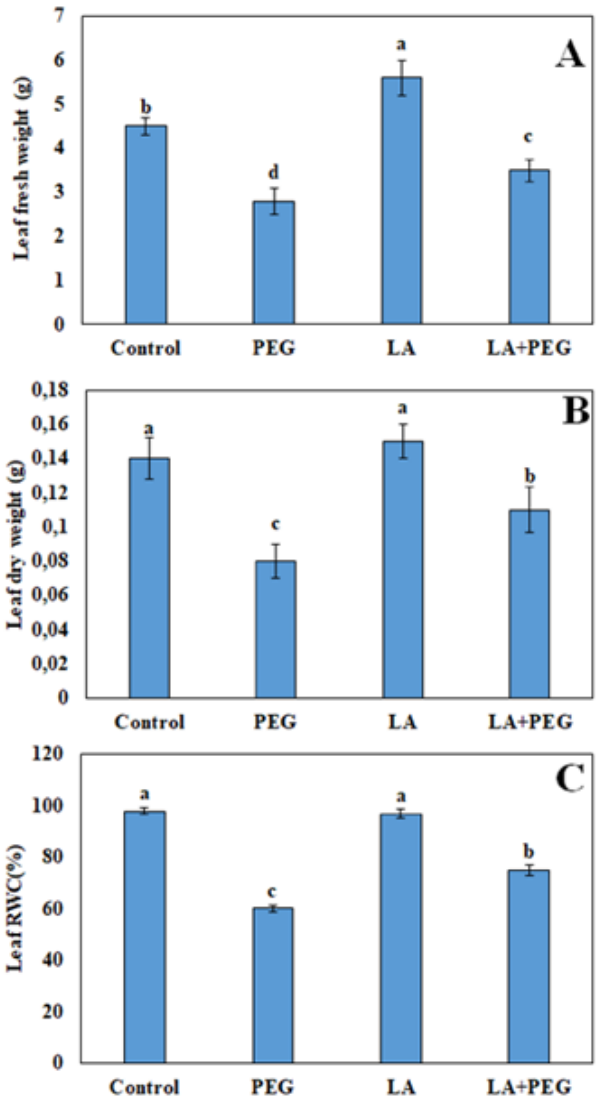

Figure 1. Effects of LA on Leaf dry weight (a), Leaf fresh weight (b), and Leaf RWC (c) under osmotic stress. The vertical bars reflect the standard deviations of three replicated means. At P <0.05, different letters denote significant differences among all treatments.

MG content increased 1.2-fold in PEG-treated seedlings compared to the control group. Moreover, MG content in LA-treated leaves decreased by 1.07-fold compared to the control group, while MG content in LA+PEG-treated leaves decreased by 1.13-fold compared to PEG-treated leaves. (Fig. 2b).

3.3. Antioxidant capacity

Under non-stressed conditions, exogenous LA increased the antioxidant system enzymes activities (except GPX and MDHAR) and antioxidant compounds. Under PEG stress, exogenous LA induced the antioxidant system enzyme activities (except DHAR) (Figure 3a-d). There was a statistically significant difference in the APX activity between control and LA groups. The APX activity of LA-treated leaves was 1.3-fold higher than the control leaves. In addition, the activity of the LA+PEG treatment was 1.2-fold higher than the PEG-treated leaves (Figure 3a). Under PEG-stressed conditions, LA significantly increased the GPX activity (1.3-fold) compared to the PEG treatment. However, there was no difference in the GPX activity

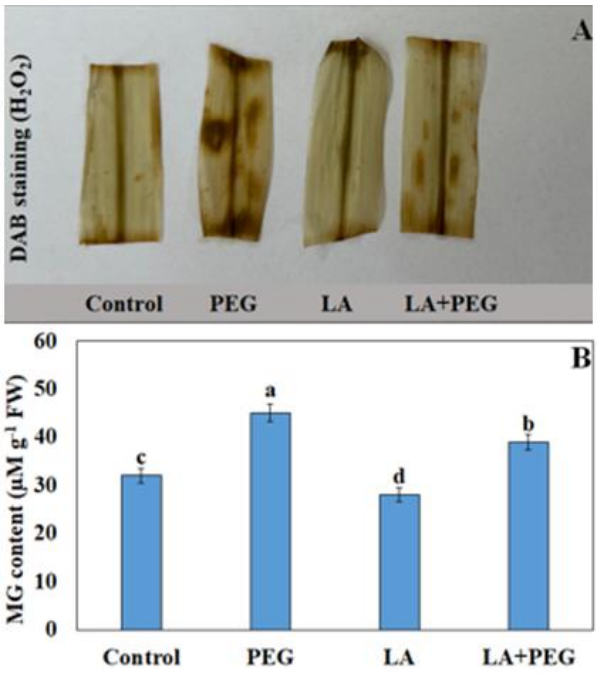

Figure 2. Effects of LA on DAB staining (H_2O_2) (a), MG content (b) under osmotic stress. The vertical bars reflect the standard deviations of three replicated means. At P <0.05, different letters denote significant differences among all treatments.

between the control and LA treatments (Figure 3a). There was a statistical difference in the GR activity between PEG and LA+PEG groups. The GR activity of the LA+PEG-treated leaves was 1.4-fold higher than the PEG alone. The LA application resulted in a 1.5-fold increase in GR activity compared to the control (Figure 3b). In addition, the MDHAR activity of the LA+PEG-treated leaves was 1.8-fold higher than the PEG treatment. However, there was no difference in the MDHAR activity between the control and LA treatments (Figure 3c). There was a statistically significant difference in the DHAR activity between control and LA groups. The LA application resulted in a 1.5-fold increase in DHAR activity compared to the control. However, there was no difference in the DHAR activity between the PEG and LA+PEG treatments (Figure 3d).

There was a statistical difference in the AsA content between control and PEG groups The PEG treatment resulted in a 2.5-fold increase in AsA content compared to the control. AsA content in the LA-treated leaves was 1.17-fold higher than the control. The LA+PEG application caused a 1.2-fold increase in AsA content compared to the PEG group (Figure 4a). The GSH content of the LA+PEG group was higher than all groups. Interestingly, the content increased 2.5-fold in the PEG-treated leaves and 1.3-fold in LA-treated leaves compared to the control. Similarly, under PEG-induced osmotic stress, exogenous LA stimulated the GSH content compared to the PEG treatment, with a 1.1-fold increase in the LA+PEG-treated leaves (Figure 4b).

3.4. Glyoxalase system enzymes

Exogenous LA under non-stressful and stressful conditions caused to significantly increase in the glyoxalase system enzymes (Gly I and Gly II) activities. Gly I activities in the PEG and LA-treated leaves were 4.0- and 3.0-fold higher than the control, respectively. The Gly I activity increased 1.3-fold in the LA+PEG treatment compared to the PEG treatment. The Gly II activity significantly increased 4.8-fold in the PEG treatment and 3.1-fold in the LA treatment compared to the control. Moreover, the activity was 1.1-fold higher in LA+PEG treatment than in the PEG treatment (Figure 5).

3.5. The levels of antioxidant enzyme gene expression

The PEG treatment resulted in a significant increase in the relative expression of the *APX* gene by 1.5-fold over the control. Under non-stressful conditions, the LA treatment contributed to the significant upregulation in the expression of the *APX* gene by 1.2-fold in comparison to the control. However, there was no difference in the *APX* gene expression between the PEG and LA-combined PEG treatments (Figure 6a). The PEG treatment significantly up-regulated the expression of the *GPX* by 2.1-fold, while the

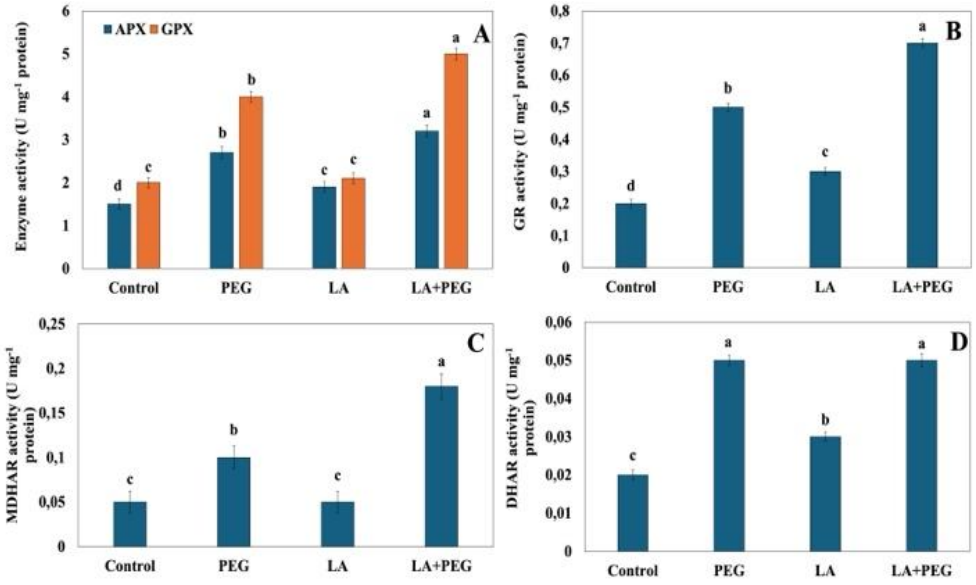

Figure 3. Effects of LA on antioxidant enzyme activities under osmotic stress: APX and GPX activities (a), GR activity (b), MDHAR activity (c), and DHAR activity (d). The vertical bars reflect the standard deviations of three replicated means. At P <0.05, different letters denote significant differences among all treatments.

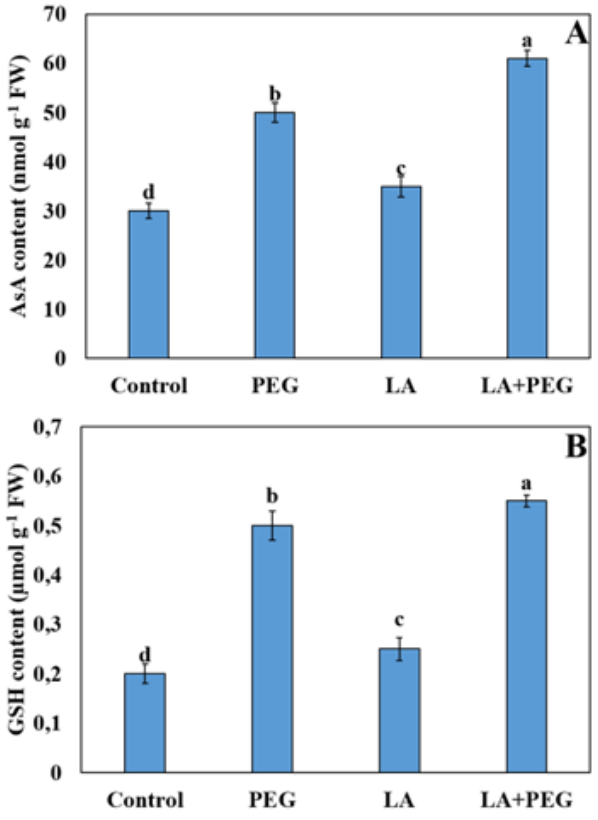

Figure 4. Effects of LA on antioxidant compounds under osmotic stress: AsA content (a), GSH content (b). The vertical bars reflect the standard deviations of three replicated means. At P <0.05, different letters denote significant differences among all treatments

LA-combined PEG treatment up-regulated this gene by 2.3-fold compared to the PEG treatment. However, there was no statistical difference in the *GPX* gene expression between the control and LA treatments (Figure 6b). The PEG treatment up-regulated *GR* expression more than the control. The *GR* expression level increased 1.2-fold in the

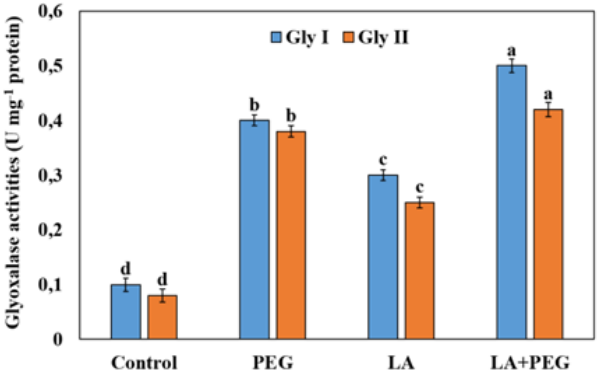

Figure 5. Effects of LA on glyoxalase system enzyme activities under osmotic stress: Gly I activity (a), Gly II activity (b). The vertical bars reflect the standard deviations of three replicated means. At P <0.05, different letters denote significant differences among all treatments.

LA-treated leaves compared to the control. Under PEG stress conditions, exogenous LA stimulated the gene expression of *GR* by 1.14-fold compared to the PEG alone (Figure 6c). The *MDHAR* gene expression level increased under PEG-induced osmotic stress compared to the control. The *MDHAR* gene was up-regulated 1.3-fold in the LA-treated leaves compared to the control. The gene expression increased 1.2-fold in the LA+PEG treatment compared to the PEG alone (Figure 6d). Exogenous LA under the non-stressed conditions contributed to the up-regulation in the expression of the *DHAR* gene by 1.2-fold in comparison to the control, while exogenous LA combined with PEG treatment up-regulated this gene by 1.1-fold over the PEG alone (Figure 6d).

3.6. The levels of gene expression of glyoxalase system enzymes

PEG-induced osmotic stress resulted in a significant increase in the relative expression of the *Gly I* gene by 2.5-fold over the control. The LA treatment contributed to a

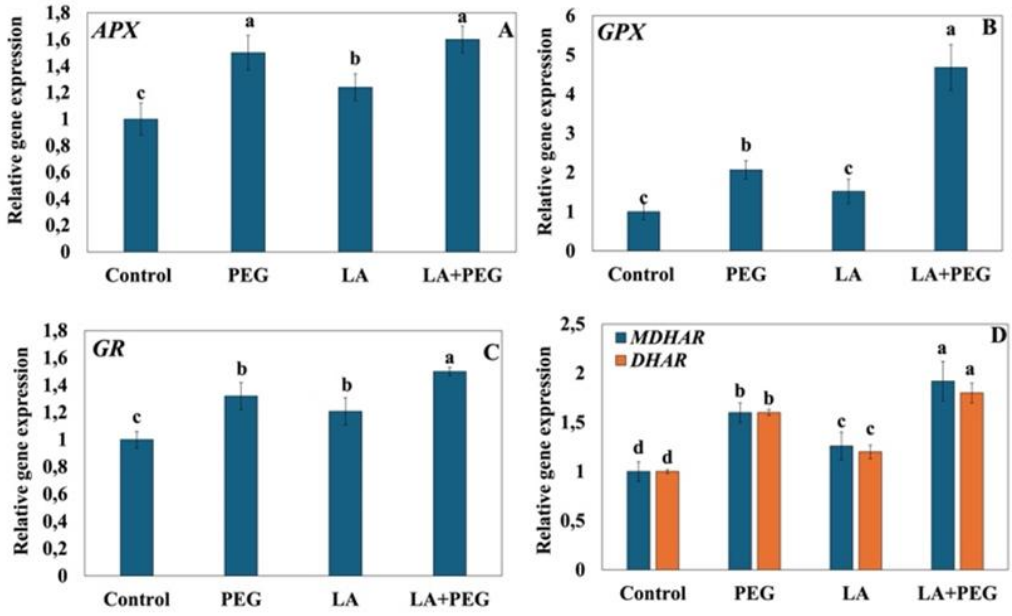

Figure 6. Effects of LA on the relative gene expressions of antioxidant enzymes under osmotic stress: *APX* gene (a), *GPX* gene (b), *GR* gene (c), *MDHAR* and *DHAR* genes. The vertical bars reflect the standard deviations of three replicated means. At P <0.05, different letters denote significant differences among all treatments.

2.0-fold upregulation in the expression of the *Gly I* gene compared to the control, while the LA+PEG treatment up-regulated this gene by 1.2-fold compared to the PEG treatment. The relative expression level of the *Gly II* gene showed a similar trend to that of the *Gly I* gene expression. The PEG treatment significantly up-regulated the expression of *Gly II* by 2.7-fold, while the LA treatment up-regulated this gene by 1.7-fold compared to the control. The LA treatment under osmotic stress up-regulated the expression of the *Gly II* gene by 1.4-fold in comparison with the PEG treatment (Figure 7).

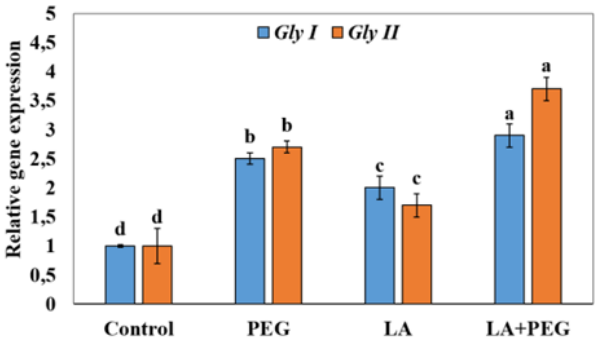

Figure 7. Effects of LA on the relative expression levels of some genes involved in the glyoxalase system under osmotic stress: *Gly I* gene (a), *Gly II* gene. The vertical bars reflect the standard deviations of three replicated means. At P <0.05, different letters denote significant differences among all treatments.

4. Discussions

It was suggested that one of the parameters that change in the plant under stress conditions and indicate the degree of water deficiency in the plant is the RWC (Flower and Ludlow, 1986). For this reason, in our study, the change in LRWC was determined to assess whether the plants were exposed to stress. It was determined that the LRWC decreased under stress conditions and exogenous LA alleviated the decrease in LRWC value. A similar study on maize reported that RWC decreased in the leaves exposed to drought (Goodarzian Ghahfarokhi et al., 2015). In the current study, the decrease in LRWC value in the LA-treated leaves was less than that of LA-untreated leaves, indicating that LA can protect plants against water loss. As another indicator of stress, leaf dry weight analyses were performed. LA treatment reduced dry weight loss in maize under PEG-induced osmotic stress. In previous investigations, LA improved water loss in maize leaves under osmotic stress (Sezgin et al., 2019; Saruhan Güler et al., 2021). Our present data showed that maintaining the efficacy of the antioxidant and glyoxalase system could reduce dry matter loss in maize during osmotic stress.

Under non-stressed conditions, various metabolic processes lead to ROS production. The production of toxic oxygen derivatives increases in response to abiotic or biotic stress (Mohammadkhani and Heidari, 2008). Under osmotic stress, the interaction between LA and ROS levels is not well known. In the current study, PEG-treated seedlings had the greatest H_2O_2 level indicating that endogenous ROS synthesis outpaced the ability of cellular antioxidant defense system to remove ROS (Liu et al., 2010). The rise in H_2O_2 levels induced oxidative stress, leading to enhanced membrane damage. However, LA application significantly reduced endogenous ROS levels by increasing enzymatic and non-enzymatic antioxidants such as AsA and GSH to enhance oxidative stress tolerance under both stress and control conditions. Our results on AsA and GSH content were consistent with the findings of Gorcek and Erdal (2015), who researched the effect of LA on salt-stressed wheat seedlings. The decrease in H_2O_2 content after LA application may be due to its ability to scavenge ROS as an antioxidant. Plants have efficient ROS-scavenging systems that keep them safe from harmful oxidative reactions. Antioxidant enzymes and chemicals play an important role in defensive systems.

Turk et al. (2018) found that applying LA dramatically lowered SOD, GPX, APX, CAT, and GR activities in wheat subjected to heavy metal stress. In our study, LA treatment increased APX, GPX, GR, MDHAR, and DHAR activities in seedlings under osmotic stress. Our findings confirmed previous research on salinity-stressed canola, wheat, and sorghum (Görçek and Erdal, 2015; Youssef et al., 2021; Khan et al., 2022). The decrease of H_2O_2 in seedlings treated with LA under both stressful and non-stressful conditions can be the outcome of increased APX activity compared to the control and PEG treatments. Similarly, another study showed that LA may have protective and antimutagenic effects against oxidants (especially H_2O_2) in sorghum plants through its antioxidant activity (Youssef et al., 2021). The application of LA+PEG significantly increased the activities of APX (1.2-fold) and GPX (1.3-fold) enzymes, which play a role in H_2O_2 scavenging, and GR (1.4-fold) and MDHAR (1.8-fold), compared to PEG treatment. GR is another important antioxidant enzyme that is vital in maintaining cellular redox balance (Dwivedi et al., 2020). In our study, maize seedlings subjected to osmotic stress exhibited a significant increase (1.4-fold) in GR activity after treatment with LA. This increase in GR activity promoted the reduction of glutathione disulfide to glutathione (GSH), indicating that GSH could be crucial for regulating redox balance and protecting cells from ROS-induced damage.

Apart from the enzyme activities, the relative expression levels of *APX*, *GPX*, *GR*, *MDHAR*, and *DHAR* genes were determined in LA-treated seedlings under unstressed and stressed conditions. The current study also determined that increased transcripts of many genes encoding antioxidant enzymes reflect genetic regulations. Moreover, it has been reported that increasing the activities of SOD, APX, and CAT caused by the application of LA, the expressions of their related genes were up-regulated in maize seedlings under osmotic stress (Gümrükçü Şimşek et al., 2024). For the first time, this study determined that LA treatment caused a significant increase of 2.3-fold in GPX gene expression under stress conditions. Similarly, we also found that GR and MDHAR enzyme activities showed notable increases of 1.4-fold and 1.8-fold, respectively.

Increased GR activity may facilitate the recycling of GSSG to GSH (Foyer and Halliwel, 1976). GSH and AsA levels increased in plants exposed to water loss (Ansari et al., 2017). D'Amico et al. (2004) discovered that AsA content increased but GSH content decreased in wheat watered with sea water over a 21-day growth period. Görcek and Erdal (2015) and Türk et al. (2018) found that exogenous LA boosted AsA and GSH levels in wheat after abiotic stress.

Our findings demonstrated that under stressful and non-stressful conditions, LA increased the accumulation of AsA and GSH, improving ROS scavenging effectiveness and reducing oxidative damage caused by ROS. The data supported that LA may operate as a signalling molecule, boosting antioxidant enzyme activity in osmotically stressed-maize seedlings.

GSH is not only the main compound of the AsA-GSH cycle but also plays an important function in the MG detoxification system, and GSH plays a role as a compound that connects these two systems (antioxidant and glyoxalase). It was reported that the antioxidant defense and glyoxalase system of plant cells work in coordination to alleviate oxidative stress by reducing toxic ROS and MG accumulation (Hasanuzzaman et al., 2019). However, the exact mechanism by which exogenous protective agent applications affect these two defense systems remains unclear. In our study, it has been determined that LA reduced the MG content, which increases under the PEG-induced stress. Also, it has been detected that Gly I and Gly II activities, included in the glyoxalase system that effectively reduce MG content, and the relative expression levels of the *Gly I* and *Gly II* genes were stimulated with LA application. The Gly I and Gly II activities increased 1.3 and 1.1-fold, respectively, in LA+PEG treatment compared to the PEG treatment. Moreover, under non-stressed conditions, LA treatment stimulated *Gly I* (2.0-fold) gene expression more than *Gly II* (1.7-fold). On the contrary, external LA application stimulated *Gly II* (1.4-fold) gene expression under stressed conditions more than *Gly I* (1.2-fold), providing osmotic stress tolerance. As a result, it has been revealed that the LA treatment induced the glyoxalase system, which includes the Gly I and Gly II, can keep the oxidative stress trigger MG under control, where GSH is used by Gly I and recycled after MG detoxification. The glyoxalase system also participates in the AsA-GSH cycle via GSH. Our study determined that increasing the GSH level by LA was effective in alleviating oxidative stress damage in maize seedlings.

In conclusion, under both non-stressful and stressful conditions, exogenous application of LA showed enhanced tolerance to oxidative damage by enhancing ROS and MG detoxification systems. Thus, an interaction relationship between ROS and MG detoxifying systems in maize seedlings treated with LA under osmotic stress was considered. The increased GSH in LA-treated seedlings was a cofactor for the glyoxalase system. As a result, this system not only detoxified MG, but also helped to preserve GSH homeostasis and subsequent ROS detoxification. In our study, we also revealed for the first time which enzymes related to these systems were more affected by exogenous LA. Under non-stress conditions, LA treatment significantly increased the activities of GR (1.5-fold), DHAR (1.5-fold), Gly I (3.0-fold), and Gly II (3.1-fold) enzymes, as well as the relative expression of *Gly I* (2.0-fold) and *Gly II* (1.7-fold) genes. Under stressful conditions, it was revealed that LA application stimulated GR (1.4-fold) and MDHAR (1.8-fold) enzyme activities, as well as the relative gene expressions of *GPX* (2.3-fold) and *Gly II* (1.4-fold), to a greater extent. The current study demonstrated that LA activated these two systems in a coordinated manner under both non-stressful and stressful conditions. The activated antioxidant and glyoxalase defense systems played a significant role in the osmotic stress tolerance of LA-treated maize seedlings through ROS and MG detoxification. It is critical to learn more about the mechanisms that drive osmotic stress tolerance induced by exogenous LA in plants and their responses. The varying responses to LA treatments, especially the significant benefits seen under non-stress conditions and escalated with increased LA concentrations, align with the findings of Navari-Izzo et al. (2002) and Saruhan Guler et al. (2021). These studies emphasize LA's powerful antioxidant properties, which help alleviate oxidative stress caused by osmotic stress and promote plant growth in challenging conditions.

Conflict of Interest

The authors declare that they have no conflict of interest.

Authors' Contribution

ASM and RT contributed to the study's conception and design. ASM conducted the experiments. ASM and AK analyzed all data and wrote the manuscript.

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References

Altansambar N, Sezgin Muslu A, Kadıoglu A (2024). The combined application of rutin and silicon alleviates osmotic stress in maize seedlings by triggering accumulation of osmolytes and antioxidants' defense mechanisms. Physiology and Molecular Biology of Plants 30(3): 513-525. https://doi.org/10.1007/s12298-024-01430-z.

Anjum SA, Ashraf U, Zohaib A, Tanveer M, Naeem M, Iftikhar ALI, Tabassum T, Nazir U (2017). Growth and developmental responses of crop plants under drought stress: a review. Zemdirbyste-Agriculture 104: 267-276.

Ansari WA, Atri N, Singh B, Pandey S (2017). Changes in antioxidant enzyme activities and gene expression in two muskmelon genotypes under progressive water stress. Biologia Plantarum 61(2): 333-341. https://doi.org/10.1007/s10535-016-0694-3.

Ashraf MFMR, Foolad MR (2007). Roles of glycine betaine and proline in improving plant abiotic stress resistance. Environmental and Experimental Botany 59(2): 206-216.

Bookout AL, Mangelsdorf DJ (2003). Quantitative real-time PCR protocol for analysis of nuclear receptor signaling pathways. Nuclear Receptor Signal 1:1-7. doi:10.1621/nrs.01012.

Bradford MM (1976) A Rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochemistry 72: 248-254.

Castillo FJ (1996). Antioxidative protection in the inducible CAM plant *Sedum album* L. following the imposition of severe water stress and recovery. Oecologia 107: 469-477.

Daler S, Kaya O (2024). Exogenous alpha-lipoic acid treatments reduce the oxidative damage caused by drought stress in two grapevine rootstocks. Physiologia Plantarum 176(4), e14437.

D'Amico ML, Navari-Izzo F, Sgherri C, Izzo R (2004). The role of lipoic acid in the regulation of the redox status of wheat irrigated with 20% sea water. Plant Physiolgy and Biochemistry 42(4): 329-334. doi: 10.1016/j.plaphy.2004.02.009.

Daudi A, O'brien JA (2012). Detection of hydrogen peroxide by DAB staining in Arabidopsis leaves. Bio-Protocol 2(18): e263-e263.

Dwivedi S, Kumar A, Mishra S, Sharma P, Sinam G, Bahadur L, Tripathi RD (2020) Orthosilicic acid (OSA) reduced grain arsenic accumulation and enhanced yield by modulating the level of trace element, antioxidants, and thiols in rice. Environ Sci Pollut Res 27:24025-24038

Elkelish A, El-Mogy MM, Niedbała G, Piekutowska M, Atia MA, Hamada MM, Ibrahim, MF (2021). Roles of exogenous α-lipoic acid and cysteine in mitigation of drought stress and restoration of grain quality in wheat. Plants 10(11): 2318-2343.

Farhad W, Saleem MF, Cheema MA, Hammad HM (2009). Effect of poultry manure levels on the productivity of spring maize (*Zea mays* L.). Journal Animal and Plant Science 19: 122-125.

Flower DJ, Ludlow MM (1986). Contribution of osmotic adjustment to the dehydration tolerance of water stressed pigeonpea (*Cajanus cajan* L.) Millsp. leaves. Plant Cell Environment 9: 33-40.

Foyer CH, Halliwell B (1976). The presence of glutathione and glutathione reductase in chloroplasts: a proposed role in ascorbic acid metabolism. Planta 133: 21-25.

Goodarzian Ghahfarokhi M, Mansurifar S, Taghizadeh-Mehrjardi R, Saeidi M, Jamshidi AM, Ghasemi E (2015). Effects of drought stress and rewatering on antioxidant systems and relative water content in different growth stages of maize (*Zea mays* L.) hybrids. Archives of Agronomy and Soil Science 61(4): 493-506.

Gorcek Z, Erdal S (2015). Lipoic acid mitigates oxidative stress and recovers metabolic distortions in salt-stressed wheat seedlings by modulating ion homeostasis, the osmo-regulator level and antioxidant system. Journal of the Science of Food and Agriculture 95:2811-2817.

Gümrükçü Şimşek SD, Terzi R, Saruhan Güler N (2024). Lipoic acid can provide homeostasis of reactive oxygen species and induce ascorbate and glutathione biosynthesis in osmotic stressed maize. Russian Journal of Plant Physiology 71. https://doi.org/10.1134/S1021443724604373

Hamdia MA, Shaddad MAK (2010). Salt tolerance of crop plants. a review. Journal of Stress Physiology and Biochemistry 6:64-90.

Hasanuzzaman M, Hossain MA, Fujita M (2011). Nitric oxide modulates antioxidant defense and the methylglyoxal detoxification system and reduces salinity-induced damage of wheat seedlings. Plant Biotechnology Reports 5: 353-365. https://doi.org/10.1007/s11816-011-0189-9.

Hasanuzzaman M, Nahar K, Hossain MS, Mahmud JA, Rahman A, Inafuku M, Oku H, Fujita M (2017). Coordinated actions of glyoxalase and antioxidant defense systems in conferring abiotic stress tolerance in plants. International Journal of Molecular Science 18: 200-228. doi: 10.3390/ijms18010200.

Hasanuzzaman M, Bhuyan MB, Anee TI, Parvin K, Nahar K, Mahmud JA, Fujita M (2019). Regulation of ascorbate-glutathione pathway in mitigating oxidative damage in plants under abiotic stress. Antioxidants 8(9): 384-434. https://doi.org/10.3390/antiox8090384.

Hoagland DR, Arnon DI (1950). The water-culture method for growing plants without soil. Circ Calif Agric Exp Stn 347(2nd edit):32

Hossain MA, Nakano Y, Asada K (1984). Monodehydroascorbate reductase in spinach chloroplasts and its participation in regeneration of ascorbate for scavenging hydrogen peroxide. Plant Cell Physiology 25:385-95.

Hossain MA, Asada K (1984). Purification of dehydroascorbate reductase from spinach and its characterization as a thiol enzyme. Plant Cell Physiology 25: 85-92.

Hossain, MA, Hasanuzzaman M, Fujita M (2010). Up-regulation of antioxidant and glyoxalase systems by exogenous glycinebetaine and proline in mung bean confer tolerance to cadmium stress. Physiology and Molecular Biology of Plants 16: 259-272.

Junaid MD, Öztürk ZN, Gökçe AF (2023). Drought stress effects on morphophysiological and quality characteristics of commercial carrot cultivars. Turkish Journal of Botany 47(2): 111-126.

Kadıoğlu A, Saruhan N, Sağlam A, Terzi R, Acet T (2011). Exogenous salicylic acid alleviates effects of long term drought stress and delays leaf rolling by inducing antioxidant system. Plant Growth Regulation 64: 27-37.

Khan R, Ma X, Hussain Q, Asim M, Iqbal A, Ren X, Shah S, Shi Y (2022). Application of 2, 4-epibrassinolide improves drought tolerance in tobacco through physiological and biochemical mechanisms. Biology 11(8):1192-1213. doi: 10.3390/biology11081192.

Krasensky J, Jonak C (2012). Drought, salt and temperatures tress-induced metabolic rearrangements and regulatory networks. The Journal of Experimental Botany 63:1593-1608.

Liso R, Calabrese G, Bitonti MB, Arrigoni O (1984). Relationship between ascorbic acid and cell division. Experimental Cell Research 150:314-320.

Liu X, Williams, CE, Nemacheck JA, Wang H, Subramanyam S, Zheng C, Chen MS (2010). Reactive oxygen species are involved in plant defense against a gall midge. Plant physiology 152(2): 985-999.

Mohammadkhani N, Heidari R (2008). Water stress induced by polyethylene glycol 6000 and sodium chloride in two corn cultivars. Pakistan Journal of Biological Sciences 11(1): 92-97.

Nahar K, Hasanuzzaman M, Alam MM, Fujita M (2015a). Exogenous glutathione confers high temperature stress tolerance in mung bean (*Vigna radiata* L.) by modulating antioxidant defense and methylglyoxal detoxification system. Environment Experimental Botany 112: 44- 54.

Nahar K, Hasanuzzaman M, Alam MM, Fujita M (2015b). Glutathione-induced drought stress tolerance in mung bean: coordinated roles of the antioxidant defence and methylglyoxal detoxification systems. AoB PLANTS 7: plv069. https://doi.org/10.1093/aobpla/plv069

Nahar K, Hasanuzzaman M, Alam MM, Fujita MJBP (2015c). Roles of exogenous glutathione in antioxidant defense system and methylglyoxal detoxification during salt stress in mung bean. Biologia plantarum 59: 745-756.

Nakano Y, Asada K (1981). Hydrogen peroxide is scavenged by ascorbate-specific peroxidase in spinach chloroplasts. Plant and cell physiology 22(5): 867-880.

Navari-Izzo F, Quartacci MF, Sgherri C (2002). Lipoic acid: a unique antioxidant in the detoxification of activated oxygen species. Plant Physiology and Biochemistry 40(6-8): 463-470.

Principato GB, Rosi G, Talesa V, Giovannini E, Uotila L (1987) . Purification and characterization of two forms of glyoxalase II from the liver and brain of wistar rats. Biochimica et Biophysica Acta 911: 349-355.

Ramadan KM, Alharbi MM, Alenzi AM, El-Beltagi HS, Darwish DBE, Aldaej MI, Ibrahim MF (2022). Alpha lipoic acid as a protective mediator for regulating the defensive responses of wheat plants against sodic alkaline stress: physiological, biochemical and molecular aspects Plants 11(6): 787-803. https://doi.org/10.3390/plants11060787.

Saruhan Guler N, Ozturk K, Sezgin A, Altuntas C, Kadioglu A, Terzi R (2021). Alpha lipoic acid application promotes water-deficit tolerance by modulating osmoprotectant metabolism-related genes in maize. Russian Journal of Plant Physiology 68(6): 1152-1160.

Sezgin A, Altuntaş C, Demiralay M, Cinemre S, Terzi R (2019). Exogenous alpha lipoic acid can stimulate photosystem II activity and the gene expressions of carbon fixation and chlorophyll metabolism enzymes in maize seedlings under drought. Journal of Plant Physiology 232: 65-73.

Sezgin Muslu A, Kadıoğlu A (2021). The antioxidant defense and glyoxalase systems contribute to the thermotolerance of *Heliotropium thermophilum*. Functional Plant Biology 48(12): 1241-1253.

Sezgin Muslu A (2024). Improving salt stress tolerance in *Zea mays* L. by modulating osmolytes accumulation and antioxidant capacity with Rutin. Anatolian Journal of Botany, 8(1): 21-29.

Sharma P, Jha AB, Dubey RS, Pessarakli M (2012). Reactive oxygen species, oxidative damage, and antioxidative defense mechanism in plants under stressful conditions. Journal of Botany 2011: 1-26. https://doi.org/10.1155/2012/217037.

Singh P, Dhaka N (2016). Glyoxalase system and salinity stress in plants. In Managing salt tolerance in plants: molecular and genomic perspectives (pp. 173-185). London: CRC Press.

Sudesh V, Linda L, Pawan S (2002). Nutrition and Hypertension. Journal Nutrition Research 22: 111-123.

Talaat NB, Ibrahim AS, Shawky BT (2022). Enhancement of the expression of ZmBZR1 and ZmBES1 regulatory genes and antioxidant defense genes triggers water stress mitigation in maize (*Zea mays* L.) plants treated with 24-epibrassinolide in combination with spermine. Agronomy 12(10): 2517.

Tanyolac D, Ekmekçi Y, Ünalan Ş (2007). Changes in photochemical and antioxidant enzyme activities in maize (*Zea mays* L.) leaves exposed to excess copper. Chemosphere 67(1): 89-98.

Taylor NL, Day DA, Millar AH (2004). Targets of stress-induced oxidative damage in plant mitochondria and their impact on cell carbon/nitrogen metabolis. Journal of Experimental Botany 55: 1-10.

Terzi R, Saruhan GN, Güven FG, Kadioglu A (2018). Alpha lipoic acid treatment induces the antioxidant system and ameliorates lipid peroxidation in maize seedlings under osmotic stress. Archives of Biological Sciences 70(3): 503-511.

Tietze F (1969). Enzymic method for quantitative determination of nanogram amounts of total and oxidized glutathione: applications to mammalian blood and other tissues. Analytical Biochemistry 27(3): 502-522.

Turk H, Erdal S, Karayel U, Dumlupinar R (2018). Attenuation of lead toxicity by promotion of tolerance mechanism in wheat roots by lipoic acid. Cereal Research Communications 46: 424-435. https://doi.org/10.1556/0806.46.2018.020

Urbanek H, Kuzniak-Gebarowska E, Herka K (1991). Elicitation of defence responses in bean leaves by *Botrytis cinerea* polygalacturonase. Acta Physiologia Plantarum 13(1): 43-50.

Yadav SK, Singla-Pareek SL, Ray M, Reddy MK, Sopory SK (2005). Methylglyoxal levels in plants under salinity stress are dependent on glyoxalase I and glutathione. Biochemical and Biophysical Research Communications 337: 61-67.

Youssef MH, Raafat A, El-Yazied AA, Selim S, Azab E, Khojah E, El Nahhas N, Ibrahim MFM (2021). Exogenous application of alpha-lipoic acid mitigates salt-induced oxidative damage in sorghum plants through regulation growth, leaf pigments, ionic homeostasis, antioxidant enzymes, and expression of salt stress responsive genes, Plants (Basel) 10(11): 2519-2535. doi: 10.3390/plants10112519.

Zaid A, Mohammad F, Wani SH, Siddique KM (2019). Salicylic acid enhances nickel stress tolerance by up-regulating antioxidant defense and glyoxalase systems in mustard plants. Ecotoxicology and Environmental Safety 180: 575-587.

Zhao L, Hu Q, Huang Y, Keller AA (2017). Response at genetic, metabolic, and physiological levels of maize (*Zea mays*) exposed to a Cu $(OH)_2$ nanopesticide. ACS Sustainable Chemistry & Engineering 5(9): 8294-8301.

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Investigation of the effect of myrtle spurge (*Euphorbia myrsinites* L.) extract on some mechanical properties of fabrics

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Mersin sütleğeni (*Euphorbia myrsinites* L.) ekstraktının kumaşların bazı mekanik özellikleri üzerine etkisinin araştırılması

Abstract: Textile products have the most direct relationship with the human body after food products. Dye residues and chemicals on our clothes can penetrate our bodies through sweating and respiration which affects our health. The purpose of this study was to investigate the effect of the extract obtained from *Euphorbia myrsinites* L. on various mechanical properties of fabrics and to find natural alternatives to currently used chemicals to improve these properties. In the study, an extract was obtained from the dried *E. myrsinites* samples by the decoction method. The extract was applied to various fabrics to determine the most suitable fabric, and a sample fabric was produced. The friction fastness, touching, flammability, crease resistance, and abrasion resistance tests were performed on the produced sample and standard fabrics. As a result of the study, it was revealed that the sample fabric produced using Euphorbia extract outperformed the standard fabric in wet and dry friction fastness, crease resistance, and mechanical friction resistance tests. It also reached the desired levels of toughness, density, and smoothness in the touching test. The fabric ignited approximately 13-14 seconds in flammability tests, and this result was considered positive as the criterion for these tests performed in advanced laboratories is 10 seconds. Based on the findings of this study, it can be suggested that fabrics which do not wrinkle, wear out quickly, or burn easily can be produced by using Euphorbia extract instead of chemicals that are harmful to health.

Key words: *Euphorbia* extract, natural fabric, dye releasing, friction resistance, fire resistance

Özet: Tekstil ürünleri gıda maddelerinden sonra insan vücuduyla en doğrudan ilişkiye sahiptir. Giysilerimizdeki boya kalıntıları ve kimyasallar terleme ve solunum yoluyla vücudumuza nüfuz edebilir ve sağlığımızı etkileyebilir. Bu çalışmanın amacı, *Euphorbia myrsinites* L.'den elde edilen ekstraktın kumaşların çeşitli mekanik özellikleri üzerindeki etkisini araştırmak ve bu özellikleri iyileştirmek için şu anda kullanılan kimyasallara doğal alternatifler bulmaktır. Çalışmada kurutulmuş *E. myrsinites* örneklerinden kaynatma yöntemiyle bir ekstrakt elde edilmiştir. En uygun kumaşı belirlemek için ekstrakt çeşitli kumaşlara uygulanmış ve bir numune kumaş üretilmiştir. Üretilen numune ve standart kumaşlar üzerinde sürtünme haslığı, dokunma, yanıcılık, kırışma direnci ve aşınma direnci testleri yapılmıştır. Çalışma sonucunda, sütleğen ekstraktı kullanılarak üretilen numune kumaşın, ıslak ve kuru sürtünme haslığı, kırışma direnci ve mekanik sürtünme direnci testlerinde standart kumaştan daha iyi performans gösterdiği ortaya çıkmıştır. Ayrıca dokunma testinde de istenilen tokluk, yoğunluk ve pürüzsüzlük seviyelerini göstermiştir. Kumaşın yanıcılık testlerinde yaklaşık 13-14 saniyede tutuşmasıyla birlikte, gelişmiş laboratuvarlarda yapılan bu testlerde kriter 10 saniye olduğu için bu sonuç olumlu olarak değerlendirilmiştir. Bu çalışmanın bulgularına dayanarak, sağlığa zararlı kimyasallar yerine sütleğen ekstraktı kullanılarak kırışmayan, çabuk yıpranmayan ve kolay yanmayan kumaşlar üretilebileceği ileri sürülebilir.

Anahtar Kelimeler: Sütleğen ekstraktı, doğal kumaş, boya dökme, sürtünme direnci, yanma direnci

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1. Introduction

People have been interested in plants since their existence. According to archaeological findings dating back to ancient times, people primarily benefited from plants for sustenance and to overcome health problems (Ersöz, 2010). *Euphorbia* L. is among such important plants. *Euphorbia*, which is among the *Euphorbiaceae* family, includes more than 2.150 species, most of which are of economic importance (Horn et al., 2012). *Euphorbia*, one of the plants used as medicines in 60 pieces of Boğazköy cuneiform text of the Hittites (1700-1200 BC), is also included in the list of plants suitable for preparing medicines by the World Health Organization (Başaran, 2012). In particular, it has been explained that *Euphorbia* species commonly used in traditional medicine contain essential oils, oxygenated sesquiterpenes, sesquiterpene hydrocarbons (Salehi et al., 2019), and macrocyclic diterpenoids (Vasas & Hohmann, 2014). Moreover, it has been reported that it contains gallic acid, phenolic compounds, quercetin, jatrophane diterpene, jatrophanepolyester, tigliane diterpene, and cycloartane-type triterpene (Chaabi et al., 2007; Haba et al., 2007; Hohmann et al., 2002). The plant is rich in flavonoids, the largest group of resin, gum, and plant phenolics. As strong antioxidants, flavonoids protect the cells against anti-radicals, inhibit the proliferation of bacteria and viruses, and provide resistance to cancer formation and heart attack (Xing et al., 2002; Dartay, 2010).

The usage areas of *Euphorbia* plants in traditional medicine

are divided into three categories: treatment of digestive system disorders, infections, and skin/subcutaneous cellular tissue disorders (Salehi et al., 2019). The specific plant parts used can vary depending on the geographical distribution of *Euphorbia* species. *E. hirta* L., *E. thymifolia* L. and *E. lathyris* L. are the most commonly used species (Ernst et al., 2015).

Generally, *Euphorbia* species are widely used for the treatment of the following disorders: dysentery, asthma, bronchitis, cough (Hargreaves, 1991; Van Sam et al., 2008), skin diseases, ringworm, gonorrhea (Lai et al., 2004; Ernst et al., 2015; Mali and Panchal, 2017b), hemorrhoids (Gürhan & Ezer, 2004; Gupta, 2011), cancer and liver diseases (Hsieh et al., 2015; Mali & Panchal, 2017a,b), snake and scorpion bites (Hsieh et al., 2015; Mali and Panchal, 2017a,b) healing of wounds (Lai et al., 2004; Kaval et al., 2014; Ahmed et al., 2016), and lowering cholesterol (Maurya et al., 2012). Ashraf et al. (2015) revealed in their study that euphorbia extract, exhibiting excellent antioxidant, antimicrobial, and anti-tumor (anti-cancer) properties, was a valuable resource for the pharmaceutical industry. It is also used in the production of latex, paint, natural rubber (Rizk, 1987; Dabholkar et al., 1991), and as a food additive to preserve foods (Toro-Vazquez et al., 2007). On the other hand, some *Euphorbia* plants, especially their latex or milky sap, can irritate the skin, mouth, and throat, causing a burning sensation, acute inflammation, and nausea (Bhatia et al., 2014). Notably, some species possess the ability to bind oxygen. Because of these characteristics, roots, branches, seeds, and milk of euphorbia are widely used in fishing (Dartay, 2010). Additionally, environmentally friendly composites with enhanced oxygen retention capacity have been produced from renewable materials by using banana polyester fiber and euphorbia (Rai et al., 2011). Research on developing composites with various properties using different types of euphorbia is ongoing (Kumari et al., 2020; Mwaikambo et al., 2024).

It is remarkable that the studies on *Euphorbia*, which grows naturally in vast areas of the earth and has a very rich species diversity, are concentrated in the field of health, while there are few studies on *Euphorbia* in our country. Besides health, people also prioritize food and clothing. As seen in the literature, euphorbia is also used for food supply purposes (e.g., fishing, honey). However, there are limited studies on the use of euphorbia in the production of fabrics that people use in many areas (clothing, coating, protection, etc.) (Bhandari et al., 2020; Koo and Park, 2022). It has been also seen that these studies are mostly related to dyeing**.** In this study, it was aimed (i) to investigate the effect of euphorbia extract on various mechanical properties of fabrics (friction fastness, fire resistance, wrinkle resistance, abrasion resistance, etc.), and (ii) to find a natural alternative to health-threatening chemicals used in fabric production.

2. Materials and Method

2.1. Material

In the study, euphorbia plants collected from Uçmakdere location of Tekirdağ province (latitude: 40°80'00.3", longitude: 27°36'60.5", altitude: 203 m) were used as the material. The collected plants were identified at the university and determined to belong to *E. myrsinites* species.

2.2. Method

2.2.1. Drying plant samples

The collected samples were sterilized by washing them first with water and then with a 5% isopropyl alcohol solution. After the samples were dried at room temperature (10-15 days), they were ground into powder and stored in airtight containers.

2.2.2. Obtaining extract from *E. myrsinites*

The Decoction (boiling) method was preferred by paying attention not to use chemical substances while obtaining the extract (Fueki et al., 2015). In the modified method, 10 g of the plant sample was weighed and mixed with 200 ml of distilled water. The mixture was boiled for 5 minutes and then filtered hot. In the method followed, it is desired to remove the water to be able to obtain the extract in a raw form. However, the water was not removed after filtration since dilution would also be performed in experiments on fabrics.

2.2.3. Determination of to which fabrics the obtained extract can be applied

Compatibility studies were performed based on Vinyl acrylic copolymer, Biopolymer, and PVA (Polyvinyl Alcohol) fabric structures to determine with which fabric structures the extract would comply.

2.2.4. Obtaining and controlling sample fabrics

To obtain a sample fabric, X (each firm has its own base structure) base structure (93.95%), Euphorbia extract (5.5%), and the protecting agent (0.55%) were stirred at 2000 rpm for 2 rad/s. The mixture was then filtered, and quality control was performed by examining its properties, such as appearance, film property, viscosity, pH, and refractive index, to determine whether it could be used as fabric.

2.2.5. The use of extract together with various fabrics

In this stage performed to determine the effect of Euphorbia extract on various fabrics, the Foulard process used in the Pad-Batch (impregnation) method was followed (Ömeroğulları Başyiğit, 2021**).** In the impregnation method, the fabric was treated in a bowl (foulard) with a solution to be impregnated in a very short time and in a short flotte ratio, and then the mangle process was performed. The fabric impregnated in the foulard was then subjected to complementary processes such as fixation and washing. Since the machine used for impregnation is foulard, the method is known by this name. In the study, knitted and polyester (PES) fabrics and 20 g/l extract were used in the foulard process. After the fabrics were dried (105 °C) in the drying-oven, other stages of the study were performed.

2.2.6. Finishing (apre) study

Finishing processes, indispensable for the textile industry, are applied to give new properties to textile products and to improve the properties available in the structure of the product. Most of the fabrics are subjected to the finishing process before they are used. Finishing processes are divided into two categories based on the application methods: Mechanical finishing (dry finishing) and chemical finishing (wet finishing).

In this study, chemical finishing was applied in an attempt to modify the retention, appearance, or handling properties of fibers by attaching the extract applied to fabric. Impregnation, the most applied method of applique in chemical finishing processes,and the foulard process described in the previous step were employed Here, the application was performed on one side of the fabric under standard finishing application conditions (Table 1).

2.2.7. Friction fastness test

This test was performed to determine whether the dyed yarn would stain the fabric rubbed by friction effect in later use. A Crockmeter (rubbing test apparatus), sufficient amount of fabric for wet and dry rubbing fastness tests, cotton rubbing cloth (pH: 5.5), and distilled water were used as materials. The tests were conducted in two stages.

Dry rubbing fastness test: The sample fabric was stretched longitudinally in the weaving direction and placed in the test apparatus. The cotton rubbing cloth was attached by being stretched on the rubbing finger with a flap. A 10 cm^2 area of the dry sample was subjected to 10 cycles of back-and-forth rubbing process within 10 seconds (Fig. 1).

Wet rubbing fastness test: A sufficient amount of the sample fabric was stretched longitudinally in the weaving direction and positioned to align with the test apparatus' path. The cotton rubbing cloth was wetted with distilled water, stretched, and attached to the rubbing finger by a flap. The wet sample was subjected to ten cycles of back-and-forth rubbing process along a 10 cm^2 straight line within 10 seconds.

The samples obtained as a result of the two tests were evaluated based on the greyscale (ISO 105-A03) (Önem et al., 2012). This scale was prepared with 1 pair of white and 4 pairs of gray and white pieces of sheet or fabric (Fig. 2). Apparent shades are available on the greyscale. To determine the degree of releasing, the material polluted as a result of the test is placed side by side with the original undyed fabric, and the difference between them is compared using the differences in the greyscale.

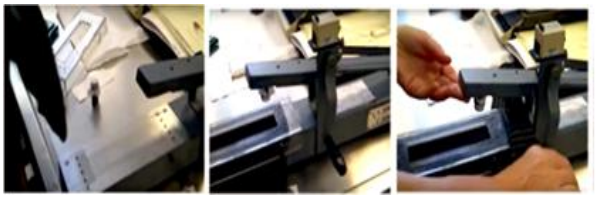


Figure 1. Dry rubbing fastness test.

2.2.8. Touching test

The subjective evaluation of a feeling such as softness, toughness, density, drapability, smoothness, thickness, and temperature perceived through the sense of touch on textile products is expressed as touching. W.S. Howorth and P.H. Oliver have revealed that there is a correlation between the touching properties and mechanical properties of fabrics (Gürcüm, 2010). Since the mechanical properties of fabrics were examined in this study, experienced personnel from the R&D department of the factory were consulted for the touching test.

2.2.9. Flammability test

Various flammability tests are performed in advanced laboratories and there are special devices for these tests. In this study, the ISO 6940 test was modified and applied. This test was performed using a setup that we created to closely simulate the standard conditions after reviewing the devices used in laboratories. The sample fabric was stretched vertically and attached to metal stands on both ends. The flame device was approximated to the fabric and held at a point for 10 seconds, and whether it ignited was observed

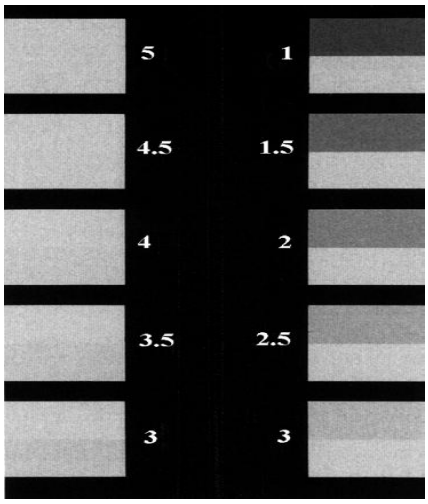


Figure 2. Greyscale (ISO 105-A03)

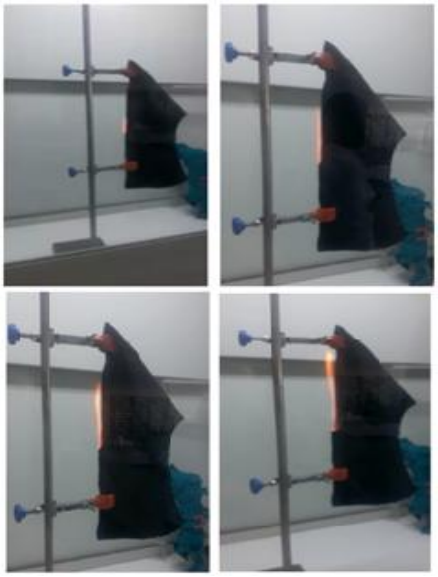


Figure 3. Flammability test

Table 1. Finishing application conditions

X base structure	20 g/l	Sample	20 g/l
Method	Foulard	Method	Foulard
pH	5 – 5.5	pH	5 – 5.5
	105 °C and N.C.	Drying	105 °C and N.C.

(Fig. 3). The flame length of the burners used as a flame source under standard conditions was set to 25 mm (our source was a little stronger).

2.2.10. Crease resistance test

The crease resistance is the process by which fabrics are wrinkled under a certain pressure to ensure ease of use and return to their original state when the pressure effect is eliminated The resistance of fabrics, which are textile products, show to wrinkles occurring during use and their ability to recover from this resistance are expressed by the crease recovery angle. The crease recovery angle is determined by measuring the untwisting and the angle of recovery on a horizontally folded fabric (TS 390 EN 22313). In this study, angle measurement was not performed due to the lack of necessary equipment, and it was evaluated by receiving help from experts.

2.2.11. Abrasion (Abrasion Resistance) test

When the fabrics used in clothing production are rubbed against another material, yarns and fibers come out of the fabric's surface, and thereby wear and tear occur on the surface. Abrasive resistance is the fabric's ability to withstand wear and tear caused by friction against other materials. Abrasion resistance, along with breaking strength, is one of the most important properties determining the overall strength of textile products. Abrasion resistance is determined depending on the number of revolutions at a time during which yarn break is observed, weight loss after a certain cycle, or color change after a certain number of cycles. The Martindale method (TS EN ISO 12947-4) was used in the abrasion test (Çatal, 2015). The sample fabric was placed on the Martindale apparatus, and the abrasion between units making a circular harmonic motion with respect to each other under 9 KPa pressure was monitored. 5000 cycles were performed by controlling every 1000 cycles.

3. Results and Discussion

In the study, the usability of the extract, obtained from *E. myrsinites* via the boiling method, in the production of various fabrics was investigated. It was seen that the extract and PVA base structure were suitable, and a sample fabric was produced (PRD 702). R&D studies of the produced sample fabric were carried out and the results were evaluated. The results were found to comply with the desired standard properties, and other tests were carried out on the sample fabric (Table 2).

First, the dry and wet rubbing fastness tests were performed on the produced sample fabric (PRD 702) and the results were compared with the X-based fabric used as standard (Kleber IR). Then, the results were evaluated by the greyscale (Fig. 2). In the dry rubbing fastness test, it was determined that the sample fabric had the same properties as the standard fabric and showed the desired value (1) in all textile products in the greyscale. This result shows that the fabric did not release the dye by abrasion and did not dye its surroundings (Fig. 4). The wet rubbing fastness test revealed that the sample fabric gave better results than the standard fabric and did not release its dye during washing (Fig. 4). Carcinogenic azo dyestuffs, allergic fabric dyes, and heavy metal-containing materials are used in many fabrics, and these substances affect our bodies through sweating when the fabric release the dye or does not retain the dye sufficiently**.** In addition to dye, substances such as chloric aromatic compounds, o-phenylphenol, biphenyl, aromatic hydrocarbons, benzylbenzoate, N-alkylphthalimide, sodium silicate, and salt are used to retain the dye and to prevent color from bleeding in fabrics. Many of these substances irritate the skin, damage the eyes, cause DNA damage and bladder cancer (Velicangil and Velicangil, 1987; Topuzoğlu and Orhun, 1993; Öztürk, 2021).

In the study, the touching test revealed that the sample fabric produced by adding extract had more desired properties than the standard fabric. The results of this test vary depending on where the fabric will be used. The fabrics to be used in cloth production are not desired to be too hard, dense, or heavy. It was observed that the sample fabric gave toughness, density, and smoothness at the desired level without weight.

In the flammability test performed, it was determined that the ignition time of the sample fabric was 13-14 seconds,

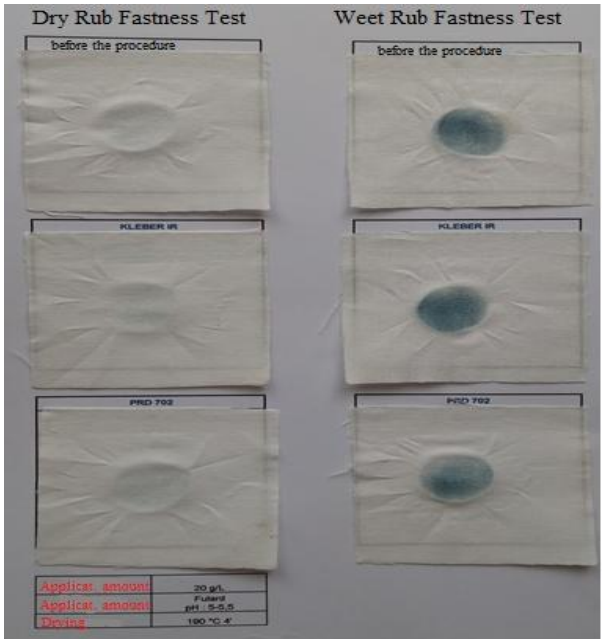


Figure 4. Results of the dry and wet rubbing fastness tests

Table 2. Quality control values of the sample fabric

R & D Study Code	Appearance	Viscosity	K.M. %	pH	K.I.	Film property
PRD702	greenish, viscose **	1.600 CPS	9.5	5	1.3460	Hard, difficult to separate from glass, not adhesive

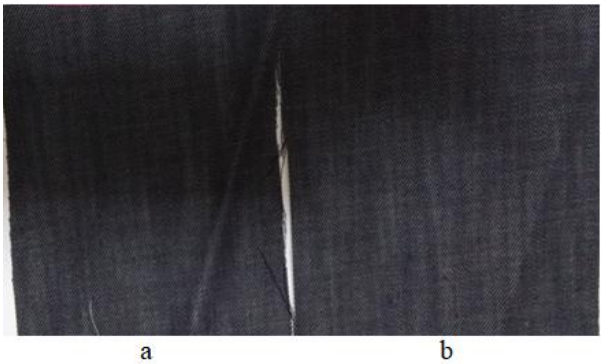
Table 3. Flammability test results

Knitted Fabric (after touching)	Knitted Fabric (After drying at 105 °C)	PES Fabric (After drying at 105 °C)	PES Fabric (after touching)
Method	Foulard	Method	Foulard

while the ignition time of standard fabric was 9-10 seconds. The flammability test was also implemented on fabrics with different structures. In the experiments carried out with knitted and PES fabrics, it was observed that the ignition times of both fabrics were similar to the sample fabric (in 13-14 s) (Table 3). However, these tests are performed with special devices kept at a certain distance from the flammable fabric at a lower severity, and 10 seconds is used as the criteria (ISO 6940). Since the intensity of the flame in the setup that we created was too high, it was normal for the fabric to ignite in 13-14 seconds.

Halogen-based additives (e.g., pen-taBDE, octaBDE, and decaBDE) are mostly used as flame retardants in fabric production. However, these substances have been banned because they accumulate in the food chain of living beings and have a negative effect on the liver, thyroid, reproductive system, and neurological development (Zhao, 2010; Gaonkar, 2021). Nowadays, inorganic-based materials are also used as additives in polymers to give flame-retardant properties. For example, zinc borate is used as an additive in polymers (such as PET, polyamide, polyethylene, and rubber) and in dyestuffs. The clustering of zinc borate in the polymer matrix and the formation of aggregates pose a problem in distributing it well in the matrix. Furthermore, it acts as waste, polluting the environment over time as it is released on the surface. This problem is not only specific to zinc borate but also applies to low molecular weight, inorganic, and organic-based additives. New flame retardants are also needed due to the effects of halogen-based additives on the environment and human health (Eren & Aşçı, 2015). For this reason, in the study, it was aimed to produce fire-resistant fabric using the oxygen retention property of Euphorbia (Rai et al., 2011). A natural flame retardant can be developed by conducting further research on the Euphorbia extract.

Crease resistance test results showed that PES fabrics in which euphorbia extract was used outperformed standard PES fabrics. After bidirectional pressure, the sample fabric exhibited fewer marks compared to the standard fabric (Fig. 5-6). The crease-resistant finishing was applied to the fabrics to prevent wrinkling, and melamine-based chemicals and formaldehyde were used in these processes. Formaldehyde was included in the list of carcinogenic chemical substances (Öztürk, 2021). It was observed that the crease resistance property of the sample fabric produced in this study was high when compared to the standard fabric. The use of natural substances should be preferred instead of health-threatening carcinogenic chemical substances.

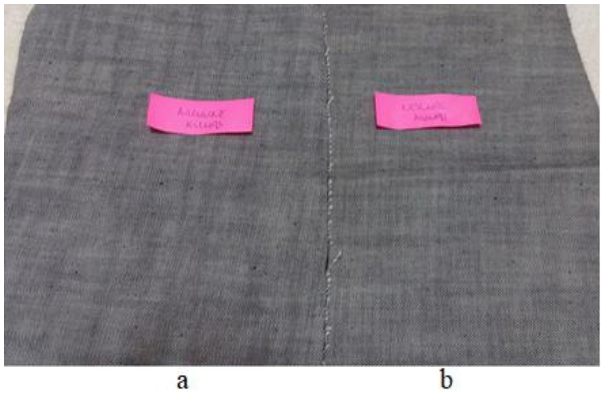


a b

Figure 5. Result of the crease resistance test carried out by the pressure applied from the front side. a: standard fabric (Kleber IR), b: sample fabric (PRD 702)

As a result of the mechanical abrasion resistance test, it was determined that the sample fabric produced using *Euphorbia* extract was more resistant to abrasion than the standard fabric. Although both fabrics were resistant to 3500-4000 cycles at 9 KPa, it was observed that the abrasions in the standard fabric started earlier and were more severe than in the sample fabric. The fabrics with which our body is in constant contact are desired to be healthy and durable. It is preferred that mechanical friction and abrasion are minimal in fabrics. Formaldehyde or formaldehyde bisulfite with the same effect is used for good retention of the dye during dying and to avoid damage to fibers (resistance to mechanical abrasion) during the use of the fabric. However, these substances are carcinogenic (Gaonkar, 2021; Öztürk, 2021).

In general, in the textile industry, various chemicals are used as additives to make fabrics or other products more durable (friction, abrasion), soft, water and oil-repellent, stain-resistant, fire-resistant, wrinkle-resistant, and resistant to bacteria and fungi. Some of these chemicals are Per-and Polyfluoroalkyl Substances (PFAS), Nonylphenol Ethoxylates (NPEOs), Decabromodiphenyl Ether (decaBDE), Organotin Compounds (OTCs), Phthalates, Bisphenol A (BPA), and Short Chain Chlorinated Paraffins (SCCPs) (Gaonkar, 2021). Many of these chemicals can enter the body through the outer layers of the skin by contacting the skin when we wear our clothes. In addition, as fabrics wear out, fibers enter the body through breathing or swallowing and cause health problems (Lensen et al., 2007; Gallagher, 2008). The chemicals used in textile products cause many disorders such as cancer, endocrine-reproductive-nerve-immune systems disruption, liver and kidney damage, increase in cholesterol levels, DNA damage, and inhibition of baby development (Gaonkar, 2021; Öztürk, 2021). Moreover, since they are toxic to aquatic and terrestrial creatures, they affect the survival, growth, development, and reproduction of these living beings (Gaonkar, 2021). Besides,living beings, the environment is also affected negatively by the chemicals used in the textile industry. Firstly, a lot of water and energy is consumed by the textile industry. As this industry is the second sector that pollutes fresh water the most worldwide. Chemicals used in production processes cause water and air pollution (Toprak & Anis, 2017). Some of the chemicals used are banned due to the harm they cause. For example, halogenated compounds used as fire retardants and brominated diphenyl derivatives have been banned because the former cause environmental problems, the latter



a b

Figure 6. The result of the crease resistance test performed with the pressure applied from the reverse side. a: sample fabric (PRD 702), b: standard fabric (Kleber IR)

cause high toxicity for humans and animals (Zhao, 2010; Van der Veen & de Boer, 2012). Many alternative substances to the chemicals used in fabric production have been suggested and many studies have been conducted on this subject. However, the suggested substances are generally chemicals and their harms are no less than these substances (Horrocks et al., 2005; Atakan et al., 2019; Gaonkar, 2021). Therefore, this current study is highly important.

In this study, the effect of the extract, obtained from the euphorbia (*E. myrsinites*) plant by applying the boiling method without using chemicals on fabrics, was examined and some mechanical properties were tried to be improved. The study revealed that fabrics with the desired properties (e.g., wrinkle-free, paint-resistant, and friction-resistant) can be produced by using euphorbia extract instead of fabrics irritating the skin and containing carcinogenic substances.

Burn-resistant fabrics can also be produced by taking advantage of the oxygen retention feature of euphorbia. However, some euphorbia species may harm health when used unconsciously or excessively (Bhatia et al., 2014). For this reason, it is recommended not to use euphorbia directly (especially as tea). In this study, euphorbia was not used directly; very little percentage (0.2 - 0.3%) of euphorbia extract was used. Even though it was used proportionately less, it created the desired effect. It is also known that euphorbia extract is used in the treatment of many skin disorders (e.g., warts, eczema, and calluses) and hemorrhoids and that it has healing, antioxidant, antimicrobial, and anti-cancer effects (Baytop, 1999; Gupta, 2011; Ashraf et al., 2015; Ahmed et al., 2016). It is believed that the data obtained from this study can be further developed in various fields such as textile, biotechnology, biology, and chemistry. This could lead to the production of fabrics by using natural, renewable, cheap, environmentally, and health-friendly substances instead of harmful chemicals. In addition, different plants could be explored in these studies**.**

Conflict of Interest

Authors have declared no conflict of interest.

Authors' Contribution

The authors contributed equally.

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References

Ahmed S, Yousaf M, Mothan RA, Al-Rehaily AJ. (2016). Studies on wound healing activity of some Euphorbia species on experimental rats. African Journal of Traditional, Complementary and Alternative Medicines 13 (5):145-152.

Ashraf A, Sarfraz RA, Rashid MA, Shahid M (2015). Antioxidant, antimicrobial, antitumor, and cytotoxic activities of an important medicinal plant (*Euphorbia royleana*) from Pakistan. Journal of Food and Drug Analysis 23(1):109-115.

Atakan R , Bical A, Celebi E, Ozcan G, Soydan N, Sarac S (2019). Development of a flame retardant chemical for finishing of cotton, polyester, and CO/PET blends. Journal of Industrial Textiles 49(2): 141-161.

Başaran AA (2012). Ülkemizdeki Bitkisel İlaçlar ve Ürünlerde Yasal Durum. MİSED 27(28): 22-26.

Baytop T (1999). Türkiye'de Bitkilerle Tedavi - Geçmişte ve Bugün. İstanbul: İstanbul Üniversitesi Yayınları.

Bhandari NL, Bist K, Ghimire J, Chaudhary S, Pandey PD, Adhikari R (2020). Feasibility study of the *Euphorbia pulcherrima* plant extract as natural dye with different mordants for fabric dyeing. Journal of Institute of Science and Technology 25(1): 30-36.

Bhatia H, Manhas RK, Kumar K, Magotra R (2014). Traditional knowledge on poisonous plants of Udhampur district of Jammu and Kashmir, India. Journal of Ethnopharmacology 152: 207-216.

Chaabi M, Freund-Michel V, Frossard N, Randriantsoa A, Andriantsitohaina R, Lobstein A (2007). Anti-proliferative effect of *Euphorbia stenoclada* in human airway smooth muscle cells in culture. Journal of Ethnopharmacology 109(1):134-139.

Çatal SR (2015). Döşemelik Kumaş Üretimi (Seminer). Bursa: Uludağ Üniversitesi.

Dabholkar DA, Kaicker PK, Diwan RK (1991). *Euphorbia* latex - Its chemistry and industrial applications. Research Industry 36: 126-131.

Dartay M, Ateşşahin T (2010). İhtiyotoksit bitkilerin bileşiminde bulunan kimyasal maddeler ve balık avcılığında yaygın olarak kullanılması. Fırat Üniversitesi Doğu Araştırmaları Dergisi 8(2): 28-34.

Eren T, Aşçı A (2015). Synthesis and Characterization of phosphonate based polynorbornene polymers derived from romp (ring opening metathesis polymerization) method and investigation their flame retardant properties. Sigma Journal Engineering and Natural Sciences 33(4):615-625.

Ernst M, Grace OM, Saslis-Lagoudakis CH, Nilsson N, Simonsen HT, Rønsted N (2015). Global medicinal uses of *Euphorbia* L. (Euphorbiaceae). Journal of Ethnopharmacology 176:90-101.

Ersöz T (2010). Bitkisel Ürünler ve Güvenilirliği. Bitkilerle Tedavi Sempozyumu, Zeytinburnu/İstanbul Bildiri Kitabı pp.89-93.

Fueki T, T Makino T, Matsuoka T, Beppu M, Sunaga T, Tanaka K, Nagamine K, Namiki T (2015). Quick and easy preparation method fordecoction of Kampo formula inspired bythe method of boiling powdered crude drugsin the Song period of China. Traditional & Kampo Medicine 2(2):1-7.

Gallagher M, Wysocki CJ, Leyden JJ, Spielman AI, Sun X, Preti G (2008). Analyses of volatile organic compounds from human skin. British Journal of Dermatology 159(4):780-791.

Gaonkar O (2021). An Overvıew of Toxic Chemicals in Textiles. Technical Report-India ·

Gupta PJ (2011). The efficacy of Euphorbia prostrata in early grades of symptomatic hemorrhoids – a pilot study. European Review for Medical and Pharmacological Sciences 15(2):199-203.

Gürcüm BH (2010). Dokuma kumaşların öznel algısı ile bazı fiziksel özellikleri arasındaki ilişkinin belirlenmesi. Tekstil ve Konfeksiyon 20 (2):101-108.

Gürhan G, Ezer N (2004). Halk arasında hemoroit tedavisinde kullanılan bitkiler-I. Hacettepe Üniversitesi Eczacılık Fakültesi Dergisi 24(1): 37-55.

Haba H, Lavaud C, Harkat H, Alabdul Magid A, Marcourt L, Benkhaled M (2007). Diterpenoids and triterpenoids from *Euphorbia guyoniana*. Phytochemistry 68(9):1255-1260.

Hargreaves BJ (1991). The spurges of Botswana. Botswana Notes and Records 23:115-130.

Hohmann J, Molnár J, Rédei D, Evanics F, Forgo P et al (2002). Discovery and biological evaluation of a new family of potent modulators of multidrug resistance: reversal of multidrug resistance of mouse lymphoma cells by new natural jatrophane diterpenoids isolated from *Euphorbia* species. Journal of Medicinal Chemistry 45(12):2425-31.

Horn WJ, van Ee BV, Morawetz JJ, Riina R, Steinmann VW et al. (2012). Phylogenetics and the evolution of major structural characters in the giant genus Euphorbia L. (Euphorbiaceae). Molecular Phylogenetics and Evolution 63 (2):305-326.

Horrocks AR, Kandola BK, Davies PJ, Zhang F, Padbury SA (2005). Developments in flame retardant textiles–a review. Polymer Degradation and Stability 88: 3-12.

Hsieh WT, Lin HY, Chen JH, Lin WC, Kuo YH et al. (2015). Latex of Euphorbia antiquorum-induced S-phase arrest via active ATM kinase and MAPK pathways in human cervical cancer HeLa cells. Environmental Toxicology 30: 1205-1215.

ISO 6940 (2004). Textile fabrics - Burning behaviour - Determination of ease of ignition of vertically oriented specimens.

Kaval I, Behcet L, Cakilcioglu U (2014). Ethnobotanical study on medicinal plants in Gecitli and its surrounding (Hakkari-Turkey). Journal of Ethnopharmacology 155(1): 171-184.

Koo H, Park Y (2022). Mechanical properties and dyeability of silk dyed with *Euphorbia humifusa* extract with addition of CNF Hydrogel. Fibers and Polymers 23: 1324-1331.

Kumari S, Kumar R, Kumar G (2020). Study on the modification of polyester resin bamboo fiber-based composite with euphorbia coagulum and their effect on mechanical and thermal properties. Journal of Composite Materials 54(24): 100-120.

Lai XZ, Yang YB, Shan XL (2004). The investigation of Euphorbiaceous medicinal plants in southern China. Economic Botany 28: 307320.

Lensen G, Jungbauer F, Gonçalo M, Coenraads PJ (2007). Airborne irritant contact dermatitis and conjunctivitis after occupational exposure to chlorothalonil in textiles. Contact Dermatitis 57(3):181-186.

Mali PY, Panchal SS (2017a). *Euphorbia tirucalli* L.: Review on morphology, medicinal uses, phytochemistry and pharmacological activities. *Asian Pacific Journal* of *Tropical* Biomedicine 7: 603-613.

Mali PY, Panchal SS (2017b). *Euphorbia neriifolia* L.: Review on botany, ethnomedicinal uses, phytochemistry and biological activities. Asian Pacific Journal of Tropical Biomedicine: 10 430-438.

Maurya AK, Tripathi S, Ahmed Z, Sahu RK (2012). Antidiabetic and antihyperlipidemic effect of *Euphorbia hirta* instreptozotocin induced diabetic rats. Scholars Research Library 4(2): 703-707.

Mwaikambo LY, Tucker N, Clark AJ (2024). Mechanical properties of hemp fibre reinforced Euphorbia composites. Macromolecular Materials and Engineering 292(9): 993-1000.

Ömeroğulları Başyiğit Z (2021). Application technologies for functional finishing of textile materials. In: Kumar P (ed.). Textiles for Functional Applications. London: IntechOpen, pp.1-20.

Önem E, Mutlu M M, Günay S, Azeri H (2012). Soğan (*Allium cepa*) yumru dış kabuğundan doğal boyarmadde ekstraksiyonu ve deri boyamada kullanımı. Tekstil ve Mühendislik Dergisi 19(88): 1-8.

Öztürk A (2021). Günlük yaşamda kimyasal madde maruziyeti ve insan sağlığına etkileri. Düzce Üniversitesi Bilim ve Teknoloji Dergisi 9:1547-1562.

Rai B, Kumar G, Diwan RK, Khandal RK (2011). Study on effect of *Euphorbia coagulum* on physico-mechanical and fire retardant properties of polyester-banana fiber composite. Indian Journal of Science and Technology 4(4):443-446.

Rizk AFM (1987). The chemical constituents and economic plants of the Euphorbiaceae. Botanical Journal of the Linnean Sociely 94:293–326.

Salehi B, Iriti M, Vitalini S, Antolak H, Pawlikowska E et al. (2019). Euphorbia-derived natural products with potential for use in health maintenance. Biomolecules 9(8): 337-350.

Toprak T, Anis P (2017). Textile industry's environmental effects and approaching cleaner production and sustainability, an overview. Journal of Textile Engineering & Fashion Technology 2(4): 429-442.

Topuzoğlu İ, Orhun H (1993). "Türk Tabipleri Birliği, İş Hekimliği Ders Notları". Ankara: Türk Tabipleri Birliği Yayını.

Toro-Vazquez JF, Morales-Rueda JA, Dibildox-Alvarado E, Charó-Alonso M, Alonzo-Macias M, González-Chávez MM (2007).Thermal and textural properties of organogels developed by candelilla wax in shower oil. Journal of the American Oil Chemists' Society 84: 989-1000.

Van der Veen I, de Boer J (2012). Phosphorus flame retardants: Properties, production, environmental occurrence, toxicity and analysis. Chemosphere 88: 1119-1153.

Van Sam H, Baas P, Keßler PJA (2008). Traditional medicinal plants in Ben En National Park, Vietnam. Blumea 53:569–601.

Vasas A, Hohmann J (2014). Euphorbia diterpenes: Isolation, structure, biological activity, and synthesis (2008-2012). Chemical Reviews 114(17) 8579-8612.

Velicangil S, Velicangil Ö (1987). "Endüstri Sağlığı (İşçi Sağlığı-İş Hijyeni) ve Meslek Hastalıkları"**.** Ankara: Çalışma Bakanlığı Yayınları.

Xing JB, Shui CH, Liui Y (2002). Determination of quercetin in the Herba Euphorbiae Humifusae. Zhongguo Zhong Yao Za Zhi, 27(10):758-760.

Zhao X (2010). Synthesis and application of a durable phosphorus/silicon flame-retardant for cotton. The Journal of The Textile Institute 101(6): 538-546.

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A new alien species record for the Flora of Türkiye: *Ipomoea indica* (Burm.) Merr. (*Convolvulaceae*)

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Türkiye Florası için yeni bir yabancı tür kaydı: *Ipomoea indica* (Burm.) Merr. (*Convolvulaceae*)

Abstract: *Ipomoea indica* (Burm.) Merr. is an invasive species with a wide distribution in tropical and subtropical regions and its natural habitat is South America. *I. indica*, which is a new record for the flora of Türkiye, was collected and photographed on 27 June 2022 from Alanya-İncekum. Additionally, the species was observed and photographed in İzmir-Foça, Manavgat-Çenger and Muğla-Bodrum in the Mediterranean and Aegean regions of Türkiye between 27 June 2022 and 02 July 2023. Although this species is morphologically similar to *I. purpurea* and *I. tricolor*, *I. purpurea* differs from *I. indica* in having smaller flowers (4-6 cm), shorter sepals (1.1-1.6 cm) with hirsute surfaces and predominantly unlobed leaves. Similarly, *I. tricolor* differs from *I. indica* by having smaller flowers (3.5-6 cm) and sepals that are glabrous, have scarious margins and shorter (0.6-0.7 cm). The spread of *I. indica*, which has the potential to exhibit invasive behavior, risks putting pressure on native plant species and the ecosystem. Therefore, it is important to conduct additional studies to understand the impacts of alien species and determine management strategies.

Key words: Exotic species, invasive species, non-native plants, Mediterranean flora

Özet: *Ipomoea indica* (Burm.) Merr. doğal yaşam alanı Güney Amerika olan, tropikal ve subtropikal bölgelerde geniş yayılım gösteren ve istilacı özelliklere sahip bir türdür. Türkiye florası için yeni kayıt niteliğinde olan *I. indica* 27 Haziran 2022 tarihinde Alanya-İncekum'dan toplanmış ve fotoğraflanmıştır. İlave olarak, bu tür 27 Haziran 2022 ile 02 Temmuz 2023 tarihleri arasında İzmir-Foça, Manavgat-Çenger ve Muğla-Bodrum'da gözlemlenmiş ve fotoğraflanmıştır. Bu tür *I. purpurea* ve *I. tricolor* türlerine morfolojik olarak benzerlik gösterse de *I. purpurea* çiçeklerin daha küçük (4-6 cm), sepallerin kıllı ve daha kısa (1.1-1.6 cm) ve yaprakların çoğunlukla lobsuz olmasıyla *I. indica*'dan ayrılır. Benzer şekilde, *I. tricolor* çiçeklerin daha küçük (3.5-6 cm) ve sepallerin çıplak, zar kenarlı ve daha kısa (0.6-0.7 mm) olmasıyla *I. indica*'dan ayrılmaktadır. İstilacı davranış sergileme potansiyeli olan *I. indica*'nın yayılımı, yerel bitki türleri ve ekosistem üzerinde baskı oluşturma riski taşımaktadır. Bu nedenle, yabancı türlerin etkilerini anlamaya yönelik ek çalışmalar yapılması ve yönetim stratejilerinin belirlenmesi önem arz etmektedir.

Anahtar Kelimeler: Egzotik türler, istilacı türler, yerli olmayan bitkiler, Akdeniz florası

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1. Introduction

As a result of intensified global mobility, plant species are able to settle in areas far from their natural range. Due to their lack of natural limitations and their high success in adapting to different habitat types, these invasive species cause significant negative impacts on ecosystems, biodiversity and national economies (Pimentel et al., 2000, 2001, 2005; Andersen et al., 2004; Cushman and Meentemeyer, 2008; Simberloff et al., 2013).

According to Uludağ et al. (2017), a total of 340 alien species have been reported in Türkiye, including 321 Angiosperms, 17 Gymnosperms and 2 ferns. Most of the alien species originated from the Americas (44.7%) and Asia (27.6%), while a small number originated from Africa (9.1%), Eurasia (4.4%), Australia and Oceania (3.8%) and the Mediterranean (3.8%) (Uludağ et al., 2017). Located at the crossroads of three continents and having a very different habitat, climate and geological structure, Türkiye is very favorable for plant invasions (Arslan et al., 2015).

Ipomea L. is the largest genus of the *Convolvulaceae* family, represented by approximately 500-600 species worldwide (Austin and Huáman, 1996). The Convolvulaceae family, which has heart-shaped leaves and funnel-shaped flowers, is mostly distributed in tropical and subtropical regions, but there are also species distributed in temperate regions. Species belonging to the genus *Ipomoea* have a wide range of growth forms and can be herbaceous, shrubs, climbers, vines or trees. *Ipomoea* species are used as ornamental plants, food, medicine and in religious rituals (Srivastava and Rauniyar, 2020; Meira et al., 2012).

Some *Ipomoea* members containing biologically active compounds such as alkaloids like ergoline, indolizidine, nortropane, phenolic compounds, coumarin, isocoumarin, diterpene, benzonoid, flavanoid, anticyanoside, glycolipid, lignan and triterpenes have pharmacologically important properties and are considered as medicinal plants (Srivastava and Rauniyar, 2020). On the other hand, some species with the ability to synthesize phytotoxic compounds have great potential for the control of invasive

plant species through their allelopathic effects (Hernández-Aro et al., 2017).

According to the latest data, 6 species belonging to *Ipomoea* are recorded in Türkiye: *I. sagittata* Poir., *I. imperati* (Vahl) Griseb., *I. purpurea* (L.) Roth., *I. tricolor* Cav., *I. triloba* L. and *I. hederifolia* L. (Parris, 1978; Aykurt, 2012; Uludağ et al., 2017; Hançerli et al., 2018). However, the newly recorded species is distinctly different from the other species in terms of some characteristics. Although this species is morphologically similar to *I. purpurea* and *I. tricolor*, *I. purpurea* differs from *I. indica* in having smaller flowers (4-6 cm), shorter sepals (1.1-1.6 cm) with hirsute surfaces and predominantly unlobed leaves. Similarly, *I. tricolor* differs from *I. indica* by having smaller flowers (3.5-6 cm) and sepals that are glabrous, have scarious margins and shorter (0.6-0.7 mm).

The natural distribution range of *I. indica* is uncertain because it appears to be pan-tropical. It is probably listed as native to the tropics of Central and South America and is probably also native to Southeast Asia and some islands in the Pacific region. It is cultivated as a crop plant in Europe, Asia, South Africa, the United States, New Zealand, Australia and a few Pacific islands, as well as naturalized (CABI, 2024). This study confirms the presence of *I. indica* in Türkiye.

2. Materials and Method

Plant specimens were collected and photographed on 27 June 2022 from Alanya-İncekum. Additionally, the species was observed and photographed in İzmir-Foça, Manavgat-Çenger and Muğla-Bodrum in the Mediterranean and Aegean regions of Türkiye between 27 June 2022 and 02 July 2023. Photographs taken in natural habitats of these regions provide evidence of the naturalization of *Ipomoea indica* in Türkiye (Fig. 1). Species identification was made according to "Flora of the Southeastern United States" (Weakley and Southeastern Flora Team, 2024). The species name was verified using the International Plant Name Index (IPNI, 2024). The description of *I. indica* is adapted from eFloras (Flora of China, 2024). The examined specimen was deposited in the GAZI Herbarium under the following details: collected by Emire Elmas (specimen number 3624) on 27 June 2022 from Alanya-İncekum, Antalya, Türkiye. The species identification was conducted by Hasan Yaşayacak in 2023.

Figure 1. *Ipomoea indica* in its natural habitat in Türkiye

3. Results

The research on all species belonging to the genus *Ipomoea* L., especially on leaf, seed and flower structure were reviewed and as a result of the examination of the plant specimen, it was determined that the species was *Ipomoea indica* (Burm.) Merr. This species, commonly referred to as "blue morning glory" or "ocean blue morning glory" in English, has been deemed appropriate to be named "Hint kahkahaçiçeği" in accordance with the Turkish Scientific Nomenclature Guidelines prepared by Menemen et al. (2021).

The diagnostic key of the species belonging to the genus *Ipomoea* distributed in Türkiye, including the newly record, has been adapted from "Flora of the Southeastern United States" (Weakley and Southeastern Flora Team, 2024) and provided below:

1. Corolla salverform; tube long, cylindrical and narrow; limb abruptly flaring at the summit of the tube ……..……...…...***I. hederifolia***

1. Corolla funnelform; tube short to long, expanding in diameter upwards from below the middle; limb gradually to abruptly flaring at the summit of the tube

2. Pedicels and peduncles with spreading, or reflexed trichomes; ovary 3-locular; sepals 8-25 (-30) mm long

3. Sepals soft-pilose on the outer surface with slender trichomes……..…….***I. indica***

3. Sepals hispid-pilose on the outer surface, with swollen-based trichomes..…….... ***I. purpurea***

2. Pedicels and peduncles glabrous or with short, appressed trichomes; ovary 2-locular; sepals 4-15 mm long

4. Stems trailing, rooting at the nodes; leaves emarginate with obtuse lobes, truncate at base.……...…...***I. imperati***

4. Stems erect or twining, not rooting at the nodes; leaf apex acute to acuminate, cordate or sagittate at base

5. Corollas blue, the throat white or yellow; sepals 3-7 mm long……***I. tricolor***

5. Corollas pink, pink-purple, or white, the throat pink or purple; sepals 4-15 mm long

6. Leaf bases sagittate…….. ***I. sagittata***

6. Leaf bases cordate…….……..***I. triloba***

Ipomoea indica (Fig. 2) is a herbaceous perennial creeper with ± densely retrorse pilose axial parts. Stems 3–6 m, sometimes rooting at the nodes. Petioles measure 2–18 cm, and the leaf blades are ovate or circular, 5–15 × 3.5–14 cm, with the abaxial surface densely short and soft pubescent, while the adaxial surface is sparsely pubescent. The base is cordate, the margins are entire or slightly 3-lobed, and the apex is acuminate or abruptly acuminate. The inflorescences are dense umbellate cymes with several flowers. Peduncles are 4–20 cm long, and the bracts are linear, occasionally lanceolate. Pedicels range from 2–5(–8) mm. Sepals are subequal, measuring 1.4–2.2 cm, with gradually linear-acuminate apices. The outer three sepals are lanceolate to broadly lanceolate, and the inner two are narrowly lanceolate. Sepals are glabrous to appressed pilose.

Figure 2. *Ipomoea indica* (Burm.) Merr.: a. General view, b. Leaves and buds, c. Front view of the flower, d. Side view of the flower, e. Twining stems with pubescence, f. Form of pubescence on the calyx

The corolla is funnelform, bright blue or bluish-purple when fresh, aging to reddish-purple or red with a paler center, measuring 5–8 cm and glabrous. Stamens and pistil are included within the corolla. The ovary is glabrous, and the stigma is 3-lobed. The capsule is nearly globose, measuring 1–1.3 cm in diameter, and the seeds are approximately 5 mm long (Flora of China, 2024).

Ipomoea indica has been taxonomically referred to by different names in the past. Homotypic synonyms of the species include *Convolvulus indicus* Burm. and *Pharbitis indica* (Burm.) Hagiw. *Convolvulus indicus* was first described by Burman (1755), while *Pharbitis indica* was renamed by Hagiwara (1938).

Specimen examined: Antalya, Alanya-İncekum, 27 June 2022 (*GAZI*-E. Elmas 3624**).**

Taxonomy:
Family : *Convolvulaceae*
Genus : *Ipomoea* L.
Species: *Ipomoea indica* (Burm.) Merr.

Lectotype:

Convolvulus indicus flore violaceo. Designated by F.R. Fosberg in Bot. Not., 129: 35-38 (1976). Based on illustration in *Hortus eystettensis*, vol. 2, folio 7, fig. 2, by B. Besler (1613) (Link: https://bibdigital.rjb.csic.es/viewer/10913/?offset=#page=348&viewer=picture&o=bookmark&n=0&q=)

Homotypic synonyms:

Convolvulus indicus Burm. in Auctuarium, 2 verso (1755).

Pharbitis indica (Burm.) Hagiw. in Bot. & Zool., 6: 1238 (1938).

Ipomoea indica is most similar to *I. purpurea* and *I. tricolor* among other species distributed in Türkiye. The characteristics that distinguish these three species are summarized in Table 1 (Flora of China, 2024; Flora of North America, 2024).

4. Discussions

Ipomoea indica (Miller et al., 2004; Eserman et al., 2014), a member of the *Ipomoea* subgenus *Quamoclit* clade, is a perennial creeper that can flower and self-fertilize throughout the year (Delgado-Dávila et al., 2016). According to Wood et al. (2020), *I. indica* can be confused with *I. purpurea* but is easily distinguished by the grey pubescence or tomentose underside of the leaves and the clustered flower state with strong bracteoles. It has also been noted that it is a highly variable species; the leaves are sometimes glabrous, lobed or entire, the bracteoles may be reduced and, rarely, the flowers may be solitary forms with prominent dark bands in the middle of the petal in colours ranging from blue to dark violet (Wood et al., 2020).

Considering the worldwide distribution rate and habitat preferences of this species, its invasive potential should be taken into account. In general, the distribution of *Ipomoea*

Table 1. Morphological comparison of *Ipomoea indica*, *Ipomoea purpurea*, and *Ipomoea tricolor*

Features	*Ipomoea indica*	*Ipomoea purpurea*	*Ipomoea tricolor*
Stems	3-6 m, retrorse pilose, sometimes rooting at the nodes	2-3 m, short pubescent and retrorse hirsute	Twining, glabrous
Leaves	Ovate or circular, 5-15 × 3.5-14 cm, abaxially densely pubescent, adaxially sparsely pubescent, cordate base	Circular-ovate or broadly ovate, 4-18 × 3.5-16.5 cm, ± strigose, cordate base	± Cordate, 6-10 × 2.5-13 cm, glabrous surfaces, cordate base
Inflorescence	Dense umbellate cymes, several-flowered, peduncle 4-20 cm	1-5-flowered, peduncle 4-12 cm	Flowers solitary or few; peduncle glabrous
Sepals	Subequal, 1.4-2.2 cm, glabrous or appressed pilose	Subequal, 1.1-1.6 cm, spreading hirsute abaxially on the lower half, apex acuminate	Lance-ovate, triangular, or oblong-triangular, 0.6-0.7 cm, scarious margins, acute apex, muriculate abaxial surface
Corolla	Bright blue or bluish-purple, aging reddish-purple or red; limb 5-8 cm in diameter and length	Red, reddish-purple, or bluish-purple, fading to white center; limb 4-6 cm in diameter and length	Usually blue to deep blue, sometimes white; limb 5-9 cm in diameter, 3.5-6 cm in length.

species is correlated with high rainfall, but some species, such as *I. indica*, can also adapt to habitats with moderate rainfall (Boyjnath et al., 2024), which facilitates its spread over large areas. The fact that it is located in an area far from its natural distribution area strengthens the possibility that this species may have been transported by anthropogenic factors rather than natural factors. Although there are no significant risk factors at the invasive level in its distribution areas in Türkiye, the fact that its preferred habitats are also the common habitats of endemic or rare important local species in the region carries the risk of suppression of these species. Therefore, their distribution should be monitored to assess whether they are being suppressed by *I. indica*.

In subtropical countries, fast-growing species such as *I. indica*, *I. purpurea*, *I. nil*, *I. quamoclit* and *I. tricolor* are used as annual ornamental plants in gardens (Wood et al., 2020). It would be appropriate to evaluate and investigate the landscape potential of the species by taking advantage of its advantageous features such as remarkable flowers, wide ecological tolerance and long vegetation period. The species may be useful as an ornamental plant in hedges and walls. The presence of its secondary metabolites such as alkaloids, flavonoids, terpenoids, glycosides, saponins, steroids, and tannins (Srivastava and Rauniyar, 2020) highlights its potential as a species suitable for ecologic and economic research.

When distribution areas of *I. indica* indicated in the present study in Türkiye are evaluated, it is likely to be found in the coastal zones of the southern and western parts of the country.

Conflict of Interest

Authors have declared no conflict of interest.

Authors' Contributions

The authors contributed equally.

References

Andersen MC, Adams H, Hope B, Powell M (2004). Risk assessment for invasive species. Risk Analysis: An International Journal 24: 787-793.

Arslan ZF, Uludag A, Uremis I (2015). Status of invasive alien plants included in EPPO Lists in Turkey. EPPO Bulletin 45: 66-72.

Austin DF, Huáman Z (1996). A synopsis of *Ipomoea* (*Convolvulaceae*) in the Americas. Taxon 45: 3-38.

Aykurt C (2012). *Ipomoea*. In: Güner A, Aslan S, Ekim T, Vural M, Babaç MT (eds.). Türkiye Bitkileri Listesi (Damarlı Bitkiler). İstanbul: Nezahat Gökyiğit Botanik Bahçesi ve Flora Araştırmaları Derneği Yayını. p. 380.

Besler B (1613). *Hortus Eystettensis*. Nürnberg: Basilius Besler Press.

Boyjnath Y, Dulloo ME, Bhoyroo V, Ranghoo-Sanmukhiya VM (2024). Ecogeographic study of *Ipomoea* species in Mauritius, Indian Ocean. Plants 13(19): 2706.

Burman NL (1755). *Auctuarium Florae Indicae*, p. 2 verso. Amsterdam: Janssonio-Waesbergios.

CABI (2024). *Ipomoea indica* (ocean blue morning-glory). https://www.cabidigitallibrary.org/doi/full/10.1079/cabicompendium.107833 / [accessed 10 November 2024].

Cushman JH, Meentemeyer RK (2008). Multiscale patterns of human activity and the incidence of an exotic forest pathogen. Journal of Ecology 96(4): 766-776.

Delgado-Dávila R, Martén-Rodríguez S, Huerta-Ramos G (2016). Variation in floral morphology and plant reproductive success in four *Ipomoea* species (Convolvulaceae) with contrasting breeding systems. Plant Biology 18(6): 903-912.

Eserman LA, Tiley GP, Jarret RL, Leebens-Mack JH, Miller RE (2014). Phylogenetics and diversification of morning glories (tribe Ipomoeeae, Convolvulaceae) based on whole plastome sequences. American Journal of Botany 101: 1-12.

Flora of China (2024). *Ipomoea indica*. http://www.efloras.org/florataxon.aspx?flora_id=2&taxon_id=210000739 / [accessed 10 November 2024].

Flora of North America (2024). *Ipomoea tricolor*. http://www.efloras.org/florataxon.aspx?flora_id=1&taxon_id=230002892 / [accessed 30 January 2024]

Fosberg FR (1976). Lectotypification of *Convolvulus indicus*. Botaniska Notiser 129: 35-38.

Hagiwara S (1938). *Pharbitis indica*. Botanica et Zoologica 6: 1238.

Hançerli L, Ayata MU, Çakan H, Uygur FN, Uygur S (2018). A new weed species record for the Flora of Turkey *Ipomoea hederifolia* L. (*Convolvulaceae*). Turkish Journal of Weed Science 21(2): 36-38.

Hernández-Aro M, Hernández-Perez R, Guadalupe-Tapia G, Guillen-Sánchez D, Castellanos-González L (2017). Phytotoxic potential of *Ipomoea batatas* extract, detected through a new type of sandwich microbioassay on three species of weeds. Planta Daninha 35: e017163066.

IPNI (2024). https://www.ipni.org/n/269086-1 / [accessed 10 November 2024].

Meira M, Silva EPD, David JM, David JP (2012). Review of the genus *Ipomoea*: traditional uses, chemistry and biological activities. Revista Brasileira de Farmacognosia 22: 682-713.

Menemen Y, Aytaç Z, Kandemir A (2021). Türkçe bilimsel bitki, mantar, suyosunu ve bakteri adları yönergesi. Bağbahçe Bilim Dergisi 8(3): 188-195.

Miller RE, McDonald JA, Manos PS (2004). Systematics of *Ipomoea* subgenus *Quamoclit* (Convolvulaceae) based on ITS sequence data and a Bayesian phylogenetic analysis. American Journal of Botany 91: 1208-1218.

Parris CS (1978). *Ipomoea* L. In: PH Davis (ed.) Flora of Turkey and the East Aegean Islands. Edinburgh: Edinburg University Press. Vol. 6, pp. 221-222.

Pimentel D, Lach L, Zuniga R, Morrison D (2000). Environmental and economic costs of nonindigenous species in the United States. BioScience 50: 53-65.

Pimentel D, McNair S, Janecka J, Wightman J, Simmonds C, O'connell C, Wong E, Russel L, Zern J, Aquino T (2001). Economic and environmental threats of alien plant, animal, and microbe invasions. Agriculture, Ecosystems and Environment 84: 1-20.

Pimentel D, Zuniga R, Morrison D (2005). Update on the environmental and economic costs associated with alien-invasive species in the United States. Ecological Economics 52: 273-288.

Simberloff D, Martin JL, Genovesi P, Maris V, Wardle DA, Aronson J, Courchamp F, Galil B, García-Berthou B, Pascal M, Pyšek P, Sousa R, Tabacchi E, Vilà M (2013). Impacts of biological invasions: what's what and the way forward. Trends in Ecology and Evolution 28(1): 58-66.

Srivastava D, Rauniyar N (2020). Medicinal plants of genus *Ipomoea*. Beau Bassin, Mauritius: LAP Lambert Academic Publishing.

Uludağ A, Aksoy N, Yazlık A, Arslan Z, Yazmış E, Üremiş İ, Cossu TA, Groom Q, Pergl J, Pyšek P, Brundu G (2017). Alien flora of Turkey: checklist, taxonomic composition and ecological attributes. NeoBiota 35: 61-85.

Weakley AS, Southeastern Flora Team (2024). Flora of the Southeastern United States Web App. University of North Carolina Herbarium, North Carolina Botanical Garden, Chapel Hill, USA. https://fsus.ncbg.unc.edu/main.php?pg=show-key.php&keyid=40395 / [accessed 10 November 2024].

Wood JRI, Muñoz-Rodríguez P, Williams BRM, Scotland RW (2020). A foundation monograph of *Ipomoea* (*Convolvulaceae*) in the New World. PhytoKeys 143: 1-823.

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Antioxidant profiling of *Anethum graveolens*: Insights into phenolic and flavonoid-rich extracts

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Anethum graveolens'in antioksidan profili: Fenolik ve flavonoid bakımından zengin ekstraktlara dair bulgular

Abstract: This study investigates the total phenolic and flavonoid contents, antioxidant activity, and metal chelation capacity of acetone and water extracts from *Anethum graveolens* L.. The total phenolic content of the acetone extract was significantly higher (173.49 ± 4.91 µg GAE/mg extract) than the water extract (98.52 ± 3.62 µg GAE/mg extract). Similarly, the flavonoid content of the acetone extract (72.81 ± 1.15 µg QE/mg extract) exceeded that of the water extract (27.69 ± 1.72 µg QE/mg extract). Concentration-dependent responses revealed higher antioxidant activity for the acetone extract across all tested concentrations (12.5–400 µg/mL), with a sharper increase in response at higher concentrations. The IC_{50} values for DPPH radical scavenging and metal chelation activities further confirmed the acetone extract's superior performance, with lower IC_{50} values for DPPH scavenging (51.56 µg/mL) and metal chelation (113.46 µg/mL) compared to the water extract (192.44 µg/mL and 268.95 µg/mL, respectively). Hierarchical clustering and 3-D surface plot analyses demonstrated strong correlations between DPPH scavenging and metal chelation activities for both extracts, with Pearson correlation coefficients of $r = 0.94$ for the acetone extract and $r = 0.99$ for the water extract. While the acetone extract displayed higher bioactivity, the water extract exhibited a more tightly linked relationship between its antioxidant and metal chelation properties. These findings highlight the potential of *A. graveolens* extracts as natural antioxidants and metal chelators, offering promising applications for oxidative stress mitigation and metal toxicity management.

Key words: Bioactive compounds, natural extracts, phytochemicals, polyphenols

Özet: Bu çalışma, *Anethum graveolens* L.'den elde edilen aseton ve su ekstraktlarının toplam fenolik ve flavonoid içeriklerini, antioksidan aktivitelerini ve metal şelatlama kapasitesini incelemektedir. Aseton ekstraktının toplam fenolik içeriği (173,49 ± 4,91 µg GAE/mg ekstrakt), su ekstraktından (98,52 ± 3,62 µg GAE/mg ekstrakt) anlamlı derecede daha yüksek bulunmuştur. Benzer şekilde, aseton ekstraktının flavonoid içeriği (72,81 ± 1,15 µg QE/mg ekstrakt), su ekstraktının flavonoid içeriğinden (27,69 ± 1,72 µg QE/mg ekstrakt) daha yüksektir. Konsantrasyona bağlı tepkiler, tüm test edilen konsantrasyonlarda (12,5–400 µg/mL) aseton ekstraktının daha yüksek antioksidan aktiviteye sahip olduğunu ve özellikle yüksek konsantrasyonlarda daha keskin bir artış gösterdiğini ortaya koymuştur. DPPH radikal süpürme ve metal şelatlama aktiviteleri için IC_{50} değerleri, aseton ekstraktının üstün performansını daha da doğrulamış, DPPH süpürme için daha düşük IC_{50} değeri (51,56 µg/mL) ve metal şelatlama için (113,46 µg/mL) değerleri bulunmuştur. Buna karşılık, su ekstraktı için DPPH süpürme (192,44 µg/mL) ve metal şelatlama (268,95 µg/mL) değerleri daha yüksektir. Hiyerarşik kümeleme ve 3 boyutlu yüzey grafiği analizleri, her iki ekstrakt için DPPH süpürme ve metal şelatlama aktiviteleri arasında güçlü korelasyonlar göstermiştir. Aseton ekstraktı için Pearson korelasyon katsayısı $r = 0,94$ iken, su ekstraktı için $r = 0,99$ olarak hesaplanmıştır. Aseton ekstraktı daha yüksek biyoaktivite sergilerken, su ekstraktı, antioksidan ve metal şelatlama özellikleri arasında daha sıkı bir ilişki ortaya koymuştur. Bu bulgular, *A. graveolens* ekstraktlarının doğal antioksidanlar ve metal şelatörler olarak potansiyelini vurgulamakta ve oksidatif stresin azaltılması ve metal toksisitesinin yönetimi için umut verici uygulamalar sunduğunu göstermektedir.

Anahtar Kelimeler: Biyoaktif bileşikler, doğal ekstraktlar, fitokimyasallar, polifenoller

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1. Introduction

Plants are a rich source of natural compounds that support human health. Among these compounds, antioxidants play a crucial role in reducing oxidative stress caused by free radicals and preventing cellular damage (Akbari et al., 2022; Göldağ and Doğan, 2024). Natural antioxidants have gained increasing interest in the food and pharmaceutical industries because of their potential to extend the shelf life of foods and protect human health (Costa et al., 2021). Free radicals are at the root of numerous health problems, including aging, chronic diseases, cancer, diabetes, and cardiovascular disorders (Kumar and Pandey, 2015). Plant-based antioxidants, including phenolic compounds, flavonoids, and vitamins, have the potential to mitigate these harmful effects (Akbari et al., 2022). Therefore, investigating the antioxidant properties of plants is of great importance for discovering new natural therapeutic compounds and developing healthy and sustainable products for the food, pharmaceutical, and cosmetic industries (Diniz do Nascimento et al., 2020; Doğan, 2020). Additionally, determining the antioxidant properties of

plants contributes to understanding the biochemical variations caused by environmental factors, cultivation conditions, and species differences (Shen et al., 2022). In recent years, many researchers have conducted studies on the antioxidant properties of plants (Kok et al., 2023; Tang et al., 2023; Collins et al., 2024; Tokgoz et al., 2024).

Commonly known as dill, *Anethum graveolens* L. is not only appreciated as a flavorful herb in culinary practices but also recognized as a healing source with significant health benefits (Al Masoody et al., 2023; Mujović et al., 2024). Its flavonoids and phenolic compounds can neutralize free radicals, reduce oxidative stress, and enhance immune function (Mohammed et al., 2019; Khan et al., 2020). Traditionally, dill has been used to alleviate digestive issues, reduce bloating, and regulate appetite (Singh et al., 2024). Furthermore, it has shown potential in regulating blood sugar levels and lowering cholesterol, positively impacting metabolic health (Haidari et al., 2020). Its antimicrobial properties may also reduce the risk of infections (Ghoname et al., 2023). These characteristics highlight dill's importance in both traditional and modern health practices.

Total phenolic compounds play a significant role in the antioxidant activity of plant-based extracts. These compounds are critical for both food preservation and human health due to their ability to neutralize free radicals (Asif, 2015; Gutiérrez-del-Río et al., 2021). Similarly, flavonoids, a group of polyphenolic compounds widely present in plants, exhibit antioxidant properties. Flavonoids act through diverse mechanisms, such as scavenging free radicals, chelating metal ions, and protecting against oxidative stress (Cotelle, 2001; Cherrak et al., 2016).

One of the common methods for determining antioxidant properties is 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging assay (Gulcin and Alwasel, 2023). This test is used to quantitatively measure a substance's capacity to neutralize free radicals and is considered an important indicator of the antioxidant activity of plant extracts (Nićiforović et al., 2010; Gupta, 2015). Additionally, metal chelating activity reflects a plant's ability to reduce the effects of metal ions that generate reactive oxygen species (Hassinen et al., 2011). Quantitative determination of total phenol and flavonoid contents is also crucial for understanding dill's antioxidant capacity.

Dill has potential applications as a natural preservative in the food industry and as an anti-inflammatory, and anticancer agent in pharmacology (Mohamed et al., 2024). However, a better understanding of factors such as environmental influences and extraction methods that affect dill's antioxidant properties is necessary. In this context, our study aims to determine the antioxidant activity of *A. graveolens* and to elucidate the relationship between this activity and its total phenol and flavonoid contents.

This study aims to provide a comprehensive analysis of the antioxidant capacity of *A. graveolens* using different solvent fractions. The findings will enhance our understanding of this plant's potential applications in food and pharmaceutical industries and serve as a foundation for future research.

2. Materials and Method

2.1. Obtaining Plant Samples and Their Extraction

Anethum graveolens samples were freshly obtained from a local producer in Konya, Türkiye for use in our research. The samples were kept under cool conditions during transportation to prevent spoilage. Before being used in the study, the plants were carefully cleaned and prepared appropriately for analysis. Plant samples were air-dried at room temperature and then transformed into powder form using an ultra-centrifuge grinder. The extraction process involved obtaining acetone and water extracts of *A. graveolens* using a Soxhlet extraction apparatus with 250 mL solvent systems. Acetone and water extracts of *A. graveolens* yielded 2.5% and 23.3% (w/w) of plant substances, respectively. The resulting crude extract from the plant sample was filtered using a Whatman No. 1 filter paper. The solvents were subsequently removed by evaporation through a rotary evaporator under vacuum conditions, leading to complete drying. The extract was further lyophilized to yield ultra-dry powders.

2.2. Determination of Total Phenol Content

To quantify the overall phenolic content in the extracts, we utilized a modified Folin-Ciocalteu method with gallic acid as a reference standard. In separate wells of a microplate, we added 20 µL of each extract (400 µg/mL) and the gallic acid standard to analyze. We then introduced 20 µL of the Folin-Ciocalteu reagent into each well and allowed the mixture to incubate in the dark for 3 min. This incubation is essential for the reduction of the Folin reagent by the phenolic compounds. Next, we added 20 µL of sodium carbonate to each well to neutralize the mixture, followed by the addition of 140 µL of distilled water (dH_2O). This step helps develop the color indicative of phenolic content. The wells were kept in the dark for an additional 10 min to allow color development. After the incubation period, we measured the absorbance at 725 nm using a spectrophotometer. The intensity of the color formed is directly proportional to the phenolic content in the samples. The phenolic content was expressed as gallic acid equivalents (GAE), calculated using a standard calibration curve generated from known concentrations of gallic acid (Kok et al., 2023).

2.3. Determination of Total Flavonoid Content

To quantify the overall flavonoid content in the extracts, we utilized a modified aluminum chloride colorimetric method with quercetin as a reference standard. In distinct wells of a microplate, we added 50 µL of each extract (400 µg/mL) and the quercetin standard. To each well, we then introduced 215 µL of 80% ethyl alcohol to help dissolve the flavonoid compounds and facilitate the reaction. Following this, 5 µL of the aluminum nitrate solution and 5 µL of potassium acetate were added to the mixture in each well. The plates were incubated at room temperature for 40 min in the dark to allow the formation of the aluminum-flavonoid complex, which is crucial for accurate detection. After incubation, the absorbance of the solutions was measured at 415 nm using a spectrophotometer. The absorbance correlates with the flavonoid content in the samples. The flavonoid content was expressed as quercetin equivalents (QE). This was calculated using a standard calibration curve created with known concentrations of quercetin (Kok et al., 2023).

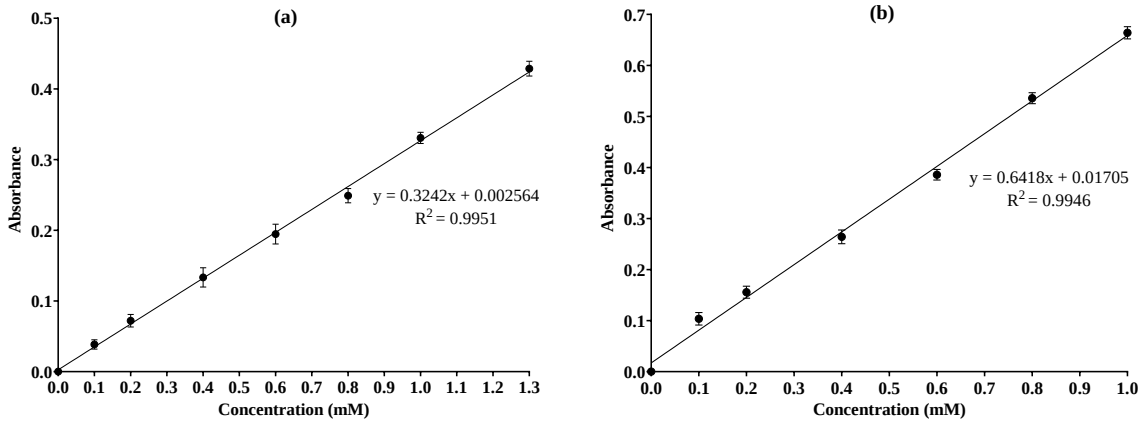

Figure 1. (a) Linear regression graphs for gallic acid, used as a standard in calculating total phenolic content, and **(b)** quercetin, used as a standard in calculating total flavonoid content (n = 3).

2.4. Free Radical Scavenging Activity

In the measurement of DPPH scavenging activity of acetone and water extracts obtained from the plants, applications were carried out with the final concentrations of the extracts in the plate wells of 12.5, 25, 50, 100, 200, and 400 µg/mL. According to the method, 20 µL of the extracts were placed in each microplate well, and 180 µL of DPPH (0.06 mM in methanol) was added. The reduction of DPPH free radical was determined by measuring the absorbance values at 517 nm after 60 minutes in the dark. The free radical scavenging activities of the extracts were calculated as a percentage using the following formula (1): Radical scavenging activity = [(Control absorbance – Extract absorbance) / Control absorbance)] × 100 (Kok et al., 2023).

2.5. Chelation of Metals

To assess the metal-binding capacity, we followed a spectrophotometric method using $FeCl_2$ and ferrozine. Various concentrations (12.5–400 µg/mL) of acetone and water extracts from both allelopathic and control plants were added to wells in a microplate. Each well received 50 µL of the plant extract, 185 µL of distilled water, 5 µL of $FeCl_2$ (2 mM), and 10 µL of ferrozine (5 mM). After 10 min incuba- tion at room temperature, the absorbance was measured at 562 nm to determine the Fe^{2+}-binding ability of the extracts. The metal chelating activities of the extracts were calculated as a percentage using the formula (2): Metal chelating activity = [(Control absorbance – Sample absorbance) / Control absorbance)] × 100 (Kok et al., 2023).

2.6. Statistical Analyses

The activities of the extracts were evaluated through a one-way ANOVA followed by the Duncan test. Probit regression analysis was applied to calculate the median inhibitory concentration (IC_{50}) values. To examine the relationships among DPPH scavenging and metal chelating activities across different extracts, three-dimensional (3D) density analysis was performed. These statistical analyses were conducted using SPSS software (version 27.0, IBM Corporation, Armonk, NY, USA). Additionally, heatmap and hierarchical cluster analyses, employing Ward's minimum variance method, were used to identify similarities and differences among DPPH scavenging and metal chelating activities. These analyses were performed in the RStudio console using the pheatmap package in R software (version 4.1.0).

3. Results and Discussion

3.1. Analysis of Antioxidant Compounds of the Extracts

The total phenolic and flavonoid contents of acetone and water extracts were determined and expressed as GAE and QE, respectively. The acetone extract demonstrated a significantly greater phenolic content (1.76 times higher than the water extract), highlighting its superior antioxidant potential. For total flavonoid content, the acetone extract also demonstrated higher values, with 72.81 ± 1.15 µg QE/mg of extract, compared to the water extract, which had a total flavonoid content of 27.69 ± 1.72 µg QE/mg of extract (Table 1). These results suggest that the acetone extract has higher concentrations of both phenolic and flavonoid compounds compared to the water extract, indicating its greater potential as an antioxidant source.

Phenolic compounds, such as flavonoids, have been widely recognized for their antioxidant properties due to their ability to neutralize free radicals and chelate metal ions (Vuolo et al., 2019; Parcheta et al., 2021). The higher phenolic and flavonoid content in the acetone extract is likely a result of acetone's ability to dissolve a wider range of non-polar and semi-polar bioactive compounds. Acetone is a less-polar solvent compared to water and is particularly effective in extracting compounds such as flavonoids, phenolic acids, and other secondary metabolites that have antioxidant and anti-inflammatory properties (Tzanova et al., 2020; Eid et al., 2023). These compounds have been associated with a broad range of biological activities, including anti-cancer, anti-inflammatory, and antioxidant effects, highlighting the acetone extract's greater potential for use in pharmaceutical and nutraceutical applications.

On the other hand, the water extract, while exhibiting lower levels of both phenolic and flavonoid compounds, may still offer beneficial bioactive properties. Water is a highly polar solvent and may extract a different spectrum of compounds, such as water-soluble vitamins, sugars, and other hydrophilic antioxidants, which may still contribute to its overall bioactivity. However, the lower concentrations of phenolic and flavonoid compounds in the water extract suggest that its potential as a potent antioxidant source is less than that of the acetone extract (Fatima et al., 2019; Shi et al., 2022). The findings also suggest that the acetone

extract may be more effective as a natural antioxidant due to the higher concentrations of these bioactive compounds. Since phenolic compounds and flavonoids are known for their ability to scavenge free radicals and reduce oxidative stress (Huyut et al., 2017), the acetone extract's superior levels of these compounds could potentially offer more robust protection against oxidative damage. This is consistent with previous research that has shown acetone extracts to possess stronger antioxidant activity compared to water extracts (Zhao et al., 2006; Ahmad et al., 2020).

In conclusion, the acetone extract of *A. graveolens* is a more promising source of antioxidants due to its higher phenolic and flavonoid content. While the water extract may have potential, particularly for applications requiring less potent antioxidant activity, the acetone extract is better suited for industries such as cosmetics, food preservation, and pharmaceuticals, where strong antioxidant properties are highly valued. Further studies are needed to investigate the specific antioxidant mechanisms of these extracts and to explore their applications in various fields.

3.2. Assessing DPPH Scavenging Abilities of the Extracts

The effects of acetone and water extracts were evaluated at different concentrations (12.5, 25, 50, 100, 200, and 400 µg/mL) on the measured responses.

For the acetone extract, the observed values increased with concentration. At the lowest concentration (12.5 µg/mL), the response was 14.17, which increased progressively to 75.5 at the highest concentration (400 µg/mL). Notably, the acetone extract exhibited a marked rise in response, especially at concentrations above 50 µg/mL, with values of 64.56 (50 µg/mL), 70.69 (100 µg/mL), and 72.80 (200 µg/mL), before stabilizing at 75.5 at 400 µg/mL. This could be attributed to the higher concentrations of phenolic and flavonoid compounds, which are known for their antioxidant and metal chelation properties (Chanda et al., 2015). The higher concentrations of these bioactive compounds in the acetone extract likely contribute to its greater ability to neutralize free radicals and chelate metal ions, which in turn leads to a stronger overall bioresponse at elevated concentrations.

In contrast, the water extract showed a more gradual increase in response across the concentrations. At 12.5 µg/mL, the response was 4.43, which increased steadily to 63.54 at 400 µg/mL. The response at 50 µg/mL was 22.52, rising to 30.61 at 100 µg/mL, and peaked at 57.60 at 200 µg/mL before reaching the highest value at 63.54 at 400 µg/mL. The gradual rise in response suggests that the bioactivity of the water extract is less concentration-dependent compared to the acetone extract. This behavior could be due to the different chemical profiles of the water extract, which may contain a lower concentration of phenolic and flavonoid compounds (Ahmad et al., 2020).

Table 1. Antioxidant compounds of the plant extracts (µg/mg)

Treatment	Total phenol (Gallic acid equivalent)	Total flavonoid (Quercetin equivalent)
Acetone extract	173.49 ± 4.91	72.81 ± 1.15
Water extract	98.52 ± 3.62	27.69 ± 1.72

Each value is expressed as mean ± standard deviation (n = 3)

The water extract may still possess antioxidant and metal chelation properties, but the presence of lower levels of bioactive compounds may result in a more gradual increase in biological response.

The data indicate a higher overall response for the acetone extract across all concentrations compared to the water extract. Additionally, the acetone extract demonstrated a sharper increase in response at higher concentrations, while the water extract exhibited a more gradual, linear rise. These findings suggest that the acetone extract may be more potent or effective at promoting the observed response, particularly at higher concentrations. The letters above the bars indicate statistical differences among the groups. Groups sharing the same letter are not significantly different ($p > 0.05$), while those with different letters indicate statistically significant differences ($p < 0.05$). For the acetone extract, the scavenging activity increased significantly with concentration, reaching the highest value at 100 µg/mL, which was not significantly different from 50 µg/mL. We found that there was no difference ($p > 0.05$) between 200 and 400 µg/mL applications of acetone extract (Fig. 2). This effect is common in biological systems, where increasing concentrations beyond a certain threshold may not result in a proportional increase in activity due to saturation of the biological response mechanism (Xie and Schaich, 2014).

3.3. Chelation of Metals by the Extracts

The effects of concentrations ranging from 12.5–400 µg/mL of acetone and water extracts were evaluated. Both extracts exhibited concentration-dependent increases in bioactivity, although the acetone extract consistently showed higher responses across most concentrations, especially at higher levels, suggesting it might be more effective in delivering antioxidant or other bioactive effects. This finding is consistent with previous studies where solvent polarity has been found to affect the extraction efficiency and bioactive potential of plant compounds (Tripathi et al., 2013).

For the acetone extract**,** the values ranged from 4.29 at 12.5 µg/mL to 67.68 at 400 µg/mL. Notably, the acetone extract showed an increasing trend in its response with higher concentrations, with the highest values observed at the 400 µg/mL concentration. The variability in the acetone extract response, as indicated by the second set of measurements, ranged from 1.31 at 12.5 µg/mL to 1.66 at 400 µg/mL. Previous studies have shown that acetone is an effective

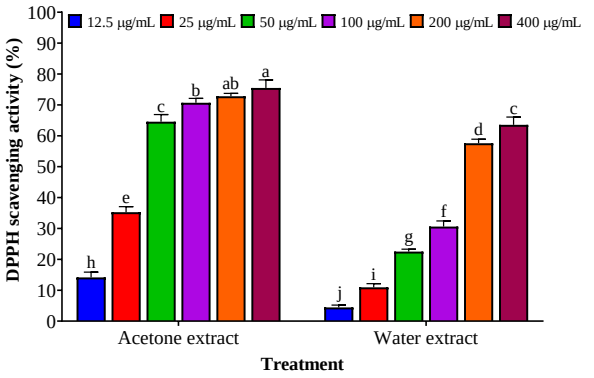

Figure 2. DPPH radical scavenging activity of different plant extracts is presented as mean ± standard deviation (n = 3). Distinct letters indicate statistically significant differences at $p < 0.05$.

solvent for extracting phenolic compounds, which are known for their antioxidant and metal chelation activities (Wang et al., 2009). The lower variability in the acetone extract also suggests that acetone-soluble compounds are more consistent in their bioactive interactions, which could be advantageous in applications requiring a predictable and reliable antioxidant effect (Salak et al., 2013).

In comparison, the water extract exhibited a similar concentration-dependent increase, with values ranging from 3.47 at 12.5 µg/mL to 56.40 at 400 µg/mL. The variability for the water extract response ranged from 1.17 at 12.5 µg/mL to 2.09 at 400 µg/mL. Overall, the water extract displayed more significant variations in response compared to the acetone extract, particularly at higher concentrations. This could be due to the more complex mixture of compounds in the water extract, including both hydrophilic and less bioavailable substances, leading to more variability in biological responses (Verma et al., 2012). Water extracts tend to contain a greater variety of compounds, but these compounds may not all contribute equally to antioxidant activity resulting in less predictable outcomes compared to acetone extracts. At all concentrations, the acetone extract generally demonstrated higher values than the water extract, with the exception of the 100 µg/mL and 200 µg/mL concentrations where water extract values were comparable or slightly higher. These findings are in line with research suggesting that water extracts can sometimes outperform organic solvent extracts at lower concentrations, particularly when hydrophilic bioactive compounds are present in substantial amounts (Matalka et al., 2007). These findings suggest that the acetone extract might be more effective at higher concentrations, whereas the water extract, although exhibiting some variability, also shows potential efficacy at different concentration levels.

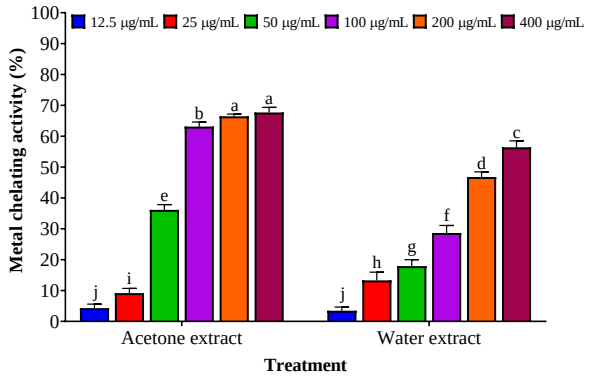

Figure 3. Metal chelating activity of different plant extracts is presented as mean ± standard deviation (n = 3). Distinct letters indicate statistically significant differences at $p < 0.05$.

When the statistical differences between the concentrations were examined, we found that there was no difference ($p > 0.05$) between 200 and 400 µg/mL applications of acetone extract (Fig. 3). Such effects are common in studies of antioxidant activity, where the bioactivity of certain compounds reaches a maximum threshold beyond which higher concentrations do not result in proportional increases in activity (Abd El-Gawad, 2016). This observation may provide valuable insight into optimizing concentrations for applications in which acetone extracts are used, such as in food preservation or therapeutic applications aimed at reducing oxidative stress.

The IC_{50} values of *A. graveolens* extracts for DPPH radical scavenging and ferrous ion chelating activities are presented in Table 2. The acetone extract exhibited a significantly lower IC_{50} value for DPPH scavenging activity (51.56 µg/mL) compared to the water extract (192.44 µg/mL), indicating stronger antioxidant activity in the acetone extract. The slope for the DPPH scavenging activity was 1.11 ± 0.06 for acetone, with a confidence interval of 0.98–1.23, suggesting a moderately steep dose-response curve.

For metal chelation, the acetone extract also showed a lower IC_{50} value (113.46 µg/mL) compared to the water extract (268.95 µg/mL), indicating superior metal chelation ability. The slope for the acetone extract's metal chelating activity was 1.48 ± 0.07 (1.34–1.62), suggesting a slightly steeper dose-response relationship than for the DPPH scavenging activity. In comparison, the water extract showed weaker activity for both DPPH scavenging and metal chelation, as reflected by the higher IC_{50} values and the less pronounced slopes (1.38 ± 0.07 for DPPH scavenging, 1.21 ± 0.07 for metal chelation). These findings demonstrate that the acetone extract of *A. graveolens* is more effective than the water extract in both DPPH radical scavenging and ferrous ion chelation activities.

The lower IC_{50} value for the acetone extract supports its ability to neutralize reactive oxygen species more efficiently, which is consistent with the general observation that acetone tends to extract more potent antioxidant compounds, such as polyphenols and flavonoids, compared to water (Ali et al., 2014). Metal ion chelation is a critical mechanism in antioxidant defense, as metal ions like iron and copper can catalyze the production of harmful free radicals (Gulcin and Alwasel, 2022). These results corroborate earlier findings in the literature, where acetone and other organic solvents were shown to be more efficient than water in extracting bioactive compounds with antioxidant and metal-chelating properties (Petkova et al., 2020).

Table 2. IC_{50} values (µg/mL) of *A. graveolens* extracts for their DPPH radical scavenging and ferrous ion chelating activities

Extract	Activity	IC_{50} (Limits)	Slope ± Standard error (Limits)
Acetone	DPPH scavenging	51.56 (45.01–58.64)	1.11 ± 0.06 (0.98–1.23)
	Metal chelating	113.46 (102.54–126.09)	1.48 ± 0.07 (1.34–1.62)
Water	DPPH scavenging	192.44 (170.10–220.85)	1.38 ± 0.07 (1.24–1.52)
	Metal chelating	268.95 (229.64–323.55)	1.21 ± 0.07 (1.07–1.36)

3.4. Analysis of Heatmap, Cluster, and 3-D Density

A hierarchical clustering analysis was conducted to group the samples based on their DPPH radical scavenging activity and metal chelation capacity. The resulting dendrogram (Fig. 4a) revealed distinct clusters, each represented by a unique color, demonstrating significant variability in bioactivities among the samples. The clustering segregated the samples into 4 groups, highlighting differences in antioxidant and metal chelation properties between the acetone extract and water extract. Applications of 100, 200, and 400 µg/mL acetone extract under Cluster 4 stood out with high DPPH and metal chelation activities. This cluster, representing the highest activity, further corroborates the strong antioxidant and metal-chelating properties of the acetone extract, which are consistent with its lower IC_{50} values for both DPPH scavenging and metal chelation (Çayan et al., 2022).

To explore the relationship between DPPH radical scavenging activity and metal chelation capacity, 3-D surface plots were generated for the acetone extract and water extract, as shown in Figures 4b and 4c, respectively. For the acetone extract, the 3-D surface plot (Fig. 4b) demonstrated a strong positive correlation between DPPH radical scavenging activity and metal chelation capacity, with a Pearson correlation coefficient of r = 0.94. This correlation was observed within the activity range of 10–60% DPPH scavenging activity and 10–50% metal chelation activity, indicating a synergistic relationship where higher antioxidant activity is consistently associated with enhanced metal chelation capacity in the acetone extract.

Similarly, the water extract exhibited an even stronger correlation, as illustrated in Fig. 4c, with a Pearson correlation coefficient of r = 0.99. This correlation was observed within the activity range of 10–70% DPPH scavenging activity and 10–60% metal chelation activity, suggesting an almost linear relationship between the two activities. The near-perfect correlation highlights the robust, tightly linked relationship and consistent, potent bioactive profile of the water extract. This is in line with previous studies indicating that antioxidants capable of scavenging free radicals are often also effective in chelating metal ions, which helps prevent oxidative damage (Di Meo and Venditti, 2020).

When comparing the two extracts, the water extract showed a slightly higher correlation coefficient compared to the acetone extract. This finding suggests that the water extract may possess a more tightly linked mechanism governing its antioxidant and metal chelation activities. The difference emphasizes the potential of water extract as a more effective extract for applications requiring both strong antioxidant and metal chelation properties. The slightly higher correlation coefficient for the water extract could reflect the presence of water-soluble compounds that more directly influence both antioxidant and chelating activities, or a unique bioactive synergy specific to the water extract (Cheng et al., 2021).

These results provide strong evidence of the interplay between antioxidant and metal chelation activities in both extracts, with the water extract demonstrating superior performance. This highlights the potential of these extracts for further bioactive compound characterization and their use in applications targeting oxidative stress and metal toxicity mitigation.

Conflict of Interest

Authors have declared no conflict of interest.

Authors' Contributions

The authors contributed equally.

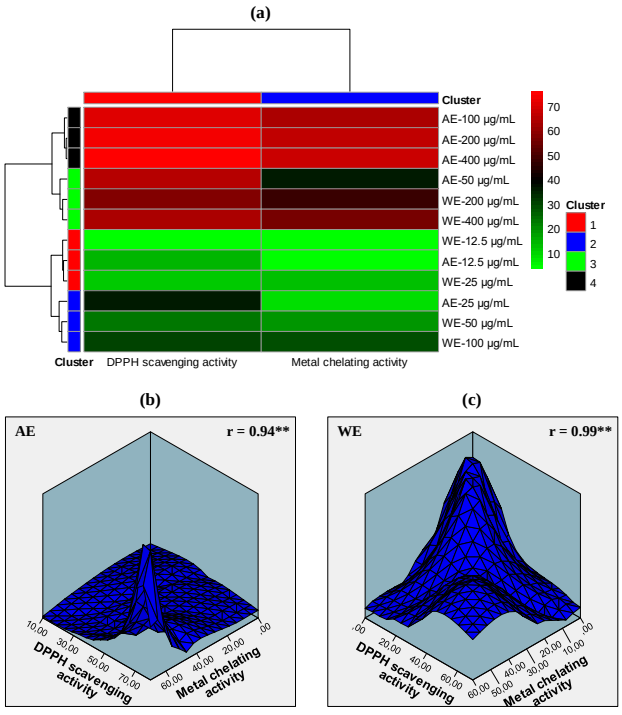

Figure 4. (a) Heatmap and hierarchical clustering analysis of various concentrations of the tested extracts, displaying DPPH scavenging and metal chelating activities, where red indicates high activity and green indicates low activity. **(b, c)** 3-D density analysis was performed to assess the DPPH scavenging and metal chelating activities of the extracts. Correlation coefficients (r) were calculated, with statistical significance denoted by a double asterisk (**) at the 0.01 level. AE: Acetone extract of *A. graveolens*, WE: Water extract of *A. graveolens*.

References

Abd El-Gawad AM (2016). Chemical constituents, antioxidant and potential allelopathic effect of the essential oil from the aerial parts of *Cullen plicata*. Industrial Crops and Products 80: 36-41.

Ahmad M, Mohammad N, Aziz MA, Alam MA, Hossain MS, Islam MR, Uddin MG (2020). Comparison of antioxidant role of methanol, acetone and water extracts of *Andrographis paniculata* Nees. Journal of Medicinal Plants Research 14(8): 428-437.

Akbari B, Baghaei-Yazdi N, Bahmaie M, Mahdavi Abhari F (2022). The role of plant-derived natural antioxidants in reduction of oxidative stress. BioFactors 48(3): 611-633.

Al Masoody IH, Alarkwazi RK, Al Yasssiry AS (2023). Pharmaceutical and biological properties of dill: a review. IOP Conference Series: Earth and Environmental Science 1158: 62005.

Ali SI, El-Baz FK, El-Emary GAE, Khan EA, Mohamed AA (2014). HPLC-analysis of polyphenolic compounds and free radical scavenging activity of pomegranate fruit (*Punica granatum* L.). International Journal of Pharmaceutical and Clinical Research 6(4): 348-355.

Asif M (2015). Chemistry and antioxidant activity of plants containing some phenolic compounds. Chemistry International 1(1): 35-52.

Çayan F, Tel-Çayan G, Deveci E, Duru ME, Türk M (2022). A detailed study on multifaceted bioactivities of the extracts and isolated compounds from truffle *Reddellomyces parvulosporus*. International Journal of Food Science & Technology 57(3): 1411-1419.

Chanda S, Moteriya P, Padaliya H, Rathod T, Baravalia Y (2015). Antioxidant and metal chelating activities of *Lagenaria siceraria* (Molina) Standl peel, pulp and aerial parts in relation to their total phenol and flavonoid content. Pharmacognosy Journal 7(1): 64-73.

Cheng Y, Xue F, Yu S, Du S, Yang Y (2021). Subcritical water extraction of natural products. Molecules 26(13): 4004.

Cherrak SA, Mokhtari-Soulimane N, Berroukeche F, Bensenane B, Cherbonnel A, Merzouk H, Elhabiri M (2016). In vitro antioxidant versus metal ion chelating properties of flavonoids: a structure-activity investigation. PloS One 11(10): e0165575.

Collins A, Santhakumar A, Latif S, Chinkwo K, Francis N, Blanchard C (2024). Impact of processing on the phenolic content and antioxidant activity of *Sorghum bicolor* L. Moench. Molecules 29(15): 3626.

Costa M, Sezgin-Bayindir Z, Losada-Barreiro S, Paiva-Martins F, Saso L, Bravo-Díaz C (2021). Polyphenols as antioxidants for extending food shelf-life and in the prevention of health diseases: encapsulation and interfacial phenomena. Biomedicines 9(12): 1909.

Cotelle N (2001). Role of flavonoids in oxidative stress. Current Topics in Medicinal Chemistry 1(6): 569-590.

Di Meo S, Venditti P (2020). Evolution of the knowledge of free radicals and other oxidants. Oxidative Medicine and Cellular Longevity 2020(1): 9829176.

Diniz do Nascimento L, Barbosa de Moraes AA, Santana da Costa K, Pereira Galúcio JM, Taube PS, Leal Costa CM, Neves Cruz J, de Aguiar Andrade EH, Guerreiro de Faria LJ (2020). Bioactive natural compounds and antioxidant activity of essential oils from spice plants: new findings and potential applications. Biomolecules 10(7): 988.

Doğan M (2020). Su teresinin (*Nasturtium officinale* R. BR.) beslenme-diyet potansiyeli ve antioksidan özellikleri: bir derleme. International Anatolia Academic Online Journal Health Sciences 6(3): 222-233.

Eid O, Elkady WM, Ezzat S, El Sayed A, Abd Elsattar E (2023). Comprehensive overview: the effect of using different solvents for barley extraction with its anti-inflammatory and antioxidant activity. Chemistry & Biodiversity 20(3): e202200935.

Fatima Z, Jin X, Zou Y, Kaw HY, Quinto M, Li D (2019). Recent trends in analytical methods for water-soluble vitamins. Journal of Chromatography A 1606: 360245.

Ghoname ESA, Hassan D, Hammad EM (2023). Antimicrobial activity of dill seeds and celery seeds on beef burger. European Journal of Nutrition & Food Safety 15(9): 106-117.

Göldağ R, Doğan M (2024). Avokado (*Persea americana* Mill.)'nun besin içeriği, antioksidan özelliği ve potansiyel sağlık faydaları. Karamanoğlu Mehmetbey Üniversitesi Mühendislik ve Doğa Bilimleri Dergisi 6(1): 62-69.

Gulcin İ, Alwasel SH (2022). Metal ions, metal chelators and metal chelating assay as antioxidant method. Processes 10(1): 132.

Gulcin İ, Alwasel SH (2023). DPPH radical scavenging assay. Processes 11(8): 2248.

Gupta D (2015). Methods for determination of antioxidant capacity: a review. International Journal of Pharmaceutical Sciences and Research 6(2): 546.

Gutiérrez-del-Río I, López-Ibáñez S, Magadán-Corpas P, Fernández-Calleja L, Pérez-Valero Á, Tuñón-Granda M, Miguélez EM, Villar CJ, Lombó F (2021). Terpenoids and polyphenols as natural antioxidant agents in food preservation. Antioxidants 10(8): 1264.

Haidari F, Zakerkish M, Borazjani F, Ahmadi Angali K, Amoochi Foroushani G (2020). The effects of *Anethum graveolens* (dill) powder supplementation on clinical and metabolic status in patients with type 2 diabetes. Trials 21: 483.

Hassinen VH, Tervahauta AI, Schat H, Kärenlampi SO (2011). Plant metallothioneins–metal chelators with ROS scavenging activity? Plant Biology 13(2): 225-232.

Huyut Z, Beydemir Ş, Gülçin İ (2017). Antioxidant and antiradical properties of selected flavonoids and phenolic compounds. Biochemistry Research International 2017(1): 7616791.

Khan K, Aishwarya S, Satapathy P, Veena SM, Melappa G, Zameer F (2020). Exploration of dill seeds (*Anethum graveolens*): an ayurpharmacomic approach. In: Rahman A, Iqbal MC, Yousuf, S (eds.). Science of Spices and Culinary Herbs-Latest Laboratory, Pre-Clinical, and Clinical Studies. Bentham Books Sharjah, pp. 116-152.

Kok O, Emsen B, Surmen B (2023). Screening of in vitro cytotoxicity and antioxidant potential of selected endemic plants in Turkey. Journal of Taibah University for Science 17(1): 2217369.

Kumar S, Pandey A (2015). Free radicals: health implications and their mitigation by herbals. British Journal of Medicine and Medical Research 7(6): 438-457.

Matalka KZ, Ali D, El Khawad A, Qa'dan F (2007). The differential effect of *Eriobotrya japonica* hydrophilic leaf extract on cytokines production and modulation. Cytokine 40(3): 235-240.

Mohamed RS, Fouda K, Maghraby AS, Assem FM, Menshawy MM, Zaghloul AH, Abdel-Salam AM (2024). Hepato-renal protective impact of nanocapsulated *Petroselinum crispum* and *Anethum graveolens* essential oils added in fermented milk against some food additives via antioxidant and anti-inflammatory effects: in silico and in vivo studies. Heliyon 10(17): e36866.

Mohammed FA, Razvi SS, Abdul WM, Mohammed K, Hakeem KR, Banaganapalli B, Shaik NA, Elkady AI (2019). Protective role of medicinal herb *Anethum graveolens* (dill) against various human diseases and metabolic disorders. In: Ozturk M, Hakeem, KR (eds.). Plant and Human Health. Springer, pp. 181-194.

Mujović M, Šojić B, Peulić T, Kocić-Tanackov S, Ikonić P, Božović D, Teslić N, Županjac M, Novaković S, Jokanović M (2024). Effects of dill (*Anethum graveolens*) essential oil and lipid extracts as novel antioxidants and antimicrobial agents on the quality of beef burger. Foods 13(6): 896.

Nićiforović N, Mihailović V, Mašković P, Solujić S, Stojković A, Muratspahić DP (2010). Antioxidant activity of selected plant species; potential new sources of natural antioxidants. Food and Chemical Toxicology 48(11): 3125-3130.

Parcheta M, Świsłocka R, Orzechowska S, Akimowicz M, Choińska R, Lewandowski W (2021). Recent developments in effective antioxidants: the structure and antioxidant properties. Materials 14(8): 1984.

Petkova NT, Popova VT, Ivanova TA, Stoyanova AS, Mazova NN, Panayotov ND (2020). In vitro antioxidant activity of *Physalis peruviana* L. fruits. Bulgarian Chemical Communications 52: 215-221.

Salak F, Daneshvar S, Abedi J, Furukawa K (2013). Adding value to onion (*Allium cepa* L.) waste by subcritical water treatment. Fuel Processing Technology 112: 86-92.

Shen N, Wang T, Gan Q, Liu S, Wang L, Jin B (2022). Plant flavonoids: classification, distribution, biosynthesis, and antioxidant activity. Food Chemistry 383: 132531.

Shi L, Zhao W, Yang Z, Subbiah V, Suleria HAR (2022). Extraction and characterization of phenolic compounds and their potential antioxidant activities. Environmental Science and Pollution Research 29(54): 81112-81129.

Singh N, Sharma U, Mishra B, Kandalkar AM, Jain SK (2024). Herbs and herbal formulations for the management and prevention of gastrointestinal diseases. In: Izah, SC, Ogwu, MC, Akram, M (eds.). Herbal Medicine Phytochemistry: Applications and Trends. Springer, pp. 657-691.

Tang Z, Wang Y, Huang G, Huang H (2023). Ultrasound-assisted extraction, analysis and antioxidant activity of polysaccharide from the rinds of *Garcinia mangostana* L. Ultrasonics Sonochemistry 97: 106474.

Tokgoz A, Emsen B, Dogan M (2024). The efficacy of allelopathy of select lichens on antioxidant potential and multiplication of *Ceratophyllum demersum* L. Plant Cell, Tissue and Organ Culture 159: 26.

Tripathi BN, Singh V, Ezaki B, Sharma V, Gaur JP (2013). Mechanism of Cu-and Cd-induced proline hyperaccumulation in *Triticum aestivum* (wheat). Journal of Plant Growth Regulation 32: 799-808.

Tzanova M, Atanasov V, Yaneva Z, Ivanova D, Dinev T (2020). Selectivity of current extraction techniques for flavonoids from plant materials. Processes 8(10): 1222.

Verma V, Rico-Martinez R, Kotra N, King L, Liu J, Snell TW, Weber RJ (2012). Contribution of water-soluble and insoluble components and their hydrophobic/hydrophilic subfractions to the reactive oxygen species-generating potential of fine ambient aerosols. Environmental Science & Technology 46(20): 11384-11392.

Vuolo MM, Lima VS, Junior MRM (2019). Phenolic compounds: structure, classification, and antioxidant power. In: Campos, MRS (ed.). Bioactive Compounds. Elsevier, pp 33-50.

Wang T, Jónsdóttir R, Ólafsdóttir G (2009). Total phenolic compounds, radical scavenging and metal chelation of extracts from Icelandic seaweeds. Food Chemistry 116(1): 240-248.

Xie J, Schaich KM (2014). Re-evaluation of the 2, 2-diphenyl-1-picrylhydrazyl free radical (DPPH) assay for antioxidant activity. Journal of Agricultural and Food Chemistry 62(19): 4251-4260.

Zhao H, Dong J, Lu J, Chen J, Li Y, Shan L, Lin Y, Fan W, Gu G (2006). Effects of extraction solvent mixtures on antioxidant activity evaluation and their extraction capacity and selectivity for free phenolic compounds in barley (*Hordeum vulgare* L.). Journal of Agricultural and Food Chemistry 54(19): 7277-7286.

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Endemic *Dactylorhiza osmanica* subsp. *osmanica:* Ex vitro symbiotic germination approach

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Endemik *Dactylorhiza osmanica* subsp. *osmanica:* Ex vitro simbiyotik çimlenme için bir bakış açısı

Abstract: In this study, the potential effects of *Ceratobasidium* sp. fungus on the ex vitro symbiotic germination of *Dactylorhiza osmanica* (Klinge) P. F. Hunt & Summerh subsp. *osmanica* seeds were evaluated. Results, *Ceratobasidium* sp. caused that about 51.95% germination rate was obtained in seeds inoculated. Protocorm and primordium formation rates were determined to be 25.27% and 26.67%, respectively. These findings indicate that the fungus promotes the germination of seeds. *Ceratobasidium sp*. on ex vitro symbiotic seed germination of *D. osmanica* subsp. *osmanica* shows the potential effect of the fungus Longer term and more detailed studies should be conducted to reach adult plants and adapt to natural conditions. These findings are very promising for protecting of endemic and rare orchid species.

Key words: *Ceratobasidium*, conservation, endemic orchid, ex vitro symbiotic germination, mycorrhiza

Özet: Bu çalışmada, *Ceratobasidium* sp. fungusunun, *Dactylorhiza osmanica* (Klinge) P. F. Hunt & Summerh subsp. *osmanica* tohumlarının ex vitro simbiyotik çimlenmesi üzerindeki potansiyel etkileri değerlendirilmiştir. Sonuçlar, *Ceratobasidium* sp. ile aşılanan tohumlarda yaklaşık %51,95 çimlenme oranı elde edildiğini göstermiştir. Protokorm ve primordiyum oluşum oranları sırasıyla %25,27 ve %26,67 olarak belirlenmiştir. Bu bulgular, fungusun tohum çimlenmesini teşvik ettiğini göstermektedir. *Ceratobasidium* sp.'nin *D. osmanica* subsp. *osmanica* üzerindeki ex vitro simbiyotik tohum çimlenmesi üzerindeki etkisi, fungusun potansiyel etkisini ortaya koymaktadır. Yetişkin bitkilere ulaşmak ve doğal koşullara adaptasyonu sağlamak için daha uzun süreli ve detaylı çalışmalar yapılması gerekmektedir. Bu bulgular, endemik ve nadir orkide türlerinin korunması için oldukça umut vericidir.

Anahtar Kelimeler: *Ceratobasidium*, koruma, endemik orkide, ex vitro simbiyotik çimlenme, mikoriza

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1. Introduction

The Orchidaceae family consists of 736 genera and about 28.000 species, and about 500 new species are defined in these two families every year (Chase et al., 2015). This family presents a wide variety of epiphytic and terrestrial specimens. It finds its habitat in all parts of the world and is famous for its morphological species diversity. Representing monocotyledons, these species impressively represent diversity with an unlimited number of variations (de Vasconcelos et al., 2023). Türkiye has a rich diversity of flora due to its geographical location and different climatic conditions, which is reflected in the diversity of orchid species (Karakaya et al., 2019) Orchidaceae species richness is quite high in Türkiye. According to current data, there are 33 taxa belonging to the genus *Dactylorhiza* Neck. in our country, 14 of which are endemic (Fırat et al., 2015). In the current climate change scenario, orchid species are threatened due to their unique habitats, complex seed germination mechanisms, and specialized pollination processes. In addition, in our country, orchid tubers are collected in an uncontrolled manner depending on economic factors such as food and medical drug production. Therefore, orchids urgently need to be protected and resettled. Seeds of all orchids form a symbiotic relationship with certain types of fungi to germinate naturally (Wani et al., 2020; Deniz et al., 2022) In nature, the seeds of almost all orchids need a certain fungal partner to germinate due to the lack of endosperm. These fungi are usually orchid mycorrhizal fungi that promote the formation of structures called pelotons in stem cells or protocorms. These fungi play an important role in in situ reproduction and conservation processes by promoting seed germination (Selosse et al., 2017). The symbiotic method may be more suitable for producing tuberous orchids for both conservation and commercial purposes to obtain fast and high-yielding results. Studies have shown that symbiotic seedlings adapt more easily to the natural environment and grow faster. Therefore, the use of symbiotic methods in orchid conservation efforts can provide a significant advantage in terms of producing orchids more effectively and adapting to natural habitats better (Quay et al., 1995; Aewsakul et al., 2013; Özdener Kömpe et al., 2022; Deniz et al., 2022).

Many orchid species, such as *D. osmanica* (Klinge) P. F. Hunt & Summerh. *var. osmanica*, require conservation efforts due to their rarity and dwindling populations. This species is classified as "Endangered" on the IUCN Red List of Threatened Species as a result of habitat loss and fragmentation caused by human activities such as agriculture and urbanization (Zhou et al., 2021). However,

conservation efforts for *Dactylorhiza osmanica* subsp. *osmanica* are unfortunately scarce in Türkiye (Sazak and Özdener 2006). For the conservation of *Dactylorhiza osmanica* subsp. *osmanica* and similar orchid species, the importance of ex vitro studies is emerging. The aim of this study is to determine the germination and development of *Dactylorhiza osmanica* subsp. *osmanica* seeds under ex vitro conditions with *Ceratobasidium* sp. to investigate the symbiotic development obtained by AG A (Accession Number: OR036462) vaccination and to provide important data for this endemic species. This study can be considered a fundamental step to develop strategies for conserving *Dactylorhiza osmanica* subsp. *osmanica*.

2. Materials and Method

2.1. Material

Dactylorhiza osmanica subsp. *osmanica* seeds were collected from East Black Sea Region in Trabzon, Türkiye (2300 m). The flowering period is between May and July, the altitude at which it grows is 1000-2400 m, its distribution area is riversides and meadows with high ground water. The mature capsules were taken, opened in the lab, dried for a few days at room temperature, and then stored at 4 °C in brown glass bottles.

2.2. Method

2.2.1. Fungal isolate

In this research, *Ceratobasidium* sp. AG A (accession number: OR036462) derived from the orchid-fungi collection at the University of Ondokuz Mayıs, Department of Biology. A segment of the fungal stock culture was aseptically transferred to a petri dish containing a meticulously prepared fungus isolation medium. Following an activation period, diligent observation was carried out to track the progressive colonization of the petri dish surface by the fungal hyphae. Upon achieving complete coverage, this cultivated fungus was utilized as the pivotal element for executing germination assessments.

2.2.2. Ex vitro symbiotic seed germination

For ex vitro seed germination, a soil mixture of ratio 2:1 (soil, perlite, respectively) was prepared. Afterwards, this mixture was sterilized in an autoclave (121 °C, 1.5Atm) and added to the pots. Previously activated fungi were inoculated into these pots. 6 seed packets were placed in each pot. About 1 mg of seeds (approx. 100 seeds) was added into the seed packets. The finished pots were placed in a climate chamber under 12:12 hours (light/dark) conditions at 23 ± 2 °C. Seed packs were placed in the control group, but the fungal isolate was not inoculated. All experimental groups were irrigated twice a week with ground oats and modified oat medium without agar. Developmental stages were evaluated according to the following stages, modified by (Clements et al., 1986): 0: Ungerminated seed, 1: Protocorm, 2: Leaf primordium, 3: First leaf, 4: Developed leaves and/or roots

2.2.3. Statistical analysis

Data were analyzed by the ANOVA method, which is analysis of variance, and the means were analyzed by Duncan's comparative test. Statistical analyzes were performed using the SPSS program. The visualizations of the statistical analyzes were made using the R program.

3. Results

3.1 Ex vitro symbiotic seed germination

Ceratobasidium sp. approximately 51.955% germination occurred in seeds inoculated with AG A (Table 1, Figure 1). However, seedling development did not occur. Protocorm and primordium formation rates differ (25.27% and 26.67%, respectively). This indicates that the fungus promotes germination of seeds. Germinated seeds are shown in Figure 2. No germination occurred in any of the fungal-free control groups.

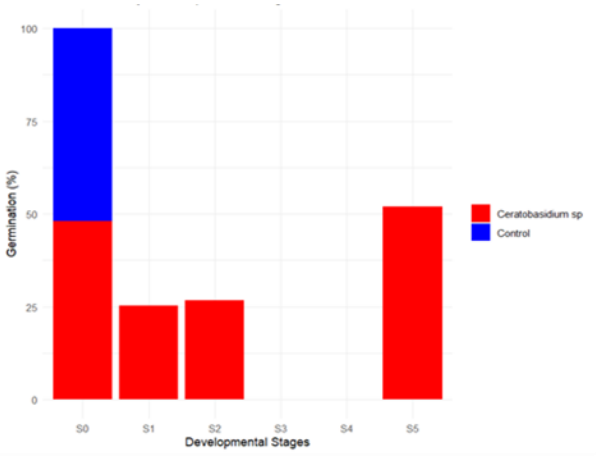

Figure 1. Column chart of *Dactylorhiza osmanica* subsp. *osmanica* development stages with R program. S0: Ungerminated seed, S1: Protocorm, S2:Leaf primordium, S3: First leaf, S4: Developed leaves and/or roots

Figure 2. *Dactylorhiza osmanica* subsp. *osmanica* protocorm and primodium stages

4. Discussions

The initial findings of our study indicate a positive impact on various stages of orchid seed germination, including the germination of *Dactylorhiza osmanica* subsp. *osmanica* seeds, protocorm development (the first germination phase), and leaf primordium formation. This is particularly remarkable considering that terrestrial orchid seeds typically possess hydrophobic and lignified seed pods, which act as a waterproof barrier, impeding water and nutrient absorption (Barsberg et al 2018; Gao et al., 2022). As a result, these seeds heavily rely on a mutually beneficial relationship with specific fungi, which provide them with essential nutrients (Zeng et al., 2012; Deniz et al., 2022). The process of symbiotic germination in orchids is

Table 1. Germination and development of *Dactylorhiza osmanica* subsp. *osmanica* seeds with *Ceratobasidiaceae*

	Developmental Stages (%)					Germination
	S0	S1	S2	S3	S4	%
Control	100±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
***Ceratobasidium* sp**	48.04± 1.52	25.27±1.46	26.67±2.98	0.00±0.00	0.00±0.00	51.95±1.52

S0: Ungerminated seed, S1: Protocorm, S2:Leaf primordium, S3: First leaf, S4: Developed leaves and/or roots

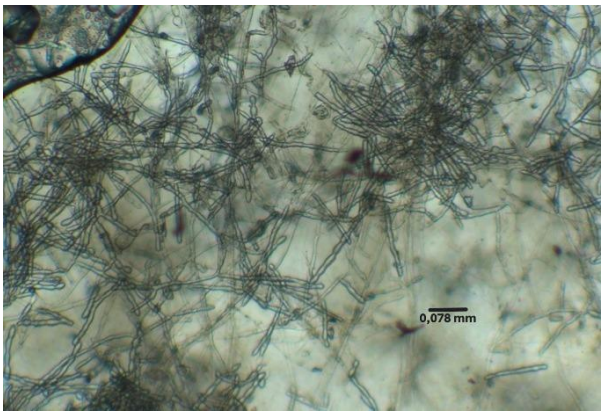

Figure 3. Isolated *Ceratobasidium* sp. derived from the orchid-fungi collection at the University of Ondokuz Mayıs, Department of Biology.

known to be exceedingly intricate and challenging. While there have been some in vitro investigations conducted on orchid species belonging to the *Dactylorhiza* genus (Rasmussen et al., 1990, Warghat et al., 2014), studies conducted ex vitro are relatively scarce (Aggarwal and Zettler 2010). Consequently, our study represents a significant contribution as the first ex vitro exploration specifically focused on *Dactylorhiza osmanica* subsp. *osmanica*. The outcomes of this study have shed light on the ex vitro germination potential *D. osmanica* subsp. *osmanica* seeds, facilitated by the mycorrhizal fungus *Ceratabaisidum* sp. However, it is worth noting that this particular fungus did not contribute to the subsequent growth and development of the seedlings. Nonetheless, the data generated from this research not only serve to aid in the conservation and propagation efforts of this species but also serve as an impetus for further investigations in this field.

The germination results observed in *Dactylorhiza osmanica* subsp. *osmanica* are consistent with the findings reported in previous studies on asymbiotic germination on *Dactylorhiza osmanica* subsp. *osmanica* (Sazak and Özdener, 2006) and other *Dactylorhiza* taxa (Hayakawa et al., 1999; Kömpe and Mutlu, 2007; Çığ and Yılmaz, 2017; Çığ et al., 2018; Fatahi et al., 2023).

By elucidating the germination dynamics and symbiotic associations in *Dactylorhiza osmanica* subsp. *osmanica*, our study expands the understanding of orchid conservation biology and the importance of fungal interactions in orchid seed germination. As seen in the control group, no germination was observed in pots without *Ceratobasidium* sp. In seeds inoculated with *Ceratobasidium* sp., a germination rate of approximately 51.95% was achieved. The protocorm formation rate was 25.27%, and the primordium formation rate was 26.67%. These findings suggest that *Ceratobasidium* sp. promotes seed germination. Future research endeavors could explore alternative mycorrhizal fungi that might exhibit a more comprehensive symbiotic relationship, encompassing both germination and subspequent seedling development. Additionally, investigates the factors that hinder the growth of *Dactylorhiza osmanica* subsp. *osmanica* seedlings in the presence of *Ceratabaisidum* sp. could provide valuable insights into the physiological and ecological aspects of orchid-fungal interactions.

In summary, our study highlights the ex vitro germination potential of *Dactylorhiza osmanica* subsp. *osmanica* seeds with the aid of the mycorrhizal fungus *Ceratabaisidum* sp. This research not only contributes to the conservation and propagation efforts of this orchid species but also paves the way for further exploration into the intricacies of orchid symbiotic germination.

Our findings provide evidence of the potential impact of *Ceratobasidium* sp. fungus on the ex vitro symbiotic germination of *Dactylorhiza osmanica* subsp. *osmanica* seeds. However, the lack of support for seedling development by this fungus raises a significant area of further investigation. These findings serve as a catalyst for future research endeavors in fields such as orchid conservation and restoration, where greater efforts are needed.

The discovery of the positive effect of *Ceratobasidium* sp. fungus on orchid seed germination represents a valuable contribution to the understanding of symbiotic interactions in orchids. While the current study focused on *Dactylorhiza osmanica* subsp. *osmanica*, it opens up avenues for exploring similar relationships in other orchid species. Investigating alternative fungal species and their potential role in facilitating both germination and subspequent seedling growth would enhance our understanding of the complexities of orchid-fungal symbiosis.

Overall, this study underscores the importance of further investigations into the ex vitro symbiotic germination of *Dactylorhiza osmanica* subsp. *osmanica*, specifically concerning the factors inhibiting seedling development in the presence of *Ceratobasidium* sp. These insights will not only advance our knowledge of orchid biology but also inform conservation and restoration strategies for orchid species. Continued research in this area will contribute to the effective management and preservation of these remarkable plants and their delicate ecological relationships.

Conflict of Interest

Authors have declared no conflict of interest.

Authors' Contributions

The authors contributed equally.

References

Aggarwal S, Zettler LW (2010). Reintroduction of an endangered terrestrial orchid, *Dactylorhiza hatagirea* (D. Don) Soo, assisted by symbiotic seed germination: First report from the Indian subcontinent. Nature and Science 8(10): 139-145.

Aewsakul N, Maneesorn D, Serivichyaswat P, Taluengjit A, Nontachaiyapoom S (2013). Ex vitro symbiotic seed germination of Spathoglottis plicata Blume on common orchid cultivation subsptrates. Scientia Horticulturae 160: 238-242.

Barsberg ST, Lee YI, Rasmussen HN (2018). Development of C-lignin with G/S-lignin and lipids in orchid seed coats–an unexpected diversity exposed by ATR-FT-IR spectroscopy. Seed Science Research 28(1): 41-51.

Clements MA, Muir H, Cribb PJ (1986). A preliminary report on the symbiotic germination of European terrestrial orchids. Kew Bulletin 41: 437-445. https://doi.org/10.2307/4102957

Chase MW, Cameron KM, Freudenstein JV, Pridgeon AM, Salazar G, Van den Berg C, Schuiteman A (2015). An updated classification of *Orchidaceae*. Botanical Journal of the Linnean Society 177(2): 151-174.

Çığ A, Yılmaz H (2017). In vitro symbiotic culture studies of some orchid species. Journal of Agricultural Sciences 23(4): 453-463.

Çığ AD, Durak E, İşler S (2018). In vitro symbiotic germination potentials of some *Anacamptis, Dactylorhiza, Orchis* and *Ophrys* terrestrial orchid species. Applied Ecology and Environmental Research 16(4):5141-5155.

de Vasconcelos FM, Andrade EHA, Teixeira LOA, Figueiredo PLB, Maia JGS (2023). Volatile constituents from *Catasetum* (*Orchidaceae*) species with occurrence in the Brazilian Amazon. Plants 12(4): 703.

Deniz İG, Kömpe YÖ, Harzli I, Aytar EC, Mutlu VA, Uysal Dİ (2022). From seed to flowering tuberous orchid using ex vitro symbiotic seed germination: A breakthrough study with Anacamptis sancta. Rhizosphere 24: 100597.

Fatahi M, Anghelescu NE, Vafaee Y, Khoddamzadeh A (2023). Micropropagation of *Dactylorhiza umbrosa* (Kar. & Kir.) Nevski through asymbiotic seed germination and somatic embryogenesis: A promising tool for conservation of rare terrestrial orchids. South African Journal of Botany 159: 492-506. https://doi.org/10.1016/j.sajb.2023.06.036

Fırat M, Karavelioğulları FA, Aziret A (2015). Geophytes of East Anatolia (Turkey). Manas Journal of Agriculture Veterinary and Life Sciences 5(1): 38-53.

Gao Y, Peng S, Hang Y, Xie G, Ji N, Zhang M (2022). Mycorrhizal fungus *Coprinellus disseminatus* influences seed germination of the terrestrial orchid *Cremastra appendiculata* (D. Don) Makino. Scientia Horticulturae 293: 110724.

Hayakawa S, Uetake Y, Ogoshi A (1999). Identification of symbiotic *Rhizoctonias* from naturally occurring protocorms and roots of *Dactylorhiza aristata* (*Orchidaceae*). Journal-Faculty Of Agriculture Hokkaido Unıversity 69: 129-141.

Karakaya S, Polat A, Aksakal Ö, Sümbüllü YZ, İncekara Ü (2019). Plants used in traditional medicine and other uses in South of Erzurum (Turkey): An ethnobotanical study. Ethnobotany Research and Applications 18: 1-18.

Kömpe YO, Mutlu VA (2017). Mycorrhizal diversity in some species of *Dactylorhiza* genus (*Orchidaceae*). Biological Diversity and Conservation 10(1): 55-64.

Özdener Kömpe Y, Akin Mutlu V, Özkoç İ, Demiray S, Serhat B (2022). Fungal diversity and ex vitro symbiotic germination of *Serapias vomeracea* (*Orchidaceae*). Acta Botanica Croatica 81(1): 108-116.

Quay L, McComb JA, Dixon KW (1995). Methods for ex vitro germination of Australian terrestrial orchids. HortScience 30(7): 1445-1446.

Rasmussen H, Andersen TF, Johansen B (1990). Temperature sensitivity of in vitro germination and seedling development of *Dactylorhiza majalis* (*Orchidaceae*) with and without a mycorrhizal fungus. Plant, Cell & Environment 13(2): 171-177.

Sazak A, Özdener Y (2006). Symbiotic and asymbiotic germination of endangered *Spiranthes spiralis* (L.) Chevall. and *Dactylorhiza osmanica* (Kl.) Soó var. *osmanica* (endemic). Pakistan journal of Biological sciences 9(12): 2222-2228.

Selosse MA, Minasiewicz J, Boullard B (2017). An annotated translation of Noël Bernard's 1899 article 'On the germination of *Neottia nidus-avis'*. Mycorrhiza 27: 611-618.

Wani I A, Kumar V, Verma S, Tasleem Jan A, Rather IA (2020). *Dactylorhiza hatagirea* (D. Don) Soo: A critically endangered perennial orchid from the North-West Himalayas. Plants 9(12): 1644.

Warghat AR, Bajpai PK, Srivastava RB, Chaurasia OP, Chauhan RS, Sood H (2014). In vitro protocorm development and mass multiplication of an endangered orchid, *Dactylorhiza hatagirea*. Turkish Journal of Botany 38(4): 737-746.

Zeng S, Wu K, da Silva JAT, Zhang J, Chen Z, Xia N, Duan J (2012). Asymbiotic seed germination, seedling development and reintroduction of *Paphiopedilum wardii* Sumerh., an endangered terrestrial orchid. Scientia Horticulturae 138: 198-209.

Zhou Z, Shi R, Zhang Y, Xing X, Jin X (2021). Orchid conservation in China from 2000 to 2020: Achievements and perspectives. Plant Diversity 43(5): 343-349.

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Comparative anatomy and palynology of two *Herniaria* L. species (*Caryophyllaceae*)

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İki *Herniaria* L. (*Caryophyllaceae*) türünün karşılaştırmalı anatomisi ve palinolojisi

Abstract: In this study, the anatomical and palynological features of *Herniaria incana* Lam. and *H. pisidica* Brummitt. species were investigated. Similarities and differences were discussed for species. Anatomy and pollen studies of these taxa are presented for the first time in this work. Scanning Electron Microscopy (SEM) and Light Microscopy (LM) studies on the pollen grains have revealed that they are prolate – spheroidal and their exine ornamentation is microechinate – punctate. The stem has a collateral vascular bundle and the mesophyll type is bifacial for both *H. incana* and *H. pisidica*.

Key words: Anatomy, *Caryophyllaceae, Herniaria*, pollen, Türkiye

Özet: Bu çalışmada *Herniaria incana* Lam. ve *H. pisidica* Brummitt. türlerinin anatomik ve palinolojik özellikleri incelenmiştir. İki tür için benzerlikler ve farklılıklar tartışılmıştır. Bu taksonlara ait anatomi ve polen çalışmaları ilk kez bu çalışmada sunulmaktadır. Taramalı Elektron Mikroskobu (SEM) ve Işık Mikroskobu (LM) çalışmaları sonucunda; polenlerin prolat – sferoidal olduğu, polen ornamentasyonlarının ise mikroekinat – punktat olduğu tespit edilmiştir. Her iki tür için; gövde, kollateral iletim demetlerine sahiptir ve yaprak mezofil tipi bifasiyaldir.

Anahtar Kelimeler: Anatomi, *Caryophyllaceae, Herniaria*, polen, Türkiye

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1. Introduction

Caryophyllaceae Juss. family includes approximately 105 genera and approximately 3000 species (POWO, 2024), mainly distributed in Holarctic regions (Bittrich, 1993; Heywood, 1998) with a center of diversity in the Mediterranean and Irano-Turanean areas (Hernández-Ledesma et al. 2015).

Caryophyllaceae family has 3 subfamilies (Alsinoideae Burnett, Caryophylloideae Arn., and Paronychioideae A.St). The genus *Herniaria* L. belongs to the subfamily Paronychioideae. *Herniaria* Linnaeus (1753: 218) includes 52 species according to POWO (2024) and have distributed in Asia, Africa and Europe (Lawrence, 2017; Bittrich, 1993).

The genus *Herniaria* was revised as eight species by Brummitt in the Flora of Turkey in 1967. In the same work, *Herniaria orientalis* F. Herm. was given as a questionable record, and *H. amoena* Çelebioğlu & Favarger was added to the 11th volume of the Flora of Turkey. Also, the subspecies *H. cinerea* D.C. subsp. *euphratica* was overlooked despite being published in 1967 and was not added to the additional volumes. However, the situation was noticed in the List of Turkish Plants and a total of 12 species of the genus *Herniaria* were included in the work. Six of these species are endemic and the endemism rate is 50% (Güner et al. 2012; Davis, 1967). According to recent studies, *Herniaria* includes 11 taxa, with *H. orientalis* F. Herm. being a questionable record.

Herniaria genus and anatomy of *Herniaria* was studied by Williams (1896) and Hermann (1937) for the first time. Later, it was presented by Van et al. (1968). Gangulee et al. (1972) and Mousaand Al-Jibouri (2019) were studied on anatomy of *H. hirsuta* L. Also, Zarinkamar (2001) surveyed on *H. hirsuta* and *H. incana*. Schweingruber (2007) has investigated of 4 *Herniaria* taxa (*H. glabra* L., *H. incana*, *H. hirsuta*, *H. alpina* Chaix.). In pollen studies, by Nowicke and Skvarla (1976), and Hutchinson (1959) on *H. hirsuta*, Punt and Hoen (1999) on *H. glabra*, Perveen and Qaiser (2003) on *H. cinerea* D.C. were studied. As a result, anatomical and palynological surveys on *Herniaria* are rare.

In this study, *H. incana* and *H. pisidica* species were studied and compared in view of anatomical and palynological characters. For these species, similarities and differences were identified. In this study, anatomy and pollen examinations of these *Herniaria* taxa are presented for the first.

2. Materials and Method

The specimens were collected from Kastamonu province (*H. incana*) and Denizli province (*H. pisidica*) in Türkiye (Table 1). The collected specimens are deposited in the herbarium of Ankara Yıldırım Beyazit University.

For anatomical studies; living material was kept in 70% ethanol. The paraffin method was used to obtain cross sections of stem and leaves. The specimens were embedded in paraffin wax. These materials were sectioned between 5

and 10 µm thickness with a Leica RM2245 rotary microtome. All species were stained with safranin–fast green. These stained sections were glued with entellan and permanent preparations were obtained according to Johansen (1940). Measurements and photos were taken using a Leica DM1000 binocular light microscope equipped with a Leica DFC280 camera.

For palynological investigations; pollen material were acquired from herbarium specimens. The pollen slides were prepared according to Wodehouse (1935) technique. For scanning electron microscopy (SEM), dry pollen samples were transferred on stubs, cover with gold, examined and photographed with a HITACHI SU5000 FE-SEM at Ankara Yıldırım Beyazıt University Central Research Laboratory Application and Research Center. Pollen morphological characters were calculated as minimum, maximum, standard deviation, average values and it presented as a table. Measurements were made on 20-30 pollen grains and based on the ratio of polar axis to equatorial axis (P/E), the pollen shape class was determined with Erdtman's system (1969). Pollen morphologies were identified by using the glossary of pollen and spore terminology of Punt et al. (2007).

3. Results

3.1. Pollen morphology

It is found that pollen grains of *H. incana* and *H. pisidica* are polyporate, isopolar, radial symmetric and pollen shape are prolate-spheroidal. Pollen ornamentation are microechinate – punctate for both taxon. Some differences were detected in terms of pollen characters such as exine and intine thickness, intense of spinule, number of punctum and these differences were presented figures and tables (Table 2, Fig. 1 and Fig. 2).

Table 1. Locality of *H. incana* and *H. pisidica*.

Species of *Herniaria*	Locality	Herbarium number
H. incana	Amasya: Lokman Mountain, 1100 m. 19.06.2024.	M. Koç - 4350
***H. pisidica* (Endemic)**	Denizli: Babadağ, 37º 39′ 52″ N, 28º 57′ 23.4″ E, 1190, 28.06.2024.	M. Koç - 3611

3.2. Anatomy

3.2.1. Stem anatomy

— ***H. incana*** has epidermis 1-layered, consisting of rectangular and oval cells and is surrounded by a cuticle layer. The cortex tissue is consist of 6−7 layered, oval or cylindrical parenchymatic cells with chloroplasts and cortex has druse crystals. The sclerenchyma tissue is composed from 2 layered. The pith is consist of circular shaped parenchymatic cells. The stem has a collateral vascular bundle (Table 3, Fig. 3).

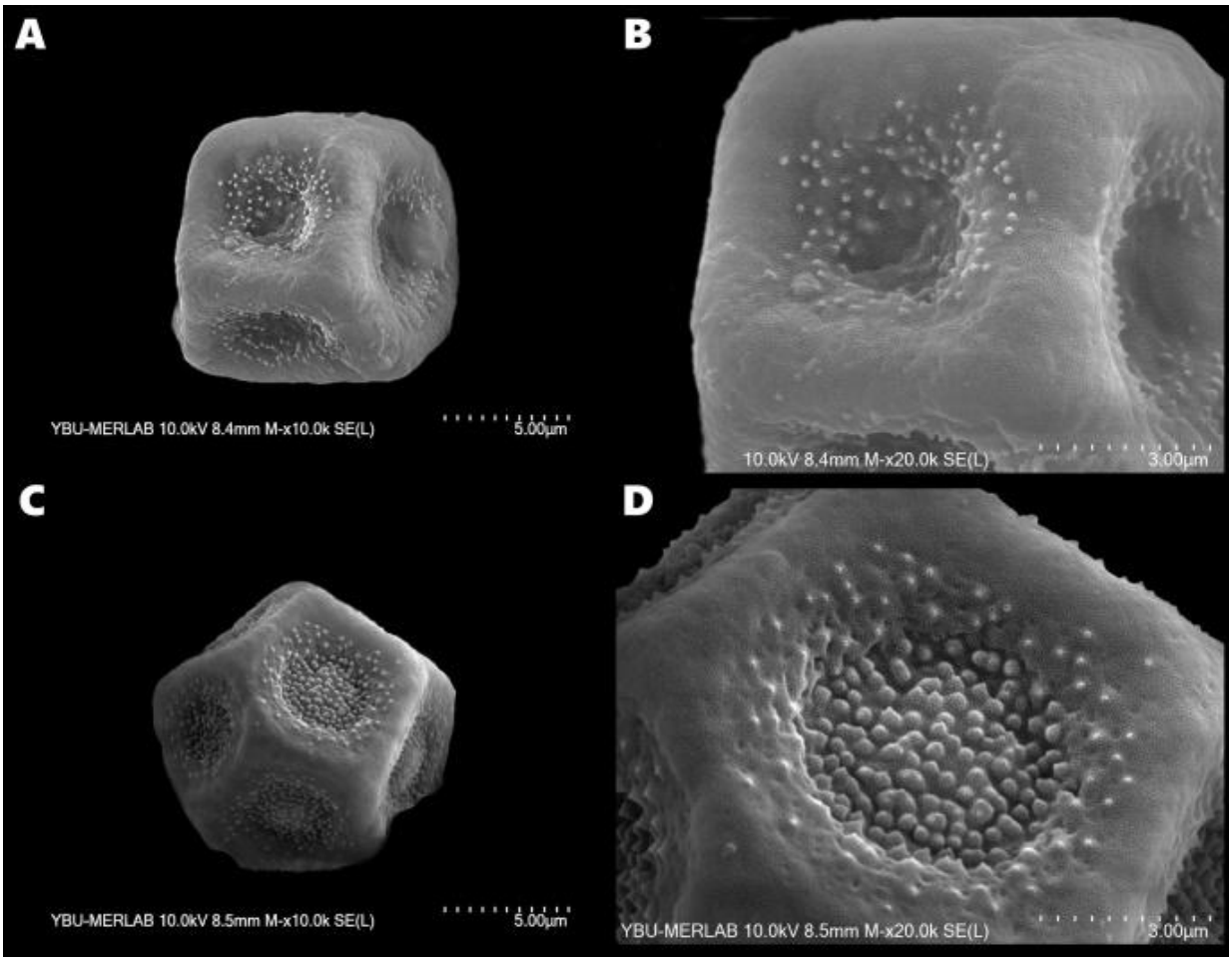

Figure 1. SEM micrographs of pollen grains. A−B: Equatorial view and exine sculpturing of *H. incana* C−D: Equatorial view and exine sculpturing of *H. pisidica*.

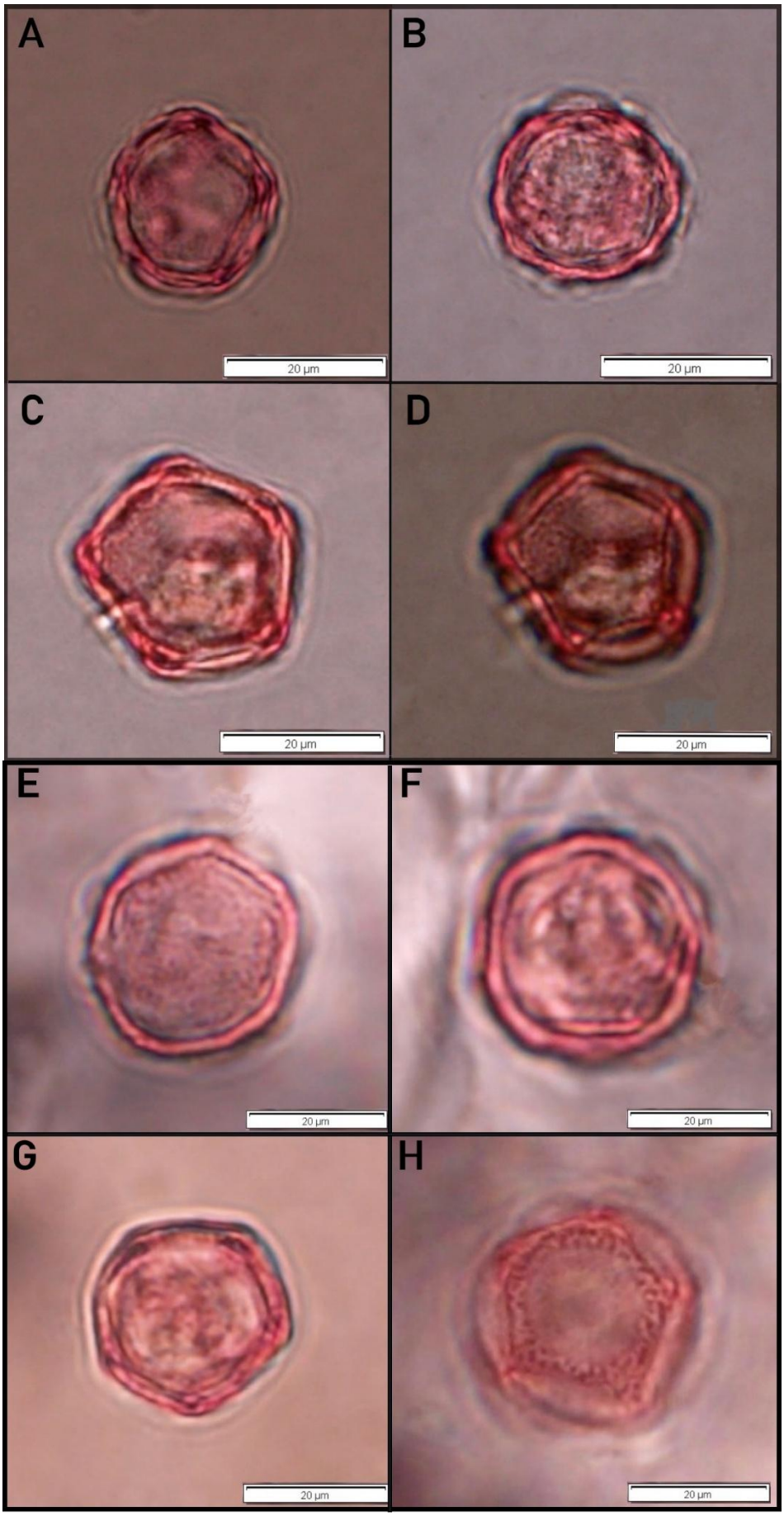

Figure 2. Light micrographs (LM) of the pollen grains. A−B: Equatorial view and ornamentation of *H. incana* C−D: Polar view and ornamentation of *H. incana*. E−F: Equatorial view and ornamentation of *H. pisidica* G−H: Polar view and ornamentation of *H. pisidica*.

Table 2. Comparative of the pollen characters *H. incana* and *H. pisidica.*

Pollen Characters Min - (M±SD) – Max [µm]	***H. incana***	***H. pisidica***
Pollen diameter (µm)	11.44 - (13.41 ± 1.68) - 16.22	13.34 - (14.21 ± 0.58) - 15.02
Polar axes (µm)	11.27 - (14.50 ± 1.45) - 16.51	11.16 - (14.14 ± 1.54) - 17.72
Equatorial axes (µm)	10.37 - (13.93 ± 1.34) - 16.96	11.49 - (13.75 ± 1.53) - 16.96
Pollen shape (µm)	Prolate spheroidal	Prolate spheroidal
Pollen ornamentation (µm)	Microechinate - Punctate	Microechinate - Punctate
Exine thickness (µm)	0.21 - (0.36 ± 0.12) - 0.63	0.74 - (0.86 ± 0.17) - 1.19
Intine thickness (µm)	0.26 - (0.36 ± 0.07) - 0.47	0.59 - (0.64 ± 0.09) - 0.89
Pore diameter (µm)	3.93 - (5.11 ± 1.78) - 9.25	4 - (6.44 ± 1.20) - 8.54
Number of pore (µm)	10 ± 3	12 ± 3
Distance between two pores (µm)	1.10 - (1.38 ± 0.23) - 2.03	0.76 - (1.36 ± 0.42) - 2.35
Operculum diameter (µm)	1.71 - (2.81 ± 1.22) - 5.68	2.38 - (3.78 ± 1.02) - 6.48
Number of spinule (10µm2)	20 - 25	>70 (intense)
Number of spinules on operculum (µm)	5 - 7	8 - 10
Number of punctum (10µm2)	20 - 25	40 - 45

Abbreviations: M: Average, SD: Standart deviation, Min: Minimum, Max: Maximum, µm: Micrometer

Table 3. Comparative anatomy of the stem *H. incana* and *H. pisidica.*

Stem Characters Min - (M±SD) – Max [µm]	***H. incana***	***H. pisidica***
Epidermis Length	7.83 - (15.62 ± 5.60) - 22.95	9.97 - (17.71 ± 3.20) - 24.52
Epidermis Width	9.33 - (19.58 ± 6.79) - 32.47	9.94 - (23.40 ± 6.3) - 41.95
Cortex Length	8.21 - (22.02 ± 7.91) - 35.83	9.73 - (19.12 ± 4.01) - 32.77
Cortex Width	14.18 - (25.59 ± 10.26) - 48.71	14.84 - (27.68 ± 6.14) - 49.55
Sclerenchyma Length	11.19 - (14.48 ± 1.76) - 17.91	17.48 - (25.02 ± 3.33) - 34.96
Sclerenchyma Width	5.59 - (8.13 ± 1.55) - 10.07	5.57 - (9.93 ± 2.42) - 15.84
Xylem Length	7.27 - (12.74 ± 2.80) - 17.54	9.51 - (18.44 ± 4.85) - 30.7
Xylem Width	5.59 - (10.69 ± 2.80) - 16.79	7.88 - (13 ± 2.19) - 18.28
Core Length	17.35 - (42.57 ± 20.01) - 81.18	9.01 - (20.22 ± 10.01) - 68.92
Core Width	6.71 - (21.31 ± 11.32) - 51.51	11.51 - (28.71 ± 16.06) - 103.9

Abbreviations: M: Average, SD: Standart deviation, Min: Minimum, Max: Maximum, µm: Micrometer

— ***H. pisidica*** has epidermis 1-layered, consisting of rectangular and oval cells and is surrounded by a cuticle layer. The cortex tissue, which is located under the epidermis, is composed 7−8 layered, oval or cylindrical parenchymatic cells with chloroplasts and cortex has druse crystals. The sclerenchyma tissue is composed from 3 layered. The pith is consist of circular shaped parenchymatic cells and has druse crystals. The stem has a collateral vascular bundle (Table 3, Fig. 3).

3.2.2. Leaf Anatomy

— *Herniaria incana* shows the lamina with upper and lower epidermis covered by a cuticle layer. Both epidermis are consist of uniseriate oval and rectangular cells. The mesophyll is consist of 2-3 layer of elongated palisade parenchyma cells. Spongy parenchyma cells are 7-8 layers. Mesophyll has druse crystals. The mesophyll type is bifacial (Table 4, Fig. 4).

— *Herniaria pisidica* shows the lamina with upper and lower epidermis covered by a cuticle layer. Both epidermis consist of uniseriate oval and rectangular cells. The mesophyll is consist of 2-3 layer of elongated palisade parenchyma cells. Spongy cells are 7-8 layers. Mesophyll has druse crystals. The mesophyll type is bifacial. (Table 4, Fig. 4).

4. Discussions

In this study, anatomical and palynological data of *H. incana* and *H. pisidica* were obtained. The anatomical and palynological findings for *H. pisidica* and *H. incana* are reported first time in this work. Data on pollen and anatomy studies are compared with other similar *Herniaria* taxa.

Compared to palynological study; exine and intine thickness of *H. pisidica* are thicker than *H. incana*. The number of spinules is very intense in *H. pisidica*. Two taxa were compared the number of punctum; *H. pisidica* is approximately twice as much in *H. incana*. Compared to anatomical studies; *H. incana* leaf characters are smaller than compared to *H. pisidica*.

Metcalfe (1946), the leaf mesophyll layer in *Herniaria* is chlorenchymatous and palisade tissue is developed. Calcium oxalate crystals (druse) generally form large and conspicuous masses in the mesophyll. These findings are similar to our study.

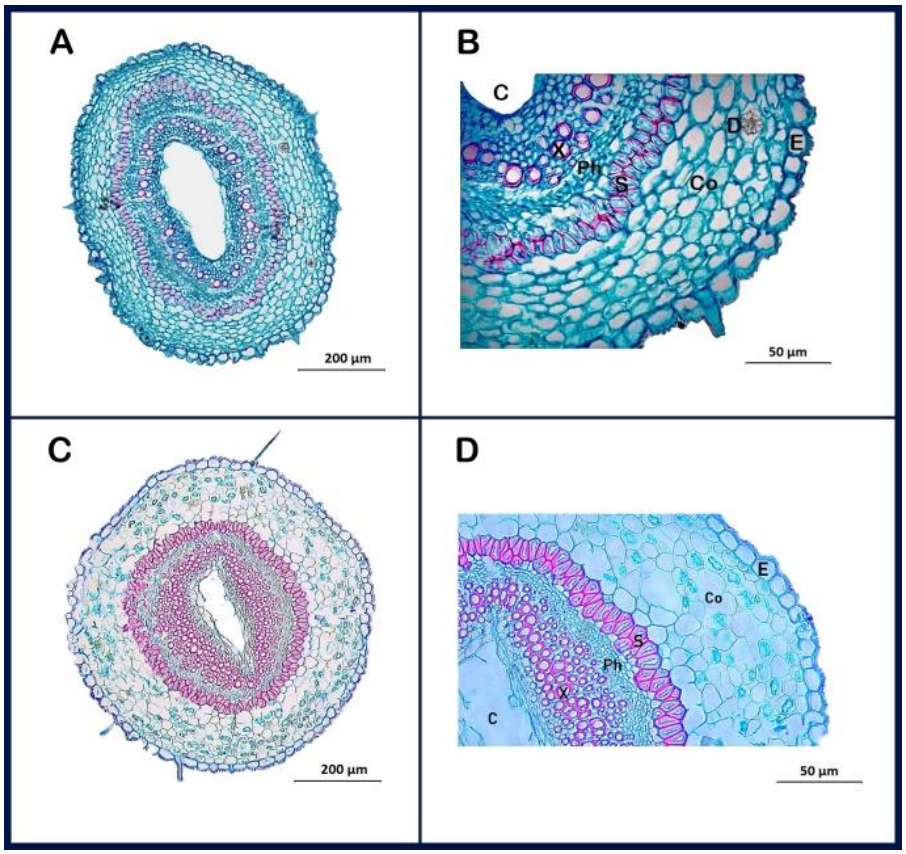

Figure 3. Cross-section of the stem. A−B: General and detailed view of *H. incana*, C−D: General and detailed view of *H. pisidica.* E: Epidermis, Co: Cortex, S: Sclerenchyma, Ph: Phloem, X: Xylem, C: Core, D: Druse crystal.

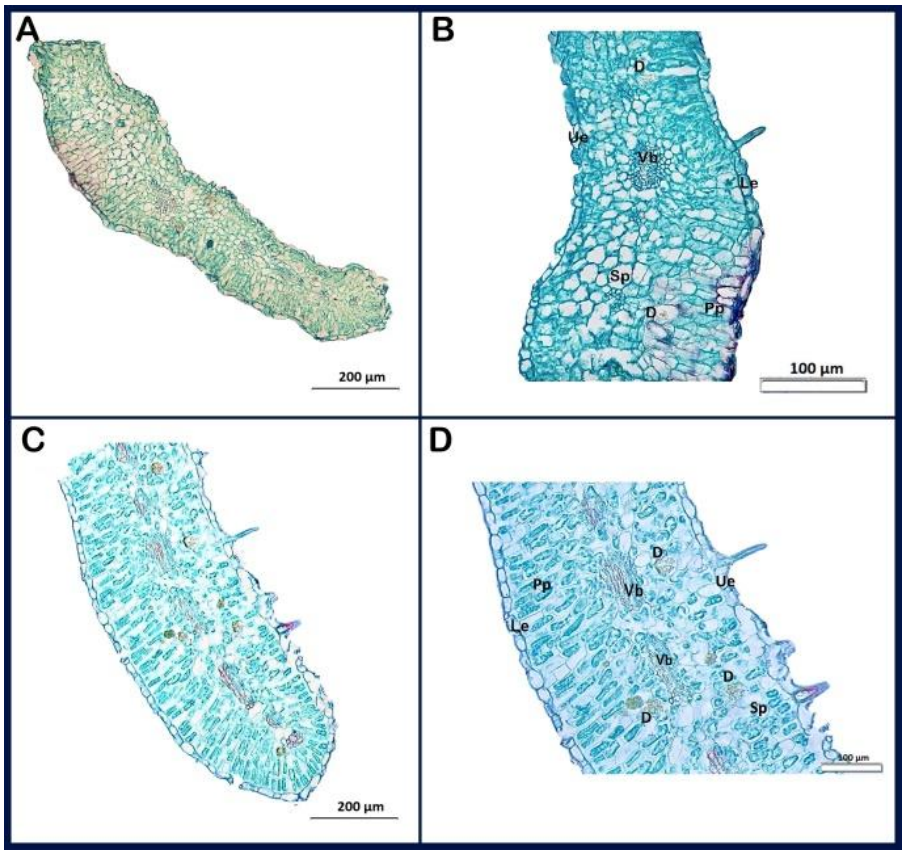

Figure 4. Cross-section of the lamina. A−B: General and detailed view of *H. incana*, C−D: General and detailed view of *H. pisidica.* Ue: Upper Epidermis, Pp: Palisade parenchyma Sp: Spongy parenchyma, Vb: Vascular bundle, D: Druse crystal, Le: Lower Epidermis.

Table 4. Comparative anatomy of the leaves *H. incana* and *H. pisidica.*

Leaf Characters Min - (M±SD) – Max [µm]	***H. incana***	***H. pisidica***
Upper Epidermis Length	4.79 - (6.60 ± 1.29) - 10.87	9.9 - (16.45 ± 4.81) - 34
Upper Epidermis Width	5.37 - (12.75 ± 4.62) - 23.67	18.03 - (27.99 ± 8.02) - 47.05
Lower Epidermis Length	4.47 - (6.35 ± 1.06) - 8.58	11.01 - (16 ± 3.41) - 25.9
Lower Epidermis Width	4.85 - (11.82 ± 3.43) - 22.76	14.02 - (26.04 ± 6.71) - 41.03
Palisade Parenchyma Length	11.19 - (17.95 ± 3.88) - 25.91	28.2 - (44.83 ± 8.34) - 66.19
Palisade Parenchyma Width	3.91 - (5.71 ± 1.48) - 9.70	9.04 - (13.7 ± 2.27) - 19.05
Spongy Parenchyma Length	6.34 - (12.11 ± 3.55) - 22.71	17.96 - (26.12 ± 5.53) - 40.9
Upper Epidermis Length	4.79 - (11.27 ± 2.60) - 16.79	20.51 - (26.19 ± 5.3) - 38

Abbreviations: M: Average, SD: Standart deviation, Min: Minimum, Max: Maximum, µm: Micrometer

Van et al. (1968), for *Herniaria* genus, the leaf is positioned in the center. Stomata is located on both surfaced of the leaf. The mesophyll is chlorenchymatous. Palisade texture is abundant and developed. Calcium oxalate crystals are usually located in the mesophyll and form large, conspicuous masses (or compound crystals). In our study, it was determined that the mesophyll layer contains intense chlorophyll and the palisade parenchyma appears in 2–3 layers, the spongy parenchyma has an average of 7-8 layers and it covers a lot of surface area, and the druse crystals are located in masses spread over the mesophyll. Our results are generally consistent this study results (apart from mesophyll layer).

Zarinkamar (2001), who examined anatomically two *Herniaria* taxa from Iran (*H. hirsuta* and *H. incana*), in terms of leaf anatomy. In these types reported: thick, granular waxy layer deposits on the epidermal cell walls and the cuticle rough on the outer surface, higher stomatal frequency, collenchyma and more mechanical sclerenchymatous tissue on the leaf edges, salt deposits in the form of large crystals around the vascular bundle in the middle of the palisade parenchyma have been reported in the literature. It has been emphasized that these characters originate from the resistance to xerophyte status in the Caryophyllaceae family. In addition, it has been reported in the literature that the variability of anatomical structures even among species of the same genus is related to the microclimatic conditions of the region where the species lives. In our study, it is observed that the epidermal cell walls are thick due to the xerophytic structure, the vascular bundle, including the sclerenchymatic tissue more mechanical in all taxa and the sclerenchymatous tissue covers a large area. Druse crystals appear as large and small structures spread throughout the mesophyll layer. The stomata has become smaller and located just under the upper and lower epidermis, and the average presence of one stoma each supports the xemorphic structure. In general, the study results are consistent with our research.

Schweingruber (2007), were examined on four *Herniaria* taxa (*H. glabra, H. incana, H. hirsuta, H. alpina*). He found the presence of thick-walled vessels (2 µm), widespread parenchyma, absence of radial symmetry, sclereids and druse crystals in the cortex were noted. In our study, the presence of widespread parenchyma and the absence of radial symmetry were detected in our study. Calcium oxalate crystals are located in the cortex for all taxa. The results are generally consistent with our research.

When compared all these studies with our work; *H. pisidica* has druse crystals both in the cortex and the core were observed. For *H. incana* druse crystals were located in cortex only.

Mousa and Al-Jibouri (2019) studied the stem anatomy of *H. hirsuta* distributed in Iraq and reported that measurements (average) of stem anatomy are: epidermis thickness 16 µm, cortex parenchyma thickness 62 µm, xylem thickness 103 µm, thickness in pith cells 288 µm. According to these results, our measurements in *Herniaria* taxa are much smaller than the study by Mousa and Al-Jibouri (2019) except for epidermis.

H. hirsuta pollen grains are pantoporate (Nowicke and Skvarla 1976; Hutchinson, 1959). These studies support the idea that the genus *Herniaria* (previously included Illecebraceae family) differs in terms of pollen grains. The genus *Herniaria* is separated from Illecebraceae family both morphologically and palynologically.

Punt and Hoen (1999) reported that 4–6 porate (rarely more) pollen grains of *H. glabra*. For *H. cinerea*, pollen shape is spheroidal, and pollen grains are 6-12 porate, exine ornamentation is scabrate-spinulose. Pollen diameter is 20.11 ± 0.09 µm, and equatorial axis 19.69 ± 0.28 µm, exine thickness 1.72 ± 0.1 µm (Perveen and Qaiser, 2003). The results of these study are not consistent with our study consequence.

When our study results was compared with the literature, we found that inconsistent findings for anatomy and pollen on *Herniaria*. This situation leads to confusion on *Herniaria*. This information pollution can be eliminated. For this purpose, the next study aims to carry out micromorphology studies on all taxa of *Herniaria*. As mentioned above, anatomical and palynological differences are very important for taxonomic descriptions. Consequently, it is predicted that the information acquired with this work will contribute to the literature.

Conflict of Interest

Authors have declared no conflict of interest.

Authors' Contribution

Y.C: Literature research, data collection, organize the execution of the study, writing the article and carrying out experimental studies, data entry and measurement. M.K: Provision of the plant material, provide a working environment and tools, create an idea.

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References

Bittrich V (1993). Caryophyllaceae. Flowering Plants· Dicotyledons: Magnoliid, Hamamelid and Caryophyllid Families. Berlin: Springer.

Davis PH (1967). Flora of Turkey and the East Aegean Islands, vol. VII. Edinburgh: Edinburgh University Press.

Erdtman G (1969). Handbook of palynology: morphology, taxonomy, ecology: an introduction to the study of pollen grains and spores. Copenhagen: København Munksgaard.

Gangulee HC, Das KS, Datta C (1972). College Botany. India: New Central Book Agency.

Güner A, Aslan S, Ekim T, Vural M. Babaç MT (2012). Türkiye Damarlı Bitkiler Listesi, İstanbul: Nezahat Gökyiğit Botanik Bahçesi ve Flora Araştırmaları Derneği Yayınları.

Hermann F (1937). Ubersicht liber die *Herniaria*- Arten des Berliner Herbars. Fedde's Repertorum 42: 203-224.

Hernández-Ledesma P, Berendsohn WG, Borsch T, Von Mering S, Akhani H, Arias S, Uotila P (2015). A taxonomic backbone for the global synthesis of species diversity in the angiosperm order *Caryophyllales*. Willdenowia 45(3): 281-383.

Heywood VH (1998). Flowering plants of the world, 3rd Edition. Oxford: Oxford University Press.

Hutchinson J (1959). The families of flowering plants. Vol. I. Dicotyledons. Clarendon: Oxford University Press.

Johansen DA (1940). Plant microtechique. London: McGraw-Hill Book Company.

Lawrence GHM (2017). Taxonomy of vascular plants. New York: McMillan Publ.

Metcalfe CR (1946). The systematic anatomy of the vegetative organs of the Angiosperms. Biological Reviews 21(4): 159-172.

Mousa MO, Al-Jibouri, KDW (2019). Anatomical study of stems for some species from *Caryophyllaceae* family in Iraq. Plant Archives 19(Supplement 1): 1433-1440.

Nowicke JW, Skvarla JJ (1976). Pollen morphology and the realtionship of the *Plumbaginaceae, Polygonaceae,* and *Primulaceae* to the order *Centrospermae*. Smithsonian Contributions to Botany. Washington: Smithsonian Institution Press.

Perveen A, Qaiser M (2003). Pollen Flora of Pakistan-XII. *Illecebraceae*. Pakistan Journal of Botany 35(2): 141-144.

POWO (2024). Plants of the World Online. Facilitated by the Royal Botanic Gardens, Kew Website: https://powo.science.kew.org/cite-us / [accessed 24 November 2024].

Punt W, Hoen P (1999). *Caryophyllaceae* in the Northwest European Pollen Flora, Vol. 7. Utrech: Utrech Univ.

Punt W, Hoen PP, Blackmore S, Nilsson S, Thomas A Le (2007). Review of Palaeobotany and Palynology143 (1-2): 1-81.

Schweingruber FH (2007). Stem anatomy of *Caryophyllaceae.* Flora 202:(4): 281-292.

Van T, Te Utrecht ADR, De Vooys DRAC, Gujarkhan TA (1968). Revision of the Paronychiinae. Mededelingen van het Botanisch Museum en Herbarium van de Rijksuniversiteit te Utrecht 285(1): 3-440.

Williams FN (1896). A systematic revision of the genus *Herniaria*, in Bulletin de l'Herbier Boissier 4: 556-570.

Wodehouse RR (1935). Pollen grains. New York: McGrawHill.

Zarinkamar F (2001). Foliar anatomy of the *Caryophyllaceae* family in Arasbaran, NW Iran. Iranian Journal of Botany 9:(1): 93-102.

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Karyotype analysis of *Paronychia dudleyi* and *Paronychia pontica*

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Paronychia dudleyi ve *Paronychia pontica*'nın karyotip analizi

Abstract: The polyphyletic genus *Paronychia* includes about 110 species that are distributed throughout the world except in the southern of Africa and Asia. Türkiye is one of the most important distribution centers of genus *Paronychia* represented by 29 species. The basic and diploid chromosome numbers were recorded in 22 species (26 taxa) of Turkish *Paronychia*. The aim of this study is to report for the first time the karyological data of *P. dudleyi* and *P. pontica*. The chromosome number and karyotype formula were $2n = 4x = 36$m in *P. dudleyi* and $2n = 2x = 18$m in *P. pontica*. The karyotype asymmetry values (especially intrachromosomal) were quite low. As a result, the new karyological data were recorded: (i) first report of the basic and diploid chromosome numbers, (ii) ploidy levels of 2*x* and 4*x*, (iii) first detailed chromosomal data, (iv) the most symmetrical karyotypes.

Key words: *Paronychia*, chromosome, karyotype asymmetry, polyploidy

Özet: Polifiletik *Paronychia* cinsi, Afrika Asya'nın güneyi dışında dünyanın her yerine dağılmış yaklaşık 110 türü içerir. Türkiye, 29 türle temsil edilen *Paronychia* cinsinin en önemli dağılım merkezlerinden biridir. Türk *Paronychia*'ların 22 türünde (26 takson) temel ve diploid kromozom sayıları kaydedilmiştir. Bu çalışmanın amacı, *P. dudleyi* ve *P. pontica*'nın karyolojik verilerini ilk kez bildirmektir. Kromozom sayısı ve karyotip formülü *P. dudleyi*'de $2n = 4x = 36$m, *P. pontica*'da $2n = 2x = 18$m'dir. Karyotip asimetri değerleri (özellikle intrakromozomal) oldukça düşük bulunmuştur. Sonuç olarak yeni karyolojik veriler kaydedildi: (i) temel ve diploid kromozom sayılarının ilk raporu, (ii) 2*x* ve 4*x* ploidi seviyeleri, (iii) ilk detaylı kromozomal veriler, (iv) en simetrik karyotipler.

Anahtar Kelimeler: *Paronychia*, kromozom, karyotip asimetrisi, poliploidi

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1. Introduction

The polyphyletic genus *Paronychia* Miller includes about 110 species that are distributed throughout the world except in the southern parts of Africa and Asia. Türkiye is one of the most important distribution centers of genus *Paronychia* represented by 29 species (Bittrich, 1993; Eroğlu et al., 2021). From the species the subject of the study, *P. dudleyi* Chaudhri; perennial, stem branched at the base (diameter 1-3 mm), decumbent to ascending, 2-7(-15) cm, white flowers 12-50. It grows on forest open areas, calcareous plains, and rocky-stony in 710-2230 m. *P. pontica* (Borhidi) Chaudhri; perennial, stem branched at the base (diameter 2-4 mm), decumbent to ascending, 2-7 cm, white flowers 4-8. It grows on forest open areas and stony in 1000-1040 m (Figure 1) (Chaudhri, 1967, 1968; Bittrich, 1993).

The basic and diploid chromosome numbers were recorded in 22 species (26 taxa) of Turkish *Paronychia*. Although the genus showed variations in the basic chromosome number such as $x = 5, 7, 9$, and 13; the basic number was $x = 9$ in the majority of Turkish *Paronychia*. There were very few taxa for which polyploidy had not been reported, namely *P. anatolica* Czecz. subsp *anatolica*, *P. kurdica* Boiss. subsp. *hausknechtii* Chaudhri, *P. kurdica* Boiss. subsp. *montis-munzur* Chaudhri, and *P. macrosepala* Boiss. ($2n = 2x = 18$). However, twenty-three taxa showed polyploidy variations such as tetraploidy ($2n = 4x = 28$, 36, and 52), hexaploidy ($2n = 6x = 54$), and octoploidy ($2n = 8x = 56$, 72, and 104). *P. echinulata* Chater ($x = 5$, 7 and $2n = 10$, 14, 28), *P. kapela* (Hacq.) Kerner ($x = 9$ and $2n = 18$, 36), and *P. polygonifolia* (Vill.) DC. ($x = 7$ and $2n = 14$, 56) were diploid and polyploid. *P. argentea* Lam., *P. chionaea* Boiss. subsp. *chionaea*, *P. chionaea* Boiss. subsp. *kemaliya* Chaudhri, and *P. polygonifolia* were taxa showing high ploidy level (8*x*). (Lorenzo Andreu and García Sanz, 1950; Blackburn and Morton, 1957; Fedorov, 1974; Löve, 1975; Diosdado and Pastor, 1994; Runemark, 1996; Eroğlu et al., 2017; Eroğlu et al., 2020; Eroğlu and Budak, 2020; Eroğlu et al., 2021).

The aim of this study is to report for the first time the karyological data listed below in *P. dudleyi* and *P. pontica*: (i) chromosome number (basic and diploid), (ii) ploidy levels, (iii) detailed chromosomal measurements, (iv) karyotype formula, and (v) karyotype asymmetry (inter- and intrachromosomal).

2. Materials and Method

2.1. Collection Information

Paronychia dudleyi: Türkiye, Ankara, Polatlı, above Sazılar village, 39˚41˙27"N 31˚56˙14"E, 710 m, 08 vii 2014, Budak 3130 & Hamzaoğlu (Bozok Hb.).

Paronychia pontica: Türkiye, Eskişehir, between Eskişehir and Söğüt, near Oluklu village, 39˚53˙28"N 30˚14˙28"E,

Figure 1. *Paronychia dudleyi* (A) and *Paronychia pontica* (B)

1020 m, 08 vii 2014, Budak 3137 & Hamzaoğlu (Bozok Hb.).

2.2. Cytogenetic Procedure

The cytogenetic procedure consists of the following steps. (i) Germination: the process was carried out by leaving the seeds between moist drying papers at room temperature. (ii) Pretreatment: the process was carried out by leaving the germinated seeds in a solution of α-monobromonaphthalene for 16 h at 4ºC. (iii) Fixation: the process was carried out by leaving the material in fixative solution (ethanol:acetic acid, 3:1) for 16 h at 4ºC. (iv) Hydrolysis: the process was carried out by leaving the material in 1 N HCl solution for 12 min at 60ºC. (v) Staining: the process was carried out by stained the material in aceto-orcein (2%). (vi) Preparation: the process was carried out by squashed the material in acetic acid (45%) (Martin et al., 2022).

2.3. Karyological Calculations

The karyological calculations were performed using 10 metaphase plates by clearly spread chromosomes (Figure 2). The short arm (SA) and long arm lengths (LA) of the chromosomes were measured with KaryoType software. Then the total length (TL) = [LA + SA], total and mean haploid length (THL and MHL), arm ratio (AR) = [LA/SA], centromeric index (CI) = [SA/(LA + SA) × 100], and relative length (RL) = [(LA + SA)/THL × 100] were determined. The karyotype formulae and asymmetry index values were detected as described by Levan et al. (1964), Paszko (2006), and Peruzzi and Eroğlu (2013).

3. Results

The chromosomal data of *Paronychia dudleyi* and *P. pontica* were herein recorded for the first time. The chromosome number and karyotype formula were $2n = 4x = 36$m in *P. dudleyi* and $2n = 2x = 18$m in *P. pontica*. The karyotype asymmetry values were quite low (Table 1).

The monoploid ideograms and detailed chromosomal data of *Paronychia dudleyi* and *P. pontica* were represented in Figure 3 and Table 2. In *P. dudleyi*, total chromosome length, relative length, and centromeric index ranged from 1.06-1.99, 4.00-7.50, and 44.09-50.00, respectively. In *P. pontica*, total chromosome length, relative length, and centromeric index ranged from 1.40-1.68, 9.96-11.95, and 46.10-48.57, respectively.

4. Discussions

Different diploid numbers as $2n = 18$ and 36 were detected in studied species. In Turkish *Paronychia*, the chromosome number variations were reported such as $2n = 10$, 14, 18, 28, 36, 52, 54, 56, 72, and 104; however, the most dominant was $2n = 36$ (Lorenzo Andreu and García Sanz, 1950;

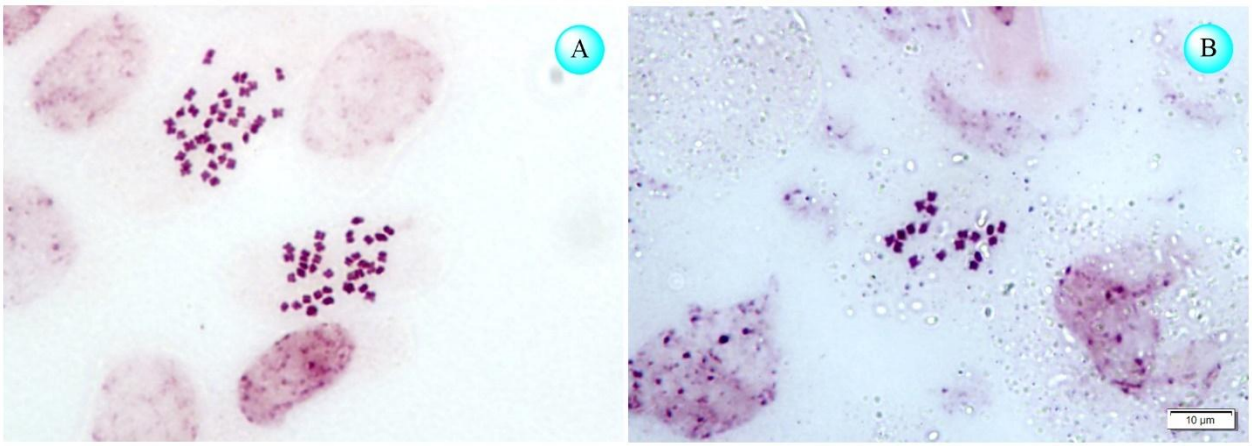

Figure 2. Metaphase chromosomes of *Paronychia dudleyi* (A) and *Paronychia pontica* (B). Scale bar 10 µm

Table 1. The karyological parameters of *Paronychia dudleyi* and *Paronychia pontica*

Karyological parameters	*P.dudleyi*	*P.pontica*
x (basic chromosome number)	9	9
2*n* (diploid chromosome number)	36	18
Karyotype formula	36m	18m
THL (total haploid length, µm)	26.53	14.06
MHL (mean haploid length, µm)	1.47	1.56
A1 (intrachromosomal asymmetry index)	0.10	0.10
A2 (interchromosomal asymmetry index)	0.16	0.06
CV_{CL} (relative variation in chromosome length)	16.46	5.91
CV_{CI} (relative variation in centromeric index)	4.08	1.83
AI (karyotype asymmetry index)	0.67	0.11
M_{CA} (mean centromeric asymmetry)	5.63	5.38

Blackburn and Morton, 1957; Fedorov, 1974; Löve, 1975; Diosdado and Pastor, 1994; Runemark, 1996; Eroğlu et al., 2017; Eroğlu et al., 2020; Eroğlu and Budak, 2020; Eroğlu et al., 2021). After the present results, the chromosome number of five Turkish *Paronychia* are still unknown, which are *P. boissieri* Rouy, *P. euphratica* (Chaudhri) Chaudhri, *P. kocii* Budak, *P. mughlaei* Chaudhri, and *P. sintenisii* Chaudhri.

The basic number was *x* = 9 in *Paronychia dudleyi* with ploidy level 4*x* and *P. pontica* with ploidy level 2*x* such as most Turkish *Paronychia* (Eroğlu et al., 2020). However, *x* = 8 was dominated in some regions such as Macaronesia, Almeria, and Granada (Diosdado and Pastor, 1994; Suda et al., 2003). In addition, the ploidy variations such as 2*x*, 4*x* (the most dominant), 6*x*, and 8*x* were reported in the genus *Paronychia* (Eroğlu et al., 2020).

All chromosomes of studied species were metacentric and the karyotype formulae were 18m and 36m. However, it was reported the submetacentric and subtelocentric chromosomes in the genus. (Diosdado and Pastor, 1994; Eroğlu et al., 2020).

In karyotype asymmetry, the values of A1 and M_{CA} refer the intrachromosomal asymmetry. Besides the values of A2 and CV_{CL} refer the interchromosomal asymmetry. The values of A1, A2, CV_{CL}, and M_{CA} were 0.10, 0.16, 16.46, and 5.63 in *Paronychia dudleyi* and 0.10, 0.06, 5.91, and 5.38 in *P. pontica*. These values referred to quite symmetric karyotypes. The only one species with lower M_{CA} value than studied species was *P. kurdica* subsp. *montis-munzur* (Eroğlu et al., 2020). The A1 value of *P. anatolica* and *P. amani* was higher than *P. pontica* and *P. dudleyi* (Eroğlu and Budak, 2020; Eroğlu et al., 2021). In intrachromosomal asymmetry, *P. pontica* and *P. dudleyi* had the most symmetric karyotypes, respectively. CV_{CL} value varied between 10.00 and 20.00 in most of the Turkish *Paronychia*. In addition, The A2 value of *P. anatolica* and *P. amani* was higher than *P. pontica* and *P. dudleyi* (Eroğlu et al., 2017; Eroğlu et al., 2020; Eroğlu and Budak, 2020; Eroğlu et al., 2021). The CV_{CL} value of *P. dudleyi* was also in this range, however it was quite lower in *P. pontica*, which had the most symmetrical karyotype in interchromosomal asymmetry. Due to low intra- and interchromosomal asymmetry, CV_{CI} and AI values were also low in *P. dudleyi* and *P. pontica*.

In the present study, the new karyological data were recorded listed below in *Paronychia dudleyi* and *P. pontica*: (i) first report of the basic and diploid chromosome number, (ii) ploidy levels of 2*x* and 4*x*, (iii) first detailed chromosomal data, (iv) the most symmetrical karyotypes.

Conflict of Interest

Authors have declared no conflict of interest.

Authors' Contributions

All authors contributed to the study's conception and design. All authors read and approved the final manuscript.

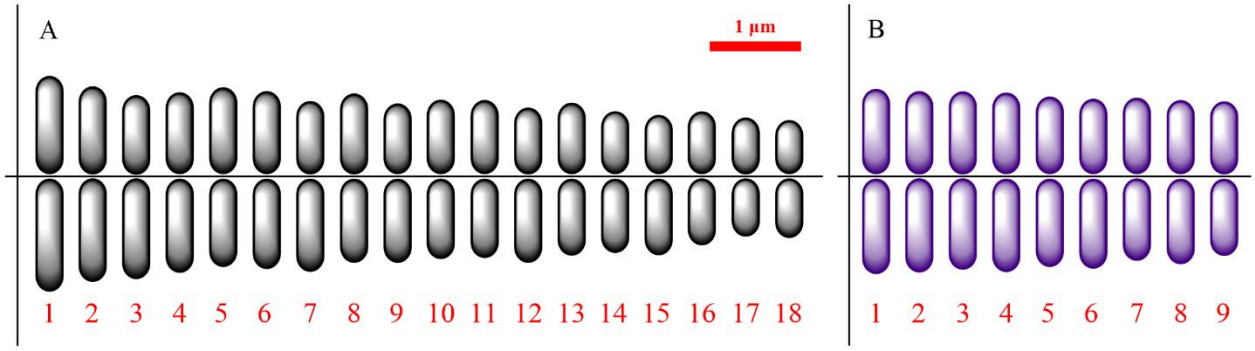

Figure 3. Monoploid ideogram of *Paronychia dudleyi* (A) and *Paronychia pontica* (B). Scale bar 1 µm

Table 2. The detailed chromosomal data of *Paronychia dudleyi* and *Paronychia pontica*

	CP	TL (µm)	LA (µm)	SA (µm)	AR	RL (%)	CI (%)	CT
Paronychia dudleyi	1	1.99	1.06	0.93	1.14	7.50	46.73	metacentric
	2	1.79	0.96	0.83	1.16	6.75	46.37	metacentric
	3	1.69	0.94	0.75	1.25	6.37	44.38	metacentric
	4	1.65	0.88	0.77	1.14	6.22	46.67	metacentric
	5	1.64	0.82	0.82	1.00	6.18	50.00	metacentric
	6	1.62	0.85	0.78	1.09	6.14	47.85	metacentric
	7	1.56	0.87	0.69	1.26	5.88	44.23	metacentric
	8	1.54	0.78	0.76	1.03	5.80	49.35	metacentric
	9	1.45	0.78	0.67	1.16	5.47	46.21	metacentric
	10	1.45	0.75	0.70	1.07	5.47	48.28	metacentric
	11	1.44	0.74	0.70	1.06	5.43	48.61	metacentric
	12	1.41	0.78	0.63	1.24	5.31	44.68	metacentric
	13	1.39	0.71	0.68	1.04	5.24	48.92	metacentric
	14	1.30	0.69	0.60	1.15	4.86	46.51	metacentric
	15	1.28	0.71	0.56	1.27	4.79	44.09	metacentric
	16	1.21	0.62	0.59	1.05	4.56	48.76	metacentric
	17	1.07	0.54	0.53	1.02	4.03	49.53	metacentric
	18	1.06	0.55	0.51	1.08	4.00	48.11	metacentric
Paronychia pontica	1	1.68	0.88	0.80	1.10	11.95	47.62	metacentric
	2	1.65	0.87	0.78	1.12	11.74	47.27	metacentric
	3	1.63	0.85	0.78	1.09	11.59	47.85	metacentric
	4	1.63	0.87	0.76	1.14	11.59	46.63	metacentric
	5	1.55	0.82	0.73	1.12	11.02	47.10	metacentric
	6	1.54	0.83	0.71	1.17	10.95	46.10	metacentric
	7	1.49	0.77	0.72	1.07	10.60	48.32	metacentric
	8	1.49	0.80	0.69	1.16	10.60	46.31	metacentric
	9	1.40	0.72	0.68	1.06	9.96	48.57	metacentric

Abbreviations: chromosome pairs (CP), total length (TL), long arm (LA), short arm (SA), arm ratio (AR), relative length (RL), centromeric index (CI), chromosome type (CT

References

Bittrich V (1993). *Caryophyllaceae*. In: Kubitzki K, Rohwer J, Bittrich V (eds.). The families and genera of vascular plants, Magnoliid, Hamamelid, and Caryophyllid Families, Vol. 2. Berlin: Springer, pp. 206-236.

Blackburn KB, Morton JK (1957). The incidence of polyploidy in the *Caryophyllaceae* of Britain and of Portugal. New Phytologist 56: 344-351.

Chaudhri MN (1967). *Paronychia* Mill. In: Davis PH (ed.). Flora of Turkey and the East Aegean Islands, Vol. 2. Edinburgh: Edinburgh University Press, pp. 250-262.

Chaudhri MN (1968). A revision of the *Paronychinae*. Utrecht: Tilburg, H. Gianotten.

Diosdado JC, Pastor J (1994). Estudio cariologico del gĕnero *Paronychia* Miller (*Caryophyllaceae*) en Andalucĭa (Espana). Acta Botanica Malacitana 19: 89-95.

Eroğlu HE, Doğan H, Altay D, Budak Ü (2017). The medicinal plant of genus *Paronychia* and the karyotype analysis of *Paronychia adalia*. International Journal of Secondary Metabolite 4(3): 103-107. https://doi.org/10.21448/ijsm.363827

Eroğlu HE, Altay D, Budak Ü, Martin E (2020). Karyotypic phylogeny and polyploidy variations of *Paronychia* (*Caryophyllaceae*) taxa in Turkey. Turkish Journal of Botany 44(3): 245-254. https://doi.org/10.3906/bot-1911-16

Eroğlu HE, Budak Ü (2020). Karyotype analysis of *Paronychia anatolica* subsp. *anatolica* (*Caryophyllaceae, Paronychioideae*). Iğdır Üniversitesi Fen Bilimleri Enstitüsü Dergisi 10(4): 2424-2430. https://doi.org/10.21597/jist.748944

Eroğlu HE, Altay D, Budak Ü, Martin E (2021). Karyotype analysis of two varieties of *Paronychia amani* (*Caryophyllaceae, Paronychioideae*). Iğdır Üniversitesi Fen Bilimleri Enstitüsü Dergisi 11(3): 1809-1816.

Fedorov AA (1974). Chromosome numbers of flowering plants. Leningrad: Komarov Botanical Institute.

Levan AK, Fredga K, Sandberg AA (1964). Nomenclature for centromeric position on chromosomes. Hereditas 52: 201-220.

Lorenzo Andreu A, García Sanz MP (1950). Cromosomas somáticos de plantas espontáneas en la estepa de Aragón. II. Anales de la Estación Experimental de Aula Dei 2: 12-63.

Löve Á (1975). IOPB Chromosome Number Reports XLIX. Taxon 24: 501-516.

Martin E, Eroğlu HE, Hamzaoğlu E, Koç M, Kelkitoğlu A, Öztürk E, Tanhaş E, Maşa M, Bozkurt H, Yavaş FN (2022). New chromosomal data of the genus *Dianthus* section *Dentati* (Caryophyllaceae, Sileneae). Anatolian Journal of Botany 6(1): 34-38. https://doi.org/10.30616/ajb.1062628

Paszko B (2006). A critical review and a new proposal of karyotype asymmetry indices. Plant Systematics and Evolution 258: 39-48. https://doi.org/10.1007/s00606-005-0389-2

Peruzzi L, Eroğlu HE (2013). Karyotype asymmetry: again, how to measure and what to measure? Comparative Cytogenetics 7: 1-9.

Runemark H (1996). Mediterranean chromosome number reports 6 (590-678). Flora Mediterranea 6: 223-243.

Suda J, Kyncl T, Freiova R (2003). Nuclear DNA amounts in Macaronesian angiosperms. Annals of Botany 92: 153-164.

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Pollen, nutlet and trichome micro-morphology of *Satureja* from flora of Iran, and their systematic implications

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İran florasından bazı *Satureja* türlerinin polen, nutlet ve trikom mikro-morfolojileri ve sistematik açıdan önemi

Abstract: Pollen, nutlet, and trichome micro-morphology of 12 species of *Satureja* (*Lamiaceae*) from the flora of Iran were examined by light and scanning electron microscopy (SEM). Our investigations indicated that these species have hexacolpate pollen with a prolate, (exceptionally sub-prolate shape) and microreticulate ornamentation. In most species the periclinal walls of the surface cells of the nutlets are wrinkled, and as a secondary sculpturing, the surface exhibits both spherical-ovoid pits and a tuberculate pattern on one seed, or a regular papillae-tuberculate pattern. In a few species, the nutlets display an undulate-reticulate pattern. *S. isophylla* and *S. khuzistanica* differ from the other studied species by the size of nutlets and *S. bachtiarica*, *S. hortensis*, and *S. kermanica* are distinguished by their nutlet sculpturing. *Satureja* species possess non-glandular trichomes together with two types of glandular trichomes, classified as sub-sessile (peltate) and capitate. Notably, the large distinctive capitate trichomes consisting of a multiseriate stalk with an enlarged, rounded multicellular head as well as the extremely long trichomes that are in two subtypes: acicular trichomes and trichomes with ridges and marked internodes are uniquely exhibits in *S. khuzistanica*. The characteristics of trichomes and nutlet were constant among different populations of *Satureja* species. The functions of both glandular and non-glandular trichomes have also been discussed. We indicated that while pollen characteristics among *Satureja* species show consistency, our findings of the micromorphological characteristics of trichomes and nutlets could provide diagnostic characters at species level and may enhance our understanding of the relationships among these species in future phylogenetic studies.

Key words: *Satureja*, Menthinae, nutlet, trichome, pollen

Özet: İran florasından 12 *Satureja* (*Lamiaceae*) türünün polen, nutlet ve trikom mikro-morfolojisi ışık mikroskobu ve taramalı elektron mikroskobu (SEM) ile incelenmiştir. Araştırmalarımız, türlerin hekzakolpat polene ve prolat (nadiren sub-prolat) şekle sahip olduğunu ve mikro-retikulat ornamentasyona sahip olduğunu göstermiştir. Çoğu türde, nutlet yüzey hücrelerinin periklinal duvarları buruşuk olup, ikincil yüzey desenlemeleri olarak hem küresel-oval çukurlar hem de tuberkulat desenler görülür ya da düzenli papillalı-tüberkül desen bulunmaktadır. Az sayıda türde, nutlerde undulat-retikulat desen görülmektedir. *S. isophylla* ve *S. khuzistanica*, nutlet boyutları açısından diğer incelenen türlerden farklıdır. *S. bachtiarica, S. hortensis* ve *S. kermanica* ise nutlet yüzey desenlemeleri bakımından diğerlerinden ayrılmaktadır. *Satureja* türleri, non-glandular trikomların yanı sıra sub-sessil (peltat) ve kapitat olmak üzere iki tür glandular trikom taşımaktadır. Ancak, çok hücreli yuvarlak bir başa sahip ve çok hücreli bir sap içeren büyük kapitat trikomlar ile iki alt tipi bulunan; asikular trikom ve çıkıntılar ve belirgin internodlar içeren uzun trikomlar yalnızca *S. khuzistanica*'da bulunmaktadır. Trikom ve nutlet özellikleri, bu türün farklı popülasyonları arasında sabit kaldığı görülmüştür. Aynı zamanda glandular ve non-glandular trikomların işlevleri de tartışılmıştır. Bu çalışma, *Satureja* türleri arasında polen özelliklerinin tutarlılık gösterdiğini, ancak trikom ve nutlet mikromorfolojik özelliklerinin tür düzeyinde ayırt edici karakterler sağlayabileceğini ve gelecekteki filogenetik çalışmalarda türler arasındaki ilişkilerin daha iyi anlaşılmasına katkı sağlayabileceğini göstermektedir.

Anahtar Kelimeler: *Satureja*, Menthinae, nutlet, trikom, polen

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1. Introduction

Satureja L. is a genus belonging to *Lamiaceae*, subfamily *Nepetoideae*, tribe *Mentheae*, subtribe *Menthinae* (Harley et al., 2004). It is indicated that the genus contains 38 species that are either annual herbs or perennial shrubs and subshrubs, widely occurring in the Mediterranean region, N Africa (Morocco and Libya), the Caucasus and W Asia (including Saudi Arabia, Iraq, and Iran) (Harley et al. 2004). According to the most recent revision (Bordbar and Mirtadzadini, 2024), *Satureja* has 12 species in Iran distributed in the northern, northwestern, western, southwestern, and southeastern regions of the country. Most of the species are endemic to the flora of Iran and distributed mainly in dry rocky limestone slopes of Irano-Turanian region (Bordbar and Mirtadzadini, 2024). This region is characterized by very dry summers and a temperate continental climate, extending from Syria and Anatolia to Turkestan and the Pamirs (Djamali et al., 2012).

The species are well-known aromatic plants rich in terpenoids, such as carvacrol, γ-terpinene, thymol, p-cymene, β-caryophyllene, linalool, and have widely used as flavoring in foods, herbal teas, and traditional medicine

(Amanlou et al., 2005; Sadeghi-Nejad et al., 2011; Alizadeh, 2015; Mazandarani and Monfaredi, 2017).

Along with morphological features, the micro-morphology of pollens, properties of nutlets, and types of trichomes have been pointed out to be useful in Lamiaceae at various taxonomic levels (Wagstaff, 1992; Navarro and Oualidi, 2000; Moon et al., 2008a,b, 2009; Salmaki et al., 2008, 2009; Krawczyk and Głowacka, 2015). The investigation of such characters can provide valuable information for the delimitation of the taxa and also is suggested to be beneficial for phylogenetic interpretations in this family (Abu-Asab and Cantino, 1987; Cantino, 1990). Until now, several studies have focused on the micro-morphology of pollens (Moon et al., 2008c), nutlets (Husain et al., 1990; Kaya et al., 2009; Semerdjieva et al., 2023), and trichomes (Malmir et al., 2014) of *Satureja* species, however, they include only a few species belonging to the flora of Iran. Therefore, a comprehensive study encompassing all Iranian species of *Satureja* is lacking. This study aims to provide detailed descriptions of the pollen, nutlet, and trichome micro-morphology of the Iranian species of *Satureja* to evaluate the systematic significance of the observed characteristics in light of the variations among the taxa of the genus.

2. Materials and Method

2.1. Plant material

The examinations were performed on the herbarium samples belonging to 12 species of *Satureja* from flora of Iran deposited in MIR herbarium (Table 1). The delimitation of the taxa follows Bordbar and Mirtadzadini (2024).

Table 1. Taxa studied and their locality information

Taxon	Locality information
S. avromanica Maroofi	W, Kordestan Prov., Auraman valley, SW of Bölbar village, 35°14'08.5"N, 46°17'36.9"E, 1012 m, 05.X.2017, *Mirtadzadini 3029* (MIR)
S. bachtiarica Bunge	W, Bakhtiari, N of Ardal, Darkash canyon, 32°03'08.7"N, 50°39'43.3"E, 2721 m, 27.X.2016, *Mirtadzadini 1991* (MIR). Kordestan Prov., Dezli to Auraman, Kalam defile, 35°20'15.52"N, 46°13'9.54"E, 1690 m, 05.X.2017, *Faruqinia 3024* (MIR). Kermanshah Prov., Kerend, near Asiab-Tanureh village, 34°29'40.14"N, 46°08'39.07"E, 1874 m, 03.XI.2016, *Bordbar 1995* (MIR). C, Yazd Prov., 13 km from Mehriz to Tang-e Tshenar, 31°30'38.0"N, 54°21'30.3"E, 1946 m, *Mirtadzadini 1987* (MIR). Esfahan Prov., Semirom, 6 km from Semirom toward Vanak, 31°26'48.34"N, 51°30'07.62"E, 26.IX.2014, *Mirtadzadini 1993* SW, Fars Prov., South west of Estahban, Kuhbehesht, 29°06'43.16"N, 54°02'03.55"E, 1829 m, 14.X.2016, *Bordbar 1952* (MIR).
S. hortensis L.	NW, West Azarbaijan Prov., Khoy to Maku, 28.VIII.2008, *Mirtadzadini 3808* (MIR). Ardabil Prov., Givi, 15.X.2007, *Mirtadzadini 1958* (MIR)
S. intermedia C.A.Mey.	NW, Ardabil Prov., Talesh area, east of Khalkhal, after Kalestan village, Soltan-e Khuni, 37°42'52.74"N, 48°36'13.28"E, 2695 m, 08.VIII.2012. *Mirtadzadini 1980* (MIR).
S. isophylla Rech.f.	N, Mazandaran Prov., between Tshalus and Karaj, 14 km to Siabishe from Tshalus, 14.VIII.2013, *Mirtadzadini 1978* (MIR). SW of Nur, 5 km from Poul to Largan village, 36°23'07.76"N, 51°32'29.4"E, 1420 m, 27.X.2011, *Mirtadzadini 1979* (MIR).
S. kermanica Payandeh, Bordbar & Mirtadz.	SE, Kerman Prov., S of Kerman, N of Mt. Jupar, 29°59'11.3"N, 57°12'10.8"E, 2289 m, 21.X.2016, *Mirtadzadini 1943* (MIR). Kerman to Jiroft, Dehbakri, Marghak, 29°07'18.39"N, 57°52'43.06"E, 05.IX.2015, *Bordbar 1962* (MIR).
S. khuzistanica Jamzad	W, Ilam Prov., 16 km to Mehran from Ilam, S of Banroshan, 33°33'17.79"N, 46°13'12.98"E, 900 m, 13.XII.2019, *Mirtadzadini, Bordbar & Doostmohammadi 3803* (MIR) (type locality of *Satureja rechingeri*). Lorestan Prov., Pol-e Dokhtar to Andimeshk, road of Emamzada Shah Ahmad, 32°48'30.0"N, 47°59'47.5"E, 830 m, 17.IX.2019, *Mirtadzadini 3805* (MIR). Pol-e Dokhtar to Andimeshk, near Emamzada Shah Ahmad, 32°48'03.4"N, 48°00'04.0"E, 1150 m, 17.IX.2019, *Mirtadzadini 3804* (MIR).
S. edmondii Briq.	W, Kermanshah Prov., 5 km from Bisotun toward Sonqor, Mt. Parow, Nojubaran defile, 34°26'20.4"N, 47°24'14"E, 1376 m, 04.XI.2016, Bordbar 2032 (MIR). Kordestan Auroman region, Speriz village, North slopes of Mt. Shahu, 1300 m, 30 Oct 2021, 04 Nov 2021, *Advay 4155* (MIR).
S. macrantha C.A.Mey.	NW, East Azarbaijan Province, NE of Tabriz, Ahar road, 28.VIII.2007, *Mirtadzadini 1981* (MIR). Aras valley, E of Siahrud, SW of Nurduz, 38°50'05.5"N, 46°11'10.4"E, 996 m, 10.VII.2016, *Mirtadzadini and student team 1971* (MIR).
S. macrosiphonia Bornm.	W, Lorestan Province, 75 km from Khoramabad to Andimeshk, 33°04'56.1"N, 48°13'57.3"E, 1230 m, 18.IX.2019, *Mirtadzadini 3811* (MIR). 20 km SW of Aleshtar, Bastam dam, 33°41'9.40"N, 48° 9'0.61"E, 1540 m, 21.IX.2022, *Mirtadzadini & Bordbar 5156* (MIR).
S. mutica Fisch. & Mey.	NE, SW of Ashkhana, S of Jauzak, 37°25'16.7"N, 56°40'49.3"E, 1333 m, 30.X.2019, *Mirtadzadini 3829* (MIR). 17 km from Tangrah to Bojnurd, 37°22'07.5"N, 55°57'38.5"E, 753 m, 29.X.2019, *Mirtadzadini 3831* (MIR). N, Gilan Prov., South of Rasht, Ganja toward Rudbar, 36°51'53.0"N, 49°29'04.2"E, 180 m, 13.IX.2019, *Mirtadzadini 3821* (MIR).
S. sahendica Bornm.	NW, East Azarbaijan, south of Qaraghaj, south west of Pir-e Saqqa village, 365140.5°N, 465613.8°E, 2459 m, 08.VII.2016. *Mirtadzadini 1984* (MIR).

2.2 Pollen morphology

Pollen grains were removed from mature anthers of the herbarium specimens (Table 1). Most of the species (10 out of 12 taxa) were represented by two or more specimens from different collections to ensure about the constancy of pollen characters among different populations of the same species. For light microscopy (LM), the pollen grains were mounted in glycerine jelly after acetolysis according to the method described by Erdtman (1960). The diameter of pollen in polar and equatorial views, P/E ratio and length of colpus were measured using 15–30 separate grains by an Olympus BH-2 light microscope equipped with camera photomicrograph system. For SEM, pollen grains were mounted on clean metallic stubs using double-sided adhesive tape and coated with gold. The whole pollen grain and the detailed surface ornamentations were photographed with TESCAN VEGA3 microscope. The terminology follows that of Erdtman (1952) for determination of the shapes and Halbritter et al. (2018) for determination of the ornamentations.

2.3. Nutlet morphology

Nutlets were collected from herbarium specimens (Table 1). When available, two or more specimens from different collections of the same species were sampled. Measurements and optical observations of color and appearance features of the nutlets were carried out with 10–15 nutlets using an Olympus BH-2 light microscope equipped with camera photomicrograph system. For SEM observations, nutlets were mounted on clean metallic stubs using double-sided adhesive tape and coated with gold and examined by means of a TESCAN VEGA3 scanning electron microscopy. The terminology for describing nutlet surface sculpturing mainly follows Stearn (1992) and Kaya et al. (2009).

2.4. Trichome morphology

For each taxon studied, one to three populations were sampled. A list of specimens examined is provided in Table 1. Trichomes were obtained from the stems, leaves, and calyces and investigated with stereo-, light and scanning electron microscopy (SEM). The epidermal surfaces were first observed by light microscopy utilizing hand cut sections. For the scanning electron microscopy, small pieces of stems, leaves and calyces were fixed on metallic stubs using double-sided adhesive tape, and then were coated with gold. The SEM micrographs were taken with a TESCAN VEGA3 scanning electron microscope. The type of indumentum was described and classified following Abu-Asab and Cantino (1987), Satil and Kaya (2007), and Atalay et al. (2016).

Results

3.1. Pollen morphology

Pollen grains of the taxa studied are isopolar and radially symmetric (Fig. 1). The mean of polar axis varies from 40.03 µm to 48.69 µm, and the equatorial axis ranges from 26.99 µm to 34.86µm. The shape of the pollen grains is prolate (P/E = 1.33 – 1.48) with the exception for *S. khuzistanica* Jamzad. In *S. khuzistanica* two types of pollen were observed: prolate (P/E = 1.42) and sub-prolate (P/E = 1.29) pollen grains (Fig. 1b). In all of the species, the pollen grains are hexacolpate, the ambs are circular, and the surface is microreticulate. Simple colpi are distributed symmetrically (Fig. 1) (Table 2). We were unable to detect the octacolpate pollen grain mentioned by Jamzad (2012) for *S. avromanica* Maroofi.

3.2. Nutlets

SEM and LM micrograph of nutlets and their surfaces of the studied taxa are presented in Figures 2 and 3. The size of the nutlets ranged from 1 mm (in *S. isophylla* Rech.f.) to 2 mm in length (in *S. khuzistanica* and *S. avromanica*) and from 0.3 mm (in *S. isophylla*) to 1.5 mm in width (in *S. khuzistanica*). Therefore, the largest nutlets are found in *S. khuzistanica* (Fig. 2g) while the smallest are those of *S. isophylla*. The basic shape of nutlets in most taxa studied is elliptic, but it is widely elliptic in *S. hortensis* L., obovate in *S. isophylla* and widely obovate in *S. khuzistanica*. Elongated elliptic nutlets are also found in *S. avromanica* and *S. macrantha* C.A.Mey. along with elliptic nutlets. In the majority of species nutlets are trigonous, with *S. khuzistanica* displaying slightly trigonous nutlets in transversal section. The areoles are bi-lobed and basal. The nutlet surfaces are glabrous and light to dark brown in color. An irregular pattern of simple or branched dark nerves can be observed under a stereomicroscope. The nerves are more evident in *S. khuzistanica*.

The nutlet surface exihibits an undulate-reticulate pattern in *S. bachtiarica* Bunge and *S. kermanica* Payandeh, Bordbar & Mirtadz. (Fig. 3b and f respectively). In other species, the cell shape (primary sculpturing) is often not discernible externally. The periclinal walls of the surface cells are wrinkled, and as a secondary sculpturing, the surface has both spherical-ovoid pits and a tuberculate pattern on one seed. However, in *S. hortensis* the pattern is regular papillae-tuberculate (Table 1, Fig. 3).

3.3. Trichome

Two basic types of trichomes are observed on different organs of the plants (Figs. 4 and 5): glandular and eglandular. Based on our observation glandular trichomes are subdivided into two main subtypes: (1) subsessile glands (Fig. 4a and b), also known as peltate hairs (Satil and Kaya, 2007), and (2) capitate trichomes (Fig. 4 d-k).

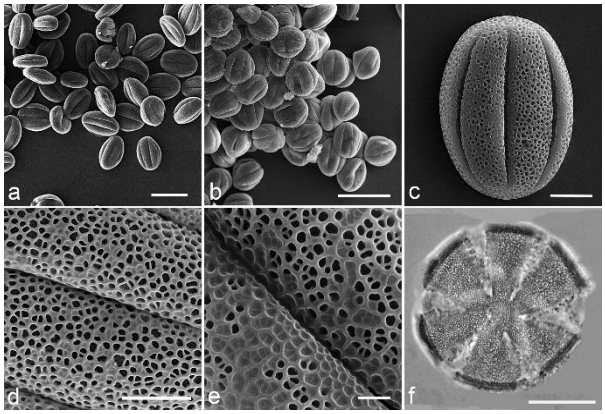

Figure 1. SEM and LM micrograph of pollens of some *Satureja* species from flora of Iran: (a) *S. avromanica.* (b) *S. khuzistanica.* (c) *S. macrantha* (equatorial view). (d) *S. kermanica* (enlarged view). (e) *S. sahendica* (enlarged view). (f) LM micrograph of *S. avromanica* (polar view). (a, b) scale bar = 50 µm, (c, d, f) scale bar =10 µm, (e) scale bar = 2 µm

subsessile glands are multicellular, yellow or red in color regularly distributed in epidermal depressions on the adaxial and abaxial leaf surfaces, on the outer surface of the calyx, and less on the stems. Their size is ranged from 55 µm in *S. isophylla* and *S. avromanica* and up to 100 µm in *S. mutica* Fisch. & Mey. and *S. macrosiphonia* Bornm. Capitate trichomes are of two different forms: small (up to 50 µm) and large (up to 180 µm). Small capitate trichomes have a short uni- or bi-, rarely tricellular stalk (in *S. khuzistanica*, Fig. 4h) with an unicellular clavate (Fig. 4d) or globular head (Fig. 4e and f). Large capitate trichomes consist of a multiseriate stalk (75-90 µm in size) with an enlarged rounded multicellular head (75-90 µm in size) (Fig. 4i-k).

Eglandular trichomes are simple, uniseriate, micro-papillate, and subdivided into three main subtypes: (1) short

Figure 2. LM micrographs of nutlets of *Satureja* species from flora of Iran: (a) *S. avromanica*. (b) *S. bachtiarica*. (c) *S. hortensis*. (d) *S. intermedia*. (e) *S. isophylla*. (f) *S. kermanica*. (g) *S. khuzistanica*. (h) *S. edmondii*. (i) *S. macrantha*. (j) *S. macrosiphonia*. (k) *S. mutica*. (l) *S. sahendica*. scale bar = 1 mm.

with one to three cells and conical (Fig. 4l) or elongated conical in shape (up to 250 µm, Fig. 5a-c), (2) long with up to six elongated cells (up to 600 µm, Fig. 5d, up to 1 mm) and (3) extremely long with up to eight elongated cells (up to 1.2 mm, in *S. khuzistanica*, Fig. 5e-f). We found some variation in morphology of extremely long trichomes and sub-divided them into two different types similar to the findings of Malmir et al. (2014): (1) acicular trichomes (Fig. 5f) and (2) trichomes with ridges and marked internodes (Fig. 5f).

The distribution of the various trichome types on the stems, leaves, and calyces of the studied taxa is presented in Table 2. Trichomes of several types can be found on the same organ. According to the results of this study, subsessile glands, small capitate trichomes and both short and long eglandular trichomes are found on the leaves, stems, and calyces of the majority of *Satureja* species, with the exception of *S. khuzistanica*. In contrast, large capitate glandular trichomes and extremely long trichomes with ridges and marked internodes are exclusive to *S. khuzistanica*. Additionally, large eglandular trichomes are only located between the teeth of the calyces of *Satureja* species. Trichomes of several types can present on the same aerial organ. However, *S. avromanica* has only subsessile glands on its leaves and lacks trichomes on stems. In addition, small glandular capitate trichomes are only visible in *S. avromanica*, *S. intermedia* C.A.Mey., *S. hortensis*, and *S. macrantha* (calyx), *S. macrosiphonia* (stem and calyx), and *S. bachtiarica*, *S. edmondii* Briq. & Hausskn. and *S. khuzistanica* (leaf, stem, calyx).

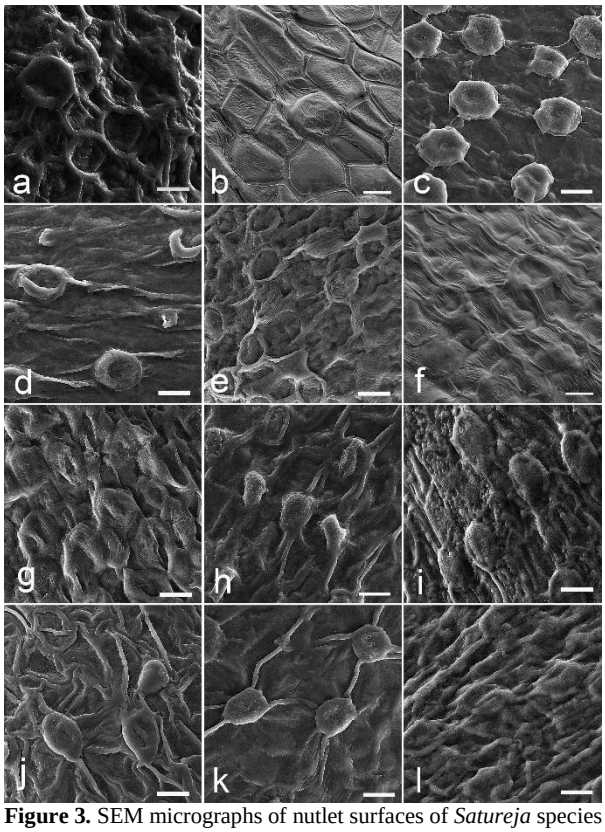

Figure 3. SEM micrographs of nutlet surfaces of *Satureja* species from flora of Iran: (a) *S. avromanica*. (b) *S. bachtiarica*. (c) *S. hortensis*. (d) *S. intermedia*. (e) *S. isophylla*. (f) *S. kermanica*. (g) *S. khuzistanica*. (h) *S. edmondii*. (i) *S. macrantha*. (j) *S. macrosiphonia*. (k) *S. mutica*. (l) *S. sahendica*. scale bar = 20 µm.

The orientation of eglandular trichomes are antrorse, retrorse or spreading. The orientation of the eglandular trichomes (if existed) on the leaves are usually antrose (Fig. 5a-c), and rarely spreading (*S. edmondii* and *S. khuzistanica*). While the orientation of the eglandular trichomes are retrose or spreading on the stems and calyces, rarely antrose on the stem (*S. kermanica*).

Although we did not qualify the density of hairs per unit area, it appears that there is little variation in the density of trichomes among different species. The only *S. avromanica* is an almost glabrous plant with eglandular trichomes absent on the leaves and stems. This type of hair is sporadically scattered on the calyx. Additionally, in *S. khuzistanica* some individuals of the same population are more densely hairy, and *S. macrosiphonia* displays an intra-specific diversity in the density of eglandular trichomes.

4. Discussions

This study provides a comprehensive investigation on the pollen, seed, and trichome micromorphology of 12 Iranian *Satureja* mostly for the first time as discussed below.

4.1. Pollen characters in taxa studied of *Satureja*

According to the results of this study, the pollens are hexacolpate in *Satureja* species. Aperture number has been considered as a useful character to define the subfamily Nepetoideae. This subfamily is characterized by hexacolpate pollen grains (Erdtman, 1945; Cantino, 1992). Prolate shape of pollens were observed in the majority of species, and both prolate and sub-prolate were identified in *S. khuzistanica*. It seems that multiple shapes of pollens within the one taxon are common in Menthinae (Moon et al., 2008c). In general, Menthinae pollens are small to medium in size (13-43 µm), oblate to prolate in shape, mostly hexacolpate (sometimes pentacolpate), and with perforate, microreticulate or bireticulate exine ornamentation types (Moon et al., 2008c). Therefore, the main characteristic features of pollen in the *Satureja* species studied here are consistent with those reported earlier for Menthinae. There are only sporadic reports of pollen characteristics in Satureja, as reports of Fırat (2015) for S. avromanica. In his work, the pollens are prolate- sub-spheroidal and reticulate; however, they appear to be underdeveloped. In another study, the pollen grains of S. montana L. and S. subspicata Bartl. ex Vis. had an oblate-spheroidal shape (Dunkic´ et al., 2007). The external morphology of pollen in Satureja is consistent, and characterized by a microreticulate pattern. Pollen characteristics of the family Lamiaceae have been reported to be of considerable taxonomic importance (Erdtman, 1945; Moon et al., 2008a,b,c); however, the results of this study are indicating that pollen morphology is stable at generic level in *Satureja* and is not useful for comparisons of the species.

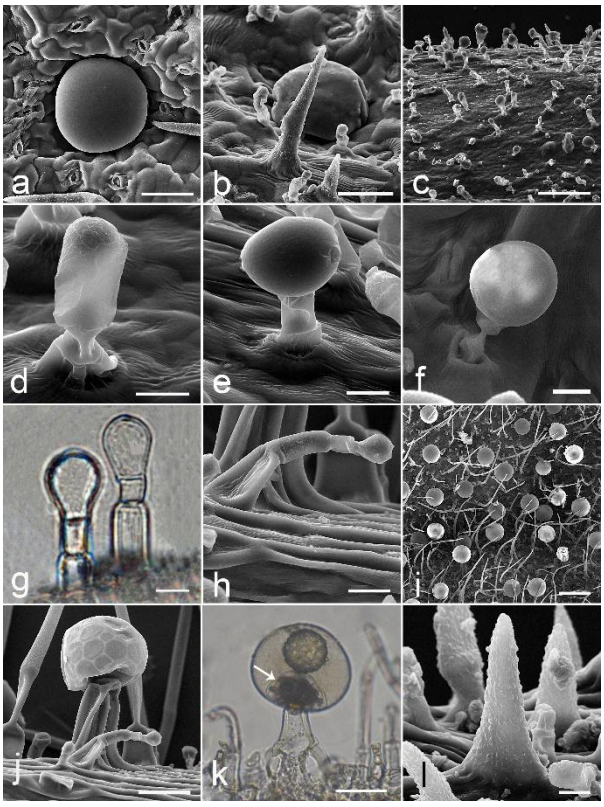

Figure 4. SEM and LM micrographs of trichomes of *Satureja* species from flora of Iran: (a) *S. intermedia*, subsessile gland (leaf). (b) *S. edmondii*, subsessile gland (leaf). (c) *S. edmondii*, small capitate trichomes (calyx). (d) *S. edmondii*, small capitate trichomes with clavate head (calyx). (e) *S. edmondii*, small capitate trichomes with globular head (calyx). (f) *S. khuzistanica*, small capitate trichomes with globular head (leaf). (g) *S. khuzistanica*, small capitate trichomes with bi-cellular stalk (leaf). (h) *S. khuzistanica*, small capitate trichomes with tri-cellular stalk (calyx). (i) *S. khuzistanica*, large capitate trichomes along with extremely long eglandular trichomes (leaf). (j) *S. khuzistanica*, large capitate trichome (calyx). (k) *S. khuzistanica*, large capitate trichome including oil storage (leaf). Secretory cells are shown with arrow. (l) *S. sahendica*, short conical eglandular trichomes (stem). (a, b, j, k) scale bar = 50 µm, (c) scale bar =100 µm, (d-g, l) scale bar = 10 µm, (h) scale bar = 20 µm, (i) scale bar = 200 µm.

4.2. Nutlet morphology in taxa studied of *Satureja*

Features of nutlet micromorphology have been widely used to study and reported to be useful as distinguishing features at different taxonomic levels in the Lamiaceae family (Husain et al., 1990; Moon and Hong, 2006; Kaya and Dirmenci, 2008; Salmaki et al., 2008; Moon et al., 2009; Siadati et al., 2019; Celep et al., 2020).

Moon et al. (2009) indicated that the nutlet characteristics are ratherly consistent at the generic level, although some variation was observed within some genera. *Salvia* L., the largest genus in Mentheae shows a considerable diversity in nutlet characteristics including size, shape, and sculpturing patterns. A similar variation in nutlet morphology is observed in *Monarda* L. and *Nepeta* L. Our results indicate that nutlets vary significantly in size, shape, and sculpturing patterns. These findings are highly consistent with those obtained for *Satureja* species from Turkey (Kaya et al., 2009). They studied 15 *Satureja* taxa from Turkey, of which *S. hortensis*, *S. macrantha* and *S. mutica* (syn. *S. spicigera* (C.Koch) Boiss.) are distributed in Iran as well. Their results identified two main types of nutlets: more or less smooth and sculptured, along with four subtypes: undulate-reticulate, reticulate, reticulate-protuberculate, and papillate-tuberculate. In their study,

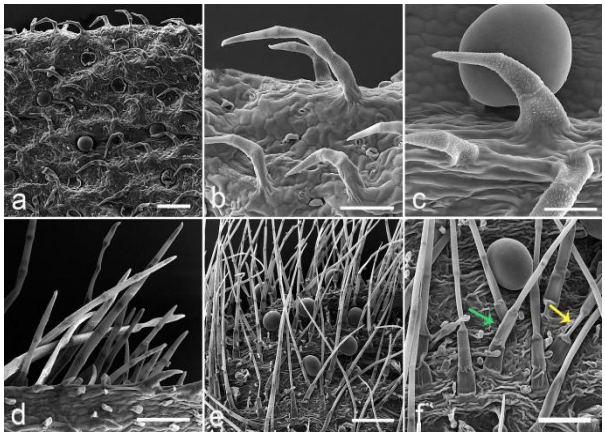

Figure 5. SEM micrographs of trichomes of *Satureja* species from flora of Iran: (a) *S. intermedia*, antrose orientation of eglandular trichomes on leaf surface (leaf). (b) *S. intermedia*, small eglandular trichomes (leaf). (c) *S. mutica*, small eglandular trichomes (calyx). (d) *S. isophylla*, long eglandular trichomes (calyx). (e) *S. khuzistanica*, extremely long eglandular trichomes over large capitate trichomes (calyx). (f) *S. khuzistanica*, acicular trichomes (green arrow) and trichomes with ridges and marked internodes (yellow arrow) (calyx). (a, e) scale bar = 200 µm, (b, d, f) scale bar =100 µm, (c) scale bar = 50 µm

Table 2. Pollen, nutlet and trichome morphological characters of *Satureja* species from flora of Iran

	Pollen morphological characters					Nutlet morphological characters						Trichome morphological characters		
Taxa	**P** (μm)	**E** (μm)	**P/E**	**shape**	**Ornamentation**	**L** (mm)	**W** (mm)	**Color**	**Shape**	**Shape of Transversal section**	**Surface sculpturing**	**Leaf**	**Stem**	**Calyx**
S. avromanica	46.53 ± 1.84	34.86 ± 2.38	1.33	prolate	microreticulate	1.5-2	0.8-1	b-db	elliptic-elongated elliptic	t	p-pt	A	-	B, D
S. bachtiarica	40.16 ± 1.51	26.99 ± 0.53	1.48	prolate	microreticulate	1.2-1.5	0.5-0.7	b-db	elliptic	t	ur	A, B, D^{an}	A, B, D$^{re\ or\ sp}$	A, B, D^{sp}, E
S. edmondii	44.92 ± 0.93	31.64 ± 2.04	1.41	prolate	microreticulate	1.5-1.8	0.6-0.8	b-db	elliptic	t	p-pt	A, B, D^{sp}	A, B, D^{sp}	A, B, D^{sp}
S. hortensis	40.03 ± 1.32	29.96 ± 2.52	1.33	prolate	microreticulate	1.2-1.3	ca. 1	b-db	widely elliptic	t	p	A, D^{an}	A, D^{re}	A, B, D^{an}, E
S. intermedia	48.69 ± 3.12	34.23 ± 2.58	1.42	prolate	microreticulate	1.4-1.6	0.7-0.8	b-db	elliptic	t	p-pt	A, D^{an}	A^{3}, D^{re}	A, B, D^{an}, E
S. isophylla	44.33 ± 1.69	32 ± 1.05	1.38	prolate	microreticulate	1-1.2	0.3-0.5	b-db	obovate	t	p-pt	A, D^{an}	A, D^{sp}	A, D^{sp}, E
S. kermanica	40.25 ± 2.1	28.13 ± 3.39	1.43	prolate	microreticulate	1.2–1.5	0.5–0.7	b-db	elliptic	t	ur	A, D^{an}	A^{3}, D^{an}	A, D, E^{an}
S. khuzistanica	40.28 ± 1.20 45.98 ± 1.0	31.23 ± 2.51 32.25 ± 2.52	1.29 1.42	sub-prolate prolate	microreticulate	1.8-2	1.2-1.5	b	widely obovate	st	p-pt	B, C, F$^{an\ \&\ sp}$	B, F^{sp}	B, F^{sp}, E
S. macrantha	46.48 ± 1.78	33.42 ± 2.24	1.39	prolate	microreticulate	1.2-1.5	0.5-0.7	b-db	elliptic-elongated elliptic	t	p-pt	A, D^{an}	D^{re}	A, B, D^{sp}, E
S. macrosiphonia	45.43 ± 0.89	32.36 ± 1.84	1.40	prolate	microreticulate	1.5-1.8	0.8-1	b-db	elliptic	t	p-pt	A, D^{an}	A, B, D^{re}	A, B, D^{an}, E
S. mutica	42.33 ± 1.05	31.66 ± 1.48	1.33	prolate	microreticulate	1.3-1.5	0.8-1	b-db	elliptic	t	p-pt	A, D^{an}	A^{3}, D^{re}	A, D^{an}, E
S. sahendica	42.71 ± 1.65	29.10 ± 1.59	1.46	prolate	microreticulate	1.2-1.5	0.7-0.8	b-db	elliptic	t	p-pt	A, D^{an}	A^{3}, D^{re}	A, D^{an}, E

Pollen characters: P; polar axis, E; equatorial axis, ± standard deviation, **Nutlet characters:** L: length, W: width. Color; b; brown, db; dark brown, Shape of transversal section; t; trigonous, st; slightly trigonous, Surface sculpturing; r; reticulate, ur; undulate-reticulate, p; pitted, pt; protuberculate, g; granulate, **Trichome characters:** Trichome types: A; subsessile glands, B; small glandular capitate trichome, C; large glandular capitate trichome, D; short eglandular micro-papillate trichome, E; long eglandular micro-papillate multi-cellular trichome, F; extremely long trichome, density: 1; dense, 2; sparse, 3; very sparse, orientation of trichome type D, E and F: an; antrorse, re; retrorse, sp; spreading.

nutlets showed a reticulate-protuberculate pattern in *S. macrantha*, papillate to tuberculate surfaces in *S. hortensis*, and reticulate-small tuberculate surfaces with shallow polygonal pits in *S. spicigera*. The latter species synonymized with *S. mutica* by Bordbar and Mirtadzadini (2024). Similarly, in our studies, most species, including *S. macrantha* and *S. mutica* indicated spherical-ovale pits and a tuberculate pattern, while *S. hortensis* revealed a papillate-tuberculate surface. The undulate-reticulate pattern of sculpturing in *S. bachtiarica* and *S. kermanica* is unique among Iranian *Satureja*. These two species also differ from others in their inflorescence and small flowers (Bordbar and Mirtadzadini, 2024). Our results showed that among the studied taxa, *S. khuzistanica* has relatively larger and wider nutlets, while the individuals of *S. isophylla* have shorter and narrower nutlets. *S. khuzistanica* is morphologically recognized from the other *Satureja* species by its large, broad, obovate or elliptic leaves, and *S. isophylla* differs by its caespitose habit and small leaves (Bordbar and Mirtadzadini, 2024). The nutlets of Turkish species of *Satureja* are typically glabrous, or in some species, apically haired (Kaya et al., 2009). In our investigations, the nutlets were exclusively glabrous. We did not observe any intraspecific variation for the taxa studied in characteristics of the nutlets.

Representative nutlet features also proved to be phylogenetically informative mainly at the generic level in Mentheae (Moon et al., 2009). The variation in surface sculpturing, nutlet shape, and size in *Satureja* is highly in consistent with the morphological diversity among species, and may provide useful diagnostic properties for future phylogenetic studies in this genus.

4.3. Trichome morphology in taxa studied of *Satureja* and their function

The significance of trichome features has been demonstrated in taxon delimitation at various levels in Lamiaceae (Giuliani and Bini, 2008; Moon et al., 2009; Atalay et al., 2016; Eiji and Salmaki, 2016). Such characteristics provide valuable criteria for comparison and serve as fundamental taxonomic tools in taxon delimitation. Trichomes of various forms are present in the studied species, and have taxonomic value for *Satureja* species. Trichome micro-morphological analyses of *Satureja* species have been described both here and in several publications (Dunkić et al., 2007; Satil and Kaya, 2007; Marin et al., 2010, 2012). In all cases, *Satureja* species have non-glandular trichomes together with two types of glandular trichomes, classified as sub-sessile (peltate) and capitate. Capitate trichomes are widespread in the Lamiaceae, but they vary significantly in stalk length and head shape (Abu-Asab and Cantino, 1987; Giuliani and Bini, 2008). In *Satureja*, these trichomes are typically small. However, the large capitate trichomes characterized by a multiseriate stalk and an enlarged, rounded multicellular head, appear to be unique and are exclusively found in *S. khuzistanica*. This type of trichome has been described in detail by Malmir et al. (2014). However, they categorized it as a peltate trichome. According to Malmir et al. (2014), this trichomes of *S. khuzistanica* comprised on average, 5 voluminous-lengthy stalk cells and 12 secretory cells. Moreover, several bundles of needle-like structures were observed in the secretory head of the trichomes, conforming to the published characteristics of calcium oxalate raphides. However, these needle-like structures were not observed in the trichomes of the samples we investigated

Subsessile glandular trichomes with multicellular heads are diagnostic for the genus *Satureja*, but they are apsent in *S. khuzistanica*. This type of trichome does not occur in some plant species belonging to Menthinae, such as *Clinopodium vulgaris* L. and *Conradina canescens* A.Gray as well (Moon et al., 2009). Sub-sessile glands on *Satureja thymbra* L. and *S. montana* consist of one basal epidermal cell, one stalk cell and a head composed of twelve secretory cells (Bosabalidis, 1990; Marin et al., 2012, respectively). In a study of leaf anatomy and trichomes of 15 *Satureja* from the flora of Türkiye by Satil and Kaya (2007), the sub-sessile trichomes (referred to as peltate trichomes) were found to be comprise one basal cell, one stalk cell, and a broad head containing 12–16-cells, of which 8–12 were described as large and peripheral, while four were noted to be small and located the central area of the head.

Non-glandular trichomes are widely distributed in Lamiaceae (Moon et al., 2009). Non-glandular trichomes likely collaborate together with the glandular trichomes in the mechanical defense against herbivores, and form a physical protection to plants against abiotic stresses. Besides, they comprise living cells that can synthesize, storage and/or release of biologically active compounds, including various classes of secondary metabolites (Tozin et al., 2016). According to the results of this study and previous reports (Satil et al. 2002, 2003; Redžić et al., 2006; Dunkić et al., 2007; Satil & Kaya, 2007; Marin et al., 2010, 2012), eglandular trichomes in *Satureja* are mainly the same and in two types: short and long. Notably, the extremely long trichomes found in two subtypes acicular trichomes and trichomes with ridges and marked internodes, are exceptionally present in *S. khuzistanica*.

In many *Labiatae*, the glandular trichomes are responsible for the secretion and storage of essential oils (Fahn, 1990; Muravnik, 2021). Different species of *Satureja* have different amount of essential oil content (Omidbaigi et al., 2007; Ghorbanpour et al., 2016) but they usually are rich in terpenoids, such as carvacrol, terpinene, thymol, *p*-cymene, *β*-caryophyllene, linalool, and other terpenoids. The variations in the quantitative composition of carvacrol and thymol compounds in the Lamiaceae family were mainly due to the geographical area, genetic diversity, climatic conditions, the existence of different chemotypes, and/or ecological differences (Salehi-Arjmand et al., 2014). For example, it has been shown that perennial winter savory (*S. montana*) accumulates more carvacrol than annual summer savory (*S. hortensis*). The highest and lowest oil yields in *S. rechingeri* were obtained during the full-flowering stage (October) and the pre-flowering stage (July), respectively (Alizadeh, 2015). Low winter temperatures cause overproduction of essential oils due to antioxidant properties that stimulate oxidative stress (Bosabalidis, 2013). These differences may also result from varying densities of glandular trichomes on the aerial parts of the plants. In *Thymus sibthorpii* Benth., glandular hairs are abundant in winter leaves and secrete a higher amount of essential oil, compared to summer leaves (Bosabalidis, 2013). According to the results of this study, *S. avromanica* and *S. isophylla* have the smallest subsessile glands among the studied taxa. *S. avromanica* is nearly glabrous and non-aromatic. The analysis of essential oils in these two species indicated that the amounts of thymol and carvacrol were very low (Sefidkon and Jamzad, 2006; Abdali et al., 2017). Conversely, *S. khuzistanica* has the largest capitate glands on its aerial parts. Alizadeh (2015) stated that a comparison of the quantities of *Satureja* species shows that *S. rechingeri* has the highest oil yield among all *Satureja*

species, whether grown in the wild or cultivated. According to Bordbar and Mirtadzadini (2024) this species is now considered a synonym of *S. khuzistanica*.

We conclude that the pollen characteristics among *Satureja* species exhibit consistency; however, the micromorphological features of trichomes and nutlets are variable and may be systematically significance. *S. khuzistanica* is distinguished from the other species by the types of trichome. Additionally, *S. isophylla* and *S. khuzistanica* differs from the other studied species in the size of nutlets and *S. bachtiarica*, *S. hortensis* and *S. kermanica* are different in their nutlet sculpturing. Nevertheless, the general characteristics of trichome and nutlet were consistent among different populations of a given species. overall, our findings are useful contribution to the taxonomy and enhance the understanding of interspecies relationships in the genus *Satureja*. However, the value of these features in *Satureja* may be more effectively assessed through a molecular phylogenetic approach used in conjunction with morphological characteristics.

Conflict of Interest

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

Authors' Contributions

FB contributed to material preparation, data collection, methodology and measured parameters. The first draft of the manuscript was written by FB. MM and MA contributed to material preparation. MM and FS commented on previous versions of the manuscript and read and approved the final manuscript.

References

Abdali E, Javadi S, Akhgari M, Hosseini S, Dastan D (2017). Chemical composition and biological properties of *Satureja avromanica* Maroofi. Journal of Food Science and Technology 54(3): 727-734.

Abu-Asab M, Cantino PD (1987). Phylogenetic implications of leaf anatomy in subtribe *Melittidinae* (*Labiatae*) and related taxa. Journal of the Arnold Arboretum 68(1): 1-34. https://www.jstor.org/stable/43782312

Alizadeh A (2015). Essential oil composition, phenolic content, antioxidant, and antimicrobial activity of cultivated *Satureja rechingeri* Jamzad at different phenological stages. Zeitschrift für Naturforschung C 70 (3-4): 51-58. https://doi.org/10.1515/znc-2014-4121

Amanlou M, Dadkhah F, Salehnia A, Farsam H (2005). An anti-inflammatory and anti-nociceptive effects of hydroalcoholic extract of *Satureja khuzistanica* Jamzad extract. Journal of pharmacy and pharmaceutical sciences 8(1): 102-106.

Atalay Z, Celep F, Bara F, Doğan M (2016). Systematic significance of anatomy and trichome morphology in *Lamium* (*Lamioideae; Lamiaceae*). Flora 225: 60-75. https://doi.org/10.1016/j.flora.2016.10.006

Bordbar F, Mirtadzadini M (2024). A morphological re-evaluation of the taxonomic status of *Satureja* L. (*Lamiaceae, Nepetoideae, Mentheae*) from flora of Iran. Adansonia 46 (7): 45-63. https://doi.org/10.5252/adansonia2024v46a7

Bosabalidis AM (1990). Glandular Trichomes in *Satureja thymbra* Leaves Annals of Botany 65(1): 71-78.

Bosabalidis AM (2013). Glandular hairs, non-glandular hairs, and essential oils in the winter and summer leaves of the seasonally dimorphic *Thymus sibthorpii* (*Lamiaceae*). Journal of Plant Development 20: 3-11.

Cantino PD (1990). The phylogenetic significance of stomata and trichomes in the *Labiatae* and *Verbenaceae*. Journal of the Arnold Arboretum 71: 323-370. https://www.jstor.org/stable/43782250

Cantino PD (1992). Evidence for a polyphyletic origin of the *Labiatae.* Annals of the Missouri Botanical Garden 79: 361-379. https://doi.org/10.2307/2399774

Celep F, Kahraman A, Guerin GR, Karabacak E, Akaydın G, Doğan M (2020). Nutlet micromorphology and its taxonomic and phylogenetic significance in *Salvia* (*Lamiaceae*). Plant Biosystems - An International Journal Dealing with all Aspects of Plant Biology 156: 271-283. https://doi.org/10.1080/11263504.2020.1852331

Djamali M, Brewer S, Breckle SW (2012). Climatic determinism in phytogeographic regionalization: a test from the Irano-Turanian region, SW and Central Asia. Flora 207: 237-249.

Dunkić V, Bezic N, Ljubesic N, Boĉina I (2007). Glandular hair ultrastructure and essential oils in *Satureja subspicata* Vis. ssp. *subspicata* and ssp. *Liburnica* Silic. Acta Biologica Cracoviensia Series Botanica 49: 45-51.

Eiji S, Salmaki Y (2016). Evolution of trichomes and its systematic significance in *Salvia* (*Mentheae; Nepetoideae; Lamiaceae*). Botanical Journal of the Linnean Society 180: 241-257.

Erdtman G (1945). Pollen morphology and plant taxonomy. IV. *Labiatae, Verbenaceae* and *Avicenniaceae*. Svensk Botanisk Tidskrift 39: 279-285.

Erdtman G (1952). Pollen morphology and plant taxonomy. An introduction to palynology, Vol 1. Stockholm: Almqvist & Wiksell.

Erdtman G (1960). The acetolysis method, a revised description. Svensk Botanisk Tidskrift 54: 561-564.

Fahn A (1990). Plant anatomy, 4th edn. Oxford: Pergamon Press.

Fırat M (2015). *Satureja avromanica* Maroofi (*Lamiaceae*): An addition to flora of Turkey with contributions to its taxonomy. Biological Sciences and Pharmaceutical Research 3(12): 123-128.

Ghorbanpour M, Hadian J, Hatami M, Salehi-Arjomand H, ALiahmadi A (2016). Comparison of chemical compounds and antioxidant and antibacterial properties of various *Satureja* species growing wild in Iran. Journal of Medicinal Plants 15(59): 58-72.

Giuliani C, Bini LM (2008). Insight into the structure and chemistry of glandular trichomes of *Labiatae*, with emphasis on subfamily *Lamioideae.* Plant Systematics and Evolution 276: 199-208.

Halbritter H, Ulrich S, Grímsson F, Weber M, Zetter R, Hesse M, Buchner R, Svojtka M, Frosch Radivo A (2018). Illustrated pollen terminology. Cham: Springer.

Harley RM, Atkins S, Budantsev A, Cantino PD, Conn BJ, Grayer R, Harley MM, De Kok R, Krestovskaja T, Morales R, Paton AJ, Ryding O, Upson T (2004). *Labiatae.* In: Kubitzki K (ed.). The Families and Genera of Vascular Plants, Vol. 7. Berlin: Springer Verlag. pp. 167-275.

Husain SZ, Márin DP, Silic C, Qaiser M, Petkovic B (1990). A micro-morphology study of some representative genera in the tribe Saturejeae (*Lamiaceae*). Botanical Journal of the Linnean Society 103: 59-80.

Jamzad Z (2012). *Satureja*. In Flora of Iran. Vol. 76. Tehran: Research Institute of Forests and Rangelands, Pp. 673-698 (in Persian).

Kaya A, Dirmenci T (2008). Nutlet surface micromorphology of the genus *Nepeta* L. (*Lamiaceae*) in Turkey. Turkish Journal of Botany 32: 103-112.

Kaya A, Satil F, Gogel F (2009). Nutlet surface micromorphology of Turkish *Satureja* (*Lamiaceae*). Biologia 64(5): 902-907. https://doi.org/10.2478/s11756-009-0156-x

Krawczyk K, Głowacka K (2015). Nutlet micromorphology and its taxonomic utility in *Lamium* L. (*Lamiaceae*). Plant Systematics and Evolution 301: 1863-1874. https://doi.org/10.1007/s00606-015-1199-9

Malmir M, Serrano R, Reza Gohari A, Silva O (2014). Characterization of *Satureja khuzestanica* leaf as a herbal medicine. Microscopy and Microanalysis 20 (5): 1425-35.

Marin M, Ascensão L, Marin PD (2012). Microscopic investigation of trichomes of wild-growing *Satureja montana* L. Archives of Biological Sciences 64: 1599-1605.

Marin M, Jasnic N, Lakusic D, Duletic-Lausevic S, Ascensao L (2010). The micromorphological, histochemical and confocal analysis of *Satureja subspicata* Bartl. ex vis. glandular trichomes. Archives of Biological Sciences 62(4): 1143-1149.

Mazandarani M, Monfaredi L (2017). Evaluation of antioxidant and antimicrobial activity of *Satureja mutica* Fisch. & C.A.Mey. collected from North Khorasan Province, Iran. Medical Laboratory Journal 11(1): 23-27.

Moon HK, Hong SP (2006). Nutlet morphology and anatomy of the genus *Lycopus* (*Lamiaceae: Mentheae*). Journal of Plant Research 119: 633-644.

Moon HK, Vinckier S, Smets E, Huysmans S (2008b). Comparative pollen morphology and ultrastructure of Mentheae subtribe Nepetinae (*Lamiaceae*). Review of Palaeobotany and Palynology 149: 174-198.

Moon H-K, Vinckier S, Smets E, Huysmans S (2008c). Palynological evolutionary trends within the tribe *Mentheae* with special emphasis on subtribe *Menthinae* (*Nepetoideae: Lamiaceae*). Plant Systematics and Evolution 275: 93-108.

Moon HK, Vinckier S, Walker JB, Smets E, Huysmans S (2008a). A search for phylogenetically informative pollen characters in the subtribe Salviinae (*Mentheae: Lamiaceae*). International Journal of Plant Sciences 169: 455-471. https://doi.org/10.1086/526463

Moon H-K, Hong S-P, Smets E, Huysmans S (2009). Micromorphology and character evolution of nutlets in tribe Mentheae (*Nepetoideae, Lamiaceae*). Systematic Botany 34(4): 760-776.

Muravnik LE (2021). The structural peculiarities of the leaf glandular trichomes: A Review. In: Ramawat KG et al. (eds.). Plant Cell and Tissue Differentiation and Secondary Metabolites. Cham: Springer. Pp. 63-96.

Navarro T, Oualidi JEl (2000). Trichome morphology in *Teucrium* L. (*Labiatae*). A taxonomic review. Anales del Jardín Botánico de Madrid 57(2): 277-297.

Omidbaigi R, Rahimi S, Kazemi Sh (2007). Comparison between essential oil content and compositions of winter (perennial) and summer (annual) savory. Journal of Essential Oil Bearing Plants 10(6): 480-485.

Redzić S, Tuka M, Pajević A (2006). Research into microscopic structure and essential oils of endemic medicinal plant species *Satureja subspicata* Bartl. ex Vis. (*Lamiaceae*). Bosnian Journal of Basic Medical Sciences 6(2): 25-31.

Sadeghi-Nejad B, Saki J, Khademvatan S, Nanaei S (2011). In vitro antileishmanial activity of the medicinal plant - *Satureja khuzestanica* Jamzad. Journal of Medicinal Plants Research 5(24): 5912-5915.

Salehi-Arjmand H, Hadian J, Khadivi-Khub A, Akramian M (2014). Comparative analysis of essential oil compositions in seven populations of Bakhtiarian savory in natural and field conditions. Acta Physiologiae Plantarum 36: 1107-1114.

Salmaki Y, Zarre S, Jamzad Z (2008). Nutlet micromorphology and its systematic implication in *Stachys* L. (*Lamiaceae*) in Iran. Feddes Repertorium 119: 607-621.

Satıl F, Dirmenci T, Tümen G (2003). The rediscovery of an extinct species: "*Satureja coerulea* Janka", The Karaca Arboretum Magazine 7(1): 19-22.

Satıl F, Tümen G, Akçelik A, Başer KHC (2002). Comparative morphological, anatomical, ecological and chemical studies on endemic *Satureja parnassica* subsp. *sipylea* from Turkey". Acta Botanica Croatia 61(2): 207-220.

Satil F, Kaya A (2007). Leaf anatomy and hairs of Turkish *Satureja* L. (*Lamiaceae*). Acta Biologica Cracoviensia Series Botanica 49(1): 67‑76.

Sefidkon F, Jamzad Z (2006). Essential oil analysis of Iranian *Satureja edmondi* and *S. isophylla*. Flavour and Fragrance Journal 21: 230‑233.

Semerdjieva I, Yankova-Tsvetkova E, Zheljazkov VD, Koleva-Valkova LH, Nikolova R (2023). Reproductive capacity and scanning electron microscopy (SEM) analyses of the micromorphological Surfaces of three endemic *Satureja* species from Bulgaria. Plants 12: 2436. https://doi.org/10.3390/plants12132436

Siadati S, Saeidi S, Salmaki Y (2019). Nutlet micromorphology of the genus *Marrubium* L. and allies and its systematic implication (*Lamiaceae:* tribe *Marrubieae*). Nova Biologica Reperta 6(3): 338‑346.

Stearn WT (1992). Botanical Latin, 4th edn. Portland Oregon: Timber Press.

Tozin LRD, De Melo Silva SC, Rodrigues TM (2016). Non-glandular trichomes in *Lamiaceae* and *Verbenaceae* species: morphological and histochemical features indicate more than physical protection. New Zealand Journal of Botany 54(4): 446‑457.

Wagstaff SJ (1992). A phylogenetic interpretation of pollen morphology in tribe *Mentheae* (*Labiatae*). In: Harley RM, Reynolds T (eds.). Advances in *Labiatae* Science. Richmond, Surrey, UK: Royal Botanic Gardens, Kew, Pp. 113‑124.

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Agaricus parvitigrinus (*Agaricaceae*): A new record for the *Agaricus* section *Xanthodermatei* from Türkiye

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Agaricus parvitigrinus (*Agaricaceae*): *Agaricus* seksiyon *Xanthodermatei* için Türkiye'den yeni bir kayıt

Abstract: *Agaricus parvitigrinus*, a member of *Agaricus* section *Xanthodermatei*, was collected under *Pinus brutia* in western Türkiye and is presented and illustrated here as a new record based on its morphological characteristics and molecular analyses of the nuclear rDNA internal transcribed spacer (ITS1-5.8S-ITS2 = ITS) region. A comprehensive description, color photographs, line drawings of microscopic features, and comparisons with phenotypically similar taxa and phylogenetically related species are provided.

Key words: *Agaricomycetes*, molecular phylogeny, nrDNA ITS, taxonomy, new record

Özet: *Agaricus* seksiyon *Xanthodermatei*'ye ait bir tür olan *Agaricus parvitigrinus*, *Pinus brutia* altında Türkiye'nin batısından toplanmış, morfolojik karakterler ve nükleer rDNA iç transkribe boşluk (ITS1-5.8S-ITS2 = ITS) bölgesini içeren moleküler analizlere dayalı olarak sunulmuş ve tanımlanmıştır. Kapsamlı bir açıklama, renkli fotoğraflar, mikroskobik özelliklerin çizimleri, fenotipik olarak benzer taksonlar ve filogenetik olarak ilişkili türlerle karşılaştırmalar sunulmaktadır.

Anahtar Kelimeler: *Agaricomycetes*, moleküler filogeni, nrDNA ITS, taksonomi, yeni kayıt

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1. Introduction

Agaricus L. is one of the most species-rich genera among agaricoid fungi, comprising more than 500 species (Nauta, 2001; Zhao et al., 2011, 2016; Callac and Chen, 2018; He et al., 2018; Medel-Ortiz et al., 2022; Tarafder et al., 2022; Bashir et al., 2023; Wang and Bau, 2024). It has a cosmopolitan distribution, occurring from sea level to mountainous regions and even in arid environments (Zhao et al., 2011, 2016; Wang and Bau, 2024). The species of this genus are saprotrophic (Zhao et al., 2011; Chen et al., 2017) and include numerous taxa of significant nutritional and medicinal value (Parra, 2013; Kerrigan, 2016; Kaygusuz et al., 2017), such as the widely cultivated and consumed *Agaricus bisporus* (J.E. Lange) Imbach (Zhao et al., 2011; Thongklang et al., 2014). However, members of *Agaricus* section *Xanthodermatei* Singer are known for their toxicity due to the presence of phenol, a toxic compound (Gill and Strauch, 1984; Wood et al., 1998; Parra, 2008).

The genus is distinguished by its pinkish lamellae, which gradually turn brown over time, chocolate-brown spore print, and the presence of a ring-like annulus on the stipe (Parra, 2008; Knudsen and Vesterholt, 2012; Parra, 2013; Kerrigan, 2016). However, species identification within *Agaricus* can be challenging due to limited phenotypic variation, environmental influences, and significant intra-species variability (Zhao et al., 2011). As a result, modern taxonomic studies now emphasize the integration of molecular data alongside traditional morphological methods (He et al., 2018; Cao et al., 2020; Ling et al., 2021; Ortiz-Santana et al., 2021). Based on both morphological and molecular phylogenetic analyses, the genus has been classified into 6 subgenera and 24 sections (Kerrigan, 2016; Chen et al., 2017).

Agaricus sect. *Xanthodermatei* was first described by Singer (1948, 1986), with *A. xanthodermus* Genev. designated as the type species. Molecular phylogenetic analyses have revealed that *A.* sect. *Xanthodermatei*, once regarded as a monophyletic group, is now recognized as polyphyletic (Zhao et al., 2016; Bashir et al., 2021). Members of *A.* sect. *Xanthodermatei* are primarily characterized by distinct chemical reactions, including a negative Schäffer reaction and a bright yellow positive KOH reaction (Parra, 2008). Additionally, many species within this section exhibit key diagnostic traits such as transient yellow discoloration on the pileus surface and stipe base when damaged, a phenolic or iodine-like odor, and the presence of toxic compounds that can induce gastrointestinal distress in humans (Parra, 2008; Parra et al., 2011; Zhao et al., 2016).

According to the checklist of macrofungi in Türkiye and recent studies, a total of 58 species belonging to *Agaricus* have been reported (Sesli et al., 2020; Solak and Türkoğlu, 2022; Aslan et al., 2024; Halıcı and Güllü, 2024). Among these, only four species, namely *A. iodosmus* Heinem., *A. menieri* Bon, *A. placomyces* Peck and *A. xanthodermus*,

have been documented from *A.* sect. *Xanthodermatei*. This study presents the first morphological and molecular description of *Agaricus parvitigrinus* from Türkiye and contributes to the mycobiota of this continent.

2. Materials and Method

2.1. Morphological studies

Three *Agaricus* specimens were collected from Aydın Province, Türkiye. Each specimen was observed in its natural habitat, photographed, and documented with sampling details, including macro-morphological characteristics, geographical location, and habitat type. The specimens were then transported to the laboratory and air-dried using an electric dryer at approximately 20°C. Dried specimens were rehydrated in 3% KOH, stained with Congo Red, and then examined microscopically. Measurements were taken from at least 30 basidiospores. In the provided list of abbreviations, "L^m" and "W^m" denote the mean values of basidiospore length and width, respectively. "Q" represents the length-to-width ratio, while "Q^m" indicates the average Q value calculated from the measured basidiospores. Following morphological and phylogenetic analyses, the specimens were preserved in the fungarium of Isparta University of Applied Sciences (ISUF).

2.2. Molecular procedures

The macrofungus was dried and used for DNA isolation with the ZR Fungal/Bacterial DNA MiniPrep Kit (Zymo Research, Irvine, California), following the manufacturer's protocol. The isolated gDNA was checked on an agorose gel electroporesis for validation. The extracted DNA was subsequently used in polymerase chain reaction (PCR) to amplify the ITS gene region, using ITS1F/ITS4 primers

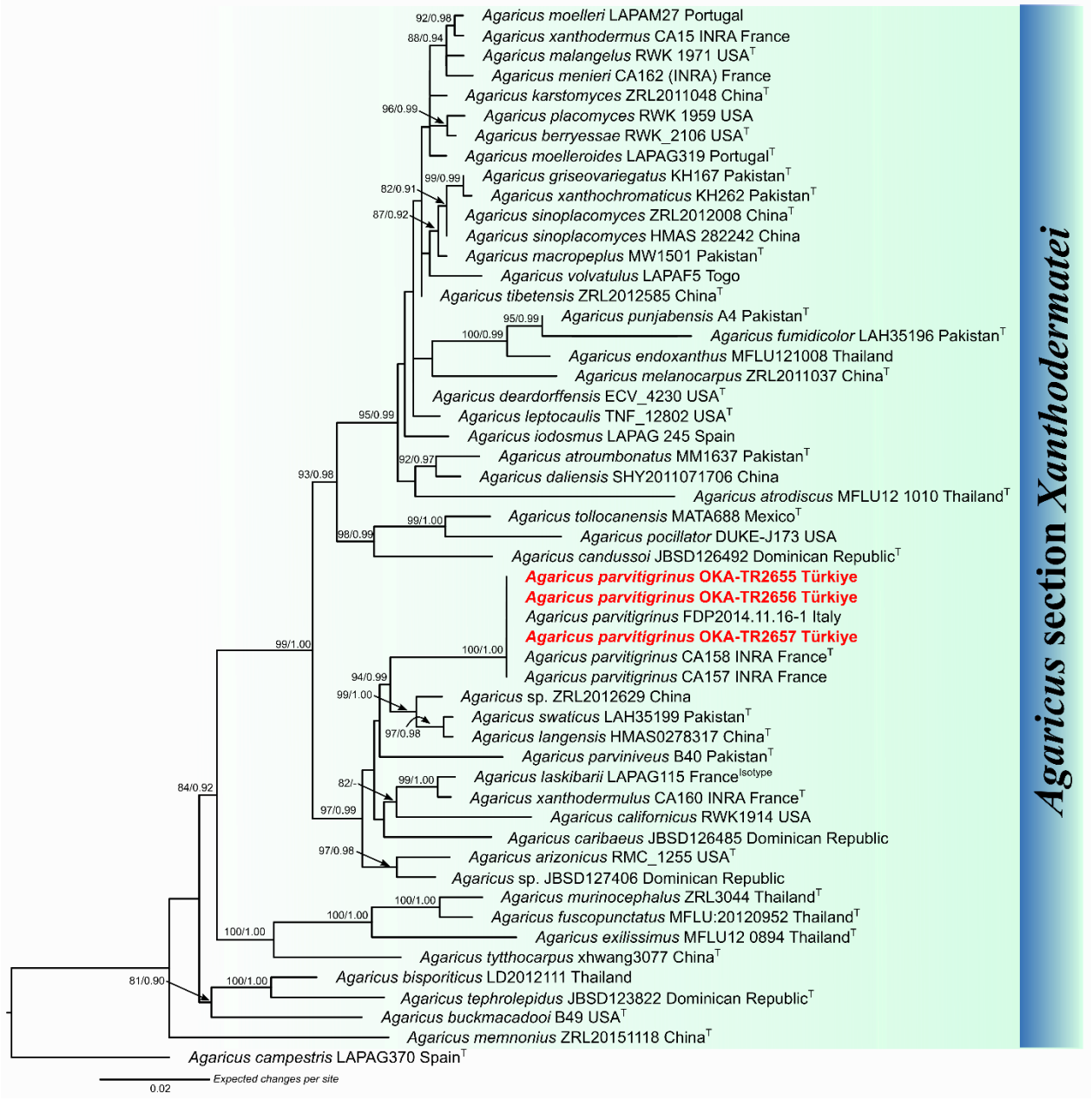

Figure 1. Maximum Likelihood (ML) phylogram of species within *A.* sect. *Xanthodermatei* based on nrDNA ITS sequence data. *Agaricus campestris* L. was used as an outgroup. Maximum Likelihood Bootstrap (MLB) values of ≥ 80% and Bayesian Posterior Probabilities (BPP) values of ≥ 0.90 are shown on the branches. Turkish collections are shown in bold red

Figure 2. Dry basidiomata of *Agaricus parvitigrinus*. Scale bar = 10 mm

(White et al., 1990; Gardes and Bruns, 1993). PCR products were checked on a 1% agarose gel electrophoresis and subsequently sequenced. The sequences were manually edited in BioEdit 7.0.5 (Hall, 1999).

A MegaBLAST search was conducted using the newly obtained sequences to identify closely related taxa in the GenBank nucleotide database (https://www.ncbi.nlm.nih.gov/genbank/). Multiple sequence alignment was performed using MAFFT version 7 with the FFT-NS-I strategy (Katoh et al., 2019). The alignment was subsequently reviewed and manually refined in BioEdit. Maximum Likelihood (ML) and Bayesian Inference (BI) methods were applied for phylogenetic analyses, following the methodology outlined by Kaygusuz (2022, 2024). FigTree version 1.4.2 was used to visualize the phylogenetic tree, which was then refined in Adobe Illustrator CS6.

3. Results

3.1. Phylogenetic analyses

Phylogenetic analyses of nrDNA ITS sequences included 53 sequences, three of which were newly generated. Since the BI and ML analyses yielded nearly identical topologies, the ML tree is presented in this study (Fig. 1). The Turkish collections of *Agaricus parvitigrinus* align phylogenetically with European specimens, confirming their taxonomic placement within *A.* sect. *Xanthodermatei*. The Turkish collections of *A. parvitigrinus* (OKA-TR2655, OKA-TR2656, and OKA-TR2657) form a strongly supported clade (MLB = 100%, BPP = 1.00, Fig. 1) alongside specimens from France (CA157, CA158) and Italy (FDP2014.11.16-1). The occurrence of *A. parvitigrinus* in Türkiye, France, and Italy suggests a broader geographical distribution of this species across Europe and Eurasia. Additionally, *Agaricus parvitigrinus* was closely related to *A. swaticus* H. Bashir, S. Jabeen, S. Ullah, Khalid & L.A. Parra (LAH35199) from Pakistan, *A. langensis* M.Q. He & R.L. Zhao (HMAS0278317), and an unidentified *Agaricus* species (ZRL2012629) from China.

3.2. Taxonomy

Agaricus parvitigrinus Guinberteau & Callac, *Mycologia* 97(2): 419 (2005) (Figs. 2, 3)

Macroscopic description: Pileus 25–60 mm diam., hemispherical to convex while young, later plano-convex to expanded, sometimes slightly depressed at center or low to flat umbo, densely covered by dark grayish appressed fibrillose squamules on a whitish background with an entire almost black to blackish grey center. Margin irregular, sometimes incurved in young, white, thick, slightly exceeding the lamellae and fimbriate, sometimes cracked. Lamellae free, unequal, crowded, straight, at first pinkish white, later greyish brown with the edge paler and eroded. Stipe 35–80 × 3–5 mm, smooth, cylindrical, curved at base, slightly bulbous or abruptly bulbous base with rhizomorphs, at first white to dull yellowish white then ferrugineous on handling. Partial veil white to pale gray, thick, membranous, forming a pendant annulus, often with radial squamules on the underside, cogwheel obvious when young. Annulus superous, double, membranous, smooth, white, the lower layer broke in a cogwheel decorating the lower surface. Context white at first, then slightly light yellowish by cutting. Odor phenolic. Taste unpleasant. KOH reaction positive, yellow. Schäffer's reaction negative.

Microscopic description: Basidiospores (4.7–)5.0–6.8(–7.0) × (3.2–)3.3–3.8(–4.2) µm, $L^m \times W^m = 5.6 \times 3.6$ µm, Q = (1.4–)1.8(–1.9), $Q^m = 1.6$, mostly ellipsoid, rarely oblong, smooth, dark brown, granular, guttulate with 1-2 guttules

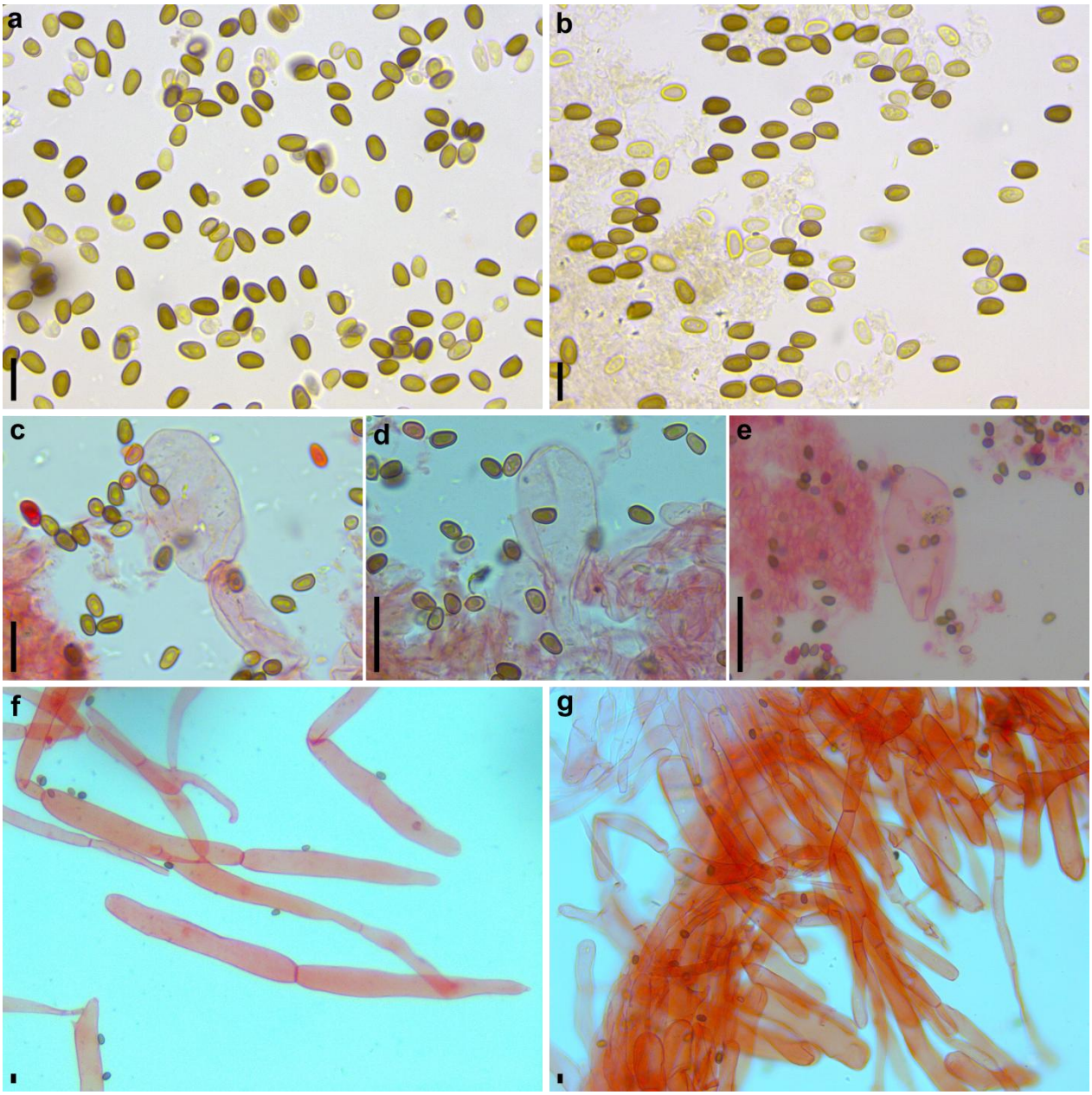

Figure 3. Micromorphological characters of *Agaricus parvitigrinus*. (a-b) Basidiospores. (c-e) Cheilocystidia. (f-g) Pileipellis hyphae. Scale bars: 10 µm

per spore, apiculate. Basidia 17–25 × 5.5–9.5 µm, tetrasporic, clavate, sterigmata up to 2.5 µm long. Cheilocystidia 15–30 × 5–10(–12) µm, clavate to spheropedunculate, rare and difficult to observe, weakly granular or hyaline. Pleurocystidia not observed. Pileipellis a cutis composed of hyphae 10–50 µm in diam., long cylindrical, sometimes curved and branched, containing light brown intracellular pigments, smooth. Clamp connections not observed.

Ecology: Occurring gregariously or in small groups on the forest floor, primarily under *Pinus brutia*, on calcareous sandy soils, predominantly in coastal areas of the Aegean region in western Türkiye.

Specimens examined*:* Türkiye. Aydın Province, Kuşadası district, in Davutlar, on calcareous sandy soil under *Pinus brutia*, alt. 52 m, 10 May 2013, leg. O. Kaygusuz (OKA-TR2655, GenBank accession no.: nrDNA ITS = PV197907); *ibid.*, alt. 45 m, 06 March 2014, leg. O. Kaygusuz (OKA-TR2656, GenBank accession no.: nrDNA ITS = PV197908); *ibid.*, alt. 48 m, 19 March 2014, leg. O. Kaygusuz (OKA-TR2657, GenBank accession no.: nrDNA ITS = PV197909).

4. Discussions

The Turkish specimen both morphologically and molecularly matches the type specimen of *Agaricus parvitigrinus* (Callac and Guinberteau, 2005) and belongs to *A.* sect. *Xanthodermatei*, as confirmed in previous studies (Zhao et al., 2011, 2016; Parra et al., 2018; Phookamsak et al., 2019; Bashir et al., 2021). According to the phylogenetic tree, *A. parvitigrinus* resides in a well-supported clade and is closely related phylogenetically to *A. swaticus* and *A. langensis*. Morphologically, *Agaricus swaticus*, a recently described species from Pakistan, differs from *A. parvitigrinus* by its larger basidiomata (up to 110 mm diam.) with grayish scattered scales, slightly larger basidiospores (on av. 6.1 × 4.0 µm), and its habitat

association with *Cedrus deodara* at high altitudes (Bashir et al., 2021). *Agaricus langensis*, originally described from China, differs from *A. parvitigrinus* by smaller basidiomata (up to 49 mm diam.), larger basidiospores (on av. 7.2 × 4.4 µm), and absence of cheilocystidia (Phookamsak et al., 2019).

Other European species within *A.* sect. *Xanthodermatei*, including *A. menieri*, *A. moelleri*, *A. pseudopratensis* (Bohus) Bohus, and *A. xanthodermus*, may be confused with *A. parvitigrinus*. However, *Agaricus menieri* and *A. moelleri* can be distinguished from *A. parvitigrinus* by larger basidiospores (Heinemann, 1978; Freeman, 1979; Capelli, 1984; Kerrigan, 1986; Nauta, 2001; Parra, 2013). *Agaricus pseudopratensis* exhibits a reddish discoloration when cut (Bohus, 1971; Heinemann, 1978). *Agaricus xanthodermus* differs by its white-toned pileus when young and larger basidiospores (Parra, 2013).

In conclusion, phylogenetic analyses and morphological characteristics confirm that *Agaricus parvitigrinus* is newly recorded from Türkiye and occupies a distinct phylogenetic position within *A.* sect. *Xanthodermatei*. It is considered a rare species, and additional reports from different localities are required to assess potential variations in its characteristics.

Conflict of Interest

Authors have declared no conflict of interest.

Authors' Contributions

The authors contributed equally.

References

Aslan AM, Uzun Y, Kaya A (2024). *Agaricus brunneofibrillosus*, A new record for Turkish Mycobiota. Mantar Dergisi 15(1): 12-15.

Bashir H, Fatima N, Izhar A, Niazi AR (2023). *Agaricus radiatosquamulosus* sp. nov. of *A.* subg. *Spissicaules* from Pakistan. Phytotaxa 579: 231-242.

Bashir H, Chen J, Jabeen S, Ullah S, Khan J, Niazi AR, Zhang MZ, Khalid, AN, Parra LA, Callac P (2021). An overview of *Agaricus* section *Hondenses* and *Agaricus* section *Xanthodermatei* with description of eight new species from Pakistan. Scientific Reports 11: 12905.

Bohus G (1971). *Agaricus* studies III. Ann. Hist.-nat. Mus Natl Hung 63: 77-82.

Callac P, Guinberteau J (2005). Morphological and molecular characterization of two novel species of *Agaricus* section *Xanthodermatei*. Mycologia 97: 416-424.

Callac P, Chen J (2018). Tropical species of *Agaricus*. In: Sanchez JE, Mata G, Royse DJ (eds.) Updates on Tropical Mushrooms, Basic and Applied Research. Chiapas: El Colegio de la Frontera Sur. Pp. 25-38.

Cao B, He MQ, Ling ZL, Zhang MZ, Wei SL, Zhao RL (2020). A revision of *Agaricus* section *Arvenses* with nine new species from China. Mycologia 113(1): 191-211.

Capelli A (1984). *Agaricus* L.: Fr. (*Psalliota* Fr.). Saronno: Liberia editrice Bella Giovanna.

Chen J, Callac P, Parra LA, Karunarathna SC, He MQ, Moinard M, Kesel AD, Raspé O, Wisitrassameewong K, Hyde KD, et al. (2017). Study in *Agaricus* subgenus *Minores* and allied clades reveals a new American subgenus and contrasting phylogenetic patterns in Europe and Greater Mekong Subregion. Persoonia (38): 170-196.

Freeman AEH (1979). *Agaricus* in the Southeastern United States. Mycotaxon 8: 50-118.

Gardes M, Bruns TD (1993). ITS primers with enhanced specificity for *Basidiomycetes*-Application to the identification of mycorrhizae and rusts. Molecular Ecology 2: 113-118.

Gill M, Strauch RJ (1984). Constituents of *Agaricus xanthodermus* Genevier: the first naturally endogenous azo compound and toxic phenilic metabolites. Zeitschrift für Naturforsch 39c: 1027-1029.

Halıcı MG, Güllü M (2024). A macrofungus taxon that is commonly eaten by the folk in Central Anatolia but never reported from Türkiye: *Agaricus pequinii* (Boud.) Konrad & Maubl. Biological Diversity and Conservation 17(1): 1-6.

Hall TA (1999). BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symposium Series 41: 95-98.

He MQ, Chuankid B, Hyde KD, Cheewangkoon R, Zhao RL (2018). A new section and species of *Agaricus* subgenus *Pseudochitonia* from Tailand. MycoKeys 40: 53-67.

Heinemann P (1978). Essai d'une clé de determination des genres *Agaricus* et *Micropsalliota*. Sydowia 30: 6-37.

Katoh K, Rozewicki J, Yamada KD (2019). MAFFT online service: multiple sequence alignment, interactive sequence choice and visualization. Briefings in Bioinformatics 20(4): 1160-1166.

Kaygusuz O (2022). First record of *Bolbitius reticulatus* (*Agaricales: Bolbitiaceae*) in Turkey, with nuclear ITS and LSU rDNA sequences data. Mantar Dergisi 13(1): 1-7.

Kaygusuz O (2024). First record of *Hygrophoropsis flavida* (*Hygrophoropsidaceae, Agaricomycetes*) from Türkiye. Anatolian Journal of Botany 8(2): 176-180.

Kaygusuz O, Kaygusuz M, Dodurga Y, Seçme M, Herken EN, Gezer K (2017). Assessment of the antimicrobial, antioxidant and cytotoxic activities of the wild edible mushroom *Agaricus lanipes* (F.H. Møller & Jul. Schäff.) Hlaváček. Cytotechnology 69: 135-144.

Kerrigan RW (1986). The *Agaricales* (Gilled Fungi) of California.Vol. 6 *Agaricaceae.* Eureka: Mad River Press.

Kerrigan RW (2016). *Agaricus* of North America; Memoirs of The New York Botanical Garden. New York, USA: New York Botanical Garden Press.

Knudsen H, Vesterholt J (2012). Funga Nordica. Agaricoid, Boletoid, Clavaroid, Cyphelloid, and Gastroid Genera. Copenhagen: Nordsvamp.

Ling ZL, Zhou JL, Parra LA, De Kesel A, Callac P, Cao B, Zhao RL (2021). Four new species of *Agaricus* subgenus *Spissicaules* from China. Mycologia 113(2): 476-491.

Medel-Ortiz R, Garibay-Orijel R, Argüelles-Moyao A, Mata G, Kerrigan RW, Bessette AE, Geml J, Angelini C, Parra LA, Chen J (2022). *Agaricus macrochlamys*, a new species from the (Sub)tropical Cloud Forests of North America and the Caribbean, and *Agaricus fiardii*, a new synonym of *Agaricus subrufescens*. Journal of Fungi (8): 664.

Nauta MM (2001). *Agaricus* L. In: Noordeloos ME, Kuyper TW, Vellinga EC (eds.), Flora Agaricina Neerlandica 5. Lisse: A.A. Balkema Publishers.

Ortiz-Santana B, Chen J, Parra LA, Angelini C, Lodge DJ, Kerrigan RW, Callac P (2021). The genus *Agaricus* in the Caribbean II. Refined phylogeny of *Agaricus* subg. *Spissicaules* with description of two new sections and eight new species. Mycological Progress 20(4): 381-411.

Parra LA (2013). *Agaricus* L. *Allopsalliota* Nauta & Bas, Fungi Europaei, Part 2,. Alassio: Candusso Edizioni.

Parra LA (2008). Agaricus L. *Allopsalliota* Nauta & Bas. Fungi Europaei 1, Italy: Edizioni Candusso.

Parra LA, Angelini C, Ortiz-Santana B, Mata G, Rojo C, Chen J, Callac P (2018). The genus *Agaricus* in the Caribbean. Nine new taxa mostly based on collections from the Dominican Republic. Phytotaxa 345(3): 219-271.

Parra LA, Mua A, Cappelli A, Callac P (2011). *Agaricus biannulatus* sp. nov., a new species of the section *Xanthodermatei* collected in Sardinia and Sicily. Micologia e Vegetazione Mediterranea 26: 3-20.

Phookamsak R, Hyde KD, Jeewon R, Bhat DJ, Gareth Jones EB, Maharachchikumbura SSN, Raspé O, Karunarathna SC, Wanasinghe DN, Hongsanan S, Doilom M, Tennakoon DS, Machado AR, Firmino AL, Ghosh A, Karunarathna A, Mešić A, Dutta AK, Thongbai B, Devadatha B, Norphanphoun C, Senwanna C, Wei D, Pem D, Ackah FK, Wang GN, Jiang HB, Madrid H, Lee HB, Goonasekara ID, Manawasinghe IS, Kušan I, Cano J, Gené J, Li J, Das K, Acharya K, Raj KNA, Latha KPD, Chethana KWT, He MQ, Dueñas M, Jadan M, Martín MP, Samarakoon MC, Dayarathne MC, Raza M, Park MS, Telleria MT, Chaiwan N, Matočec N, De Silva NI, Pereira OL, Singh PN, Manimohan P, Uniya P, Shang QJ, Bhatt RP, Perera RH, Alvarenga RLM, Nogal-Prata S, Singh SK, Vadthanarat S, Oh SY, Huang SK, Rana S, Konta S, Paloi S, Jayasiri SC, Jeon SJ, Mehmood T, Gibertoni TB, Nguyen TTT, Singh U, Thiyagaraja V, Sarma VV, Dong W, Yu XD, Lu YZ, Lim YW, Chen Y, Tkalčec Z, Zhang ZF, Luo ZL, Daranagama DA, Thambugala KM, Tibpromma S, Camporesi E, Bulgakov TS, Dissanayake AJ, Senanayake IC, Dai DQ, Tang LZ, Khan S, Zhang H, Promputtha I, Cai L, Chomnunti P, Zhao RL, Lumyong S, Boonmee S, Wen TC, Mortimer PE, Xu J (2019). Fungal diversity notes 929–1035: taxonomic and phylogenetic contributions on genera and species of fungi. Fungal Diversity 95:1-273.

Sesli E, Asan A, Selçuk F (eds.) Abacı Günyar Ö, Akata I, Akgül H, Aktaş S, Alkan S, Allı H, Aydoğdu H, Berikten D, Demirel K, Demirel R, Doğan HH, Erdoğdu M, Ergül C, Eroğlu G, Giray G, Halikî Uztan A, Kabaktepe Ş, Kadaifçiler D, Kalyoncu F, Karaltı İ, Kaşık G, Kaya A, Keleş A, Kırbağ S, Kıvanç M, Ocak İ, Ökten S, Özkale E, Öztürk C, Sevindik M, Şen B, Şen İ, Türkekul, İ, Ulukapı M, Uzun Ya, Uzun Yu, Yoltaş A (2020). The Checklist of Fungi of Turkey. İstanbul: Ali Nihat Gökyiğit Vakfı Yayını.

Singer R (1986). The Agaricales in modern taxonomy. 4th ed. Koenigstein, Germany: Koeltz Scientific Books.

Singer R (1948). Diagnoses fungorum novorum Agaricalium. Sydowia 2: 26-42.

Solak MH, Türkoğlu A (2022). Macrofungi of Turkey (Checklist Volume III). İzmir: Kanyılmaz Matbaacılık Kağıt ve Ambalaj San. Tic. Ltd. Şti.

Tarafder E, Dutta AK, Acharya K (2022). New species and new record in *Agaricus* subg. *Minores* from India. Turkish Journal of Botany (46): 183-195.

Thongklang N, Rizwana N, Abdul NK, Jie C, Kevin DH, Ruilin Z, Luis AP, Muhammad H, Magalie M, Philippe C (2014). Morphological and molecular characterization of three *Agaricus* species from tropical Asia (Pakistan, Thailand) reveals a new group in section *Xanthodermatei*. Mycologia 106: 1220-1232.

Wang SE, Bau T (2024). Six new species of *Agaricus* (Agaricaceae, Agaricales) from Northeast China. Journal of Fungi (10): 59.

White TJ, Bruns T, Lee S, Taylor J (1990). Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ (eds.) PCR Protocols: A Guide to Methods and Applications. New York: Academic Press, Pp. 315-322.

Wood WF, Watson RL, Largent DL (1998). Phenol, the odor compound from *Agaricus praeclaresquamosus*. Biochemical Systematics and Ecology 26: 793-794.

Zhao RL, Karunarathna S, Raspé O, Parra LA, Guinberteau J, Moinard M, De Kesel A, Barroso G, Courtecuisse R, Hyde KD, Guelly AK, Desjardin ED, Callac P (2011). Major clades in tropical *Agaricus*. Fungal Diversity 51: 279-296.

Zhao RL, Zhou JL, Chen J, Margaritescu S, Sánchez-Ramírez S, Hyde KD, Callac P, Parra LA, Li GJ, Moncalvo JM (2016). Towards standardizing taxonomic ranks using divergence times - A case study for reconstruction of the *Agaricus* taxonomic system. Fungal Diversity 78: 239-292.

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Glaucomaria rupicola: A new lichen record from Gilgit Baltistan (Pakistan), its DNA barcoding, morphology, ecology and distribution

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Glaucomaria rupicola: Gilgit Baltistan (Pakistan)'dan yeni bir liken kaydı, DNA barkodlaması, morfolojisi, ekolojisi ve yayılışı

Abstract: During an examination of lichen specimens in the Darel Valley, one new record of genus *Glaucomaria* was identified for the lichen flora of Pakistan. *Glaucomaria rupicola* (L.) P.F. Cannon has been collected from Gilgit Baltistan. Diagnostic characters of the species, distribution, ecology and phylogenetic analyses are given. Here, it has been reported first time on ITS-based phylogeny. It is a new record for Gilgit Baltistan, Pakistan.

Key words: *Ascomycota*, Darel, *Lecanoraceae*, phylogenetic analyses, taxonomy

Özet: Darel Vadisi'ndeki liken örneklerinin incelenmesi sırasında, Pakistan'ın liken florası için *Glaucomaria* cinsinin yeni bir kaydı tanımlandı. *Glaucomaria rupicola* (L.) P.F. Cannon, Gilgit Baltistan'dan toplandı. Türün tanısal karakterleri, dağılımı, ekolojisi ve filogenetik analizleri verilmiştir. Burada, ilk kez ITS tabanlı filogenide bildirilmiştir. Pakistan, Gilgit Baltistan için yeni bir kayıttır

Anahtar Kelimeler: *Ascomycota*, Darel, *Lecanoraceae*, filogenetik analizler, taksonomi

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1. Introduction

Maurice Gustave Benoît Choisy (1897-1966) first introduced the genus *Glaucomaria* M. Choisy in 1929. When Choisy discovered the genus *Glaucomaria* in (1929), at that time this genus consisted of three species which are ranked: *Lecanora glaucoma* (= *L. rupicola*, *L. angulosa* (= *L. carpinea* and *L. albella*. Though none of the three constituent species were formally united into *Glaucomaria*, and Choisy did not designate a type, the genus was given a description that satisfies the standards for legitimate publication. Hafellner (1984) well-defined the genus and lectotypified it with *L. rupicola*, but the requisite combination was not completed. Similarly, even though Kondratyuk et al. (2019) designated *G. rupicola* as a binomial in their summary appearance of the genus, no formal grouping was recognized. According to latest survey, the number of species of the genus *Glaucomaria* has now reached 10, whose names are classified as; *G. bicincta*, *G. carpinea*, *G. cinerella*, *G. leptoplaca*, *G. leptyrodes*, *G. lojkaeana*, *G. rupicola*, *G. subcarpinea*, *G. swartzii* and *G. swartzii subsp. swartzii* (http://www.indexfungorum.org/names/Names.asp).

The genus *Glaucomaria* segregated from *Lecanora* with strongly pruinose, often semi-immersed apothecia; containing sordidone, the disc pruina C+ yellow or orange (Cannon et al., 2022). In the current study, just one species of this genus was reported and identified using comparative morpho-anatomical, molecular, and chemical investigations. *G. rupicola* has never been documented in Darel Valley, Gilgit Baltistan, Pakistan. This is the first time it has been reported on an ITS-based phylogeny. This is a new record for Darel Valley, Gilgit Baltistan, Pakistan.

2. Materials and Method

Morpho-anatomical characteristics were examined using a stereomicroscope (Meiji Techno, EMZ-5TR, Japan) and a compound microscope (MX4300H, Meiji Techno Co., Ltd., Japan). Spot tests were conducted according to the protocol established by Orange et al. (2010). DNA was extracted from dried and purified thalli using a GF1 Plant DNA extraction kit, adhering to the manufacturer's instructions (Vivantis, Selangor Darul Ehsan, Malaysia). The cladogram was developed using MEGA 6.0 (Tamura et al., 2013). The sample referenced in this study (DR–198) clustered with *G. rupicola* (L.) P.F. Cannon and formed a sister branch with the same specimen of *G. rupicola* from Austria and the USA (AY541257, MZ243628 and MZ243629) (Fig. 1).

3. Results

Glaucomaria rupicola (L.) P.F. Cannon, in Cannon, Malíček, Ivanovich, Printzen, Aptroot, Coppins, Sanderson, Simkin & Yahr, Revisions of British and Irish Lichens 25: 75, 2022 (Fig. 2).

Thallus crustose, 0.5-1 cm broad, rimose, up to 1 mm thick, irregular. **Areoles** thin or thick, flat to convex, and slightly shiny. **Soredia** not found. **Surface** whitish grey to brownish grey, epruinose, unclear at the borders. **Prothallus** whitish at the edges. **Alga** subglobose to globose, chlorococcoid, 14-24 µm in diameter. **Apothecia** lecanorine, highly pruinose, frequent, scattered, initially immersed, and

emerged when mature. **Disc** 0.5-1.8 mm in diameter, grayish brown to greenish brown, contiguous, grey to pale grey pruinose, planar to convex. **Epihymenium** 15-25 µm tall. **Hymenium** 95-120 µm tall. **Hypothecium** 50-75 µm tall. **Paraphyses** hyaline, simple, branching apically, 2.5-4 µm wide, apices 3 µm in diameter. **Asci** clavate, *Lecanora*-type, 60-72 × 24-34 µm broad, 8-spored. **Ascospores** 11-16×6-8 µm, simple, hyaline.

Spot tests: thallus; K+ (yellow); apothecial margin; C+ (yellow); TLC: atranorin

Ecology: The valley receives an annual rainfall ranging from 100 to 300 mm, with the majority falling as snow in the winter and early spring months.

Specimen examined: PAKISTAN: Gilgit Baltistan, Darel Valley, 35°37'N, 73°27'E, 2,000 m a.s.l., Aug. 10, 2022, Muhammad Shahid Iqbal, DR–198 (ITS genbank accession number OR751654) (LAH38347).

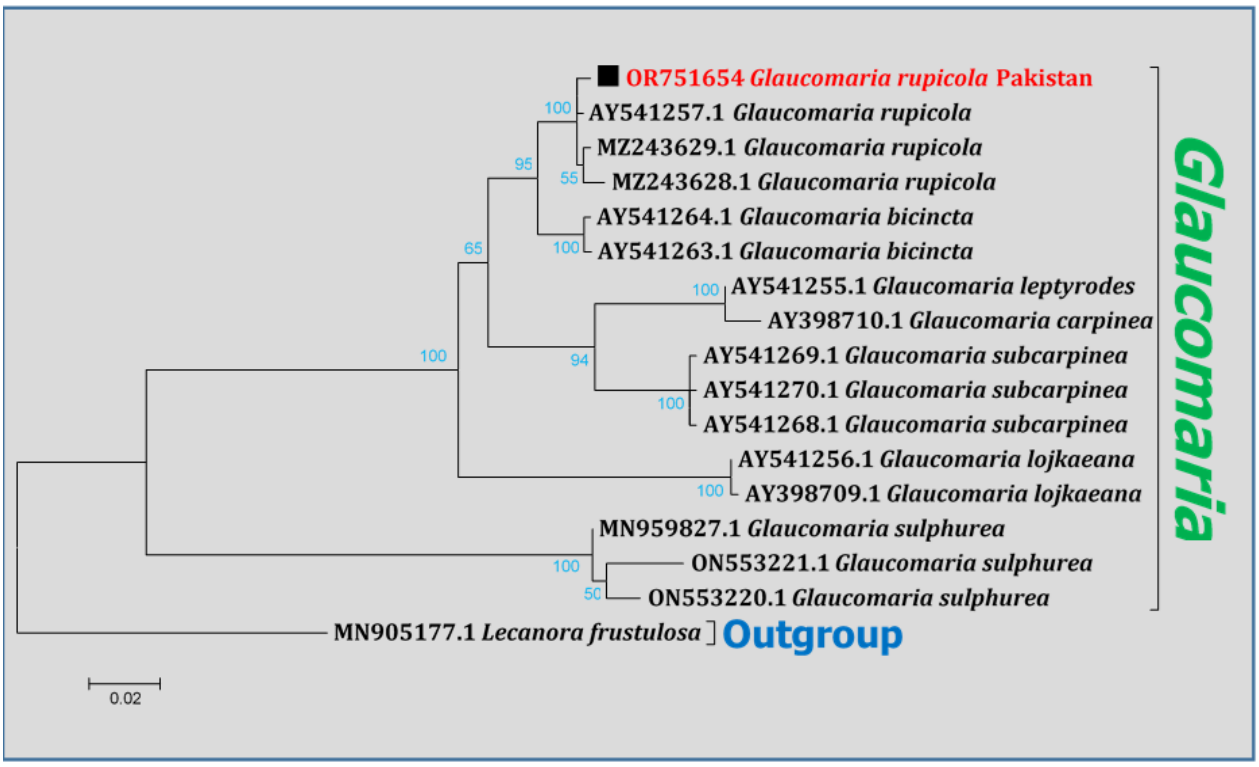

Figure 1. Molecular phylogenetic analysis of *Glaucomaria rupicola* using the Maximum Likelihood

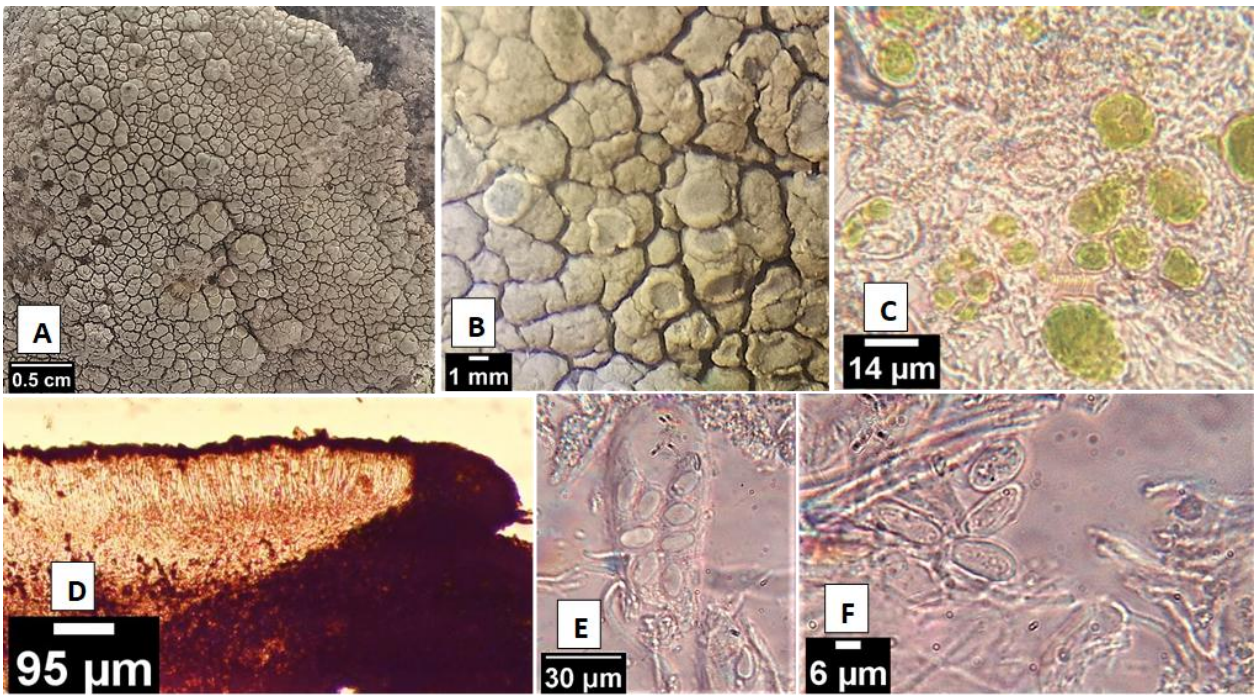

Figure 2. *Glaucomaria rupicola,* **A:** Thallus, **B:** Apothecia, **C:** Algal cells, **D:** Cross section of apothecium, **E:** Asci with ascospores, **F:** Ascospores

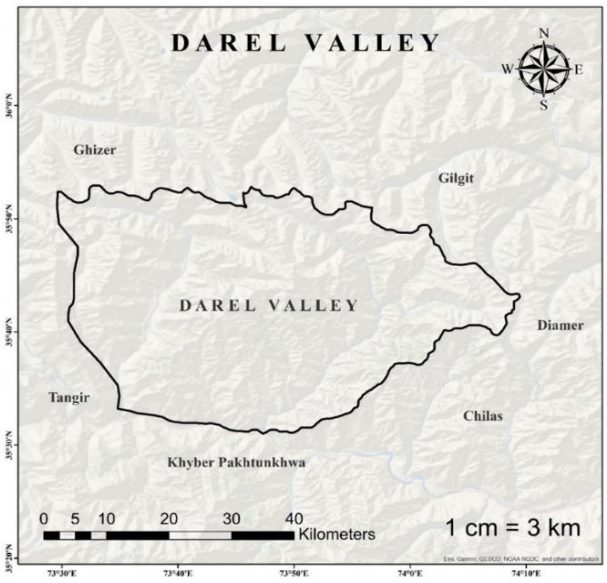

Figure.3 Map of sampling site, Darel Valley, Gilgit-Baltistan, Pakistan (Iqbal and Khalid, 2024b).

Distribution: *Glaucomaria rupicola* is discovered for the first time in Darel Valley, Gilgit Baltistan, Pakistan, growing on calcareous rocks. The species occurs in Austria, England, Ireland, and the United States. It occurs on hard exposed siliceous rocks, frequent in the xeric-supralittoral zone on coastal rocks, but also locally frequent inland, widespread in North and West Britain and Ireland, but relatively local in South and East England, where it is primarily confined to churchyards (Cannon et al., 2022).

4. Discussions

Our species' morpho-anatomical characteristics match those of *G. rupicola* from Great Britain and Ireland, with the exception of a taller hymenium (95-120 µm vs. 80-90 µm) and larger ascospores (11-16 × 6-8 µm vs. 9-14.5 × 5.5-7 µm). According to ecological behaviour, the specimen *G. rupicola* found in Great Britain and Ireland grows on hard, exposed siliceous rocks. It is commonly found in the xeric supralittoral zone on coastal rocks, but it is also occasionally found inland. For some forms of the species, it has strong maritime tendencies. Additionally common throughout the aerohaline zone, however certain types are less common inland. *G. rupicola* growing on siliceous rocks and walls, widespread and fairly frequent, particularly in northern and western Britain. The species exhibits significant variation across its range, with the identification of multiple subspecies; all specimens originating from the British Isles are consistent with *Glaucomaria rupicola* (Cannon et al., 2022). *Glaucomaria rupicola* has been reported for the first time from Pakistan.

In conclusion, with the discovery of this one species, the total number of species in Darel Valley has increased to 30 (Afshan et al., 2024; Asghar et al., 2023; Aptroot and Iqbal, 2012; Din et al., 2023a,b, 2024 ; Fayyaz et al., 2023; Iqbal et al., 2022; 2023a, b, c; 2024a, b, c, d, e; 2025; Iqbal and Khalid, 2022, 2023, 2024a,b,c,d,e; Riaz et al., 2024; Zulfiqar et al., 2023, 2024).

Conflict of Interest

Author has declared no conflict of interest.

References

Afshan NUS, Fayyaz I, Iftikhar F, Habib K, Iqbal MS, Wang XY, Khalid AN (2024). New species and new records of genus *Buellia* (lichenized *Ascomycota, Caliciaceae*) from Pakistan using electron microscopy and DNA barcoding techniques. Microscopy Research and Technique 87: 31-41. DOI: 10.1002/jemt.24403

Aptroot A, Iqbal SH (2012). Annotated checklist of the lichens of Pakistan, with reports of new records. Herzogia 25: 211-229. DOI: 10.13158/heia.25.2.2010.211

Asghar HS, Fayyaz I, Iqbal MS, Afshan NUS, Khalid AN (2023). Two new records of genus *Diploschistes* (Lichenized *Ascomycota, Thelotremataceae*) from Pakistan. Biology Bulletin 50: 870-875. DOI: 10.1134/S1062359023600071

Cannon P, Malíček J, Ivanovich C, Printzen C, Aptroot A, Coppins B, Yahr R (2022). *Lecanorales: Lecanoraceae* including the genera *Ameliella, Bryonora, Carbonea, Claurouxia, Clauzadeana, Glaucomaria, Japewia, Japewiella, Lecanora, Lecidella, Miriquidica, Myriolecis, Palicella, Protoparmeliopsis, Pyrrhospora* and *Traponora*. Revisions of British and Irish Lichens 25: 1–83. https://britishlichensociety.org.uk/sites/default/files/Lecanoraceae

Choisy M (1929). Genres nouveaux pour la lichénologie dans le groupe des *Lécanoracées*. Bulletin De La Societe Botanique De France 76: 521-527. DOI.org/10.1080/00378941.1929.10837179

Din AU, Iqbal MS, Khalid AN, Niazi AR (2024). *Chlorangium ahmadii* sp. nov. and *Circinaria darelensis* sp. nov. two new species of lichenized *Ascomycetes* from Pakistan. Plant Systematics and Evolution 310: 1-8. DOI: 10.1007/s00606-024-01893-3

Din AU, Iqbal MS, Niazi AR, Khalid AN (2023a). Additions to the list of *Megasporaceae* (Lichenized *Ascomycetes*) in Pakistan. Biology Bulletin 50: 598-601.

Din AU, Iqbal MS, Niazi AR, Khalid AN (2023b). Two new records of lichens from Pakistan. Biology Bulletin 50:1195-1200.

Fayyaz I, Iqbal MS, Afshan NUS, Niazi AR, Khalid AN (2023).Taxonomic and phylogenetic study of the genus *Diploschistes* (*Ostropales, Thelotremataceae*) reveals one new species from Pakistan. Acta Botanica Brasilica 37: e20220125. DOI: 10.1590/1677-941x-abb-2022-0125

Hafellner J (1984). Studien in richtung einer natürlicheren gliederung der sammelfamilien *Lecanoraceae* und *Lecideaceae*. Beihefte Zur Nova Hedwigia 79: 241-371. https://pascal-francis.inist.fr/vibad/index.php?action=getRecordDetail&idt=9129792

Iqbal MS, Abbas MZ, Khalid AN (2022). A new species of lichen genus *Acarospora* (*Acarosporaceae*, Lichen forming *Ascomycota*) from Darel Valley, Gilgit Baltistan, Pakistan. Biology Bulletin 49: S45-S50. DOI: 10.1134/S1062359022150110

Iqbal MS, Usman M, Habib K, Khalid AN (2023a). *Oxneriaria pakistanica* sp. nov. (*Megasporaceae, Pertusariales, Ascomycota*) from Darel Valley, Gilgit Baltistan, Pakistan. Phytotaxa 579: 125-131. https://doi.org/10.11646/phytotaxa.579.2.6

Iqbal MS, Asghar HS, Khalid AN (2023b). A new species and a new record of family *Ramalinaceae* (Lichenized *Ascomycetes*) from Pakistan. Biology Bulletin 50: 1187-1194. DOI: 10.1134/S1062359023602203

Iqbal MS, Fayyaz I, Afshan NUS, Iftikhar F, Khalid AN (2023c). A new species of *Circinaria* (*Pertusariales, Megasporaceae*) from Pakistan. Botanica Serbica 47: 235-239. DOI: 10.2298/BOTSERB2302235I

Iqbal MS, Ashraf A, Asghar HS, Khalid AN (2024a). A new species and a new record of the genus *Candelariella* (*Candelariaceae*, lichenized *Ascomycota*) from Pakistan. Nova Hedwigia 119: 221-231. DOI: 10.1127/nova_hedwigia/2024/0942

Iqbal MS, Nadeem M, Khalid AN, Niazi AR (2024b). Some new records of lichens from Pakistan. Nova Hedwigia 119:457-468. DOI: 10.1127/nova_hedwigia/2024/0975

Iqbal MS, Nadeem M, Din AU, Khalid AN, Niazi AR (2024c). Two new species of genus *Acarospora* (Lichenized *Ascomycota: Acarosporaceae*) from Pakistan. Journal of Asia-Pacific Biodiversity. https://doi.org/10.1016/j.japb.2024.10.002

Iqbal MS, Din AU, Khalid AN, Niazi AR (2024d). A new species in the lichen genus *Circinaria* (*Megasporaceae*) From Pakistan based on morphology and DNA sequence data. Journal of the Botanical Research Institute of Texas 18: 333-340. https://doi.org/10.17348/jbrit.v18.i2.1370

Iqbal MS, Din AU, Khalid AN, Niazi AR (2024e). Morphology and muti-gene phylogenetic analyses reveal *Immersaria pruinosa* sp. nov. (Lichenized *Ascomycota, Lecideaceae*) from Pakistan. Current Research in Environmental & Applied Mycology 14: 333-344. DOI: 10.5943/cream/14/1/16

Iqbal MS, Din AU, Khalid AN, Niazi AR (2025). Morphology and DNA barcoding analyses reveal *Acarospora s albicans* sp. nov. (Lichenized Ascomycota, Acarosporaceae) from Pakistan. Journal of Asia-Pacific Biodiversity. https://doi.org/10.1016/j.japb.2025.01.010

Iqbal MS, Khalid AN (2022). *Acarospora sultanii* sp. nov. (*Acarosporaceae*, lichen forming *Ascomycota*) from Darel Valley, Gilgit Baltistan, Pakistan. Biology Bulletin 49: S40-S44. DOI: 10.1134/S1062359022150122

Iqbal MS, Khalid AN (2023). *Rusavskia dasanensis* (*Teloschistaceae*), a new record for Pakistan. Lindbergia 2023: 1. https://doi.org/10.25227/linbg.025179

Iqbal MS, Khalid AN (2024a). *Diploschistes iqbalii* sp. nov. (*Ascomycota: Graphidaceae*), a new lichen species from Darel Valley, Gilgit Baltistan, Pakistan. Nova Hedwigia 118: 477-485. DOI.org/ 0.1127/nova_hedwigia/2024/0936

Iqbal MS, Khalid AN (2024b). *Lecanora darelensis*, a new species of *Lecanoraceae* from Darel Valley, Gilgit Baltistan, Pakistan. Telopea 27: 117-124. DOI.org/10.7751/telopea17708

Iqbal MS, Khalid AN (2024c). *Aspicilia fumosa* (*Megasporaceae*), a new record for Pakistan and the first in Eurasia. Journal of the Botanical Research Institute of Texas 18: 187-191. DOI.org/ 10.17348/jbrit.v18.i1.1347

Iqbal MS, Khalid AN (2024d). First report of poorly known genus *Gloeoheppia* (*Ascomycota: Gloeoheppiaceae*), reveals one new species from Pakistan. Journal of Asia-Pacific Biodiversity. DOI.org/10.1016/j.japb.2024.08.006

Iqbal MS, Khalid AN (2024e). *Verrucaria aptrootii* sp. nov. (*Verrucariaceae, Ascomycota*), a new lichen species from Darel Valley, Gilgit Baltistan, Pakistan. Acta Botanica Hungarica 66: 193-202. DOI: 10.1556/034.66.2024.3-4.4

Kondratyuk SY, Lőkös L, Jang SH, Hur JS, Farkas E (2019). Phylogeny and taxonomy of *Polyozosia, Sedelnikovaea* and *Verseghya* of the *Lecanoraceae (Lecanorales*, lichen-forming *Ascomycota*). Acta Botanica Hungarica 137-184. DOI.org/10.1556/034.61.2019.1-2.9

Orange A, James PW, White FJ (2010). Microchemical methods for the identification of lichens. London: British Lichen Society.

Riaz S, Nadeem M, Iqbal MS, Niazi AR, Khalid AN (2024). *Circinaria russus* sp. nov.(Lichenized *Ascomycota: Megasporaceae*) from Pakistan. Biology Bulletin 51(1): 98-103. DOI: 10.1134/S1062359023603579

Tamura K, Stecher G, Peterson D, Filipski A, Kumar S (2013). MEGA6: molecular evolutionary genetics analysis version 6.0. Molecular Biology and Evolution 30: 2725-2729. DOI.org/10.1093/molbev/mst197

Zulfiqar R, Razzaq F, Iqbal MS, Khalid AN (2023). A new species of *Lobothallia* (*Megasporaceae, Pertusariales, Ascomycota*) from Pakistan. Plant Systematics and Evolution 309: 35.

Zulfiqar R, Shaheena M, Iqbal MS, Ahmad UF, Naseer A, Khalid AN (2024). New additions to the list of lichenized fungi of Pakistan. Nova Hedwigia 119: 233-249. DOI: 10.1127/nova_hedwigia/2024/0953